CHAPTER 1

INTRODUCTION

1. Inflammation

Inflammation is a protective response intended to abolish the initial cause of cell injury as well as the necrotic cells and tissues from the original insult[1]. Pathogens,abrasions,chemical irritations, distortion or disturbances of cells and extreme temperatures causes inflammation. It is an attempt to dispose of microbes,toxins or foreign materials, at the site of injury, to prevent their spread to other tissues, and to prepare the site for tissue repair in an attempt to restore tissue homeostasis

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1.2Signs of inflammation

The four cardinal signs and symptoms of inflammation,described by Roman writer Celsus 1st century A.D are as follows[2][3]

- ✓ *rubor* (redness);
- ✓ *tumor* (swelling);
- ✓ *calor* (heat); and
- ✓ *dolor* (pain).

Inflammation can also cause a loss of function in the injured area depending on the site and extent of the injury and thus a fifth sign *functio laesa* (loss of function) was also added by Virchow[4]

1.3Types of inflammation

According to the defence capacity of the host and duration of response , inflammation is classified as;

- ➢ Acute inflammation
- ➢ Chronic inflammation

1.3.1 Acute inflammation

Acute inflammation which has a short duration lasting from few minutes to a few days which is characterised by fluid and plasma protein exudation and neutrophilic leukocyte accumulation[5]. Acute inflammation has two major components

Vascular changes:-

Changes in vessel calibre resulting in increased blood flow (vasodilation) and structural changes that permits plasma proteins to leave the circulation (increased vascular permeability)

Cellular events:-

Movement of leukocytes from the microcirculation and accumulation in the focus of the injury(cellular recruitment and activation)

1.3.1.1 Vascular changes in acute inflammation

- **Changes in vascular calibre and flow**

Changes in blood vessels begin rapidly after infection or injury but may develop at variable rates,depending on the nature and severity of the inflammatory stimulus.

- After transient vasoconstriction, arteriolar vasodilation occurs,which increase the blood flow locally and engorgement of the downstream capillary beds. This vascular expansion is the cause of the erythema and warmth characteristically seen in acute inflammation.
- As the microvasculature becomes more permeable,protein rich fluid moves into the extravascular tissues.This causes the red blood cells to become more concentrated thereby increasing blood viscosity and slowing the circulation,known as stasis
- As stasis develops, leucocyteswill start to accumulate along the vascular endothelial surface, a process called margination..

- **Increased vascular Permeability.**

In the early phase of inflammation, arteriolar vasodilation and increased volume of blood flow lead to a rise in intravascular hydrostatic pressure ,resulting in movement of fluid from capillaries into thetissues. This fluid, called as transudate is essentially an ultra filtrate of blood plasma and contains little protein. However, transudation is soon eclipsed by increasing vascular permeability that allows the movement of protein rich fluid and even cells(called an exudate) into the interstitium. The loss of protein rich fluid into the perivascularspace reduces the intravascular osmotic pressure and increases the osmotic pressure of the interstitial fluid. The net result is the outflow of water and ions into the extravascular tissues. Fluid accumulation in extravascular spaces is called edema, The fluid may be a transudate or exudates. Whereas exudates are typical of inflammation, transudates accumulate in various non inflammatory conditions.

1.3.1.1.1Mechanisms contributing vascular permeability

a.Endothelial cell contraction leading to intercellular gaps in postcapillary venules

-An immediate transient process which is a reversible process elicited by histamine,bradykinin,leukotrienes and many other chemical mediators when bound to the specific receptors due to endothelial contraction.

b.Endothelial injury results in vascular leakage by causing endothelial cell necrosis and detachment.

-Direct injury to endothelial cells causes vascular leakage which begins immediately after the injury and persist for several hours until the damaged vessels are thrombosed or repaired.

c.Leukocyte-mediated endothelial injury.

-Occur as a consequence of acculation of leukocyte along the vessel wall and the activated leukocytes release many toxic mediators that cause endothelial injury or detachment

d.Increased transcytosis of proteins via an intracellular vesicular pathway.

-Augments venular permeability especially after exposure to certain mediators such as vascular endothelial growth factor.(VEGF).

e.Leakage from new blood vessels.

-Tissue repair involves angiogenesis and these vessel sprout remain leaky until proliferating endothelial cells mature sufficiently to form intracellular junctions.

1.3.1.1.2 Chemistry of vasodilation in acute inflammatory response

The haemodynamics in capillaries and venules are controlled by the precapillary arteriole and the post capillary venule. The vascular tone is dependant upon the smooth muscle coat in the media and is under the contol of the control of a number of neuroendocrine influences.some of these vasoregulatory agents include

- Vasoconstrictors:-Leukotrienes, Endothelin, Thromboxane, sympathetic stimuli, catecholamines
- Vasodilators:- PGI_2,PGD_2, PGE_2, Nitric oxide, Histamine, Parasympathetic stimuli, Serotonin

1.3.1.1.3 Microvascular thrombosis

The contact between the plasma and the collagen of the basement membrane as well as the platelets and the basement membrane activates the clotting cascade. The result is platelet aggregation, fibrin deposition causing a small thrombus to form in the capillary. The result of such thrombi is to further obstruct the blood flow thus causing more capillary distension, more accumulation of leukocytes and red cells and greater movement of these elements out of the capillary. Another major role of the fibrin precipitation is that the fibrin threads provide a scaffolding for the leukocytes to move like "climbers on the rope".

1.3.1.1.4 Relative tissue hypoxia and lymph nodes

The stagnation of blood quickly drains out the oxygen from the red cells and allow accumulation of carbon dioxide and with delay in the arrival of more cells,there develops a state of local hypoxia and anoxia.The change in the milieu promotes increased capillary permeability.

1.3.1.1.5 Involvement of lymphatics and lymph nodes

Normally the passage of fluids into the tissue spaces causes greater drainage of fluid into the lymphatics to restore the balance. Since the inflammatory fluid is plasma, and contains blood cells it is an exudates. Not only do the capillaries find it difficult to absorb the larger molecules but the clotting of the fluids in the tissue further obstructs the lymphatic flow. But in most acute inflammations, the lymphatics also get dilated and may become red as they transport the cells and fluids to the regional lymph node. This presents sometimes with very painful thin streaks of inflamed lymphatics.

1.3.1.2 Cellular events : leukocyte recruitment and activation.

An important function of the inflammatory response is to deliver leukocytes to the site of injury and to activate them. Leukocytes ingest offending agents,kill bacteria and other microbes and eliminate necrotic tissue and foreign substances. A price that is paid for the defensive potency of leukocytes is that, once activated, they may induce tissue damage and prolong inflammation, since the leukocyte products that destroy microbes can also injure host tissues. Therefore key to normal function of leukocytes in host defence is to ensure that they are recruited and activated only when needed.

1.3.1.2.1 Leukocyte recruitment

The sequence of events in the recruitment of leukocytes from the vascular lumen to the extravascular space consist of

a. Margination, adhesion to endothelium and rolling along the vessel wall.
b. Firm adhesion to the endothelium
c. Transmigration between endothelial cells; and
d. Migration in interstitial tissues towards a chemotactic stimulus.

- **Margination and rolling**

As blood flows from capillary venules, circulating cells are swept by laminar flow against the vessel wall. In addition,the larger white cells tends to move slower than the smaller red cells. As a result, leukocytes are pushed out of the central axial column and thus have a better opportunity to interact with lining endothelial cells, especially as stasis sets in. This process of leukocyte accumulation at the periphery of the vessels is called margination. Subsequently leukocytes tumble on the endothelial surface, transiently sticking along the way, a process called rolling.

The weak and transient adhesions which are involved in rolling are mediated by the selectin family of adhesion molecules. Selectins are receptors expressed on leukocytes and endothelium which contain an extracellular domain that binds sugars. Selectins binds sialyted oligosaccharides,that are attached to mucin like glycoproteins on various cells. The three members of this family are

- ✓ E- selectin(CD62E) , expressed on endothelial cells;
- ✓ P-selectins(CD62P), present on endothelium and platelets.
- ✓ L-selectins(CD62L), present on the surface of leukocytes.

- **Adhesion and transmigration**.

The adhesion is mediated by integrins expressed on leukocyte cell surfaces interacting with their ligands on endothelial cells. Integrins are transmembrane heterodimeric glycoproteins (composed of different α andβ chains) that also functions as cell receptors for extracellular matrix.Chemokines are chemoattractant cytokines that are secreted by many cells at site of inflammation and are displayed bound to proteoglycans on the endothelial surface.When the adherent leukocytes encounter the displayed chemokines, the cells are activated, and their integrins undergoes conformational changes and cluster together, thus converting to a high affinity form. At the same time, other cytokines notably TNF and IL-1, activate endothelial cells

to increase their expression of ligands for integrins. The result of cytokine stimulated increasedintegrin affinity and increased expression of integrin ligands is the stable attachment of leukocytes to endothelial cells at sites of inflammation.

After getting detained on the endothelial surface, leukocytes drift through the vessel wall primarily by squeezing between cells at intercellular junctions. This movement of leukocytes called diapedesis, which occurs mainly in the venules of the systemic vasculature. movement of leukocytes is driven by chemokines produced in extravascular tissues, which stimulate movement of the leukocytes in the direction of their chemical gradient. After passing through endothelium, leukocytes cross vascular membranes by focally degrading them with secreted collagenases.

- **Chemotaxis**

The chemotactic factor-mediated transmigration of leucocytes after passing several barriers (endothelium, basement membrane, perivascular myofibroblasts and matrix) arrive at the interstitial tissues is known as chemotaxis. The following agents operate as potent chemotactic substances or chemokinesfor neutophils:

i) Leukotriene B4 (LT-B4), a product of lipooxygenase pathway of arachidonic acid metabolites

ii) Components of complement system (C5a and C3a in particular)

iii) Cytokines (Interleukins, in particular IL-8)

iv) Soluble bacterial products (such as formylated peptides).

Different types of leukocytes react differently to chemotactic influences. For example:

- Most bacteria attract the PMNL
- Foreign bodies, whether exogeneous or endogeneous like the infarct attract PMNL
- Viruses are chemotactic for lymphocytes, usually utilising the MHC antigenon the surface of the infected cells.
- Foreign cells as in a transplant attract lymphocytes and macrophages with the mediation of the MHC
- Mycobacteria like tuberculosis and *lepra bacilli* attract macrophages and lymphocytes.
- Allergens attract eosinophils,basophils and mast cells.

1.3.1.2.2 Leukocyte activation

Different receptors are there in the surface of leukocytes which can sense the presence of microbes. The term leukocyte activation stands for a number of responses by microbial products or various mediators of inflammation with the engagement of receptors..

Leukocyte activation leads to

- ✓ Phagocytosis
- ✓ Production of substances which destroys phagoytosed microbes and remove dead tissues.
- ✓ Production of mediators that enhance inflammatory response.

1.3.1.2.3 Phagocytosis

Phagocytosis is defined as the process of engulfment of solid particulate material by the cells (cell-eating). The cells performing this function are called *phagocytes*. There are 2 main types of phagocytic cells:

i) Polymorphonuclear neutrophils (PMNs) which appear early in acute inflammatory response, sometimes called as microphages.

ii) Circulating monocytes and fixed tissue mononuclear phagocytes, commonly called as macrophages.

Neutrophils and macrophages on reaching the tissue spaces produce several proteolyitc enzymes—lysozyme, protease, collagenase, elastase, lipase, proteinase, gelatinase,

and acid hydrolases. These enzymes degrade collagen and extracellular matrix. The microbe undergoes the process of phagocytosis by polymorphs and macrophages and involves

the following 3 steps

1. Recognition and attachment
2. Engulfment
3. Killing and degradation[6] [7] [8]

- **Recognition and attachment**

Phagocytosis is initiated by the expression of surface receptors on macrophages which recognise microorganisms: mannose receptorand scavenger receptor. The process of phagocytosis is further improved when the microorganisms are coated with specific proteins, opsonins, from the serum or they get opsonised. Opsonins create a bond between bacteria and the cell membrane of phagocytic cell. The major opsonins present in the serum and their matching receptors on the surface of phagocytic cells (PMNs or macrophages) are as under:

i) ***IgG opsonin***is the Fc fragment of immunoglobulin G; it is the naturally occurring antibody in the serum that coats the bacteria while the PMNs possess receptors for the same.

ii) ***C3b opsonin***is the fragment produce by activation of complement pathway. It is strongly chemotactic for attracting PMNs to bacteria.

iii) *Lectins* are carbohydrate-binding proteins in the plasma which bind to bacterial cell wall.

- **Engulfment**

The opsonised particle bound to the surface of phagocyte is equipped to be engulfed. This is accomplished by development of cytoplasmic pseudopods around the particle due to activation of actin filaments under cell wall, enveloping it in a phagocytic vacuole. Eventually, the plasma membrane enclosing the particle breaks from the cell surface so that membrane lined phagocytic vacuole or phagosome lies internalised and free in the cell cytoplasm. The phagosome fuses with one or more lysosomes of the cell and form larger vacuole called phagolysosome.

- **Killing and degradation**

It is the stage of killing and degradation of microorganism to dispose it off justifying the function of phagocytes as scavenger cells. The microorganisms after being killed by antibacterial substances are degraded by hydrolytic enzymes. However, this mechanism fails to kill and degrade some bacteria like tubercle bacilli.

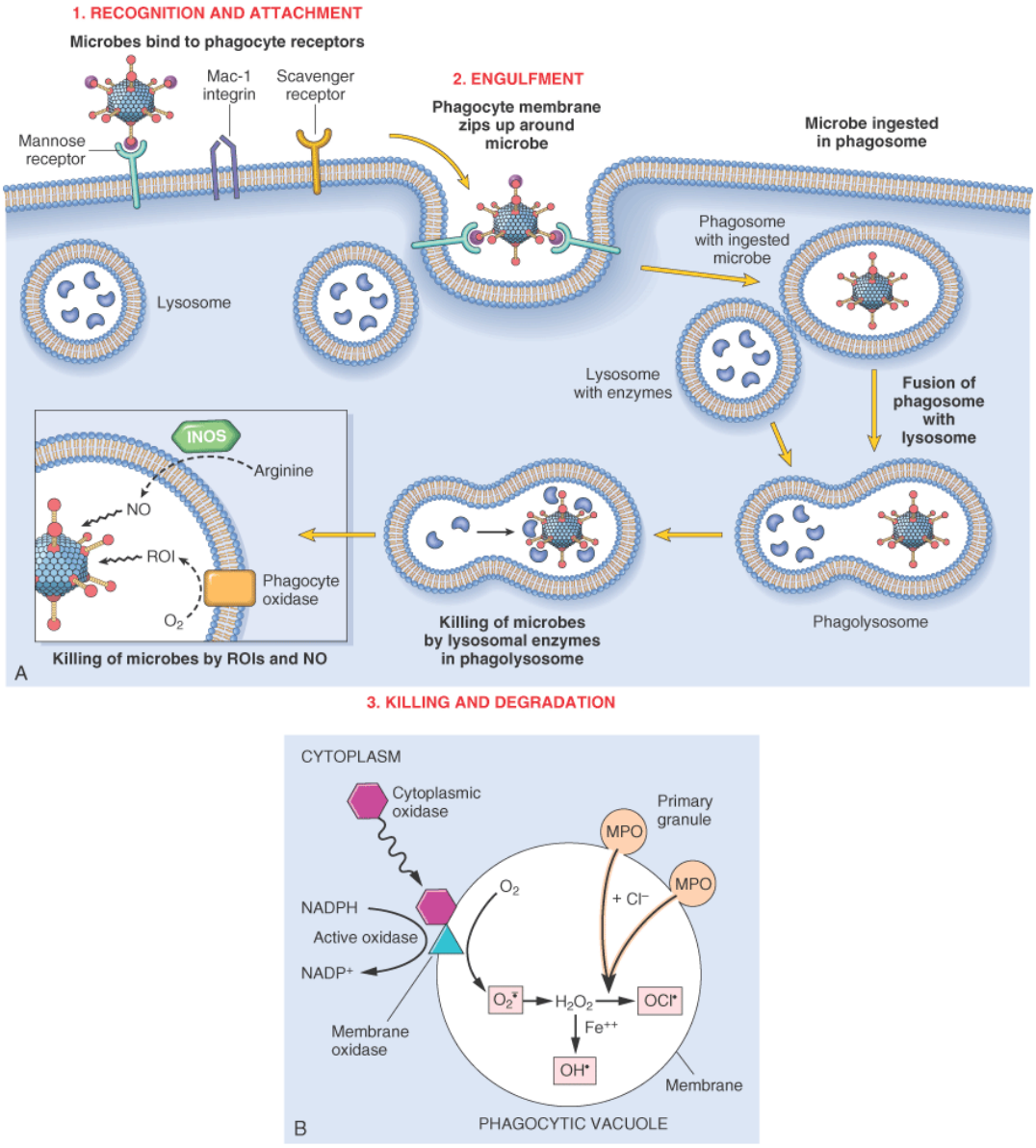


Figure 1.1 Phagocytosis

There are intracellular metabolic pathways which generally killmicrobes by oxidative mechanism and less often nonoxidativepathways. They include

i) Oxidative bactericidal mechanism by oxygen free radicals

a) MPO-dependent

b) MPO-independent

ii) Oxidative bactericidal mechanism by lysosomal granules.

iii) Non oxidative bactericidal mechanism

B) Extracellular mechanisms.

1.3.1.3 Systemic effects of acute inflammation

The account of acute inflammation given up to now above is based on local tissue responses. However, acute inflammation is associated with systemic effects also. These include fever, leucocytosis and lymphangitis-lymphadenitis

- **Fever** occurs due to bacteraemia. It ismediated through release of factors like prostaglandins, interleukin-1 and TNF-α in response to infection[9].
- **Leucocytosis** commonly follows the acute inflammatory reactions, usually in the range of 15,000- 20,000/µl. When the counts are higher than this with 'shift to left' of myeloid cells, the blood picture is defined as leukaemoid reaction.
- **Lymphangitis-lymphadenitis**

The lymphatics and lymph nodes that drain the inflamed tissue show reactive inflammatory changes in the form of lymphangitis and lymphadenitis.

- **Shock**

Enormous release of cytokine TNF-α, a mediator of inflammation, in response to severe tissue injury or infection results in profuse systemic vasodilatation, amplified vascular permeability and intravascular volume loss. The net effect of these alterations is hypotension and shock.

1.3.1.4 Morphology of acute inflammation

- **Pseudomembranous inflammation.** It is inflammatory response of mucous surface (oral, respiratory, bowel) to toxins of diphtheria or irritant gases. As a result of denudation of epithelium, plasma exudes on the surface where it coagulates, and along with necrosed epithelium, forms false membrane.
- **Ulcer.** In the acute stage, there is infiltration by polymorphs with vasodilatation while long-standing ulcers build up infiltration by lymphocytes, plasma cells and macrophages with associated fibroblastic proliferation and scarring.
- **Suppuration (abscess formation).**When acute bacterial infection is followed by intense neutrophilic infiltrate in the inflamed tissue, it results in tissue necrosis. A cavity is formed which is called an abscess and contains purulent exudate or pus and the process of abscess

formation is known as suppuration. Pyogenic bacteria are the bacteria which cause suppuration.

- **Cellulitis.** It is a diffuse inflammation of soft tissues resulting from distribution effects of substances like hyaluronidase released by some bacteria.
- **Bacterial infection of the blood.** This includes the following 3 conditions:
  - Bacteraemia
  - Septicaemia
  - pyaemia

1.3.1.5 Basic laboratory parameters in acute inflammation.

- **Acute phase reactants.**The increase in C reactive protein level is consistent feature in inflammation.
- **Raised erythrocyte sedimentation rate.** The raised reactive proteins cause increased clustering of red cells of rouleaux formation that determines ESR. This is prominent in 36-48 hours
- **Leukocytosis.** The total WBC count goes up from the normal of 4500-10000 percmm to an average of about 15000 but may go as high as 30000.[10]

1.3.1.6 Pathological outcomes acute inflammation

- **Resolution.** It means complete return to normal tissue following acute inflammation. This occurs when tissue changes are slight and the cellular changes are reversible e.g. Resolution in lobar pneumonia.
- **Healing.** Healing by fibrosis takes place when the tissuedamagein acute inflammation is extensive so that there is no tissue regeneration. When tissue loss is superficial, it is restored by regeneration.
- **Suppuration.** When the pyogenic bacteria causing acute inflammation turn out in severe tissue necrosis, the processleadsto suppuration. Initially, there is intense neutrophilicinfiltration. Subsequently, mixture of neutrophils, bacteria, fragments of necrotic tissue, cell debris and fibrin comprise pus which is contained in a cavity to form an abscess. The abscess, if not drained, may get structured by Dense fibrous tissue, and in time, get calcified.

- **Chronic inflammation.** Persisting or recurrent acute inflammation may progress to chronic inflammation in whichthe processes of inflammation and healing proceed side by Side.

1.3.2 Chronic inflammation

Chronic inflammation is inflammation of prolonged duration(weeks to months to years), in which active inflammation, tissue injury, and healing proceed simultaneously. In contrast to acute inflammation,which is characterised by vascular changes, edema and a predominantly neutrophilic infilterate, chronic inflammation is distinguished by

- Infiltration with mononuclear cells, including macrophages,lymohocytes and plasma cells
- Tissue destruction, largely induced by the products of the inflammatory cells.
- Repair, involvingnew vessel proliferation (angiogenesis) and fibrosis.

Acute inflammation may progress to chronic inflammation. This change occurs when the acute response cannot be resolved, either because of the persistence of the inflammatory agent or because of the intervention with the normal process of healing.

1.3.2.1 Types of chronic inflammation

- **Non-specific,** when the irritant substance give result to a nonspecific chronic inflammatory reaction with formation of granulation tissue and healing by fibrosis e.g. chronic osteomyelitis, chronic ulcer.
- **Specific,** when the injurious agent causes a characteristic histologic tissue response e.g. tuberculosis, leprosy, syphilis.

According to histopathological changes chronic inflammation can be classified as:

- **Chronic non-specific inflammation.** It is characterised by non-specific inflammatory cell infiltration e.g. chronic osteomyelitis, lung abscess. A alternative of this type of chronic inflammatory response is chronic suppurative inflammation in which infiltration by polymorphs and abscess formation are additional features e.g. actinomycosis.
- **Chronic granulomatous inflammation.** It is characterised by formation of granulomas e.g. tuberculosis, leprosy, syphilis, actinomycosis, sarcoidosis etc.

1.3.2.2 Granulomatous inflammation.

It is a distinctive pattern of chronic inflammation characterised by the aggregates of activated macrophages that assume an epithelioid appearance. [11]The causes of or conditions in which granuloma develops are

a. Bacterial infections. (Mycobacteria:leprosy, Tuberculosis; Spirochaete: syphilis)
b. Fungal infections (Coccidomycosis, Histoplasmosis, Cryptococcosis)
c. Parasites (ova of schistosomiasis)
d. Foreign bodies(minerals, dust,I nsect stings, ruptured cyst in tissues etc)
e. Immune conditions(crohn's disease, thyroiditiss, Aschoff body of rheumatic heart disease)

1.3.2.2.1 Pathogenesis of granuloma

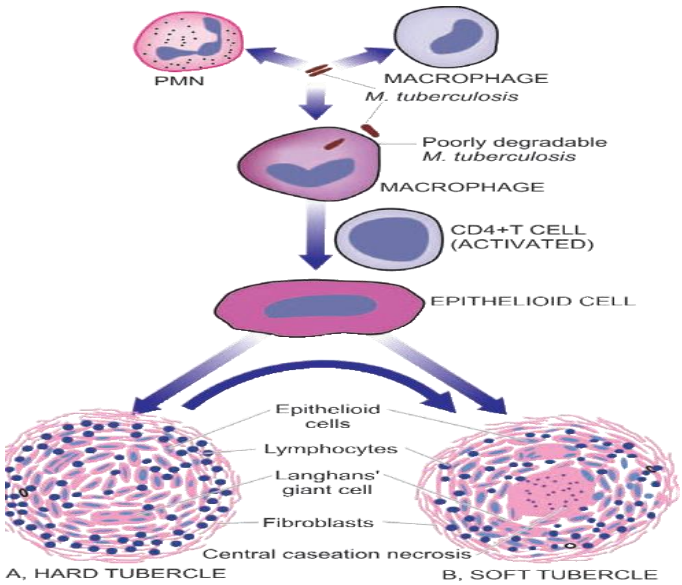


Figure 1.2 Pathogenesis of granuloma

1.3.2.2.2 Composition of granuloma

1. **Epithelioid cells**. It is one of the modified histocyte and are weakly pathogenic.
2. **Multinucleate giant cells.** They are formed by fusion of adjacent epithelioid cells and may have 20 or more nuclei. These nuclei may be arranged at the periphery like

horseshoe or ring, or are clustered at the two poles (Langhans' giant cells), or they may be present centrally (foreign body giant cells).

3. **Lymphoid cells**
4. **Necrosis**
5. **Fibrosis.** It is a feature of healing by proliferatingfibroblasts at the periphery of granuloma.

1.3.2.2.3 Morphological features of foreign body granulomas.

Such granulomas may develop in any organ or skin or mucous membrane where a particulate foreign body may enter. Visible small nodules may form. It differ from a tubercle in that the macrophages are smaller not typically epithelioid and the multinucleated giant cells too are not of the Langhan's typebut of the foreign body type. The reaction is not organised in compact tubercles,but more in focal clusters of macrophages and few to many foreign body type giant cell. There is no caseation, there is considerable vascularisation and much heavier lymphocyte and plasma cell response around. Depending on the foreign body, neutrophils may also be associated with it.

1.3.2.3 Systemic effects of chronic inflammation

1. Fever. Invariably there is mild fever, often with loss of weight and weakness.

2. Anaemia. chronic inflammation is accompanied by anaemia of varying degree.

3. Leucocytosis. As in acute inflammation, chronic inflammation also has leucocytosis but generally there isrelative lymphocytosis in these cases.

4. ESR. ESR is elevated in all cases of chronic inflammation.

5. Amyloidosis. Long-term cases of chronic suppurative inflammation may cause secondary systemic (AA) amyloidosis.

1.4 Mediators of inflammation

Biochemical mediators released during inflammation strengthen and broadcast the inflammatory response. These mediators are soluble, diffusible molecules that can act locally and systemically. They are classified as

1.4.1.Cell derived mediators

Cell derived mediators are derived from injured tissue cells or leukocytes recruited to the site of inflammation. Mast cells, platelets, and basophils produce the vasoactive amines serotonin and histamine.

Histamine causes arteriolar dilation, increased capillary permeability, contraction of nonvascular smooth muscle, and eosinophil chemotaxis and can stimulate nociceptors responsible for the pain response. Its release is stimulated by the complement components C3a and C5a and by lysosomal proteins released from neutrophils. Histamine activity is mediated through the activation of one of four specific histamine receptors, designated H_1, H_2, H_3, or H_4, in target cells. Most histamine-induced vascular effects are mediated by H_1 receptors. H_2 receptors mediate some vascular effects but are more important for their role in histamine-induced gastric secretion. Less is understood about the role of H_3 receptors, which may be localized to the CNS. H_4 receptors are located on cells of hematopoietic origin, and H_4 antagonists are promising drug candidates to treat inflammatory conditions involving mast cells and eosinophils (allergic conditions).

Serotonin (5-hydroxytryptamine) is a vasoactive mediator similar to histamine found in mast cells and platelets in the GI tract and CNS. Serotonin also increases vascular permeability, dilates capillaries, and causes contraction of nonvascular smooth muscle. In some species, including rodents and domestic ruminants, serotonin may be the predominant vasoactive amine.

Cytokines, including interleukins 1–10, tumor necrosis factor α (TNF-α), and interferon γ (INF-γ) are produced mainly by macrophages and lymphocytes but can be synthesized by other cell types also. These polypeptides alter the activity and function of other cells to coordinate and control the inflammatory response. Two of the more important cytokines, interleukin-1 (IL-1) and TNF-α, mobilize and activate leukocytes, enhance proliferation of B and T cells and natural killer cell cytotoxicity, and are involved in the biologic response to endotoxins. IL-1, IL-6, and TNF-α mediate the acute phase response and pyrexia that may accompany infection and can induce systemic clinical signs, including sleep and anorexia. In the acute phase response, interleukins stimulate the liver to synthesize acute-phase proteins, including complement

components, coagulation factors, protease inhibitors, and metal-binding proteins. By increasing intracellular Ca^{2+} concentrations in leukocytes, cytokines are also important in the induction of PLA_2. Colony-stimulating factors (GM-CSF, G-CSF, and M-CSF) are cytokines that promote expasnsion of neutrophil, eosinophil, and macrophage colonies in bone marrow. In chronic inflammation, cytokines IL-1, IL-6, and TNF-α contribute to the activation of fibroblasts and osteoblasts and to the release of enzymes such as collagenase and stromelysin that can cause cartilage and bone resorption.[12]

Lipid-derived autacoids play vital roles in the inflammatory response and are a main focus of research into novel anti-inflammatory drugs. These compounds include the eicosanoids such as prostaglandins, prostacyclin, leukotrienes, and thromboxane A and the modified phospholipids such as platelet activating factor (PAF). Eicosanoids are synthesized from 20-carbon polyunsaturated fatty acids by many cells, including activated leukocytes, mast cells, and platelets and are therefore widely distributed. Hormones and other inflammatory mediators (TNF-α, bradykinin) stimulate eicosanoid production either by direct activation of PLA_2, or indirectly by increasing intracellular Ca^{2+}concentrations, which in turn activate the enzyme. Cell membrane damage can also cause an increase in intracellular Ca^{2+}. Activated PLA_2 directly hydrolyzes AA, which is rapidly metabolized via one of two enzyme pathways—the cyclooxygenase (COX) pathway leading to the formation of prostaglandin and thromboxanes, or the 5-lipoxygenase (5-LOX) pathway that produces the leukotrienes.

Cyclooxygenase catalyzes the oxygenation of AA to form the cyclic endoperoxide PGG_2, which is converted to the closely related PGH_2. Both PGG_2 and PGH_2 are inherently unstable and rapidly converted to various prostaglandins, thromboxane A_2 (TXA_2), and prostacyclin (PGI_1). PGE_1, PGE_2, and PGI_1 are potent arteriolar dilators and augment the effects of other mediators by increasing small vein permeability. Other prostaglandins, including $PGF_{2\alpha}$ and thromboxane, cause smooth muscle contraction and vasoconstriction. Prostaglandins sensitize nociceptors to pain-provoking mediators such as bradykinin and histamine and, in high concentrations, can directly stimulate sensory nerve endings. TXA_2 is a potent platelet-aggregating agent involved in thrombus formation.

Found predominately in platelets, leukocytes, and the lungs, 5-LOX catalyzes the formation of unstable hydroxyperoxides from AA. These hydroxyperoxides are subsequently converted to the peptide **leukotrienes**. Leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetranoate (5-HETE) are strong chemoattractants stimulating polymorphonuclear leukocyte movement. LTB_4 also stimulates the production of cytokines in neutrophils, monocytes, and eosinophils and enhances the expression of C3b receptors. Other leukotrienes facilitate the release of histamine and other autacoids from mast cells and stimulate bronchiolar constriction and mucous secretion. In some species, leukotrienes C_4 and D_4 are more potent than histamine in contracting bronchial smooth muscle.[13][14]

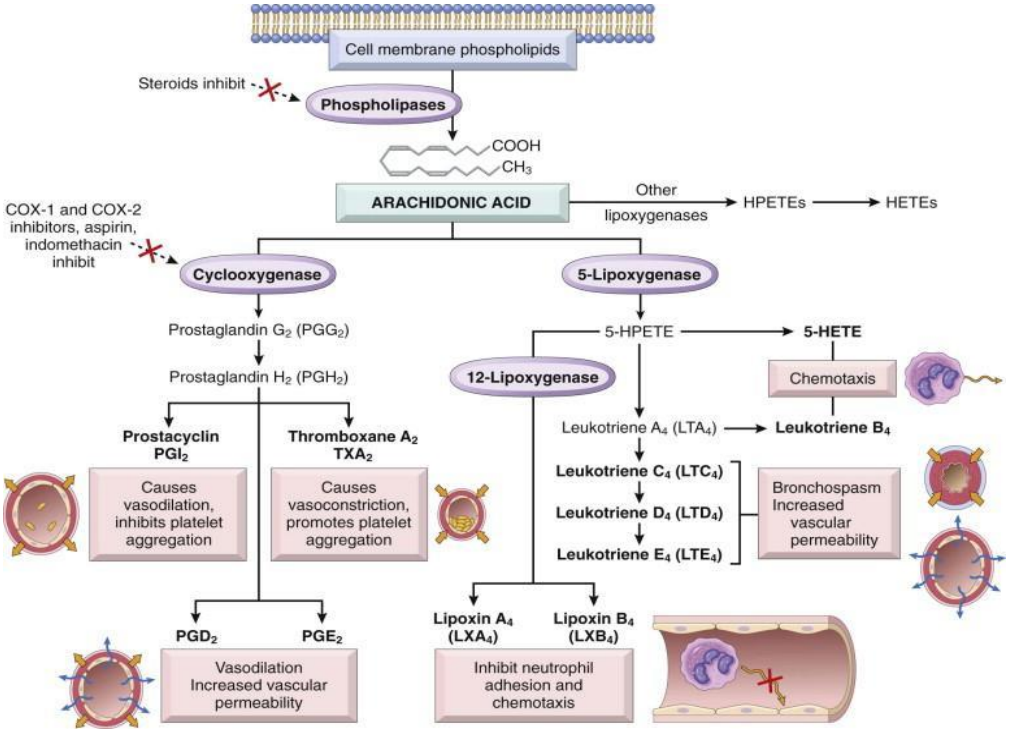


Figure 1.3 Generation of arachidonic acid metabolites and their roles in inflammation

Platelet activating factor (PAF) is also derived from cell membrane phospholipids by the action of PLA_2. PAF, synthesized by mast cells, platelets, neutrophils, and eosinophils, induces platelet aggregation and stimulates platelets to release vasoactive amines and synthesize thromboxanes. PAF also increases vascular permeability and causes neutrophils to aggregate and degranulate.

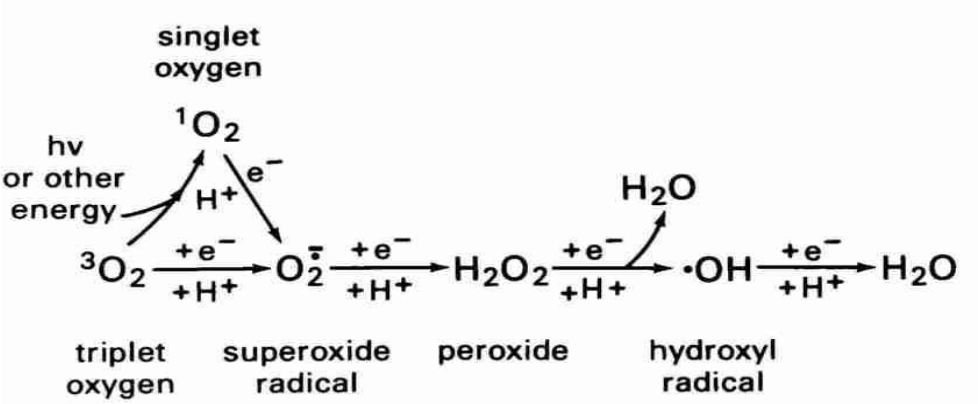
The role of free radical in inflammation is inevitable. A free radical is any species capable of independent existence containing one or more unpaired electrons. The unpaired electron alter the chemical reactivity of the molecule/atom, making it more reactive.[15][16] .

The oxygen free radicals include;

- superoxide anion radical (O_2·-),
- singlet oxygen (O_2),
- hydroxyl radical (·OH) and
- perhydroxyl radical (HO_2·)

These all are together known as "reactive oxygen species" (ROS). With a single electron reduction several free radicals and hydrogen peroxide (H_2O_2) are formed. Reactive oxygen species are generated by oxidant enzymes, phagocytic cells, ionizing radiation etc.[11]

Superoxide anion is the first radical formed, by the electron transport chain when O_2 picks up a single electron. Radicals such as ·OH, HO_2· and H_2O_2 are formed from $O_2^{\cdot-}$. $O_2^{\cdot-}$ undergoes a dismutation reaction catalysed by the enzyme superoxide dismutase (SOD) to form H_2O_2, which by itself is not reactive enough to cause damage to macromolecules.



Among the reactive oxygen species, ·OH is the most potent destructive radical which can react with all biological macromolecules (lipids, proteins, nucleic acids and carbohydrates). It is extremely reactive and can lead to formation of DNA-protein cross-links, single and double-strand breaks, base damage, lipid peroxidation and protein fragmentation . It may also be produced by ionizing radiation .

$$H_2O \rightarrow H_2O^{\cdot+} + e^-$$

$$H_2O + H_2O^{\cdot+} \rightarrow H_3O^+ + {}^{\cdot}OH$$

The cellular generation of ·OH may occur in two steps :

(i) Reduction of H_2O_2 by the Fenton reaction:

$$Fe_2^{+} + H_2O_2 \rightarrow \cdot OH + OH^{-} + Fe_3^{+}$$

$$Cu^{+} + H_2O_2 \rightarrow Cu_2^{+} + {}^{\cdot}OH + OH^{-}$$

(ii) Interaction of $O_2^{\cdot -}$ with H_2O_2 by the Haber–Weiss reaction:

$$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + H_2O + {}^{\cdot}OH$$

The oxygen-derived free radicals have the following role in inflammation;

- Endothelial cell damage and increased vascular permeability.
- Activation of protease and inactivation of antiprotease which caude tissue matrix damage.
- Damage to other cells.

Increased generation of free radicals and reactive oxygen species leads to several pathological conditions like rheumatoid arthritis, myocardial infarction, malignancy, aging, alzheimer's disease, cancer etc.[17]

The role of the free radical gas **nitric oxide** (NO) in inflammation is well established. NO is an important cell-signaling messenger in a wide range of physiologic and pathophysiologic processes. Small amounts of NO play a major role in maintaining resting vascular tone, vasodilation, and antiaggregation of platelets. In response to certain cytokines (TNF-α, IL-1) and other inflammatory mediators, the production of relatively high quantities of NO is stimulated. In larger quantity, NO is a potent vasodilator, facilitates macrophage-induced cytotoxicity, and may contribute to joint destruction in some types of arthritis.[18][19]

1.4.2.Plasma derived mediators.

These include the various products derived from activation and interaction of 4 interlinked systems: kinin, clotting, fibrinolytic and complement. Hageman factor (factor XII) of clotting system plays a key role in interactions of the four systems. Activation of factor XII *in vivo* by contact with basement membrane and bacterial endotoxins, and *in vitro* with glass or kaolin, leads to activation of clotting, fibrinolytic and kinin systems. In inflammation, activation of factor XII is brought about by contact of the factor leaking through the endothelial gaps. The end-products of the activated clotting, fibrinolytic and kinin systems activate the complement system that generate permeability factors. These permeability factors, in turn,further activate clotting system.[20]

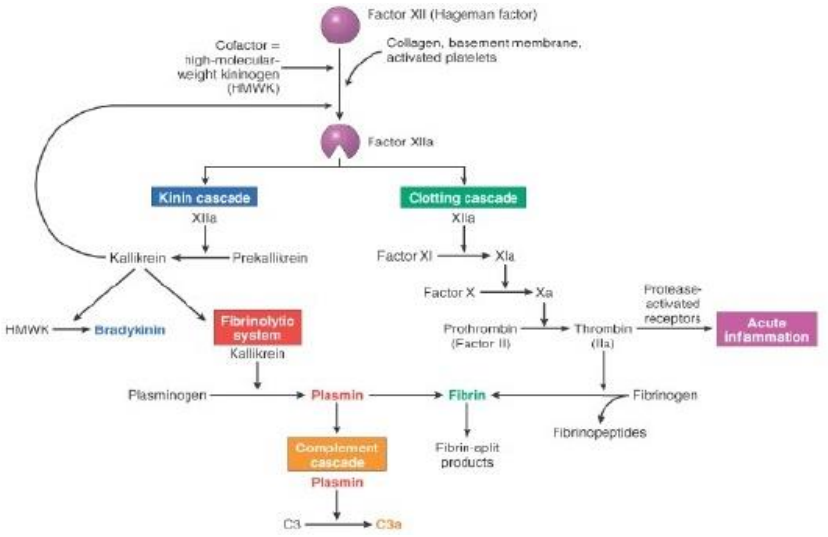


Figure 1.4Interrelationships between the four plasma mediator systems triggered by activation of factor XI

1.4.3 Inflammatory cells

The cells involved in acute and chronic inflammation are circulating leukocytes, plasma cells and tissue macrophages.

1.4.3.1 Circulating leukocytes

a.**Polymorphonuclear neutrophils** are acute inflammatory cells, which are involved in initial phagocytosis of bacteria and foreign bodies.

b. **Monocytes** are chronic inflammatory cells which are involved in bacterial phagocytosis and regulates lymphocyte response.

c. **Lymphocytes** are chronic inflammatory cells which are involved in humoral and cell mediated immune responses and regulate macrophage responses..

d. **Eosinophils** are chronic inflammatory cells which are involved during allergic states and parasitic infestations.

e**. Basophils** containing electron dense molecules andfunctions as receptor for IgE molecules

1.4.3.2 Plasma cells

These cells are bigger than lymphocytes with more abundant cytoplasm and an eccentric nucleus which has cart-wheel pattern of chromatin. They develop from Blymphocytes and are rich in RNA and Ɣ-globulin in theircytoplasm. These cells are active in antibody synthesis.

1.4.3.3 Mononuclear-Phagocyte System (Reticuloendothelial System)

This cell system includes cells derived from 2 sources with common morphology, function and origin. These are as under:

a.Blood monocytes. These comprise 4-8% of circulating leucocytes.

b. Tissue macrophages. These include the following cells in different tissues:

i) Macrophages in inflammation.

ii) Histiocytes which are macrophages present in connective tissues.

iii) Kupffer cells are macrophages of liver cells.

iv) Alveolar macrophages (type II pneumocytes) in lungs.

v) Macrophages/histiocytes of the bone marrow.

vi) Tingible body cells of germinal centres of lymph nodes.

vii) Littoral cells of splenic sinusoids.

viii) Osteoclasts in the bones.

ix) Microglial cells of the brain.

x) Langerhans' cells/dendritic histiocytes of the skin.

xi) Hoffbauer cells of the placenta.

xii) Mesangial cells of glomerulus.

The mononuclear phagocytes are the scavenger cells of the body as well as participate in immune system of the body; their functions in inflammation are as under:

i) Phagocytosis (cell eating) and pinocytosis (cell drinking).

ii) Macrophages on activation by lymphokines released by T lymphocytes or by non immunologic stimuli elaborate a variety of biologically active substances like proteases, plasminogen activator,products of complement,coagulation factors etc.

1.4.3.4 Giant Cells

A few examples of multinucleate giant cells exist in normal tissues (e.g. osteoclasts in the bones, trophoblasts in placenta, megakaryocytes in the bone marrow). However, in chronic

inflammation when the macrophages fail to deal with particles to be removed, they fuse together and form multinucleated giant cells. Besides, morphologically distinct giant cells appear in some tumours also. Some of the common types of giant cells are

i) Foreign body giant cells.

ii) Langhans' giant cells..

iii) Touton giant cells..

iv) Aschoff giant cells.[21][22]

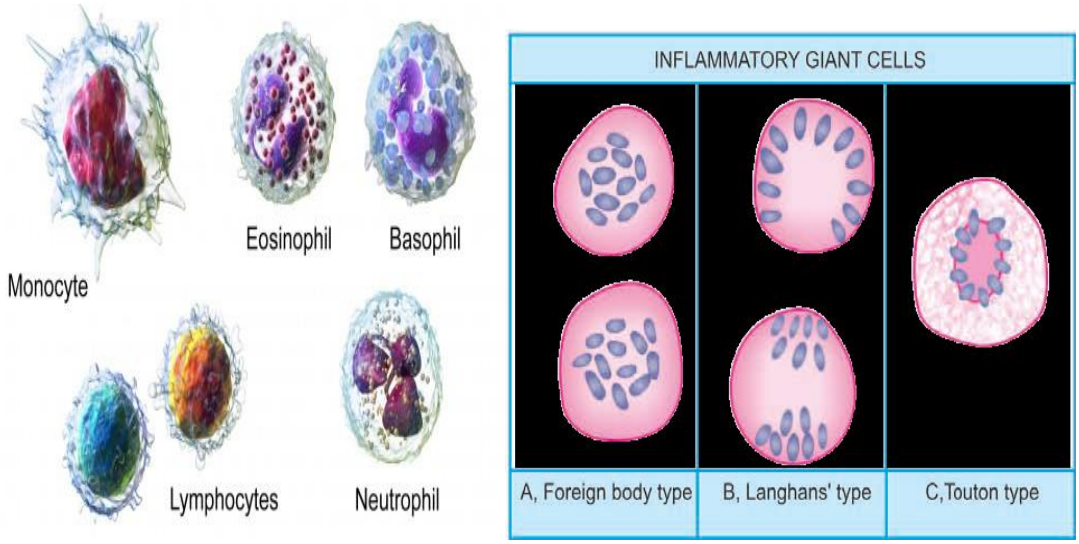


Figure 1.5 Inflammatory cells

1.5 Pain

Pain is a multidimensional occurrence that is essential for the maintenance and preservation of an individual. It warns of the danger of bodily harm and alerts to trauma and injury. Pain is a specific enteroceptive sensation; it can be perceived as arising from a particular portion of the body, its temporal properties can be detailed, it can be differentiated qualitatively (for example, as stinging, pricking, burning, throbbing, dull or aching), and it involves dedicated subsets of peripheral and central neurons. The experience of pain has a distinctly unpleasant character, that is, an affective or motivational aspect that can be distinguished from its discriminative sensory aspects and from the long-term emotional experience of 'suffering'. The unpleasantness ranges in intensity from the discomfort of a cold room, fatigued muscles or colonic tension to the excruciating agony of a severe burn, toothache, gallstone or migraine.

Under normal circumstances, primary afferent pain fibres activate particular central pathways that engage protective mechanisms at many functional levels: autonomic, homeostatic, motor, behavioural and mnemonic. However, injury or disease can change the balance of this system and result in persistent, pathological pain. Analgesic substances, such as aspirin and morphine, which interact with the transmitters and modulators of the pain system are helpful for many people with pain, but there is a great need for the development of better methods for the lessening and control of both acute (immediate) and chronic (long-term, pathological) pain.[23]

During the process of inflammation, a mixture of inflammatory mediators such as peptides (bradykinin), lipids (prostaglandins), neurotransmitters (ATP), protons and neurotrophins (NGF) are released.The release of neuropeptides from peripheral neurones supports plasma extravasation of further mediators and chemoattraction of inflammatory cells, a process called as "neurogenic inflammation". The sensitisation of the primary afferent neurones by local inflammatory mediators can occur by a direct action on the sensory neurones, or it can involve an indirect action on non-neuronal cells, especially but not exclusively on immune cells, from which further inflammatory mediators can then be released.[24]

1.5.1 Pain pathways

Nociceptors, or pain receptors, are free nerve endings that respond to painful stimuli which transmit information to the brain. Pain perception occurs when electrical, thermal, mechanical, biological and chemical stimuli are transmitted to the spinal cord and then to the central areas of the brain. Pain impulses travel to the dorsal horn of the spine, where they synapse with dorsal horn neurons in the substantia gelatinosa and then ascend to the brain. The basic sensation of pain occurs at the thalamus. It continues to the limbic system and the cerebral cortex, where pain is perceived and interpreted.[25]

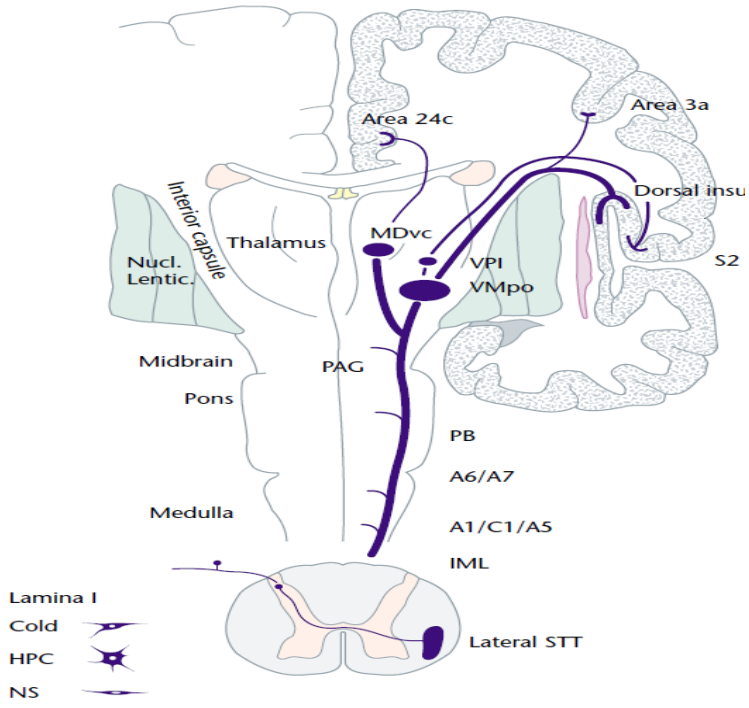


Figure 1.6 Pain pathway

1.5.2 Pain theories:

- Specificity theory.
- Pattern theory.
- Gate control theory.
- Anodal blocking theory and,
- Central inhibition theory.

1) Specificity Theory:

It proposes that a mosaic of specific pain receptors in body tissue projects to a main center in the brain. It maintain that free nerve endings are pain receptors which generate pain impulses that are carried by A-delta and C-fibers in peripheral nerves and by the lateral spinothalamic tract in the spinal cord to a pain center in the thalamus."Pain receptor" implies that stimulation of one type of receptor elicits a single psychological or physiological response.

2) Pattern Theory:

According to this theory, pain is the result of the stimulation of certain nerve impulses that form a pattern and are then brought together and deserted into the spinal cord as a lump sum of pain, a process called "central summation."

3) Gate Control Theory:

Melzack and Wall published this theory in 1965. According to this theory, a mechanism in the brain acts as a gate to increase or decrease the flow of nerve impulses from the peripheral fibers to the Central nervous system. An "open" gate allows the flow of nerve impulses, and the brain can perceive pain. A "closed" gate does not allow flow of nerve impulses, decreasing the perception of pain.

4) Anodal Blocking Theory:

This theory proposes maintaining the absolute refractory period in the nerve by rapidly and repeatedly stimulating the nerve fiber, thus preventing pain impulses from being transmitted.

5) Central Inhibition Theory:

It suggests that large-fiber and small-fiber activity do not result in presynaptic effects of opposite polarity as suggested by the gate control theory. The concept of inhibitory balance depends on minor inhibitory feedback from small fiber input that activates the nociceptive marginal neuron and major inhibitory feedback to the marginal neuron from large non-nociceptive fibers. Modulation of pain producing input thus depends on the balance between large fiber and small fiber activity via a postsynaptic inhibitory mechanism acting on the nociceptive relay neurons.[26]

1.6 Anti-inflammatory drugs and analgesics

Anti-inflammatory drugs are the drugs which are used for the treatment of inflammation. They are mainly used in infections like rheumatoid arthritis and gout . Analgesic is a drug that selectively relieves pain by acting in the CNS or on peripheral pain mechanisms, without significantly altering consciousness.

Analgesics are divided into two groups;

- Opioid / Narcotic / Morphine like analgesics

- ❖ Nonopioid / Non-narcotic /Antipyretic or anti-inflammatory analgesics.

1.6.1 Narcotic analgesics:

1.6.1.1 Classification:

I) Narcotic Agonist Analgesics

i) Natural opium alkaloids: Morphine, codeine

ii) Semi synthetic opiates: Diacetylmorphine, Pholcodeine, Ethylmorphine

iii) Synthetic opioids: Pethidine, Fentanyl, Methadone, Tramadol

II) Narcotic Agonist-Antagonist Analgesics

i) Phenanthrenes: Buprenorphine, Nalbuphine

ii) Morphinan: Butorphanol

iii) Benzomorphans: Phenazocine , Pentazocine

1.6.1.2 General mechanism Of Action Of Narcotic Analgesics:

It exerts its effects by interacting with opioid receptors (μ, κ and δ) located on prejunctional neurons. Activation of opioid receptor reduces intracellular cAMP formation and opens potasium channels or suppresses voltage gated N type Calcium channels. These actions leads to neuronal hyperpolarisation and reduced availability Of intracellular calcium, decreased neurotransmitter release by CNS and myenteric neurons.[27]

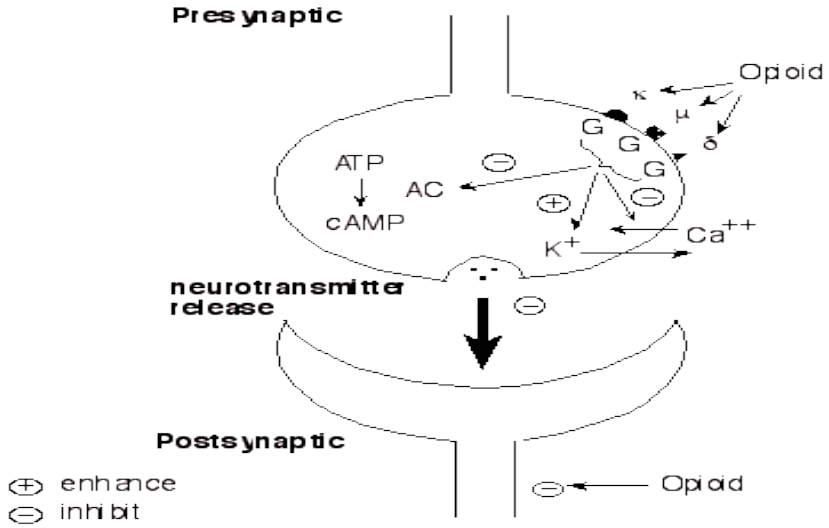


Figure 1.7 Opioid receptor transducer mechanism

1.6.1.3Adverse Effects associated with opiods.

The various adverse effects associated with opioids include respiratory depression, drowsiness, nausea, vomiting, endocrine disturbances, tolerance to analgesic effect, physical dependence, abuse potential and interaction with CNS depressant drugs. [28]

1.6.2 Nonsteroidal Anti-inflammatory Drugs and Antipyretic-Analgesics :

1.6.2.1 Classification:

A) Nonselective COX inhibitor

1. Salicylates: Aspirin.
2. Propionic acid derivatives: Ibuprofen , Ketoprofen.
3. Fenamates: Mephenamic acid.
4. Oxicams: Piroxicam, Tenoxicam.
5. Pyrazolones: Phenylbutazone, Oxyphenbutazone.
6. Indole derivatives: Indomethacin, Sulindac.
7. Aryl-acetic acid derivatives: Diclofenac.
8. Pyyrrolo-pyrrole derivatives: Ketorolac.

B) Preferential COX-2 Inhibitors: Nimesulide.

C) Selective COX-2 Inhibitors: Celecoxib, Etoricoxib.

D) Analgesic-antipyretics with poor anti-inflammatory action.

1. Paraaminophenol derivative: Paracetamol.
2. Pyrazolone derivatives: Metamizol, Propiphenazone.
3. Benzoxazocine derivative: Nefopam.

1.6.2.2 General mechanism Of Action Of NSAIDs:

Nonsteroidal anti-inflammatory drugs exert their anti-inflammatory effect through inhibition of cyclooxygenase, which is the enzyme catalyzing the transformation of arachidonic acid to prostaglandins and thromboxanes.NSAIDs also inhibit expression of cell adhesion molecules and directly inhibit activation and functions of neutrophils.[29]

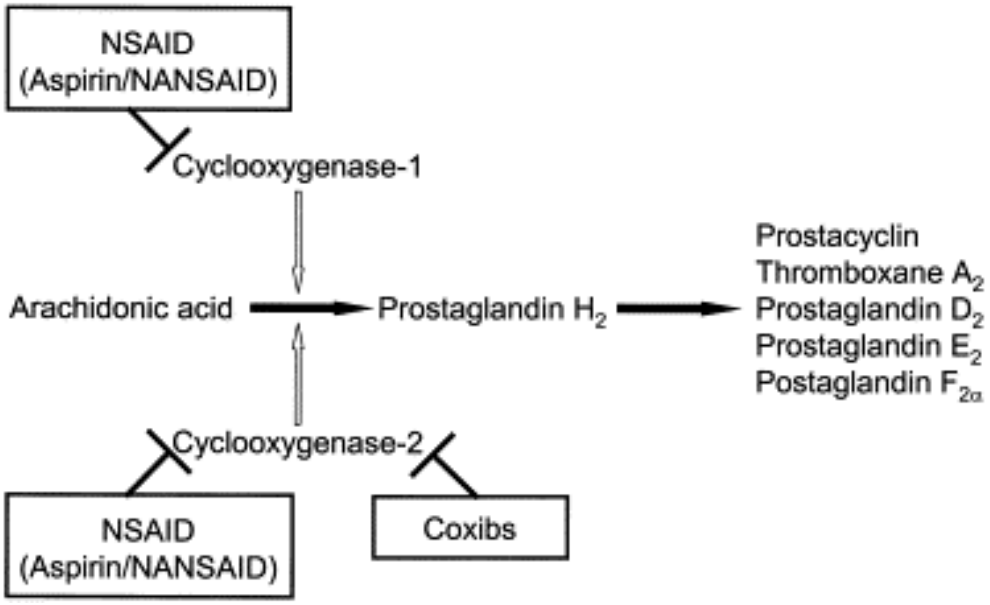


Figure 1.8 : Mechanism of action of NSAIDs.

1.6.2.3 Adverse effects of NSAID Therapy

NSAIDs produces various adverse effects as it interfere with various pathways that maintain homeostasis.The manifestations are as follows[30]

Table 1.1 Adverse drug reactions of NSAIDs

SYSTEM	MANIFESTATIONS
GI	Abdominal pain, nausea, anorexia, gastric erosions, anemia, GI hemorrhage, perforation, diarrhea
Renal	Salt and water retention, hyperkalemia, Decreased effectiveness of diuretic and antihypertensive medications
CNS	Vertigo, dizziness, lowering of seizure threshold ,hyperventilation, depression.
Platelets	Inhibited platelet activation
Uterus	prolongation of gestation and possible prolongation of labor
Hypersensitivity	Vasomotor rhinitis, angioedema, asthma, urticaria, flushing, hypotension, shock
Vascular	Closure of ductus arteriosus

1.7 Cyclo oxygenase as a drug target

Three isoforms of COXs have been identified. Cyclooxygenase-1 (COX-1) is a glycoprotein of 71kDa, which is constitutively expressed in different tissues. COX-1 is encoded by a gene on chromosome 9 and plays a role in tissue homeostasis by modulating several cellular processes ranging from cell proliferation to angiogenesis or platelet aggregation due to thromboxane production . Cyclooxygenase-2 (COX-2) is the inducible isoform, which is regulated by growth factors and different cytokines such as IL1β, IL6, or TNFα , therefore overexpressed during inflammation. The COX-2 gene is located on chromosome 1 and its promoter displays an NFκB response element as well as other cytokine-dependent (i.e., IL6) response elements . The protein shows a 60% homology with COX-1 [31]; in addition, COX-2 presents a C-terminal extension and a different binding site for NSAIDs, which makes COX-2 a preferential target compared to COX-1, thus being specifically inhibited at lower doses . Finally, COX-3 has been identified as a splice variant of COX-1, and it is present mainly in brain and spinal cord . Currently, the role of COX-3 is not known. Some pieces of evidence suggest a possible role in pain sensitivity, based on studies focused on the mechanism of action of acetaminophen (paracetamol), recently evoked as a selective inhibitor of COX-3[32].

The COX molecule consists of three independent folding units: an epidermal growth factor-like domain, a membrane binding site, and an enzymatic domain . The active COX site is a hydrophobic channel with a series of amino acids. Aspirin binds irreversibly to serine 580 by acetylation, whereas most other NSAIDs bind sterically and reversibly to Tyrosine 385 or Arginine 120, blocking the COX action resulting in the alleviation of pro-inflammatory ARA metabolites, and in particular, prostaglandin E2(PGE2)[33]. The use of NSAIDs is often limited by side effects emanating from disrupting the levels of these protective COX metabolites. Following the discovery of a second form of COX enzyme (COX-2) ,COX-2 selective inhibitors (coxibs) such as celecoxib and rofecoxib were subsequently developed in an effort to circumvent these problems. Coxibs are effective against inflammation and pain, with comparatively less risk of severe GI toxicity associated with conventional NSAIDs. However, there are safety concerns with coxib use due to an increase in the risk of cardiovascular events associated with the imbalance of the PGI2 and thromboxane (TXA2) metabolite levels . Inhibition of COX-1 decreases platelet-derivedTXA2, an eicosanoid which functions as a vasoconstrictor and facilitates platelet aggregation. Selective inhibition of COX-2 affects the

PGI2/TXA2 ratio to favor TXA2, increasing the risk of mortality from ischemic heart disease.Recently, a metabolomic approach to study the relationship between adverse cardiovascular eventsand the use of rofecoxib suggested that this drug acts, in part,through accumulation of 20-hydroxyeicosatetraenoic acid (20-HETE) which is a potent vasoconstrictor among ARA metabolites. Treatment of inflammation and pain constitutes significant medical needs because more people are prone to these conditions than any other disease state. Thus, there is a growing demand for safer but efficacious NSAIDs or coxibs . Upcoming concepts and approaches forthe treatment of inflammation and pain have moved towards simultaneously targeting multiple enzymes in the ARA cascade through combination therapy and multi target inhibitors such as dual inhibitors with the aim of overcoming the risks in single enzyme or pathway inhibition[34]

1.8 Inflammation and cancer

Inflammatory conditions in selected organs augment the risk of cancer. An inflammatory component is present also in the microenvironment of tumors which are not epidemiologically related to inflammation[35]. Over expression of COX-2 has been detected in a number of tumors, such as colorectal breast as well as pancreatic and lung cancers which can be correlated with a poor prognosis. Moreover, over expression of COX-2 has been reported in hematological cancer models like RAJI (Burkitt's lymphoma) and U937 (acute promonocytic leukemia) as well as in patient's blast cells. Data suggested that COX-2 may play a role in different steps of cancer progression, by increasing proliferation of mutated cells, thus favoring tumor promotion as well as by affecting programmed cell death and affecting the efficacy of anticancer therapies to be, finally, concerned in metastasis formation, for example, by affecting apoptosis induced by loss of cell anchorage (anoikis). Combination of preferential orselective COX-2 inhibitors with anti-cancer agents already used in clinics were tested with the goal to improve the efficiency of anti-cancer protocols.

1.9 *In silico* drug design

Pharmaceutical research has successfully incorporated a wealth of molecular modelling methods, within a variety of drug discovery programs, to study complex biological and chemical systems. The integration of computational and experimental strategies has been of great value in the

identification and development of novel promising compounds. Broadly used in modern drug design, molecular docking methods explore the ligand conformations adopted within the binding sites of macromolecular targets. This approach also estimates the ligand-receptor binding free energy by evaluating critical phenomena involved in theintermolecular recognition process.

Two approaches are used in drug design

1.Structure based drug design

2.Ligand based drug design

1.9.1 Structure Based drug design

If reliable information about the 3-D structure and active sites of the target protein can be obtained from X-ray crystallography, nuclear magnetic resonance, or 3-D structure databases, and integrated into a computer model, compounds binding to the target can be designed. This approach is called “structure-based drug design”. Commonly used techniques in this approach are docking and molecular dynamics simulation. Potent ligands can be found by screening a molecule database with docking software. Molecular dynamics simulation can be useful to determine not only how a molecule interacts with the target protein, but also to determine some other properties of the molecule itself, such as membrane permeability.[36]

1.9.2 Ligand Based drug Design

When the receptor structure is unknown but the ligand structures are known, a ligand based approach is used. An extension of the QSAR approach is used to study the active ligands, also known as pharmacophore based drug design. The pharmacophore refers to an ensemble of steric and electronic features that enables it to exhibit specific biological activity.generally, this method depend on the application of descriptors of molecular structure and properties, including structure or- descriptor- based queries, finger print queries, clustering and partitioning [37].

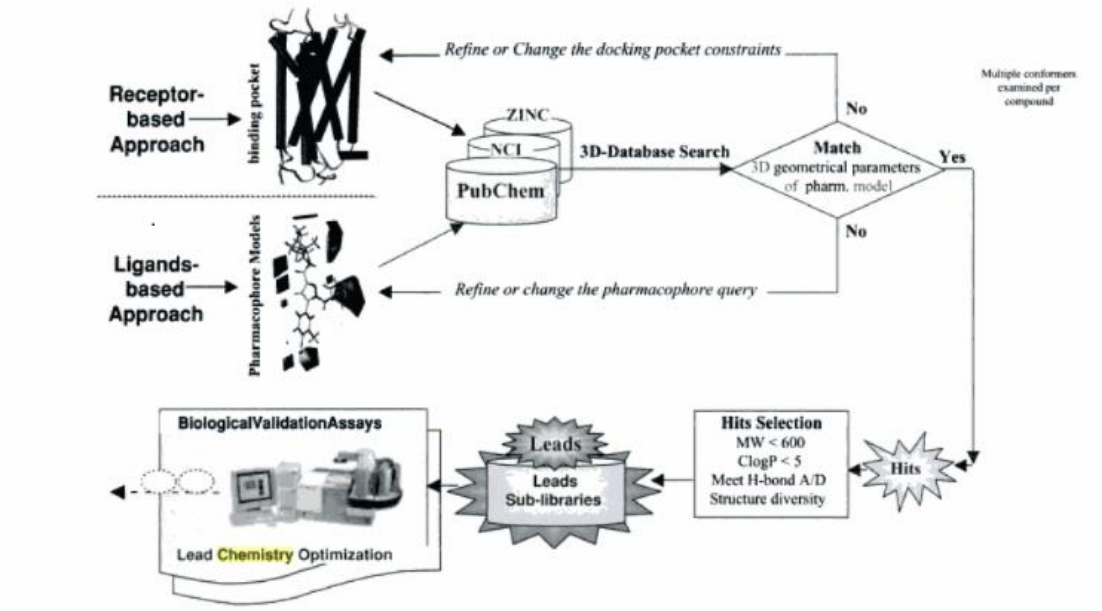


Figure1.9 Workflow of various approaches in *in silico* drug design

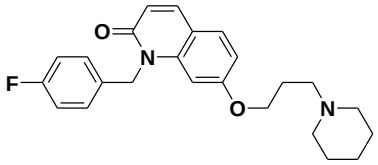
Heterocycles always remain as a potential scaffold with variety of pharmacological significance. Numerous research reports have indicated the coumarin nucleus as a potential candidate for development of anti-inflammatory drugs.[37]As an important class of compounds, quinolin-2-ones are isomeric to 4-quinolones and isosteric to coumarins. The compounds that have quinolin-2-one moiety are associated with biologic activities such as antibacterial, anticancer, antiviral, cardiotonic, and N-methyl-D-aspartate receptor inhibitor functions, among others. Thiazole, oxazoles ,N-substituted piperazines and aminopyridine containing scaffolds are proven to be promising entities with good anti inflammatory, analgesic and antioxidant potential. In the current study, based on the rational approach, various analogs of 2-quinolones were designed for binding to COX2 enzyme with less ulcerogenic effects.

CHAPTER 2

LITERATURE REVIEW

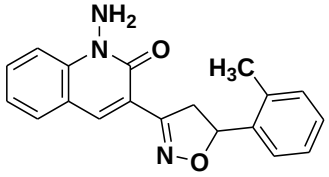
Quinolinone derivatives

1. Lan *et al.*,(2014)[39]; synthesised and evaluated the analgesic potential of 3,4-dihydroxy-2(1H)-quinolinone derivatives as novel sigma-1 receptor antagonists. The compounds were evaluated *in vitro* in sigma-1 and sigma-2 receptor-binding assays in guinea pig brain membranes.7-(3-(piperidin-1-yl)propoxy)-1-(4-fluorobenzyl)-3,4-dihydro-2(1H) quinolinone **(1)** was profiled with highest affinity and greatest selectivity which possess a high binding constant for sigma-1 receptor and high sigma-1/2 selectivity (1066-fold). In formalin test, compound **(1)** produced dose-dependent anti-nociception in both phases with ED50 values 49.4 ± 4.1 and 50.5 ±2.5 mg/kg for phases I and II, respectively.



(1)

2.Kumar *et al.*,(2014)[40]; synthesised and evaluated the antimicrobial and anti-inflammatory activity of isoxazolineincorporared 2 quinolones.Among the synthesised compounds compound **(2)** and **(3)** showed maximum percentage inhibition of edema volume at 4th hour when compared to the standard drug diclofenac sodium in the carrageenan induced paw edema model at dose of 200mg/kg



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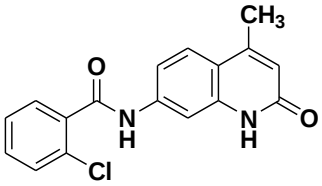
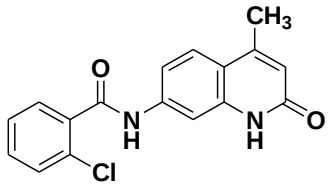


(3)

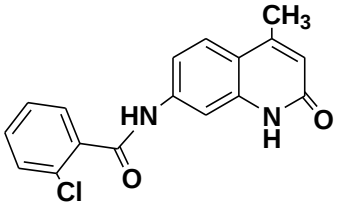
3.Pudlo *et al.*, (2014)[41]; designed, synthesised and evaluated hybrids of quinolinone-benzylpiperideine as novel acetylcholinesterase inhibitors and antioxidants. Among the

synthesised compound **(4)** was found to possess high free radical scavenging activity in DPPH (EC_{50} = 12.2 µM) and superoxide anion assays (EC_{50} =138.8µM)



(4)

4.Nitesh *et al.*,(2012)[42]; elucidated the anticancer activity of synthesized 2-quinolone derivatives without N-methyl or 3-aryl substitution. Significant cytotoxicity was observed in MCF-7 cells treated with **(5)** and **(6)**.Both the derivatives' treatment showed damage to the DNA. *In vivo* studies for (5) and (6)were performed at two doses 100 and 200 mg/kg using Ehrlich ascites carcinoma (liquid) and Dalton lymphoma ascites (solid) models. Both derivatives showed a significant reduction in the tumor progression by increasing the mean life span and by improving the haematological profile and antioxidant status of the liver in a liquid tumor model. More prominent effect was observed in a solid tumor model by reduction in solid tumor weight and tumour volume. The CTC_{50} of (5) and (6) were found to be, 83.04µM and 22.04 µM respectively



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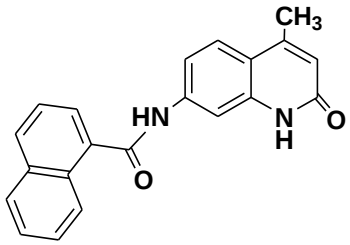
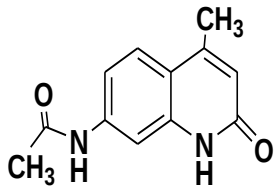


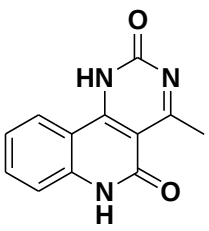
(6)

5.Jayashree *et al.*,(2010)[43]; designed and synthesised a series of 2-quinolone derivatives as anti oxidants and antimicrobials. Based on the docking stimulation and QSAR studies, the 7-amino-4-methylquinolin-2(1H)-one parent compound and its carboxamides) were synthesized using Conrad Limpach synthesis. Among the compounds synthesised compound **(7)** showed highest anti oxidant activity with IC_{50} 170.01 µg/mL.



(7)

6.Sankaran *et al.,* (2010)[44]; synthesised and conducted antioxidant and toxicological study of novel pyrimido quinolone derivatives from 4-hydroxy-3-acyl quinolin-2-one. They were screened for their *in vitro* antioxidant activities against radical scavenging capacity using DPPH, Trolox equivalent antioxidant capacity (TEAC), total antioxidant activity by FRAP, superoxide radical scavenging activity, metal chelating activity and nitric oxide scavenging activity. Among the compounds screened, **(8)** and **(9)** exhibited significant antioxidant activities.



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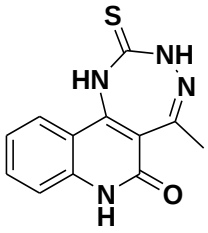
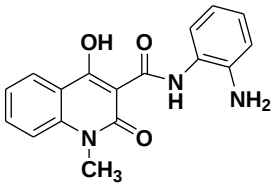


(9)

7.Detsi *et al.*,(2007)[45]; designed and synthesised a series of N-substituted-quinolinone-3-aminoamides and their hybrids containing the R-lipoic acid functionality as potential bifunctional agents combining antioxidant and anti-inflammatory activity. The compounds were evaluated for their antioxidant activity and for their ability to inhibit *in vitro* lipoxygenase as well as for their anti-inflammatory activity *in vivo*. Among the compounds synthesised compound **(10)** was found to be most potent anti-inflammatory agent with 85.3% inhibition of paw edema volume in carrageenan induced paw edema method.

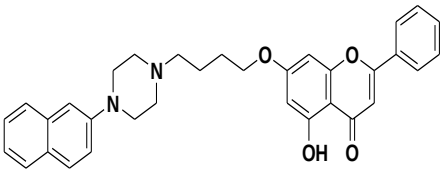


(10)

Piperazine derivatives

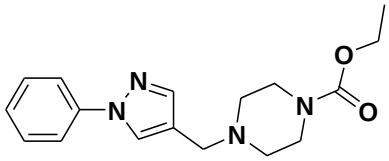
8.Patel *et al.*,(2016)[46]; established the antioxidant and anti cancer potential of newly synthesised chrysin-piperazine conjugates. Among the synthesised compounds, **(11)** was found

to be the most potent antioxidant giving an IC_{50} of 20.30 µg/mL and 5.62 µg/mL in DPPH and ABTS assay method respectively.



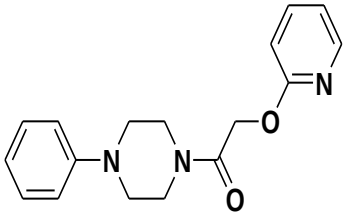
(11)

9.Silva *et al*.,(2015)[47]; investigated the anti-nociceptive and anti-inflammatory effects of piperazine derivative 4-[(1-phenyl-1H-pyrazol-4-yl) methyl]1-piperazine carboxylic acid ethyl ester **(12)** and the involvement of the serotonergic pathway. In the formalin test, treatments with this compound (15 and 30 mg/kg p.o.) reduced the licking time in both neurogenic and inflammatory phases. In the tail flick and hot plate tests, **(12)** treatment increased latency to thermal stimulus and in the carrageenan-induced paw edema test, at the doses of 15 and 30 mg/kg reduced the edema at all tested time points, while the dose of 7.5 mg/kg reduced the edema only for the first hour.Compound **(12)** (30 mg/kg p.o.) reduced both cell migration and protein exudation in the carrageenan-induced pleurisy test.



(12)

10.Ghorbani *et al.*, (2015)[48]; synthesised a series of novel piperazine analogues bearing quinolin-8yloxy-butan-1-ones/pyridine-2-yloxy-Ethanone. The analogues were evaluated for *in vitro* antioxidant activity against DPPH and ferrous ion radical scavenging activities and anti-inflammatory activity by inhibition of *Vipera russelli* venom (PLA_2) and gastric K+/H+ ATPase activities.It was found that pyridine ring with phenyl and nitro-phenyl group of (**13**) and (**14**) showed potent inhibition against all the assays



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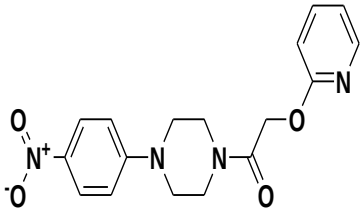
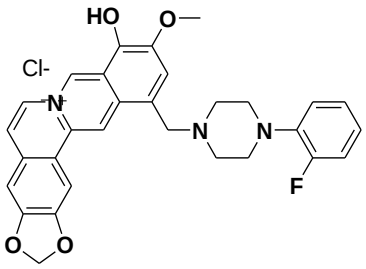


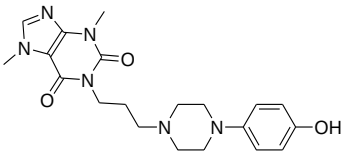
(14)

11.Mistry *et al.*, (2015)[49] synthesised new Mannich base series of piperazine linked berberine analogues and were evaluated for antioxidant and anti-cancer activities. The radical scavenging potential of the final derivatives was found excellent with IC_{50}s, <13 lg/mL and<8 lg/mL in DPPH and ABTS assay, respectively, whereas some analogues showed significant Fe+3 reducing power with absorption at around 2 nmin the FRAP assay.compound**(15)** was found to be more potent. Anticancer effects of titled compounds were inspected against cervical cancer cell line Hela and Caski adapting SRB assay, in which the analogues presented <6 lg/mL of IC50s, and>30 of therapeutic indices, thus exerting low cytotoxic values against Malin–Darby canine kidney (MDCK) cell lines at CC_{50}s >125 lg/mL.



(15)

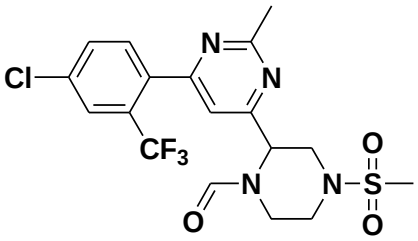
12.Andonova *et al.*, (2014)[50]; synthesised and evaluated the antioxidant activity of some 1-aryl/aralkylpiperazine derivatives with xanthine moiety at N4. All compounds were *in vitro* screened for their activity as antioxidants using DPPH, and FRAP methods. The antioxidant activity of the studied compounds against lipid peroxidation was also measured. The highest antioxidant activity was demonstrated by compound **(16)** with IC_{50} values . 189.42 µmol/L^2,3.45 µmol/L^2and173.99 µmol/L^2 for DPPH, ABTS and FRAP assays respectively.



(16)

13.Mohan *et al.*, (2014)[51]; synthesised and evaluated the antibacterial, anthelmintic and anti-Inflammatory activity of some novel methylpyrimidinesulfonylpiperazine derivatives.

Compounds **(17)**and **(18)** were proven to be good anti-inflammatory agents at adose 100mg/kg in carrageenan induced paw edema method.



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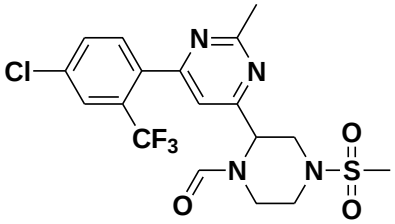
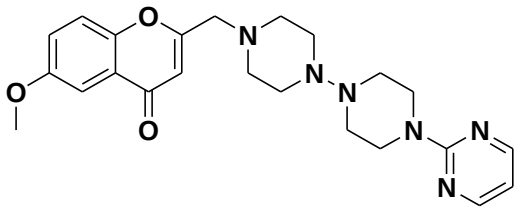


(18)

14.Girish *et al.*,(2012)[52]; synthesised a series of novel 6-methoxy-2-(piperazin-1-yl)-4*H*-chromen-4-one and 5,7-dimethoxy-2-(piperazin-1-ylmethyl)-4*H*-chromen-4-one derivatives of biological interest and screened for their pro-inflammatory cytokines (TNF-α and IL-6) and antimicrobial activity (antibacterial and antifungal). Among all the compound screened, the compounds **(19)(82%**TNF-α and 87 %IL-6 inhibitory activities) and **(20) (85%**TNF-α and 91 %IL-6 inhibitory activities) **were** found to have promising anti-inflammatory activity at concentration of 10 μM with reference to standard dexamethasone (71% TNF-a and 84% IL-6 inhibitory activities at 1 μM)



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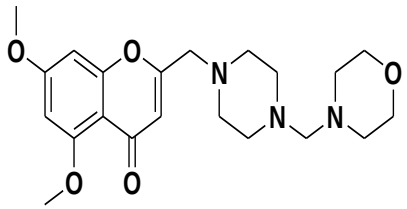
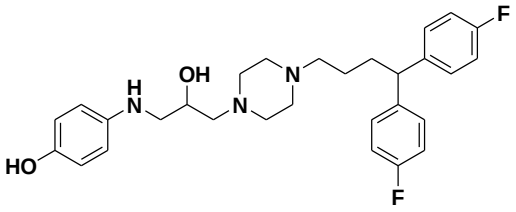


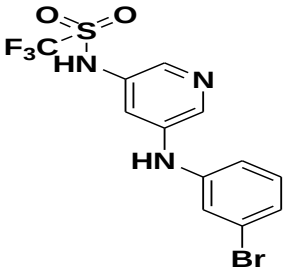
(20)

15.Kimura *et al.*, (2004)[53]; synthesised a new series of diphenylalkylpiperazine derivatives with high affinities for the dopamine transporter (DAT), which were modified at both the diphenylalkyl moiety and the phenyl ring in the phenylamino moiety of 1-[4,4-bis(4-fluorophenyl)butyl]-4-[2- hydroxy-3-(phenylamino)propyl]piperazine and was evaluated for their inhibitory activities against auto-oxidative lipid peroxidation in canine brain homogenates.The 4-hydroxyphenyl derivative **(21)** showed the most potent anti-oxidative activity with an IC_{50} value of 0.32 lM, exhibiting approximately 5-fold more potent activity than a-tocopherol.



(21)

16.Renard *et al.*,(2014)[54]; synthesised a series of analogs of nimesulide resulting from isosteric replacement of the nitrobenzene ring by the pyridine nucleus, was synthesized and their ability to inhibit both cyclooxygenases (COXs) isoforms was evaluated *in vitro* using a human whole blood model Among the synthesised compounds, those bearing a bulky halogen atom (compounds **22** and **23**) displayed the most important activity (COX- 2 IC_{50} values for **(22)**and**(23):** 0.12 and 0.26 mM, respectively) and selectivity against COX-2 (COX-2 selectivity ratio for 7 and 19c: 7.48 and 7.46, respectively)



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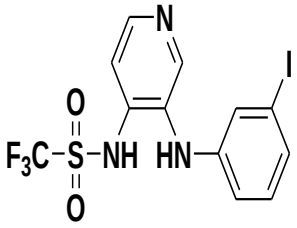
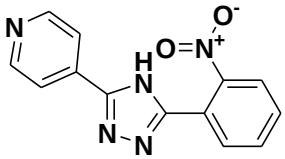


(23)

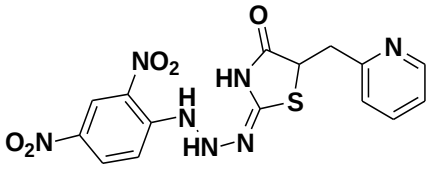
17.G Nigade *et al.*, (2012)[55]; synthesised a series of heterocyclic compounds containing pyridine nucleus and was evaluated for its analgesic activity by using Eddy's hot plate method and formalin induced licking test. Among the compounds synthesised compound **(24)**was found to be produce the highest inhibition of inflammation at a dose 25mg/kg



(24)

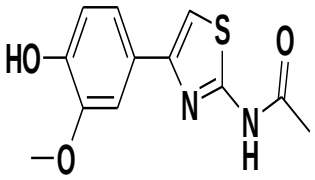
18.Ranga *et al.*,(2012)[56]; synthesised and evaluated new thiazolidinylanalogs containing pyridine ring and QSAR and molecular docking studies were performed for the same. Among

the compounds synthesised compound **(25)** exhibited the highest docking score and was also found to be the most active compound of the series with an dual anti-inflammatory and analgesic activity at a dose 10mg/kg p.o



(25)

19. G.A. El-Achkar *et al.*,(2015)[57]; investigated the *in vitro* and *in vivo* effects of two novel thiazole derivatives**(26)** (N-[4-(4-hydroxy-3-methoxyphenyl)- 1,3-thiazol-2-yl]acetamide) and **(27)**(4-(2-amino-1,3-thiazol-4-yl)-2-methoxyphenol)on prostaglandin E2 (PGE2) productionand COX activity in inflammatory settings. The results revealed the potent inhibition of both compound **(25)** (IC50 9.01±0.01 mM) and **(26)** (IC_{50} 11.65±0.20 mM) on COX-2-dependent PGE2 production.It was found that compound **(26)** is a specific inhibitor of COX-1 withIC_{50}($5.56*10^{-8}$±$2.26*10^{-8}$mM), whereas compound **(27)**did not affect COX-1 using HEK-293 cells stably over-expressing COX-1and human blood platelets.Both compounds produced inflammatory effect in the dorsal air pouch model of inflammation



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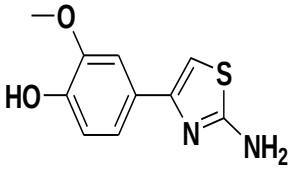
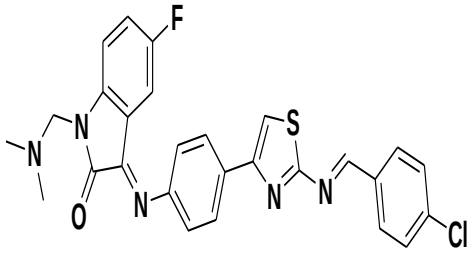
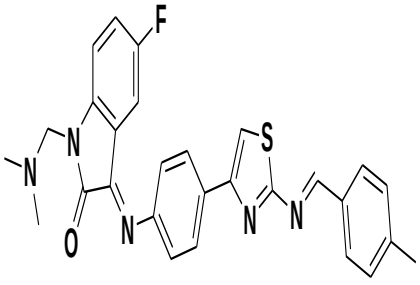


(27)

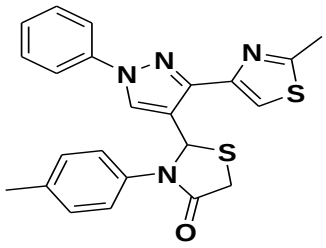
20.Prakash *et al.*,(2015)[58]; synthesised series of novel 3-(4-(2-(substituted benzylideneamino) thiazol-4-yl)phenylimino)-1-((dimethylamino)methyl)-5-fluoroindolin-2-one Schiff and Mannich base derivatives and evaluated for their analgesic anti-inflammatory , antimicrobial and ulcerogenic potential.Among the synthesised compounds **(28)** and **(29)** found to be more potent





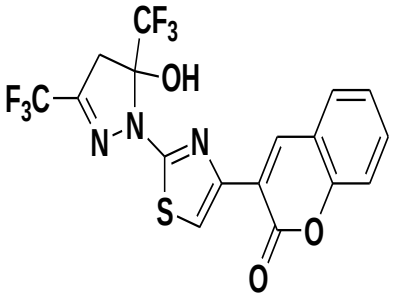
(28) **(29)**

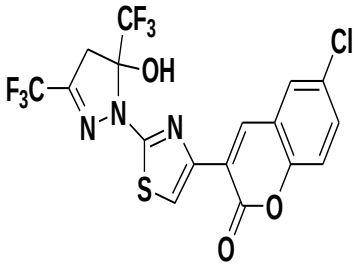
21.Khillare *et al.*,(2014)[59]; synthesised and evaluated new trisubstitutedpyrazoles bearing thiazolyl and thiazolidinonyl moieties from 5-acetyl thiazoles as starting materials. Among the synthesised compounds compounds, after 4thanf 6th hour **(30)** showed good anti-inflammatory at a dose equimolar to 50mg/kg of celcoxib.



(30)

22.Aggarwal *et al.*,(2013)[60]; synthesised a series of novel 2-(5-hydroxy-5-trifluoromethyl-4,5-dihydropyrazol-1-yl)-4-(coumarin-3-yl)thiazoles and evaluated for their anti-inflammatory and ant microbial activities. Among the synthesised compounds, Compounds (**31)**and**(32)** showed the highest (83 and 86%, respectively) anti-inflammatory activity, when compared to standard anti-inflammatory drug indomethacin (94%).

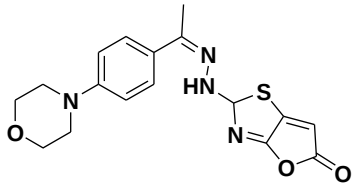




(31) **(32)**

23.Helal *et al.*,(2013)[61]; synthesised and evaluated the anti- inflammatory activity of some novel thiazole compounds. Furo[2,3-d]thiazol-5(2H)-one **(33)** was obtained from reaction of thiosemicarbazone derivative with diethyl acetylene dicarboxylate and 4-(4-Morpholino phenyl) thiazol-2-amino **(34**) was obtained via the reaction of acetophenone derivative with thiourea in

presence of iodine. Among the synthesised compound these compounds showed a higher degree of inhibition of paw edema with a percent inhibition of 85 and 87 respectively.



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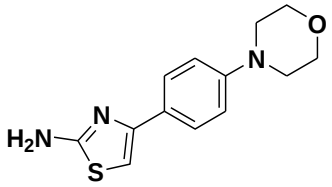
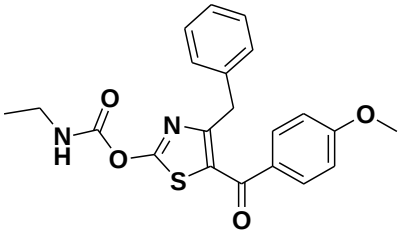


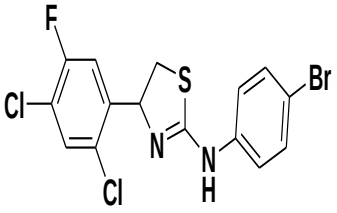
(34)

24. R.N Sharma *et al.*,(2009)[62]; applied an analogue based drug design approach for synthesising 4-benzyl-1,3-thiazole derivatives as potential anti-inflammatory agents. Among the compounds synthesised compound **(35)** emerged as the most potent compound in carrageenan induced inflammatory model with a percentage inhibition of 60.8% at a dose 50mg/kg



(35)

25.Holla *et al*. (2003)[63]; reported different series of arylaminothiazoles, arylidene/5-aryl-2-furfurylidene hydrazine thiazoles and screened them for their antibacterial and anti-inflammatory activities. Two of the newly synthesized compounds **(36)** and **(37**) showed anti-inflammatory activity comparable with that of ibuprofen at a dose 20mg/kg i.p



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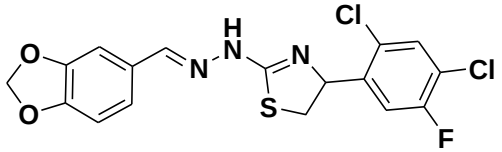
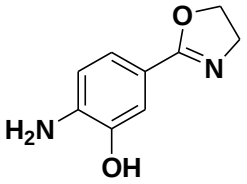


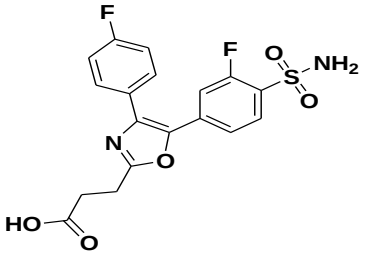
(37)

26.Mathew *et al.*, (2012)[64]; synthesised some novel 2,4-diphenyloxazole derivatives and evaluated their*in vitro* antioxidant and anticancer activity. Among the synthesised compounds, **(38)** showed nitric oxide scavenging activity with IC_{50} at 461.28 mg/mL.



(38)

27.Zhou *et al.*,(2009)[65]; designed and synthesized a series of 4,5-diaryloxazole analogs as a novel anti-inflammatory drug and the interaction between oxaprozin and cyclooxygenase- 2 was studied by docking method to improve the biological activity and reduce the gastrointestinal side effects of oxaprozin. Among the compounds analysed, 3-(4-(4-fluorophenyl)-5-(4-aminosulfonyl-3-fluorophenyl)-oxazole-2-yl) propanoic acid **(39)**, was selected for synthesis and pharmacological evaluation.Compound39 showed anti-inflammatory activity up to 31.7% at 80 mg/kg after 3 h of drug treatment in carrageenan induced paw edema and showed an inhibitory value of up to 54.0% at 100 mg/kg over 15 min in acetic acid induced writhing response test in mice .



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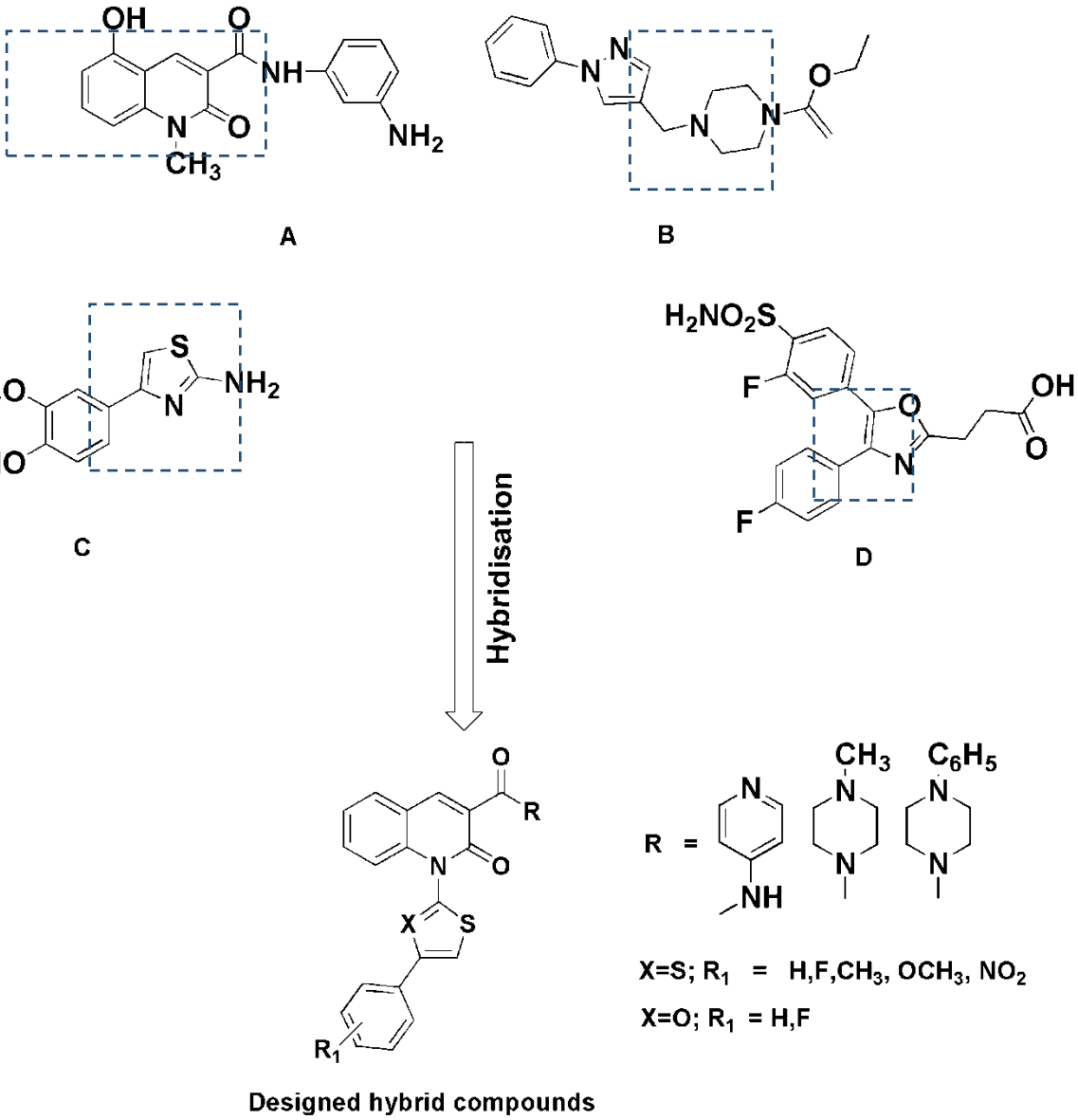
CHAPTER 3
AIM AND OBJECTIVES

Inflammation is a universal host defence process involving a complex network of cell-cell mediator and tissue interactions. Inflammatory diseases cover a broad spectrum of conditions including autoimmune diseases (eg:rheumatoid arthritis),osteoarthritis, inflammatory bowel disease, multiple sclerosis, asthma, chronic obstructive pulmonary disease, allergic rhinitis, infectious diseases, various types of cancers and cardiovascular diseases. Until a few years ago, inflammatory disorders were treated primarily with relatively non selective anti- inflammatory drugs such as such as corticosteroids and various NSAIDs , however, now a days several mediator antagonists alone or in combination and gene therapy are also bring tried.

Research in last few decades has shown that inflammation is regulated by a large number of pro and anti-inflammatory mediators. Advent of genomic era has emphasised the role of altered gene expression as fundamental to aetiology of inflammation and immune disorders..Efforts to develop new safer and more effective anti-inflammatory drugs are based on the role of key mediators identified as the key culprits in this condition. Inhibitors which specifically interfere with different components of different intracellular signalling pathways or inhibit the activation of transcription factors responsible for the expression of disease related genes have applications as novel therapeutic agents in inflammation. Inorder to search newer inhibitors of inflammatory signals, several chemical entities are being cloned with the help of *in silico* drug design stratergies.

Quinolinones are the conventional scaffolds with antibacterial activity.As an important class of compounds, quinolin-2-ones are isomeric to 4-quinolones and isosteric to coumarins. The compounds that have 2-quinolone moiety are associated with interesting biologic activities such as antibacterial, anticancer, antiviral, cardiotonic, and N-methyl-D-aspartate receptor inhibitor functions, among others. This study aimed to design, synthesis and evaluate various quinolin-2-one analogs, which contain thiazole, oxazole, aminopyridine, methyl piperazine and phenylpiperazine substitutions. The study also targeted in designing a molecule which is a selective COX2 inhibitor and thus can overcome the side effects caused by existing NSAIDs.

The study focuses on designing compounds with quinolin-2-one nucleus as potent COX-2 inhibitor and thereby evaluating *in vitro* antioxidant and *in vivo* anti-inflammatory and analgesic activity followed by evaluating the ulcerogenic potential of the compounds.



Designed hybrid compounds

CHAPTER 4

PLAN OF WORK

The plan of present work is

- Review of literature and scientific survey of the title compounds.
- Selection of target protein and ligand nucleus for anti-inflammatory and analgesic activity assessment.
- Design of molecules based on selected nucleus by molecular modeling software and determination of potent molecule by docking study.
- To synthesize quinolin-2-one analogs based on the reported literature procedure.
- To check the purity of the recrystallized compounds by melting point, TLC.
- Characterization of synthesized compounds by various analytical techniques like UV, FT-IR, ^{1}H-NMR and Mass spectral analysis.
- Screening of synthesized compounds for *in vitro* anti-oxidant activity
    - ✓ DPPH radical scavenging assay
    - ✓ ABTS method.
- Screening of synthesized compounds for their
    - ✓ *In vivo* anti-inflammatory activity.
        - Carrageenan induced paw edema method
        - Cotton Pellet induced granuloma method
    - ✓ *In vivo* analgesic activity
        - Eddy's hot plate method
        - Acetic acid induced writhing method
    - ✓ Ulcerogenic potential studies
    - ✓ Antibacterial activity

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CHAPTER 5

EXPERIMENTAL WORK (CHEMISTRY)

5.1 Materials

The chemicals, reagents, solvents, glassware and other necessary things which are used for the synthesis, purchased from various companies (Hi media and Spectrochem) stored in well closed container. Stored chemicals were used without further purification.

5.2 Methods

5.2.1 Docking studies

5.2.1.1 Autodock

Autodock is a suite of C programs used to simulate interactions between small flexible ligands and macromolecules of known structure. Docking is achieved through a search of conformational space using a Lamarckian genetic algorithm coupled with energy assessments using auto dock method. The combination of these two functions produces a family of molecular coordinates detailing possible docked ligand conformations which can then be used as a starting point for theoretical ligand design and study. Confidence in the docked conformation is represented by an energy value based on both quantum and molecular mechanical modelling of atomic forces. The success of a docking program depends on the Lamarckian genetic algorithm.[66]

- **Lamarckian genetic algorithm**

The vast majority of genetic algorithms mimics the major characteristics of darwinian evolution and apply mendelian genetics. This is illustrated on the oneway transfer of information from the genotype to the phenotype. However, in those cases where an inverse mapping function exists, one which yields a genotype from a given phenotype, it is possible to finish a local search by replacing the individual with the result of the local search. This is called the Lamarckian genetic algorithm (LGA). The most important issues arising in hybrids of local search (LS) techniques with the GA revolve around the developmental mapping, which transforms genotypic representations into phenotypic ones. The genotypic space is defined in terms of the genetic operator's mutation and cross over in our experiments by which parents of one generation are perturbed. The phenotypic space is defined directly by the problem, namely, the energy function being optimized.[67]

In our case, the fitness or energy is calculated from the ligand's coordinates, which together form its phenotype. The genotypic representations of the ligand, and its mutation and cross over operators, have already been described. The developmental mapping simply transforms a molecule's genotypic state variables into the corresponding set of atomic coordinates. This means that the developmental mapping does not need to be inverted. Nonetheless, this molecular variation of the genetic algorithm still qualifies as Lamarckian, because any ''environmental adaptations'' of the ligand acquired during the local search will be inherited by its offspring. At each generation, it is possible to let a user defined fraction of the population undergo such a local search. We have found improved efficiency of docking with local search frequencies.[68]

5.2.1.2 Autodock procedure.[69]

Docking studies of compounds S5a-o; s6a-f were performed using one cyclooxygenase-2 protein (PDB ID: 1CX2) obtained from the RCSB protein data bank,http://www.rcsb.org/pdb. Experiments were performed using the program Autodock module version 4.0.

AutoDock includes 3 steps:

1. Preparation of receptor & ligand files.
2. Calculation of affinity maps by using a 3D grid around the receptor & ligand.
3. Defining the docking parameters and running the docking simulation.

The preparation step starts with pdb files of receptor (R.pdb) and ligand (L.pdb), which are added hydrogens and then saved as RH.pdb & LH.pdb. The calculation of affinity maps in the "Grid" section requires the above pdb files to be assigned charges & atom types, and also that the nonpolar hydrogens are merged. This is done automatically by ADT, and the resulting files need to be saved as RH.pdbqt & LH.pdbqt, which is the only format AutoGrid &AutoDock can work with. Calculation of affinity maps is done by Auto Grid, and then docking can be done by AutoDock. The newest docking algorithm is LGA (Lamarckian Genetic Algorithm).

Preparing and Running a Docking

1. Preparing the protein

- Opening file: [Right-click "PMV molecules"] → [choose file].
- Color by atom: [Click under "Atom"].

- Eliminate water: Select → Select from string → [write HOH in "Residue" line and in the "Atom" line] → Add → Dismiss → Edit → Delete → Delete Atom Set.
- Find missing atom and repairing them: File → Load module → [Pmv; repair Commands] → Edit → Misc. → Check for missing atoms → Edit → Misc. → Repair missing atoms.
- Add hydrogen's: Edit → Hydrogen's → Add → [choose "All hydrogen's", "no bond order", and "Yes" to renumbering].
- Hide protein: [Click on the gray under "show Molecules"]. (Note: if you are planning rigid docking (i.e. no flexible parts in the protein), save the protein as RH.pdb for now)

2. Preparing the ligand

- Make sure the ligand has all hydrogens added before working with ADT.
- Toggle the "AutoDock Tools" button.
- Opening file: Ligand → Input → Open → All Files → [choose file] → Open. (ADT now automatically computes Gasteiger charges, merges nonpolar hydrogens, and assigns Autodock Type to each atom.)
- Define torsions:

  * Ligand → Torsion Tree → Detect Root (this is the rigid part of the ligand)

  * Ligand → Torsion Tree → Choose Torsions → [either choose from the viewer specific bonds, or use the widget to make certain bond types active (rotatable) or inactive (non-rotatable). Amide bonds should NOT be active (colored pink)] → done.

  * Ligand → Torsion Tree → Set Number of Torsions → [choose the number of rotatable bonds that move the 'fewest' or 'most' atoms].

  Save ligand file:

  * Ligand → Output → Save as PDBQT → [save with L.pdbqt].

  Hide the ligand, as explained in (A5) for the protein.

3. Preparing the flexible residue file (Note: if you are planning rigid docking, ignore this section and do the following: Grid → Macromolecule → Open → [choose RH.pdb]. AutoDock will

automatically add charges and merge hydrogens. Save the object as RH.pdbqt and move to next section.)

- Flexible residues → Input → Choose molecule → [choose the original protein R.pdb] → Yes to merge nonpolar hydrogens (AutoDock assigns charges + atom types to R.pdb, and merges nonpolar hydrogens).
- Select the residues to be flexible: Select → Select from string → ARG8 → Add → Dismiss.
- Define the rotatable bonds: Flexible residues → Choose torsions in currently selected residues → [click on rotatable bonds to inactivate them, or vice versa].
- Save the flexible residues: Flexible residues → Output → Save flexible PDBQT → [save as R_flex.pdbqt].
- Save the rigid residues: Flexible residues → Output → Save rigid PDBQT → [save as R_rigid.pdbqt].
- Delete this version of protein: Edit → Delete → Delete Molecule → [choose protein (R)] → Delete → Dismiss.

4. Running AutoGrid calculation the purpose of this section is to define the search grid and produce grid maps used later by Autodock.

- Open the rigid protein: Grid → Macromolecule → Open → [choose the rigid protein] → Yes to preserving the existing charges. (Note: if you are doing rigid docking, choose RH.pdbqt)
- Prepare grid parameter file: Grid → Set Map Types → Choose Ligand → [choose the ligand already opened] → Accept.
- Set grid properties: Grid → Grid Box → [Set the grid dimensions, spacing, and center] → File → Close Saving Current.
- Save the grid settings as GPf file: Grid → Output → Save GPF → [save as R.gpf].
- Running: Run → Run AutoGrid → [make sure the program name has the right path, and that it is where the input files are] → Launch → [in the command prompt prompt, type "tail –f hsg1.glg" to follow the process] (Note: the AutoGrid calculation can be started directly from the command prompt by typing "autogrid4 –p hsg1.gpf –l hsg1.glg ").

5. Preparing the docking parameter file (.dpf)

- Specifying the rigid molecule: Docking → Macromolecule → Set Rigid Filename → [choose R_rigid.pdbqt]. (or RH.pdbqt for rigid docking)
- Specifying the ligand: Docking → Ligand → Choose → [choose L.pdbqt] → [here you can set the initial location of the ligand] → Accept.
- Specifying the flexible residues: Docking → Macromolecule → Set flexible Residues Filename → [choose R_flex.pdbqt].
- Setting the parameters for the chosen docking method: Docking → Search Parameters → Genetic Algorithm → [for 1st time, use the short number of evaluations (50,000), and for other runs choose the medium or long] → Accept. 5. Setting docking parameters: Docking → Docking Parameters → [choose the defaults].
- Specifying the name of the ligand dpf file to be formed, containing the docking instructions: Docking → Output → Lamarckian GA → [type L.dpf].
- Confirming the details of docking: Docking → Edit DPF → [make sure the right ligand pdbqt file name appears after the word "move", and that the right number of active torsions is specified].

6. Running AutoDock

- Make sure the AutoDock executable is in the same directory as the macromolecule, ligand, GPF, DPF and flex files (in case of flexible docking).
- Running: Run → Run AutoDock... → Launch.

7. When RH and LH already exist

- Protein: Grid → Macromolecule → choose RH.pdb → (charges & atom types assigned, nonpolar hydrogen merged) → File → save → write PDBQT → save as RH.pdbqt

  Ligand: Ligand → Input → Open → All Files → choose LH.pdb → (charges & atom types assigned, nonpolar hydrogen merged) → save as LH.pdbqt
- Set the rest of the grid parameters & calculate map
- Setting Docking parameters: Docking → Macromolecule → Set Rigid Filename → choose either RH.pdbqt or RH_rigid.pdbqt → Docking → Ligand → Choose → choose LH.pdbqt → set the rest of the docking parameters.
- Running docking simulation.

8. Viewing Docking Results

A. Reading the docking log file (.dlg)

- Analyze → Dockings → Open → [choose L.dlg].
- Analyze → Conformations → Load → [double-click on each conformation to view it on screen].

B.Visualizing docked conformations

- Analyze → Conformations → Play... (Note: & allows changing the ligand's color)

5.2.2 Experimental protocol

The experimental work conducted the following sub headings.

5.2.2.1 Scheme:

Scheme I :-

Step (i): Synthesis of various substituted 4-phenylthiazol-2-amines**(Compounds S8a-e)**

Step(ii): Synthesis of various substituted4-phenyloxazol-2-amines**(Compounds S9a-b)**

Scheme II :-

Step (i): Synthesis of ethyl 2-oxo-2H-chromene-3-carboxylate**(Compound S2).**

Step (ii): Synthesis of 2-oxo-2H-chromene-3-carboxylic acid**(Compound S3).**

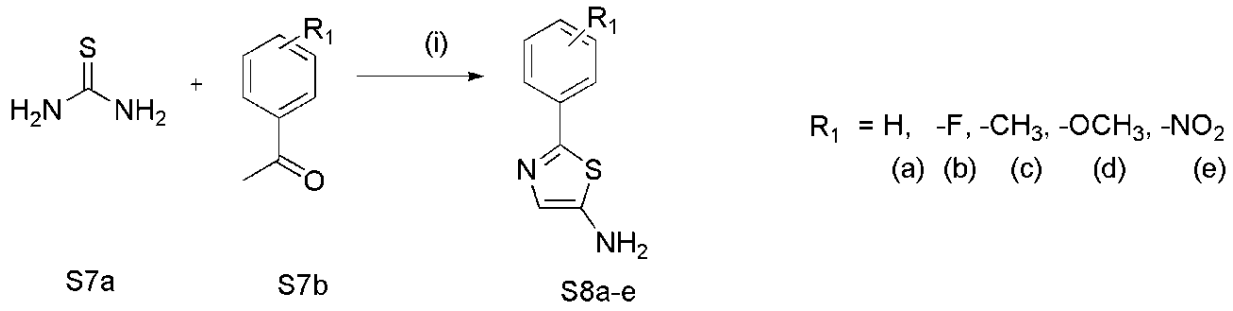
Step (iii): Synthesis of various amide derivatives of 2-oxo-2H-chromene-3-carboxylic acid **(Compound S4a-c).**

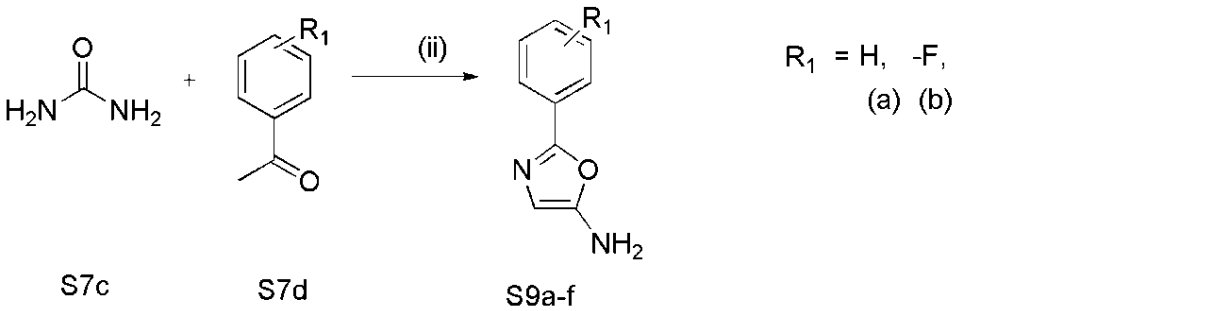
Step (iv): Synthesis a series of 1-heterocyclic substituted quinoline-2-one derivatives **(Compounds S5a-o; S6a-f).**

5.2.2.2 Physicochemical studies of synthesized compounds.

5.2.2.3 Spectral analysisof synthesized compounds.

5.2.2.1 SCHEME -I:

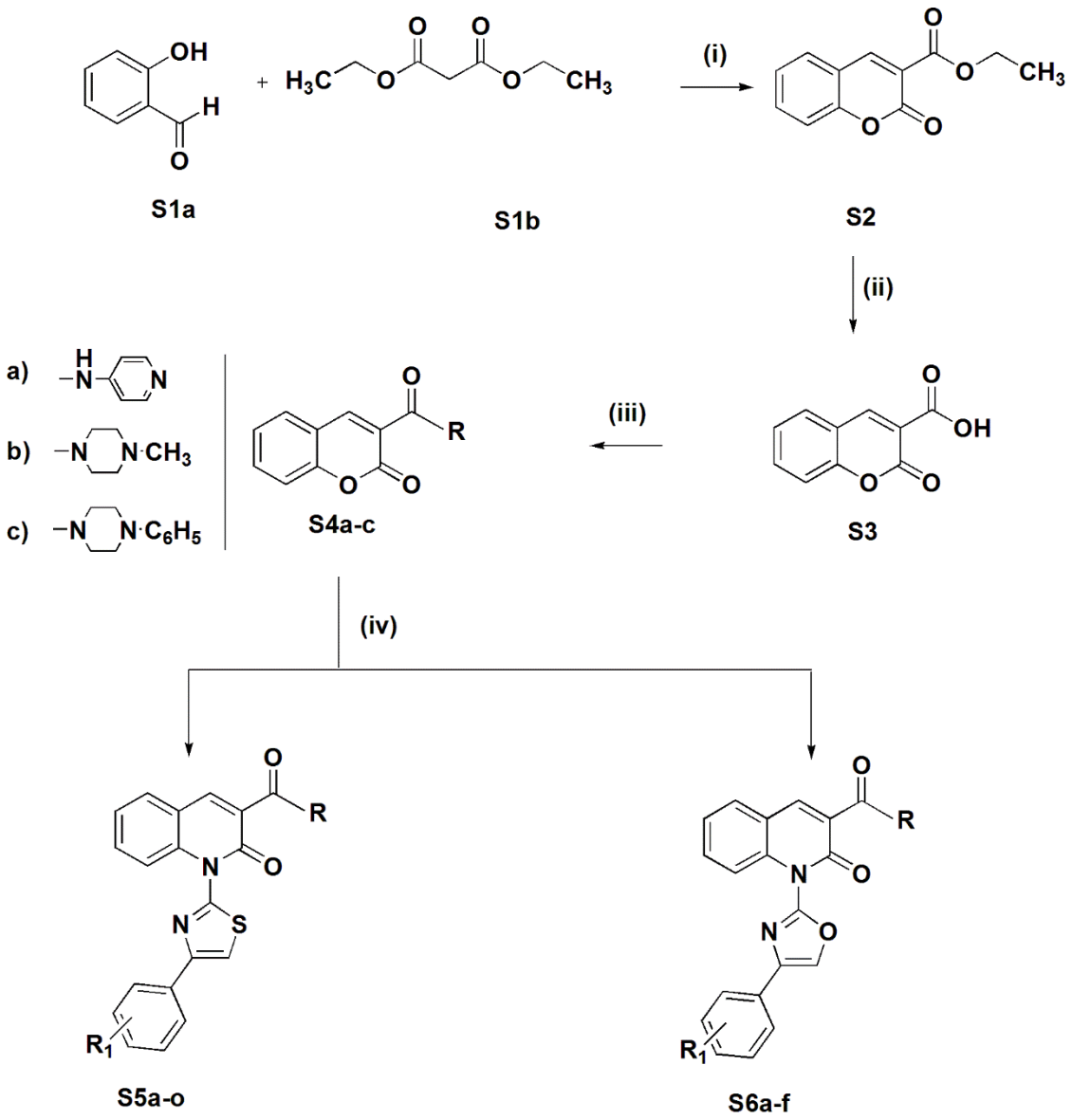




Reagents and condition:

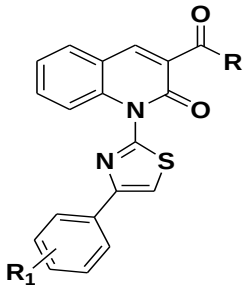
(i) Thiourea, S7a-e, I_2, hot H_2O, ether, NH_3;
(ii) Urea, S7a-b, I_2, hot H_2O, ether, NH_3.

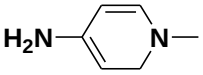
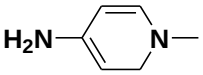
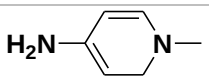
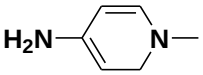
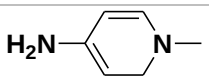
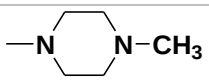
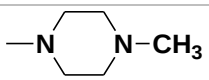
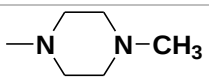
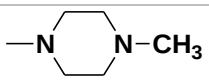
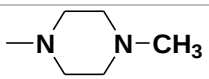
Scheme-II



Reagents and Conditions:(i) Salicylaldehyde, Diethyl malonate, pyridine, 0-5°C, stirring 20 min; **(ii)**Compound **(S2),**0.1N NaOH stirring for 45min, 1N HCl;**(iii)**Compound (**S3),**DMF,DCC Microwave irradiation at 300W for 2-3min; **(iv)**Compounds(**S4a-c**), Compounds (**S8a-e**;**S9a-b**)in glacial acetic acid,Microwaveirradiation at 450W for 10-12 min**.**

Table 5.1 Compounds (S5a-o)



Cpd code	R	R_1
S5a		H
S5b		-F
S5c		$-CH_3$
S5d		$-OCH_3$
S5e		$-NO_2$
S5f		-H
S5g		-F
S5h		$-CH_3$
S5i		$-OCH_3$
S5j		$-NO_2$

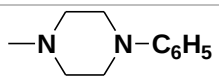
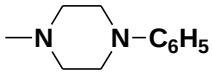
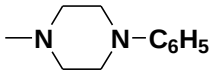
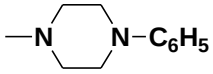
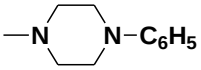
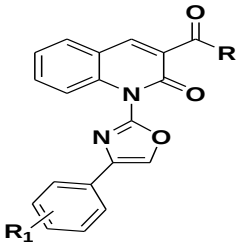
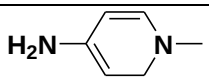
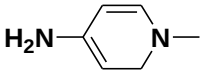
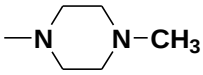
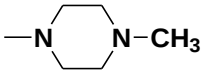
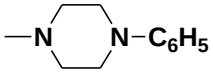
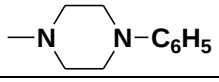
Cpd code	R	R_1
S5k		-H
S5l		-F
S5m		$-CH_3$
S5n		$-OCH_3$
S5o		$-NO_2$

Table 5.2 Compounds (S6a-e)



Cpd code	R	R_1
S6a		-H
S6b		-F
S6c		-H
S6d		-F
S6e		-H
S6f		-F

5.2.2.2 PROCEDURE:

Scheme 1 :-[70]

Step (i): Synthesis of various 4-phenylthiazol-2-amines (Compounds S8a-e;)

A mixture of various substituted acetophenone (0.1 mole), thiourea(0.2 mole) and iodine (0.1 mole) was heated on a steam bath for 4-5 h. The hydroiodide, thus separated, was filtered, afforded pale yellow powder, washed with ether and dried. Resulting product was dissolved in hot water, filtered while hot and the clear solution neutralized with a strong solution of ammonia. The solid separated was filtered,dried and recrystallized.

Step (ii) Synthesis of various 4-phenyloxazol-2-amines (Compounds S9a-b).

A mixture of various substituted acetophenone (0.1 mole), urea (0.2 mole) and iodine (0.1 mole) was heated on a steam bath for 4-5 h. The hydroiodide, thus separated, was filtered, afforded pale yellow powder, washed with ether and dried. Resulting product was dissolved in hot water, filtered while hot and the clear solution neutralized with a strong solution of ammonia. The solid separated was filtered,dried and recrystallized.

Scheme II:-

Step (i) :Synthesis of ethyl 2-oxo-2H-chromene-3carboxylate (**Compound S2**): [71]

To a cold mixture of salicylaldehyde (0.2Mole) and diethyl malonate (0.2Mole), 2 mL of piperidine was added by rapid stirring. After 20 min the yellow solid separated was filtered off subsequently washed with ethanol and recrystallized from water: ethanol (2:8).

Step(ii) :Synthesis of 2-oxo-2H-chromene-3-carboxylic acid (Compound S3).

Compound**(S2**)was dissolved in 0.1N NaOH and stirred in a magnetic stirrer for about 45 min. The resulting solution was filtered, filtrate collected, acidified with 1N HCl to give compound**(S3).**

Step (iii): Synthesis of various amide derivatives of 2-oxo-2H-chromene-3-carboxylic acid (Compound S4a-c).[72]

A mixture of 2-oxo-2H-chromene-3-carboxylic acid**(compound S3)** (1mole) and N,N' di-cyclohexylcarbo-di-imide (DCC) (2mole) in 20mL of dichloromethane and was stirred for 10min at 0-5ºC. To this mixture the respective secondary amines were added and irradiated in a

microwave at 300W for 2-3min. The solid urea separated was filtered off and the organic layer was washed with ice cold water to yield the desired product.

Step (iv) : Synthesis of series of quinoline-2-one derivatives from various amide substituted 2-oxo-2H-chromene-3-carboxylic acid by coupling with synthesised 4-phenylthiazol-2-amines (Compounds S5a-o;S5a-e).[73]

Equivalent mole(0.1mole) of compounds **(S8a-e; S9a-b)** andcompounds **(S4a-c)**in presence of glacial acetic acid were irradiated with microwave at 450 watts for 10 -12 min. The excess solvent was distilled off under reduced pressure and poured into crushed ice to get the solid. The product so obtained was filtered under suction and dried at room temperature. It was purified by recrystallisation from absolute ethanol.

5.2.3 Physicochemical studiesof synthesized compounds.

5.2.3.1 Melting point analysis:

The melting point of the compounds were determined in one end fused capillary tubes on a THERMONIC MODEL-C-LMP-1, Campbell melting point apparatus was used to evaluated the purity of the synthesized compounds and are uncorrected.

5.2.3.2 Clog P

Molecular formula, molecular weight and clog P were determined by using the software Chem Draw 0.8 April 2003.

5.2.3.3 Thin layer chromatography:

TLC is an important analytical tool in the separation, identification and estimation of different classes of natural and synthetic compounds. Thin layer chromatography is a method of analysis in which the stationary phase is a finely divided solid, spreaded as a thin layer on a rigid supporting plate and the mobile phase.

Principle:

The principle involved in this technique is "ADSORPTION", which involves the separation and identification of the compound, by a differential migration of the compound between two phases.

a) Stationary phase: Finely divided solid, is spread as a thin layer on a rigid supporting plate.
b) Mobile phase: It is a liquid allowed to migrate across the surface of the plate.

Need for TLC in identification of synthesised compounds:

- To establish the purity and authenticity of starting materials and reagents.
- To monitor reactions, particularly in the case of new reactions.
- Assessment of purity of a crude reaction product.
- The optimization of experimental conditions to achieve the highest possible yield of product.

Procedure:

Stationary phase : Silica gel-G (Merck-E).

Mobile phase : Ethyl aceto acetate : Hexane (1:1).

Detecting agent : Iodine vapour.

Method : Ascending TLC

Supporting plate : Glass (5x 20 cms)

Preparation of plate:

Uniform slurry of silica gel G was prepared by addition of distilled water, poured into the spreading trough and drawn across the glass plate of size 5 x 20 cm, depositing a uniform layer of 0.25 mm thickness. The plates were dried and then activated by heating at 110°C for 1h and stored over a dessicator until used.

Selection of mobile phase:

Evaluation of various mobile phase was tried, alone or in combination in which ethyl acetate : Hexane (1:1) was found to be suited.

Sample application and development:

Sample was applied as a small spot at about 2cm from the base of plate. For ascending development, the plate was placed in the TLC chamber which was saturated previously with mobile phase .The solvent was allowed to move until it travelled a distance of about 15cm from the point of application. The plate was then removed and the solvent front was marked and it was allowed to evaporate.

Detection:

For detection of solute spots, the plates were placed in a chamber containing iodine crystals which sublimes to produce an atmosphere saturated by the vapours. The solute spot was visible as brown spots.

5.2.4 Spectral studies:

The structures of the compounds were confirmed by Infrared Spectroscopy, Proton Nuclear Magnetic Resonance and Mass Spectroscopy analysis.

5.2.4.1 Ultraviolet spectral analysis (UV)):

Molecular absorption in the ultraviolet and visible region of spectrum is dependent onthe electronic structure of molecule. Characteristic group with diverse electronic environment absorbs at selective wavelength, and helps in recognising characteristic groups in a molecule of widely varying complexity.

UV spectra are recorded on SHIMADZU 1700A, spectrophotometer. Spectral grade ethanol was used as solvent.

5.2.4.2 Infrared spectral analysis (IR):

The range of electromagnetic radiation between 0.8 and 500μm is referred as infrared radiation, which is represented with percent transmittance as the ordinate and the wave number(cm^{-1}) as the abscissa.

Applications:

- Determination of identity of a compound by means of spectral comparison with that of authentic sample.
- Verification of the presence of functional groups in unknown molecules.

Procedure:

Pellet technique is used .Solid samples (0.5-1mg) were intimately mixed with appropriate solid sample of dry powdered potassium bromide. The mixture was pressed between a punch and disc under pressure of 1,00000 -15,000 psi to form a transparent disc. The IR spectral study was done on JASCO FTIR 4100.

5.2.4.3 Nuclear Magnetic Resonance spectral analysis (^{1}H NMR):

The NMR spectra of the synthesized compounds were recorded by Bruker Fourier, Transform-NMR using TMS (Tetramethylsilane) as internal standard. The PMR (Proton Magnetic Resonance) spectroscopic values were measured in δ ppm in DMSO-d_6.

5.2.4.4 Mass Spectral analysis:

The mass spectra of newly synthesized compounds were recorded on JEOL GCMATE instrument. The mass of the compounds are expressed in m/z values.

EXPERIMENTAL WORK (PHARMACOLOGICAL ACTIVITY)

5.2.5. *In vitro* antioxidant activity

5.2.5.1 DPPH radical scavenging assay. [74][75]

Reagents

- 0.3mM Diphenyl-2-picrylhydrazyl (DPPH).
- Ethanol.
- Ascorbic acid (1mg/mL).

Principle

The molecule of 1,1-diphenyl-2-picrylhydrazyl (α, α-diphenyl-β-picrylhydrazyl; DPPH) is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet colour, characterized by an absorption band in ethanol/methanol solution centred at about 520 nm.When a solution of DPPH is mixed with a substance that can donate a hydrogen atom,this gives rise to the reduced form with the loss of violet colour; although there would beexpected to be a residual pale yellow colour from the picryl group still present. Representing theDPPH radical by Z• and the donor molecule byAH, the primary reaction

$$Z^{\bullet} + AH \longrightarrow ZH + A^{\bullet}$$

Procedure

The antioxidant activity of the compound was measured in terms of hydrogen donating or radical scavenging ability, using the DPPH method. 0.3mM solution of DPPH in ethanol was prepared and 1mL of this solution was added to 500 µL of various concentrations of sample (50,100, 200, 300, 400 and 500 µg/mL)and thereference compound.The mixture was shaken vigorously and left to stand in thedark at room temperature for 30 min. Then the absorbance was measured at 517 nm against a blank. Reference compounds used were ascorbic acid and quercetin. A control reaction was carried out without the test sample. The percentage of inhibition was calculated by comparing theabsorbance valuesof the control and test samples. Antiradical activity was expressed as percentage inhibition and calculated using the following equation:

$$\text{Percentage inhibition (I \%)} = (Abs_{control} - Abs_{sample} / Abs_{control}) \times 100$$

Where '$Abs_{control}$' was the absorbance of the control reaction and 'A_{test}' was the absorbance in the presence of the sample/ standard. The antioxidant activity of the compounds was expressed as $IC_{50.}$ (IC_{50} - concentration required to obtain a 50% radical scavenging activity).

5.2.5.2 ABTS*$^{+}$ Radical Cation Assay.[76][77]

Reagents

- ✓ 7 mM 2,2,- azinobis (3-ethylbenzoline-6-sulfonic acid) ABTS salt
- ✓ 2.45 mM ammonium per sulphate.
- ✓ Ascorbic acid (1mg/mL).

Principle

ABTS decolorization assay is an inhibition method. The peroxidase substrate 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), forms a relatively stable radical ($ABTS^{+}$) upon one electron oxidation. The formal reduction potential for ABTS is high enough for it to act as anelectron donor for the reduction of reactive oxygen species such as molecular oxygen and hydrogen peroxide, particularly at extreme pH values encountered in biological catalysis. Under this condition, the sulphonate groups are fully deprotonated and the mediator exists as a "dianion".

$$ABTS + e^{-} \longrightarrow ABTS^{-}.$$

$$ABTS^{-} + e^{-} \longrightarrow ABTS^{2-}.$$

Procedure

ABTS radical cation (ABTS*$^{+}$) was produced by reacting equal volumes of 7mM ABTS salt and 2.45 mM ammonium per sulfate and the mixture was allowed to stand in the dark for 16 h at room temperature. The resultant solution was diluted with ethanol until an absorbance of 0.70 ± 0.02 at 734 nm was obtained. Varying concentrations(0.5, 1, 2.5, 5, 10 µg/mL) of the samples were allowed to react with 900 µl of ABTS*$^{+}$ solution. After 20 min the absorbance reading were recorded at 734 nm and compared with the control ABTS solution. A control reaction was carried out without the sample. The ABTS*$^{+}$ scavenging capacity of the compound was compared with that of ascorbic acid and quercetin. All the tests were performed in triplicate in order to get the mean values. The percentage inhibition of $ABTS^{+}$ by the sample was calculated according to the formula:

$$\text{Percentage inhibition (I \%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

Where '$Abs_{control}$' was the absorbance of the control reaction and 'A_{test}' was the absorbance in the presence of the sample/ standard. The antioxidant activity of the compound was expressed as $IC_{50.}$ (IC_{50} - concentration required to obtain a 50% radical scavenging activity).

5.2.6. Toxicological evaluation. [78]

Acute toxicity study

Guideline: OECD-423

Method: Acute Toxic Class

Principle:

This method allows for the determination of LD50 value only when two doses result in mortality higher than 0% and lower than 100%.The principle of the test, based on a stepwise procedure with the use of a minimum number of animals, each step using three animals of a single sex (normally females) from which sufficient information is obtained regarding the acute toxicity of test substance. Absence or presence of compound- related mortality of the animals dosed at

One step will determine the next step, i.e;

- no further testing is needed,
- dosing of three additional animals, with the same dose
- dosing of three additional animals at the next higher or the next lower dose level.

Procedure:

Selection of animals:

- Species : Rats
- Strain : Albino Wistar
- Age : 8-12 weeks
- Weight: 20-25 g

Conditions to be maintained:

- Humidity: 30-70%
- Temperature: 22 ± 3^0C
- Lighting:12hr dark and 12 hr light cycle

- Feed : Standard pellet diet

Grouping of animals:

The animals are randomLy selected, marked to permit individual identification, grouped (3 animals per group and kept in their cages for at least 5 days prior to dosing to allow for acclimatisation to the laboratory conditions.

Dose : 5, 50,300,2000mg/kg

Preparation of dose:

The drug substance is suspended in the suitable solvent i.e. 1% CMC (whose toxicity characteristic is evaluated and proved to be safe).Doses must be prepared shortly prior to administration.

Administration of dose:

The animals should be kept fasting (food is with held but free access to water) 4h prior to the treatment. The test substance is administered in a single dose by oral route. The dose is gradually increased with each step starting with 5, 50.300, 2000mg/kg. After the substance has been administered, food may be withheld for further 1-2 h in rats.

Observation

Animals were observed individually after dosing at least once during the first 30 min, periodically during the first 24 h, with special attention given during the first 4 h and daily thereafter, for a total of 14 days. Observations included any change in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous system, somatomotor activity and behaviour pattern. Attention was directed to observations of mortality, tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. If mortality is observed in 2/3 or 3/3 animals, then the dose administered is assigned as toxic dose (LD_{50}), mortality observed in one animal out of three animals, then the same dose was repeated again to confirm the toxic effect. If the mortality is not observed in the given dose, then the treatment proceeds with the next dose to another group making the observations as mentioned.

5.2.7 Pharmacological evaluation[79]

5.2.7.1 Screening of anti-inflammatory activity

Experimental methods:

Anti-inflammatory activity was assessed by carrageenan induced paw oedema method and cotton pellet induced granuloma method.

5.2.7.1.1 Carrageenan induced paw edema in rats.[80][81]

Experimental design:

Wistar albino rats weighed around 150-250g were used for the study. 24 rats were divided into 6 groups of 6 rats each for various treatments.

Group I : Served as negative control which received carrageenan 1%w/v (0.1mL, S.C)

Group II : Served as positive control which received Diclofenac (20 mg/kg, i.p)

Group III : Served as test group which received test compound (**S5i)** (62.5 mg/kg p.o).

Group IV : Served as test group which received test compound (**S5i)** (125mg/kg p.o).

Group V : Served as test group which received test compound (**S5m)** (62.5mg/kg p.o).

Group VI : Served as test group which received test compound (**S5m)** (125mg/kg p.o).

Procedure

Anti-inflammatory activity was determined according to the method of Winter et al. The rats were divided into groups of 6 animals each. The different groups were treated withcompound **S5i** and **S5m** (62.5 and 125 mg/kg p.o) and Diclofenac (20 mg/kg i.p). After 30 min, the rats were challenged with subcutaneous injection of 0.1 mL of 1% w/v solution of carrageenan into the sub plantar region of left paw. The paw was marked with ink at the level of lateral malleolus and immersed in mercury up to the mark. The paw volume was measured at 0,1,2,3,4 and 5h after carrageenan injection using a volume displacement method using Plethysmometer by immersing the paw in mercury cell. The inflammation in paw volume is calculated as percentage compared with the basal volume. The difference of average values between treated animals and control group is calculated for each time interval and evaluated statistically. The percentage inhibition of paw edema was calculated by using the following formula;

$$\textbf{Percentage of edema inhibition} = (Vc - Vt/Vc) \times 100$$

Vc- Volume of paw edema in control group.

Vt- Volume of paw edema in treated groups.

5.2.7.1.2 Cotton pellet induced granuloma method.[82][83]

Experimental design:

Wistar albino rats weighed around 150-250g were used for the study. 24 rats were divided into 4 groups of 6 rats each for various treatments.

Group I : Served as negative control which received 1% CMC (0.1mL/10g p.o)

Group II : Served as positive control which received Diclofenac (20 mg/kg, i.p)

Group III : Served as test group which received test compound (**S5i)** (62.5 mg/kg p.o).

Group IV : Served as test group which received test compound (**S5i)** (125 mg/kg p.o).

Group V : Served as test group which received test compound (**S5m)** (62.5 mg/kg p.o).

Group VI : Served as test group which received test compound (**S5m)** (125 mg/kg p.o).

Procedure

The method of Winter and Porter was used to study chronic inflammation. Animals were divided into four groups of six each, anaesthetized with ether .The axillary skin was shaved and disinfected with 70% ethanol. An incision wasmade and by a blunt forceps subcutaneous tunnels were formed and a sterilized cotton pellet (20±5mg) was placed in axilla. The test compound, diclofenac and vehicle were administered for 7 consecutive days starting from day of cotton implantation. At 8^{th} day rats were anaesthetized again and cotton were removed surgically and freed from extraneous tissue. The pellets were weighed immediately for wet weight. Then pellets were dried in an incubator at 60°C until weight become constant. The net dry weight of each cotton pellet was determined (after subtracting the initial weight of the cotton pellet). The mean weights of the cotton pellet of the control and treatment groups were calculated. The percentage of anti-inflammatory activity was calculated by inhibition of increase in the weight of the cotton pellet was estimated.The percentage inhibition increase in the weight of the cotton pellets was calculated by:

$$\text{Percentage inhibition} = \frac{Wc - Wt}{Wc} \times 100$$

Where, Wc = Pellet weight of the control group

Wt = Pellet weight of the drug treated group

5.2.7.2 Screening of analgesic activity

Experimental methods

Analgesic activity was assessed by hot plate and acetic acid induced writhing method.

5.2.7.2.1 Hot plate method. [84][85]

Experimental design:

Swiss Albino mice weighing around 20-25g were used for this study. They were divided into 4 groups of 6 mice each.

Group I : Served as positive control which received morphine (5mg/kg, i.p).

Group II : Served as test group which received test compound (**S5i)**(62.5 mg/kg p.o).

Group III : Served as test group which received test compound (**S5i)** (125 mg/kg p.o).

Group IV : Served as test group which received test compound (**S5m)**(62.5 mg/kg p.o).

Group V : Served as test group which received test compound (**S5m)** (125 mg/kg p.o).

Procedure

Evaluation of analgesic activity of test compound was carried out using hot plate method. Mice were screened by placing them on a hot plate (Medicraft analgesiometer Mark III, Medicraft electro medicals (P) Ltd; Lucknow, India) maintained at 55± 1^0C and the reaction time in seconds for hind paw licking or jumping were recorded. Only mice which reacted within 15 sec and which did not show large variation when tested on four separated occasions, each 15 min apart, were used in this study. Morphine (5mg/kg, s.c) was used as standard.

After administration of test drugs and standard drug, the animals are placed on the hot plate and the time until either licking or jumping occurs is recorded by a stop-watch. The latency was recorded before and after 0.15,30,45 and 90min. The latency period for hind paw licking or jumping on the heated plate of analgesiometer was taken as reaction time. The prolongation of the latency times comparing the values before and after administration of the test compounds was used for statistical comparison.

5.2.7.2.2 Acetic acid induced writhing method

Experimental design:

Swiss Albino mice weighing around 20-25g were used for this study. They were divided into 4 groups of 6 mice each.

Group I : Served as negative control which received acetic acid (0.1mL/10g i.p)

Group II : Served as positive control which received diclofenac (20 mg/kg, i.p)

Group III : Served as test group which received test compound (**S5i)**(62.5 mg/kg p.o).

Group IV : Served as test group which received test compound (**S5i)** (125 mg/kg p.o).

Group V : Served as test group which received test compound (**S5m)**(62.5 mg/kg p.o).

Group VI : Served as test group which received test compound (**S5m)** (125 mg/kg p.o).

Procedure:

Animals were divided into five groups of six each. The control group received 0.1mL 1% CMC solution p.o. The test group was treated with 62.5 and 125 mg/k.p.o of compounds **S5i** and **S5m**. The standard group received diclofenac at a dose of 20mg/kg/i.p. After 30min of drug administration 1% acetic acid was given to each mouse at the dose of 0.1mL/10g body weight i.p. Number of writhing was counted for 15 min. The percentage inhibition of writhing offered by the test samples to the animals was calculated and compared with the control.

For all the tests, statistical analysis was performed using one way ANOVA followed by Dunnet test in graphpad prism 5 software.

5.2.7.3 Ulcerogenic effect[86]

Experimental design

Wistar albino rats weighed around 150-250g were used for the study. Rats were divided into 6 groups of 6 rats each for various treatments.

Group I : Served as negative control which received CMC(0.1mL/10g p.o)

Group II : Served as positive control which received indomethacin (20 mg/kg, p.o)

Group III : Served as test group which received test compound (**S5i)**(125 mg/kg p.o).

Group IV : Served as test group which received test compound (**S5i)** (62.5mg/kg p.o).

Group V : Served as test group which received test compound (**S5m)** (62.5 mg/kg p.o).

Group VI : Served as test group which received test compound (**S5m)** (125 mg/kg p.o)

Procedure

The animals were starved overnight (water ad libitum) prior to drug administration. Doses which showed action in the anti-inflammatory tests in rats were chosen(62.5mg/kg and 125 mg/kg). The animals are sacrificed 5h post drug administration. Stomachs were removed and placed on saline-soaked filter paper until inspection. A longitudinal incision along the greater curvature was made with fine scissors. The stomach was inverted over the index finger and the presence or absence of

gastric irritation was determined. The presence of a single or of multiple lesions (erosion, ulcer or perforation) was considered to be positive. The number of ulcers and the occurrence of hyperaemia were noted.

5.2.8Anti-bacterial activity[87]

5.2.8.1 Zone of inhibition

Preparation of inoculums

The inoculums for the experiment were prepared in fresh Nutrient broth from the preserved slant culture. The turbidity of the culture can be adjusted by the addition of broth or sterile saline (if it is excessive) or by further incubation to get the required turbidity, And the newly prepared inoculums were standardized by adjusting the turbidity of the culture to that of McFarland standards.

Preparation of sterile swabs

Cotton wool swab on wooden applicator or plastics were prepared and sterilized by autoclaving or by dry heat (only for the wooden swabs). It was sterilized by packing the swabs in culture tubes.

Sterilization of forceps

Forceps was sterilized by dipping in alcohol and burning off the alcohol.

Procedure

The standardized inoculums were inoculated in the sterilized plates prepared earlier (aseptically) by dipping a sterile loop into the inoculums The excess inoculum was removed by passing and rotating the swab firmLy against the side of the culture tube above the level of the liquid. Finally the swab was all over the surface of the medium 3 times rotating the plate through an angle of 60º after each application, further moving the swab round the edges of the plate. The inoculated plates were kept closed and were allowed to dry at room temperature. The sterile discs (Himedia lab.Pvt.Ltd.,Mumbai) soaked in the test solutions were placed over the solidified media by using a sterile forceps. Ciprofloxacin (10µg) discs were used as standard. The plates were incubated at 37ºC for 24 h and zone of inhibition of each sample was measured and noted

Table 5.3Bacterial strain used for the study with NCIM

Sl no.	Organism	Strain	NCIM
1	Gram $^{+ve}$ bacteria	*Bacillus subtilis*	*2063*
2		*Staphylococcus aureus*	2079
3	Gram $^{-ve}$ bacteria	*Vibrio cholerae*	*1738*
4		*Salmonella Paratyphi*	2501
5		*Escherichia coli*	2065
6		*Klebsilla Pneumoniae*	2707
7		*Pseudomonas Aeuroginosa*	2200

5.2.8.2 Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) is the lowest concentration of a chemical that prevents the visible growth of the organism.ie, the lowest concentration at which it has bacteriostatic activity.

Preparation of test drug:

The selected test drugs were prepared in DMSO at a concentration 100µg/mL

Preparation of inoculum:

*Bacillus subtilis*and *Pseudomonas aeuroginosa* were the two strains of organisms selected for the study. Overnight culture are grown at 37^0C Kirby- Bauer procedure and diluted to Muller Hinton Broth. This overnight culture was diluted to 10^{-2}.

Inoculation

The sterile tubes were labelled 1-8 and 8^{th} tube was taken as control.1mL of Muller Hinton Broth was transferred to all tubes. 1mL of drug solution was added to 1^{st} tube and mixed well.From the 1^{st} tube 1mL of solution was transferred to the 2^{nd} tube and was repeated up to 7^{th} tube. From the final 2 mL volume of 7^{th} tube 1mL of solution was pipette out.0.01mL of culture was added to all the test tubes and all the tubes were incubated at 37^0C for 18-24hrs.After incubation the turbidity was observed visually. The highest dilution without growth is the minimal inhibitory concentration.

CHAPTER 6

RESULT AND DISCUSSION (DOCKING AND CHEMISTRY)

Based on the literature review of quinolin-2-ones, compounds were designed and docked with COX-2 protein. The general structure for the designed synthesised compounds are as follows.

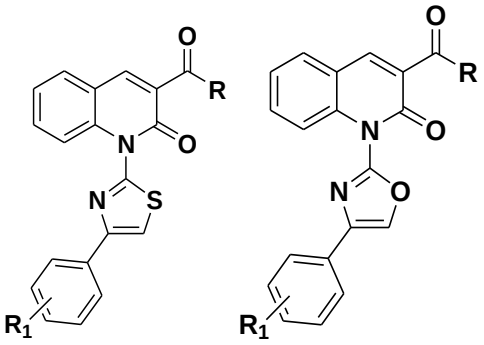


Figure 6.1 General structures of synthesised compounds

The X-Ray structure of COX-2 (PDB ID 1CX2)[88] in complex with selective inhibitor SC-58 was obtained from RCSB protein bank and used as template for flexible ligand docking with AutoDock 4.2. [89]The enzyme was considered rigid fixed in their crystallographic conformation and ligand in the binding was considered fully flexible. Prior to the calculations, a monomer was separated and ligand molecule was removed from the template structure and ligand molecule was removed from the template structure and hydrogen atoms were added using Molecular operating environment (MOE 2013.08)[90]. Atomic partial charges were added with AutoDock Tools[91]. Three dimensional grids for calculating the binding energy of the inhibitors were computed within a grid in a grid box of 60x60x60 points with a spacing of 0.37A, centred on the co-crystallised ligand. The inhibitors designed for COX-2 were docked into the active site of the enzyme using Lamarckian Genetic Algorithm in AutoDock 4.2. 50 independent docking runs for the ligands molecule were run into binding site of COX-2. The other parameters for search optimisation and scoring function were set to their default values. Putative compound binding modes were selected by visual inspection of high scoring docking poses.

The crystallographic structure of COX-2 is as shown in the Figure 6.2.Docking stimulations with AutoDock 4.2 successfully reproduced the crystallographic pose of COX-2. The ligand **S5i** in the COX-2 binding pocket is stabilised within the binding site through H-bond interactions with residues Met 522, Val 523, Gly526 and electronic interactions with residues His90, Thr94, Val349, Ser353. The ligand **S5m** in the COX-2 is stabilised within the binding site through H-

bond interactions with residues Met 522, Val 523, Ala 527, Ser 353 and electronic interactions with amino acid residues His90, Ser 353, Arg 513, Ala 516, Gly 52.

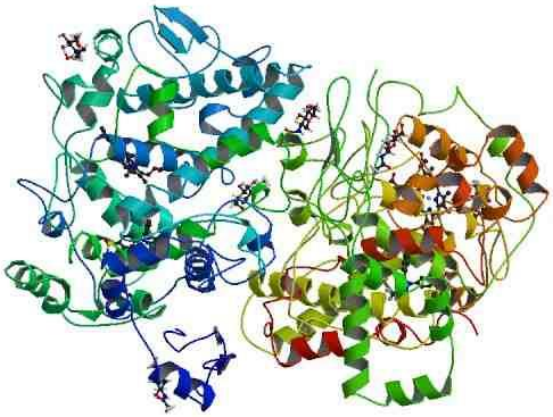


Figure 6.2 Crystallographic structure of COX-2 (PDB ID: 1CX2)

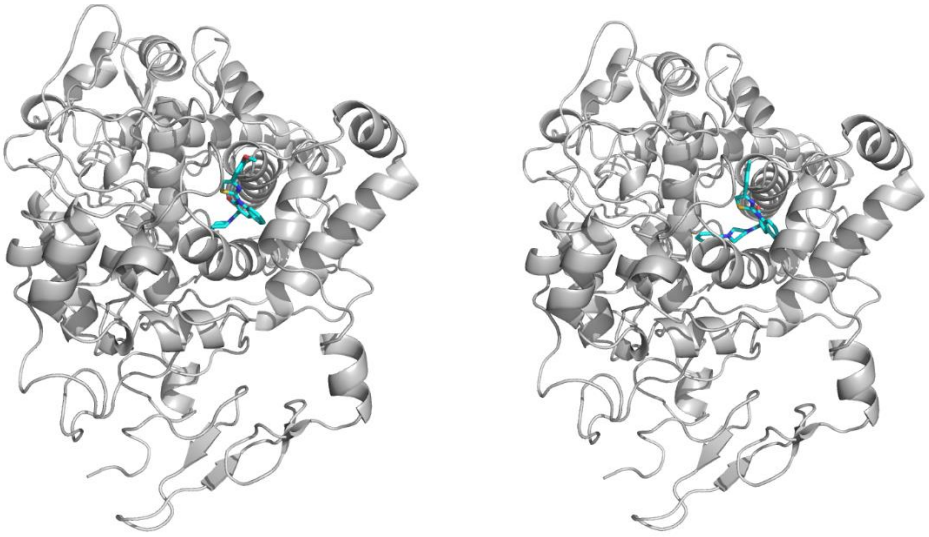


Figure 6.3 Binding pocket of compound S5i and S5m in COX-2

Table 6.1 Docking score of the designed ligands

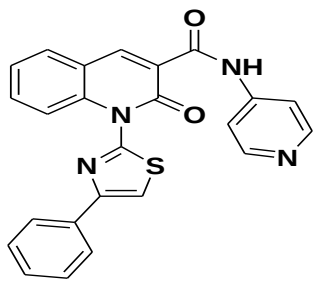
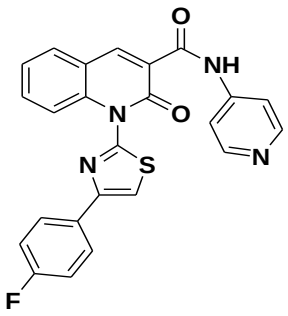
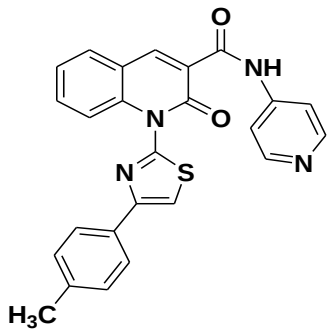
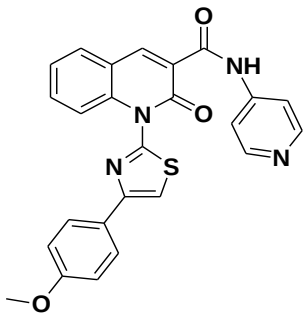
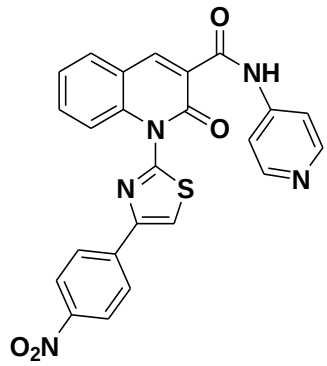
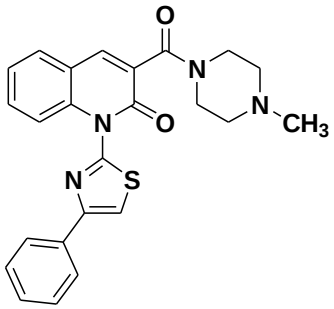
Compound code and structure	Docking score	Compound code and structure	Docking score
S5a 	-7.21	S5b 	-5.49
S5c 	-5.91	**S5d** 	-7.32
S5e 	-6.07	**S5f** 	- 9.27

Table 6.1a Docking scores of designed ligands

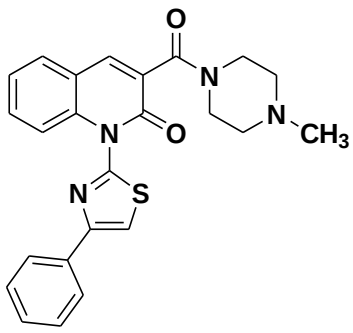
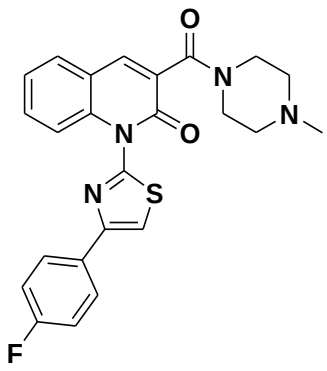
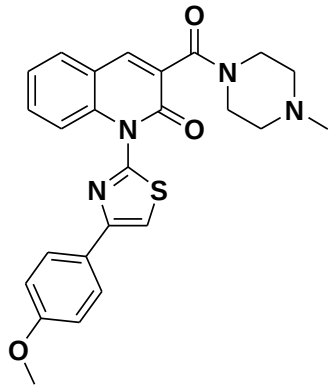
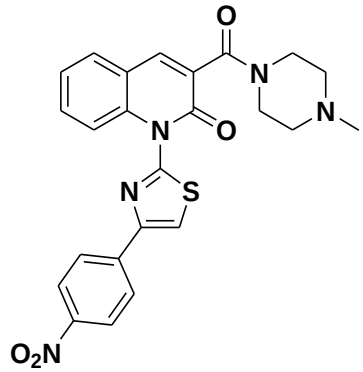
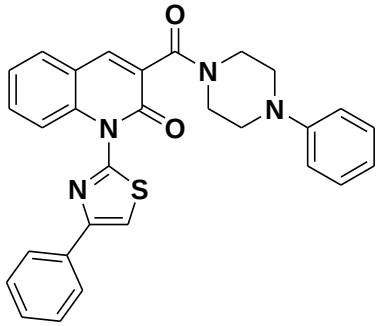
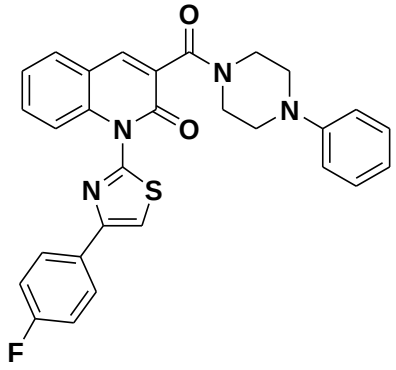
Compound code and Structure	Docking score	Compound code and structure	Docking score
S5g 	-5.40	S5h 	-6.61
S5i 	-6.00	S5j 	-7.98
S5k 	-1.38	S5l 	-0.80

Table 6.1b Docking score of designed ligands

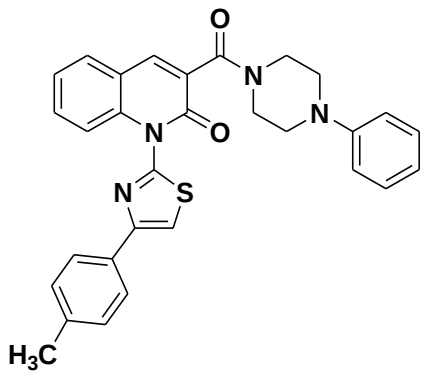
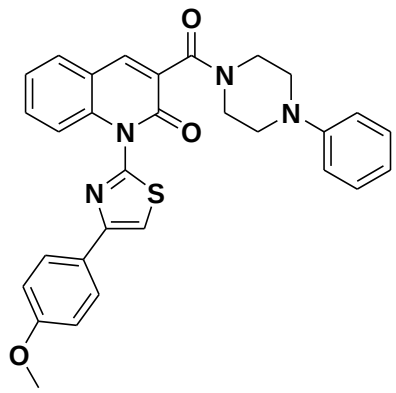
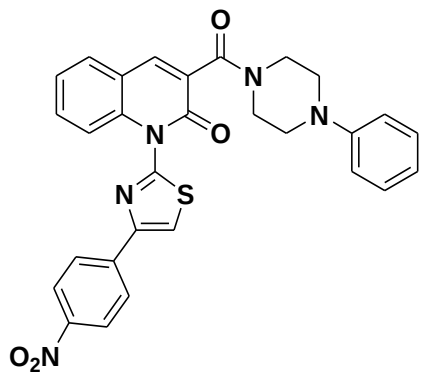
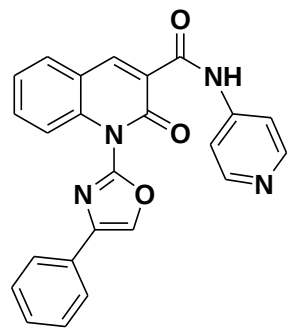
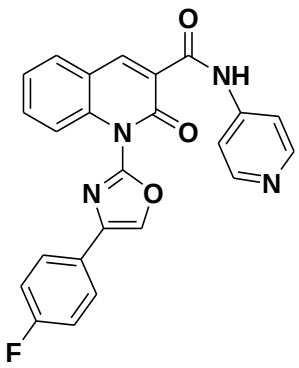
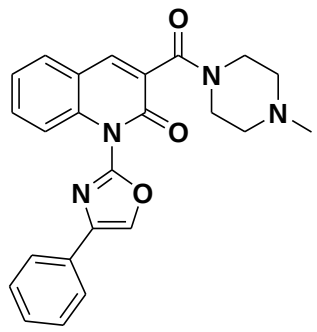
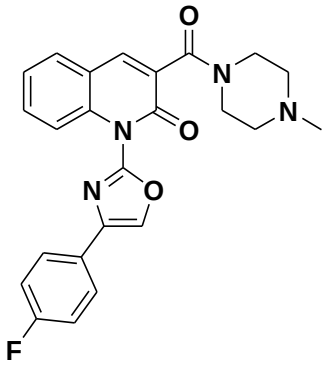
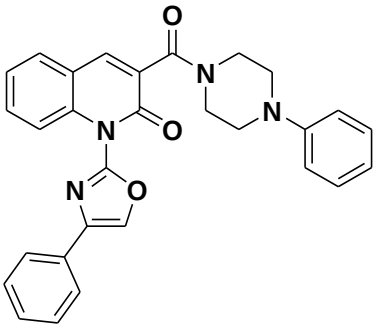
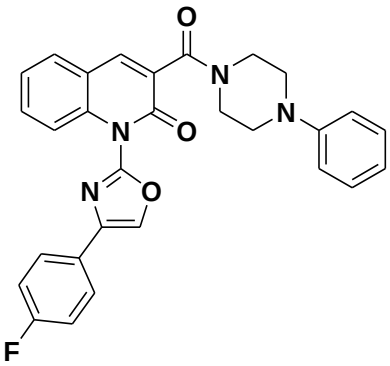
Compound code and structure	Docking score	Compound code and structure	Docking score
S5m 	-4.15	S5n 	7.72
S5o 	7.71	S6a 	-6.82
S6b 	-8.41	S6c 	-8.99

Table 6.1c Docking score of designed ligands

Compound code and structure	Docking score	Compound code and structure	Docking score
S6d 	-8.49	S6e 	2.35
S6f 	2.03		

Taking into consideration that coumarins are well known scaffolds for the anti-oxidant analgesic and anti-inflammatory activity, quinolin-2-one analogues which are isosteric to coumarins were designed,docked and synthesised according to the mentioned general procedure

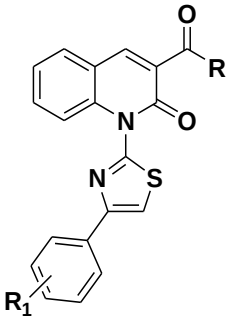
Ester of coumarin derivative (**S2**) was obtained by cyclization of salicylaldehyde and diethyl malonate in presence of pyridine as catalysts. Compound (**S2**) was hydrolysed to give coumarin-3-carboxylic acid, resulting compound containing carboxylic was further activated by using DCC, then convert into various 2-amide derivatives of coumarin by using various amines (**Compounds S4a-c**). The key intermediate 2-oxo-2H-chromene-3- amide derivatives convert into titled compounds (**S5a-o, S6a-f**) by microwave irradiation with primary amine like 2-amino thiazole (**S8a-e**) and 2-amino oxazole (**S9a-b**).

Physical parameters like percentage yield, molecular weight, molecular formula, melting point, Clog-P and R_f value of the synthesized compounds were determined. The titled compounds were in moderate yields from 20-70 percentages. Melting point of all newly synthesized compounds were determined and are incorrect. The purity for all the synthesized compounds was confirmed by a single spot in TLC. Clog P values of all the newly synthesized compounds were determined by using Chem draw 12.

IR spectra of synthesized compounds showed characteristic absorption band around 3690-3600 cm^{-1} (NH in hetero aromatic ring), 3000-3100 cm^{-1} (C-H stretching), 1750-1700 cm^{-1} (C=O stretching), 1690-1650 cm^{-1} (CN stretching), 1570-1500 cm^{-1} (amide C=O stretching), 800-600 cm^{-1} (substituted benzene).

^{1}H NMR spectra of the synthesized compound (**S5i**)showed peaks at δ 7.8-6 (m, Ar-H); 3.8 (d, 3H,-OCH_3); 2.3 (s, 3H, CH_3 Piperazine); 2.3 (s, 3H, CH_3 Piperazine); 1.4 (d, 8H, CH_2-Piperazine) and compound (**S5m**) showed peaks at δ 8.2-6.5 (m, Ar-H); 3.8 (s, 3H, -CH_3 phenyl); 1.4 (d, 8H, CH_2-Piperazine). Mass spectra showed the desired M + 1 peak.

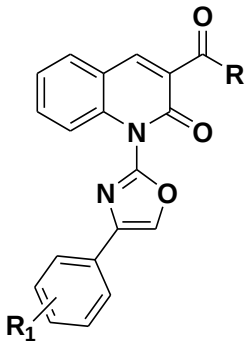
6.1 Physicochemical data



Compounds S5a-o

Table 6.2 Physicochemical data of synthesized compounds(S5a-o)

Cpd Code	R	R_1	Mol. Formula	Mol. Wt	% Yield	M.P (° C)	R_f Value	C logP
S5a	4-aminopyridine	-H	$C_{24}H_{16}N_4O_2S$	424.1	70	168	0.7	3.367
S5b	4-amino pyridine	-F	$C_{24}H_{15}FN_4O_2S$	442.09	62	173	0.75	3.510
S5c	4-amino pyridine	$-CH_3$	$C_{25}H_{18}N_4O_2S$	438.12	69	171	0.80	3.86
S5d	4-amino pyridine	$-OCH_3$	$C_{25}H_{18}N_4O_3S$	456.51	54	145	0.81	3.38
S5e	4-amino pyridine	$-NO_2$	$C_{24}H_{15}N_5O_4S$	469.08	58	141	0.85	
S5f	N-methyl piperazine	-H	$C_{24}H_{22}N_4O_2S$	430.52	44	183	0.95	3.53
S5g	N-methyl piperazine	-F	$C_{24}H_{21}FN_4O_2S$	448.51	40	170	0.90	3.67
S5h	N-methyl piperazine	$-CH_3$	$C_{25}H_{24}N_4O_2S$	444.54	38	186	0.81	4.03
S5i	N-methyl piperazine	$-OCH_3$	$C_{25}H_{24}N_4O_3S$	460.54	30	162	0.62	3.42
S5j	N-methyl piperazine	$-NO_2$	$C_{24}H_{21}N_5O_4S$	475.51	34	169	0.68	
S5k	N-phenyl piperazine	-H	$C_{29}H_{24}N_4O_2S$	492.59	32	183	0.91	5.98
S5l	N-phenyl piperazine	-F	$C_{29}H_{23}FN_4O_2S$	510.58	31	168	0.76	4.29
S5m	N-phenyl piperazine	$-CH_3$	$C_{30}H_{26}N_4O_2S$	506.61	28	172	0.71	4.64
S5n	N-phenyl piperazine	$-OCH_3$	$C_{30}H_{26}N_4O_3S$	522.61	24	165	0.69	4.16
S50	N-phenyl piperazine	$-NO_2$	$C_{29}H_{23}N_5O_4S$	537.58	29	159	0.73	



Compounds 6a-f

Table 6.2 Physicochemical data of synthesized compounds (S6a-f)

Cpd code	R	R_1	Mol. Formula	Mol. weight	%yield	M.P (°C)	R_f Value	CLogP
S6a	4-amino pyridine	H	$C_{24}H_{16}N_4O_3$	408.12	24	158	0.90	2.73
S6b	4-amino pyridine	F	$C_{24}H_{15}FN_4O_3$	426.11	28	159	0.76	2.88
S6c	N-methyl piperazine	H	$C_{24}H_{22}N_4O_3$	414.45	20	144	0.90	2.49
S6d	N-methyl piperazine	F	$C_{24}H_{21}FN_4O_3$	432.44	22	140	0.85	3.04
S6e	N-phenyl piperazine	H	$C_{29}H_{24}N_4O_3$	476.52	29	170	0.87	4.57
S6f	N-phenyl piperazine	F	$C_{29}H_{23}FN_4O_3$	494.51	21	160	0.82	4.73

6.2 Spectral analysis

6.2.1 Spectral data of compound S5a-o; S6a-f

Compound S5a **λmax:**258; **IR (cm^{-1}):**3473.65 (amide NH stretching); 1718.26 (C=N stretching);1562.54(C=O stretching);879.38(-C-S stretching); 620.96(mono substituted aromatic ring).

Compound S5b **λmax:**260**; IR (cm^{-1}):**3478.95 (amide NHstretching); 1718.75(C=N stretching);1627.14(C=O stretching);1563.02(C=Cstretching);899.50(C-S stretching); 1040.89(C-F stretching); 769.94 (di-substituted aromatic ring)

Compound S5c **λmax:**281**; IR (cm^{-1}):**3468.83 (amide NHstretching); 2926.45(C-H stretching in aliphatic chain); 1718.26(C=N stretching); 1624.14(C=N stretching); 1562.06(C=C stretching); 769.94(di-substituted aromatic ring); 889.50 (-C-S stretching)

Compounds S5d **λmax:**257**; IR (cm^{-1}):**3473.65 (amide NHstretching); 3057.1 (C-H aromatic stretching); 1717.26 (C=N stretching); 1626.66(C=O stretching); 1562.54 (C=Cstretching); 1046.19(C-O-C); 894.8(-C-S stretching); 764.63(di-substituted aromatic ring)

Compound S5e **λmax**:207**; IR (cm^{-1}):**3468.83(-NH_2 stretching); 3067.23 (C-H stretching in aromatic ring); 1717.78 (C=N stretching); 1626.66 (N=O stretching); 1562.54 (C=C); 764.637 (di-substituted aromatic ring); 1374.03(C-N stretching).

Compound S5f **λmax:**262**; IR(cm^{-1}):**3036.23(aromatic C-H stretching); 2927.87(aliphatic C-H stretching); 1627.14 (C=0 stretching); 1573.63 (C=C stretching); 1311.84 (C-N stretching); 892.39(C-S stretching); 639.76 (mono-substituted aromatic ring)

Compound S5g **λmax**:265; **IR (cm^{-1}) :** 2928.28 (aliphatic C-H stretching); 1627.63(C=O stretching); 1574.59 (C=C stretching); 1312.32(C-N stretching); 892.39(C-S stretching) 1088.17(C-F stretching); 641.69(di-substituted aromatic ring)

Compound S5h **λmax**:209; **IR (cm^{-1}):** 2927.41 (aliphatic C-H); 1627.63 (C=O stretching); 1574.59 (C=C stretching); 1311.84 (C-N Stretching); 866.6(C-S stretching); 641.21 (di-substituted aromatic ring)

Compound S5i **λmax**:245**; IR (cm^{-1}):** 2927.41 (aliphatic C-H stretching); 1627.63(C=0 stretching); 1574.59 (C=C stretching); 1311.84(C-N stretching); 886.6(C-S stretching); 641.21(di-substituted aromatic ring). **^{1}H NMR** (δ values): 7.8-6 (m, Ar-H); 3.8 (d, 3H,-OCH_3); 2.3 (s, 3H, CH_3 Piperazine); 2.3 (s, 3H, CH_3 Piperazine); 1.4 (d, 8H, CH_2-Piperazine).

Compound S5j **λmax**:266**; IR (cm^{-1}) :** 1313.77(C-N in tertiary amine); 3026.73(aromatic C-H stretching); 2927.80 (aliphatic C-H stretching); 1627.63(N=O stretching); 1575.08(C=C stretching); 842.25(C-S stretching); 717.3(di-substituted aromatic ring).

Compound S5k **λmax**:268**; IR(cm^{-1}):**3044.09 (aromatic C-H stretching); 2929.34(aliphatic C-H); 1627.6 (C=N stretching); 1576.52 (C=O);

1448.76(C=C stretching); 1312.3(C-N stretching); 893.36(C-S stretching); 642.17(mono-substituted aromatic ring)

Compound S5l **λmax**:269**; IR(cm^{-1}):**3059.75(aromatic C-H stretching); 2928.36 (aliphatic C-H stretching); 1627.63(C=N); 1576.04(C=O stretching); 1450.21(C=C stretching); 1312.32 (C-N in tertiary amines); 1089.1(C-F stretching)

Compound S5m **λmax:** 262**; IR (cm^{-1}):**3063.3(aromatic C-H stretching); 2927.8(aliphatic C-H stretching); 1627.6(C=Nstretching); 1575.56(C=C stretching); 1311.8(C-N stretching); 892.39(C-S stretching); 642.17(di-substituted aromatic ring). **^{1}H NMR (δ values)**: 8.2-6.5 (m, Ar-H); 3.8 (s, 3H, -CH_3 phenyl); 1.4 (d, 8H, CH_2-Piperazine).

Compound S5n **λmax:**246**; IR(cm^{-1}):**3035.4 (aromatic C-H stretching); 2928.86(aromaticC-H stretching); 1627.6(C=N stretching); 1576.5(C=O stretching); 1438.15(C=C stretching); 1312.32 (C-N stretching); 1048.19(C-O-C); 835.99(C-S stretching); 758.37(di-substituted aromatic ring); 641.6(mono-substituted aromatic ring).

Compound S5o **λmax:**267**; IR (cm^{-1}):**2927.89 (aliphatic C-H stretching); 1627.63(C=N stretching); 1577.5(C=O stretching); 1312.32(C=N stretching); 1088.19(C-O-C); 642 (mono substituted aromatic ring)

Compound S6a **λmax:**268**;IR (cm^{-1}):**3469.7(amide NHstretching); 2827.89(aliphatic C-H stretching); 1718.75 (C=N stretching); 1627.14(C=O stretching); 1573.15(C=C stretching); 1088.14(C-O-C); 695.21(mono substituted aromatic ring); 1311.84(C-N stretching)

Compound S6b **λmax:**263**;IR (cm^{-1}):**3469.7 (amide NHstretching);3057.1(aromatic C-H stretching); 1627.14(C=O stretching); 1508.35(C=C stretching); 1311.84(C-N stretching); 1088.62(C-O-C); 1042.82(C-F stretching); 758.8(di-substituted aromatic ring).

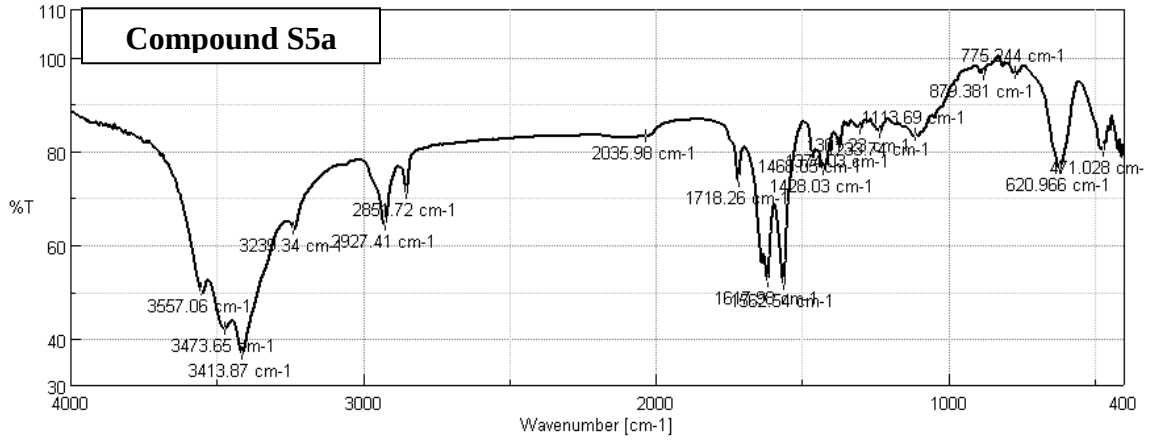
Compound S6c **λmax:**208**; IR (cm^{-1}):**3061.44(aromatic C-H stretching); 1627.14(C=O stretching); 1572.18(C=C stretching); 2927.89(aliphatic C-H stretching);1312.8(C-N stretching); 1098.55(C-O-C); 695.21(di-substituted aromatic ring)

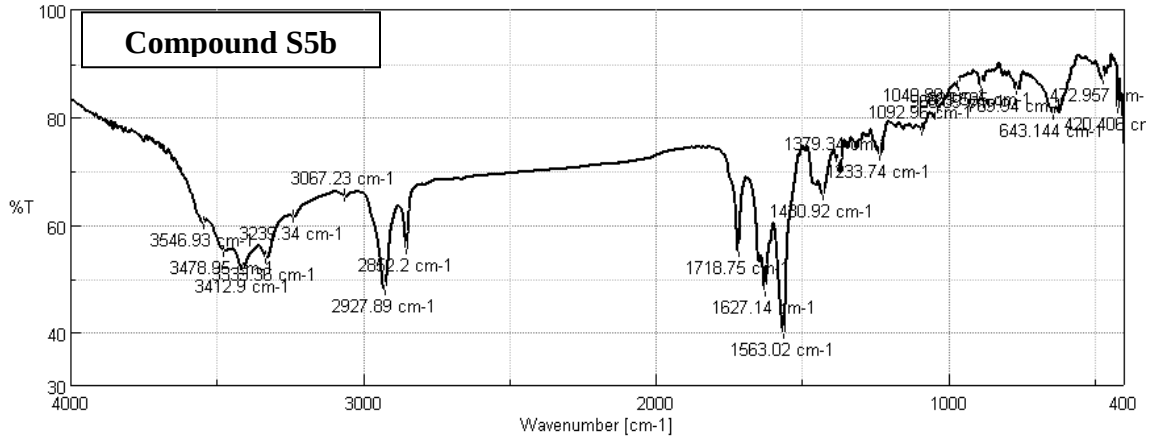
Compound s6d **λmax:**250**; IR (cm^{-1}):**3057.1(aromatic C-H stretching); 2927.8 (aliphatic C-H stretching); 1720.19(C=N stretching); 1627.14(C=O stretching); 1574.59(C=C stretching); 1311.84 (C-N stretching); 1088.62(C-O-C); 1042.82(C-F stretching);757.88(di-substituted aromatic ring).

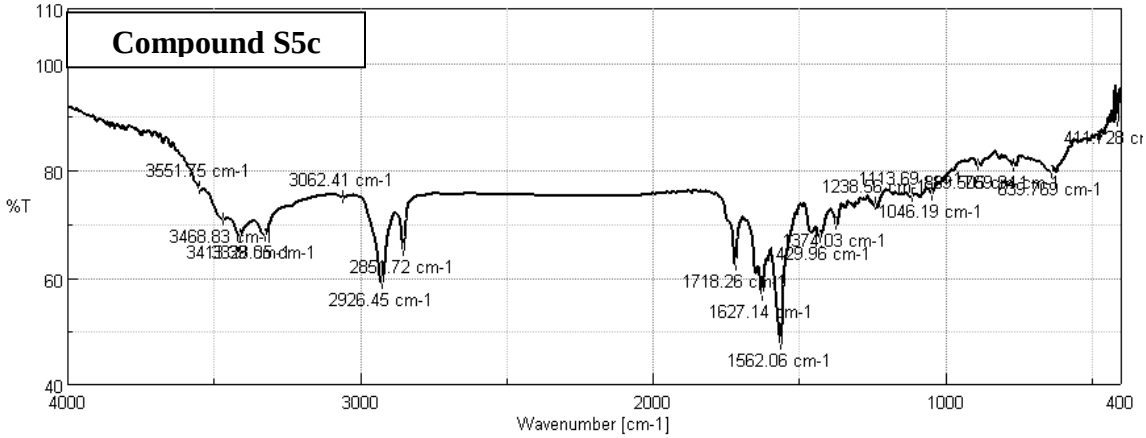
Compound S6e **λmax:**251**; IR (cm^{-1}):**2927.8 (aliphatic C-H stretching); 1627.14 (C=O stretching); 1573.63(C=C stretching);1312.32 (C-Nstretching); 1088.62 (C-O-C); 642.17(mono-substituted aromatic ring)

Compound S6f **λmax:**238**; IR (cm^{-1}):**3070.12(aromatic C-H stretching); 2927.8(aliphatic C-H stretching) ; 1572.66(C=N stretching) ; 1627.14(C=O stretching); 1508.44(C=C stretching); 1311.84 (C-N stretching); 1088.62(C-O-C) ; 1038.48(C-F stretching);765.48(mono-substituted aromatic ring)

6.2.2 IR spectra of synthesised compounds






Compound S5c
%T
Wavenumber [cm-1]
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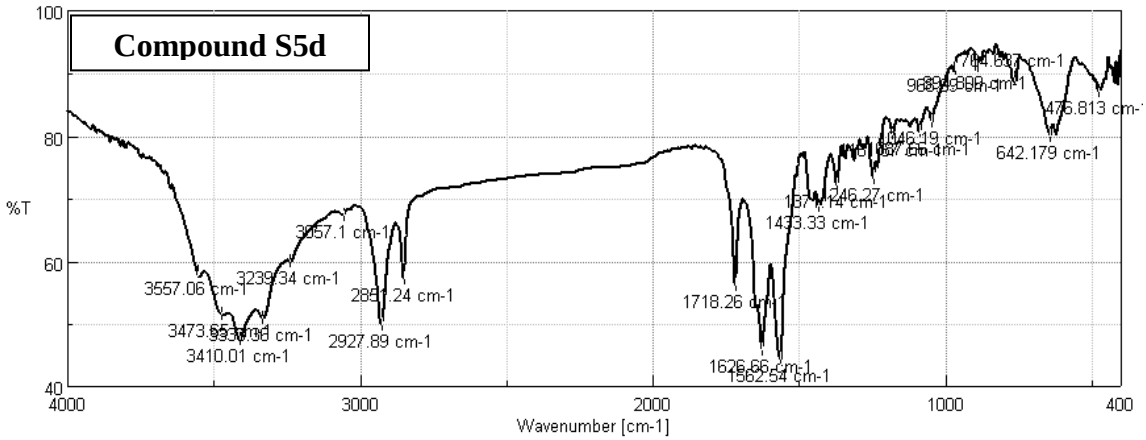
3468.83 cm-1
3062.41 cm-1
2926.45 cm-1
1718.26 cm-1
1627.14 cm-1
1562.06 cm-1
1374.03 cm-1
1046.19 cm-1
839.769 cm-1


Compound S5d
%T
Wavenumber [cm-1]
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3410.01 cm-1
3057.1 cm-1
2927.89 cm-1
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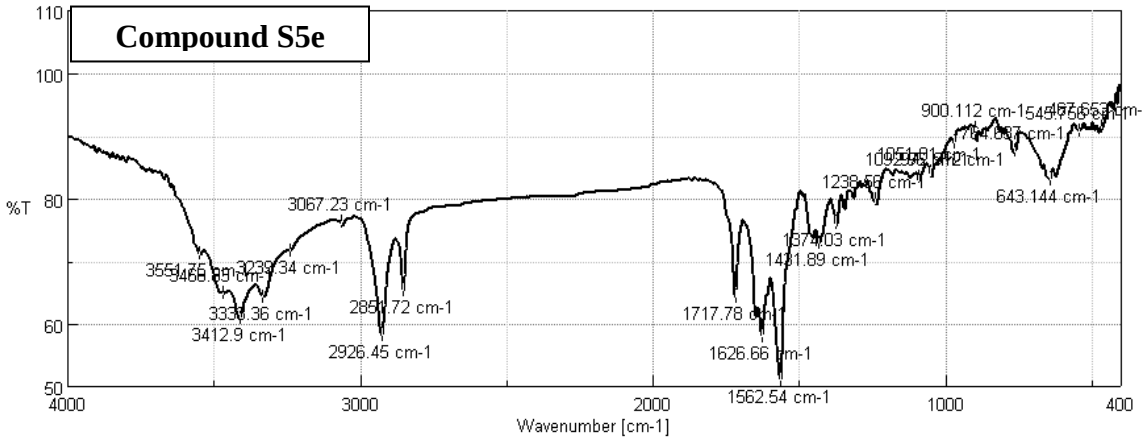
1626.66 cm-1
1562.54 cm-1
1433.33 cm-1
1046.19 cm-1
642.179 cm-1


Compound S5e
%T
Wavenumber [cm-1]
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2926.45 cm-1
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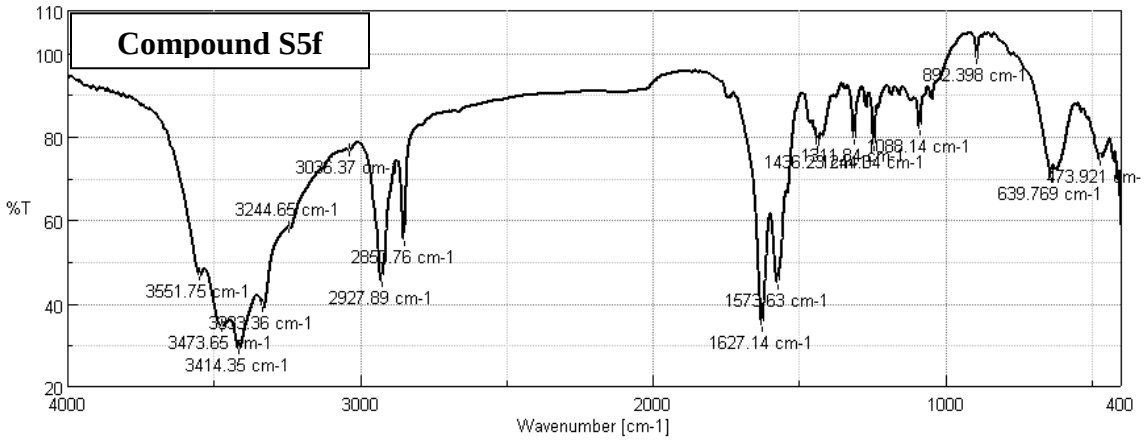
1562.54 cm-1
1431.89 cm-1
1374.03 cm-1
900.112 cm-1
643.144 cm-1


Compound S5f
%T
Wavenumber [cm-1]
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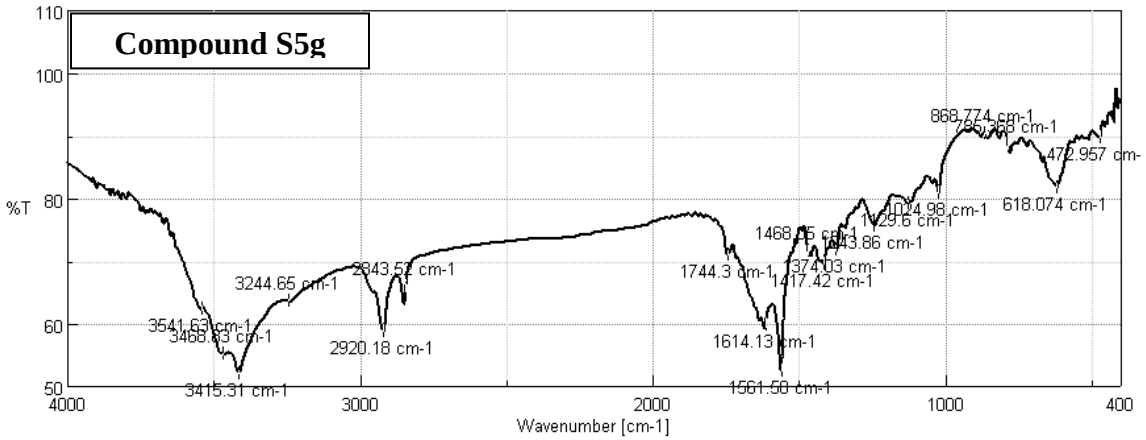
639.769 cm-1


Compound S5g
%T
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3244.65 cm-1
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1744.3 cm-1
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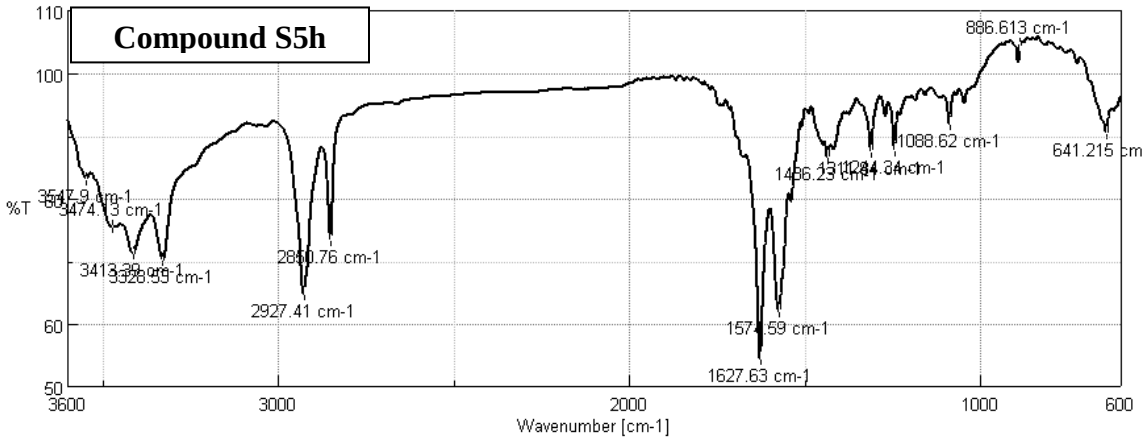
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%T
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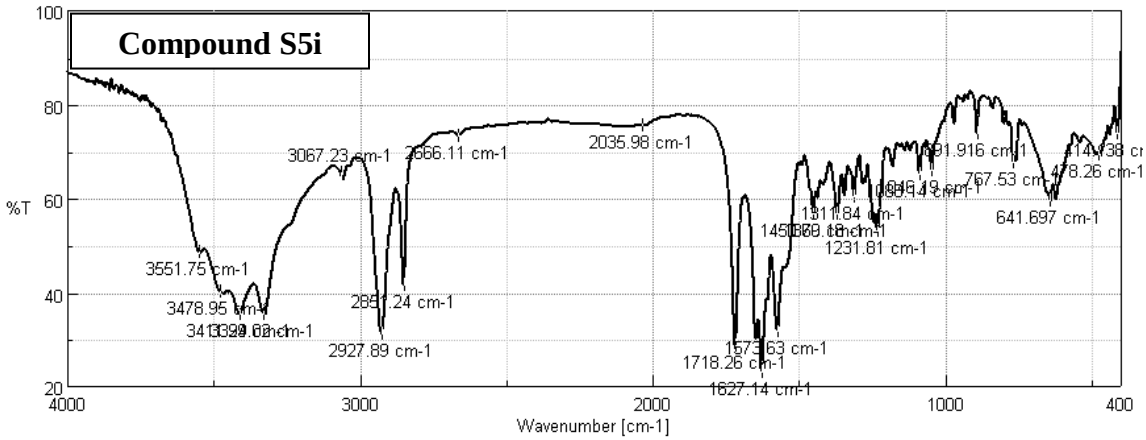
1627.63 cm-1
1486.23 cm-1
1088.62 cm-1
886.613 cm-1
641.215 cm


Compound S5i
%T
Wavenumber [cm-1]
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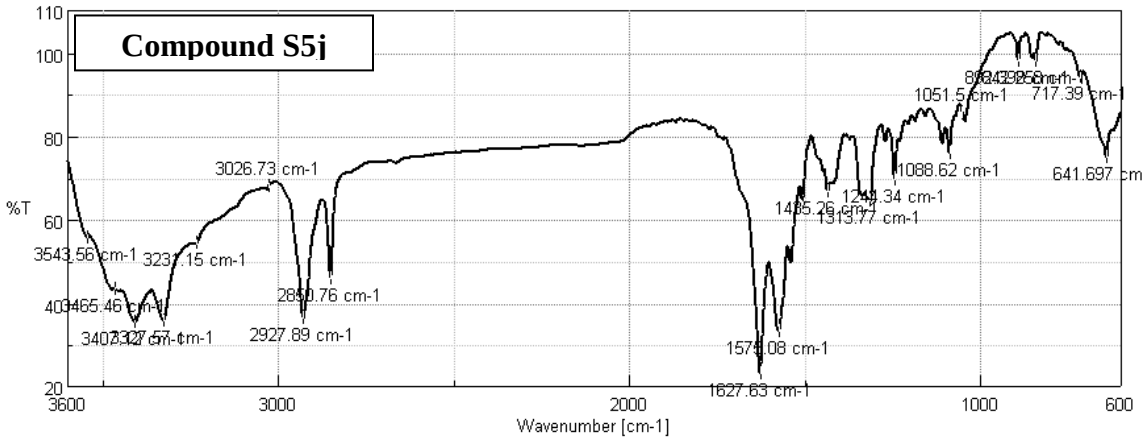
3478.95 cm-1
3067.23 cm-1
2927.89 cm-1
2035.98 cm-1
1718.26 cm-1
1627.14 cm-1
1231.81 cm-1
641.697 cm-1
767.53 cm-1
478.26 cm-1


Compound S5j
%T
Wavenumber [cm-1]
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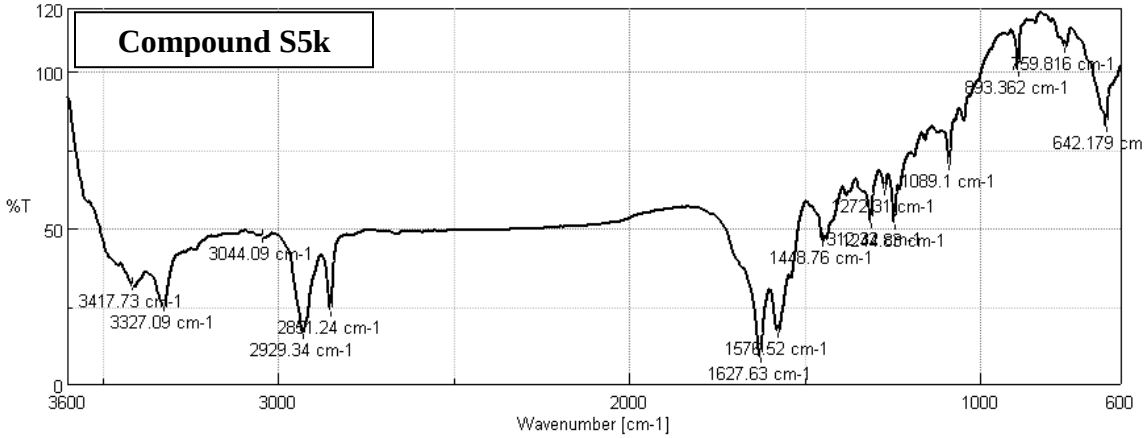
1627.63 cm-1
1313.77 cm-1
1088.62 cm-1
1051.5 cm-1
717.39 cm-1
641.697 cm


Compound S5k
%T
Wavenumber [cm-1]
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3044.09 cm-1
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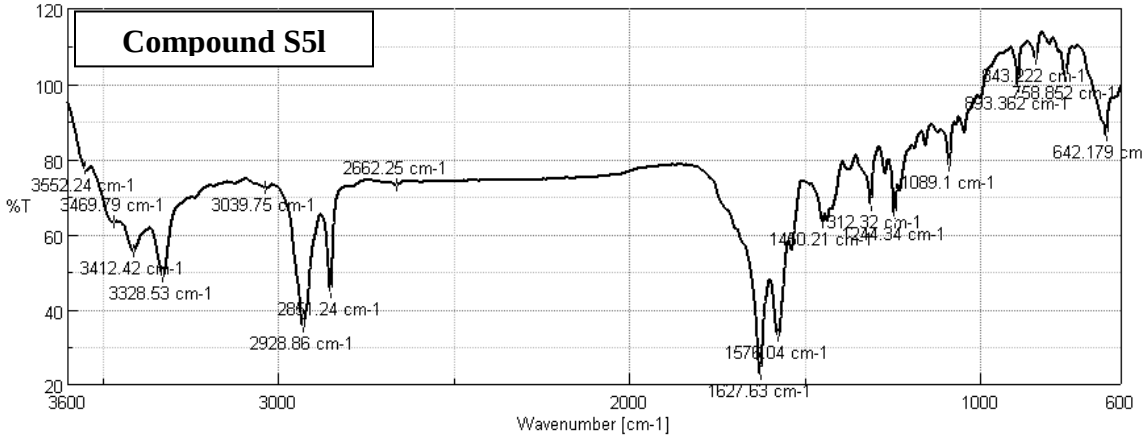
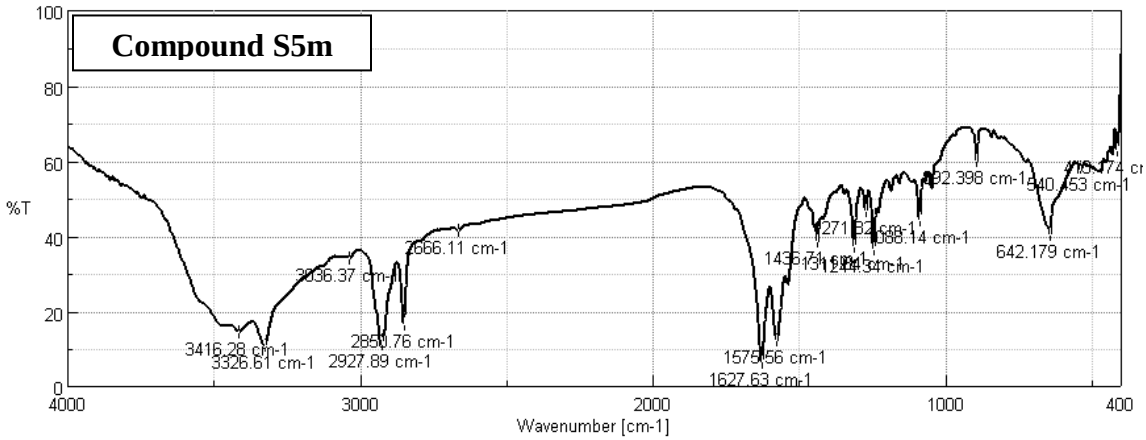
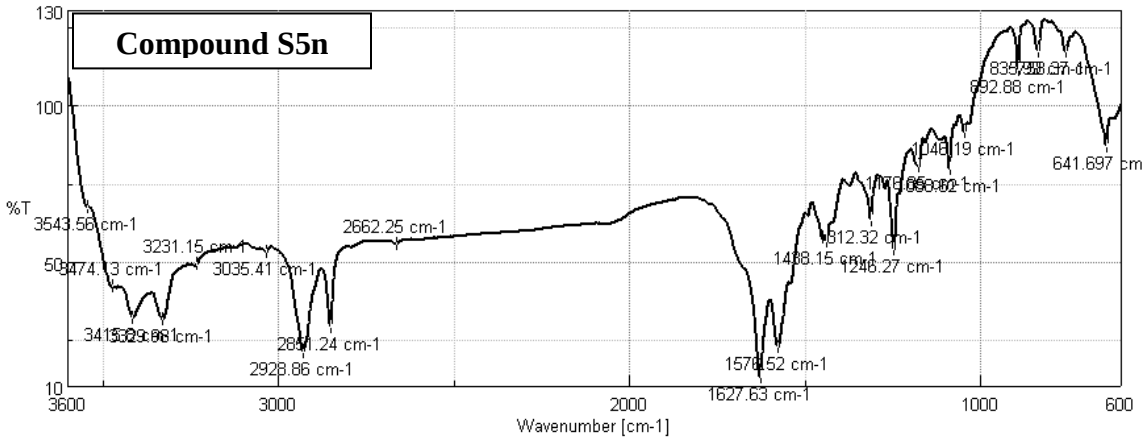
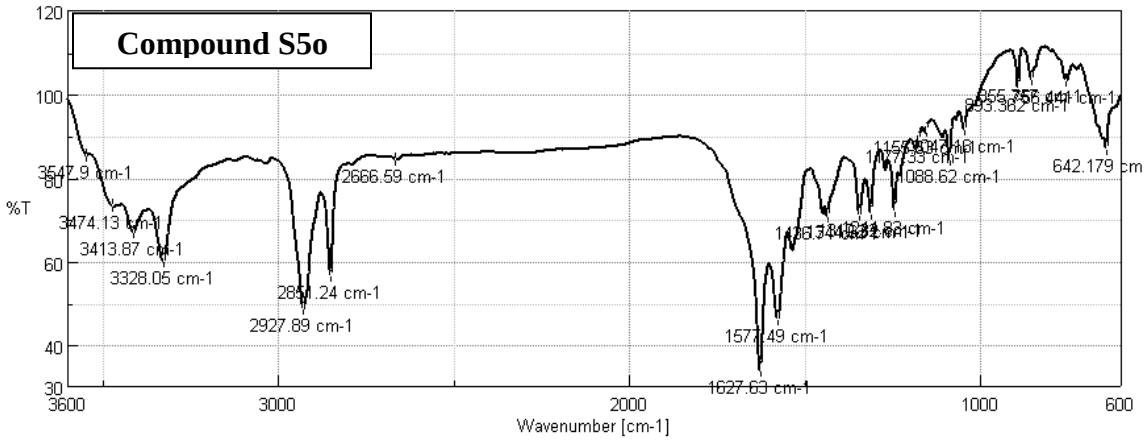
893.362 cm-1
759.816 cm-1
642.179 cm


Compound S5l
%T
3552.24 cm-1
3469.79 cm-1
3412.42 cm-1
3328.53 cm-1
3039.75 cm-1
2928.86 cm-1
2662.25 cm-1
1627.63 cm-1
1460.21 cm-1
1312.32 cm-1
1244.34 cm-1
1089.1 cm-1
993.362 cm-1
758.852 cm-1
642.179 cm
Wavenumber [cm-1]


Compound S5m
%T
3416.28 cm-1
3326.61 cm-1
3036.37 cm-1
2927.89 cm-1
2850.76 cm-1
2666.11 cm-1
1627.63 cm-1
1244.34 cm-1
992.398 cm-1
642.179 cm-1
Wavenumber [cm-1]


Compound S5n
%T
3543.56 cm-1
3231.15 cm-1
3035.41 cm-1
2928.86 cm-1
2662.25 cm-1
1627.63 cm-1
1488.15 cm-1
1312.32 cm-1
1246.27 cm-1
692.88 cm-1
641.697 cm
Wavenumber [cm-1]


Compound S5o
%T
Wavenumber [cm-1]
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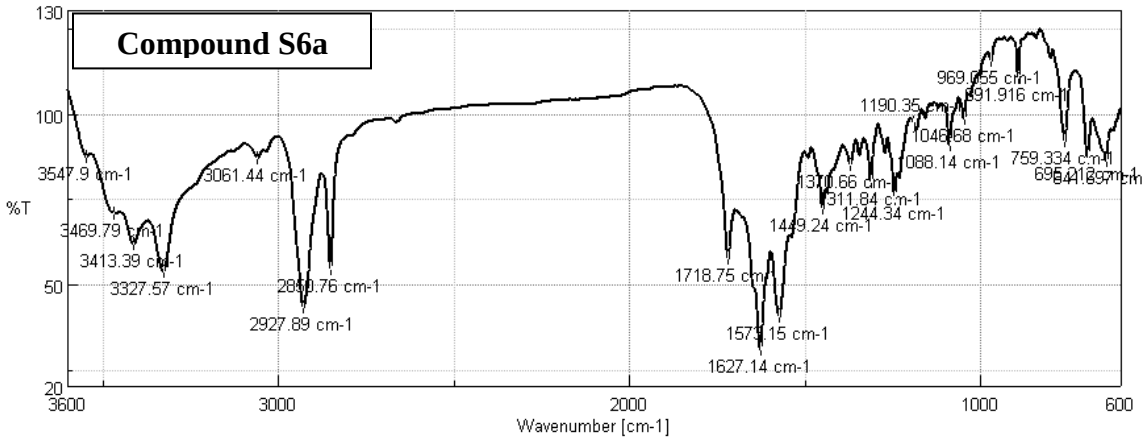
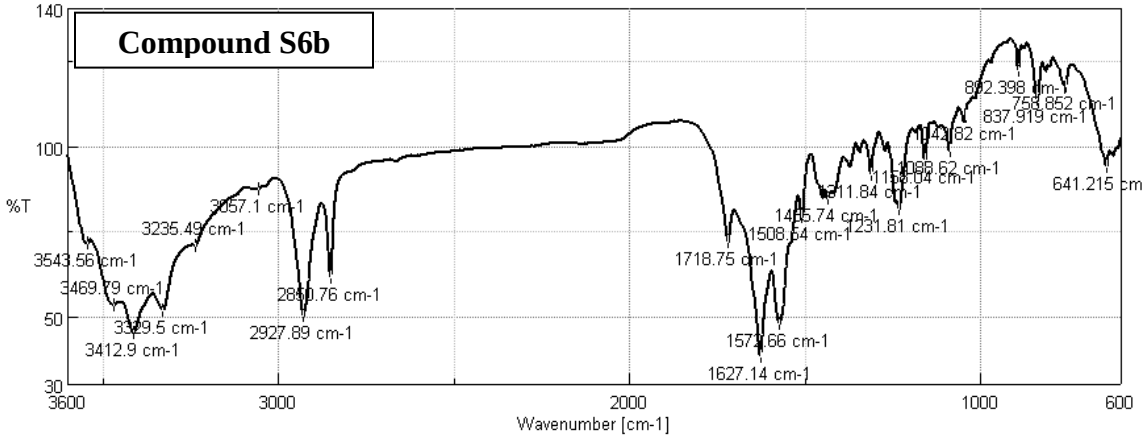
3474.13 cm-1
3413.87 cm-1
3328.05 cm-1
2927.89 cm-1
2666.59 cm-1
1627.63 cm-1
1088.62 cm-1
642.179 cm


Compound S6a
%T
Wavenumber [cm-1]
3547.9 cm-1
3469.79 cm-1
3413.39 cm-1
3327.57 cm-1
3061.44 cm-1
2927.89 cm-1
1718.75 cm-1
1627.14 cm-1
1449.24 cm-1
1370.66 cm-1
1311.84 cm-1
1244.34 cm-1
1190.35 cm-1
1088.14 cm-1
1046.68 cm-1
969.055 cm-1
891.916 cm-1
759.334 cm-1


Compound S6b
%T
Wavenumber [cm-1]
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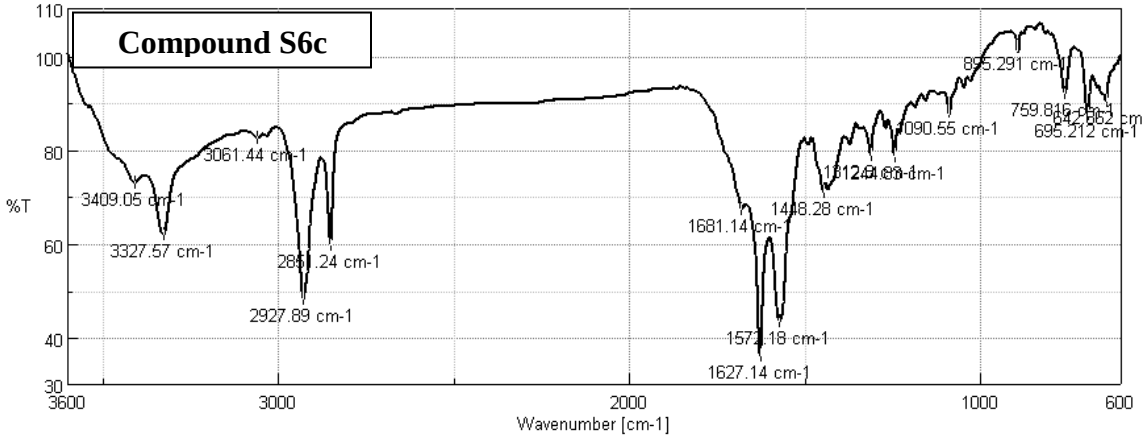
3469.79 cm-1
3412.9 cm-1
3235.49 cm-1
3057.1 cm-1
2927.89 cm-1
1718.75 cm-1
1627.14 cm-1
1508.64 cm-1
1465.74 cm-1
1311.84 cm-1
1231.81 cm-1
1088.62 cm-1
892.398 cm-1
837.919 cm-1
758.852 cm-1
641.215 cm


Compound S6c
%T
Wavenumber [cm-1]
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3061.44 cm-1
2927.89 cm-1
2851.24 cm-1
1681.14 cm-1
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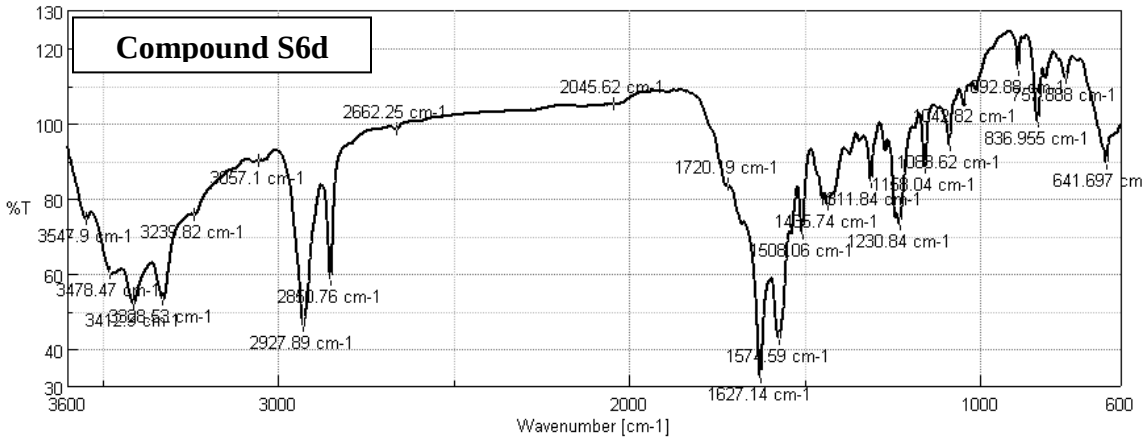
1090.55 cm-1
855.291 cm-1
759.816 cm-1
695.212 cm-1


Compound S6d
%T
Wavenumber [cm-1]
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3057.1 cm-1
2927.89 cm-1
2850.76 cm-1
2662.25 cm-1
2045.62 cm-1
1720.19 cm-1
1627.14 cm-1
1574.59 cm-1
1508.06 cm-1
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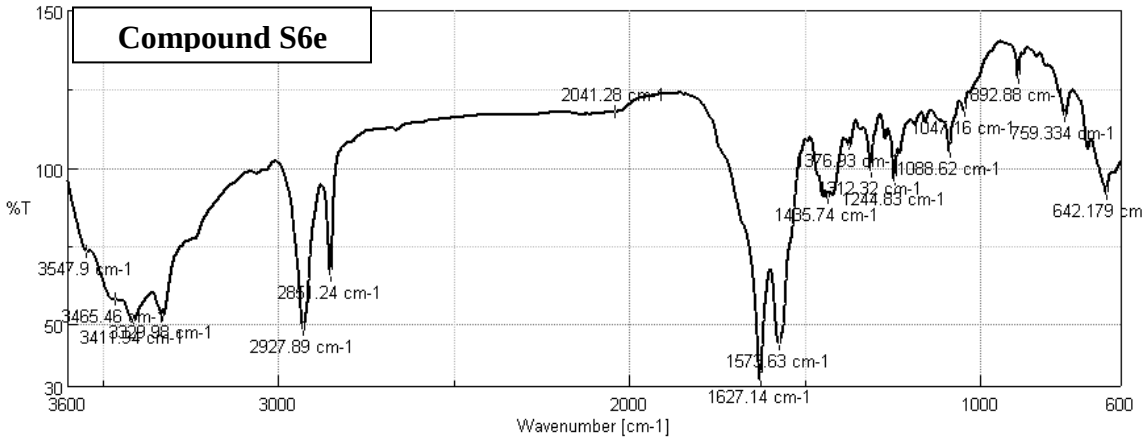
1088.62 cm-1
836.955 cm-1
641.697 cm


Compound S6e
%T
Wavenumber [cm-1]
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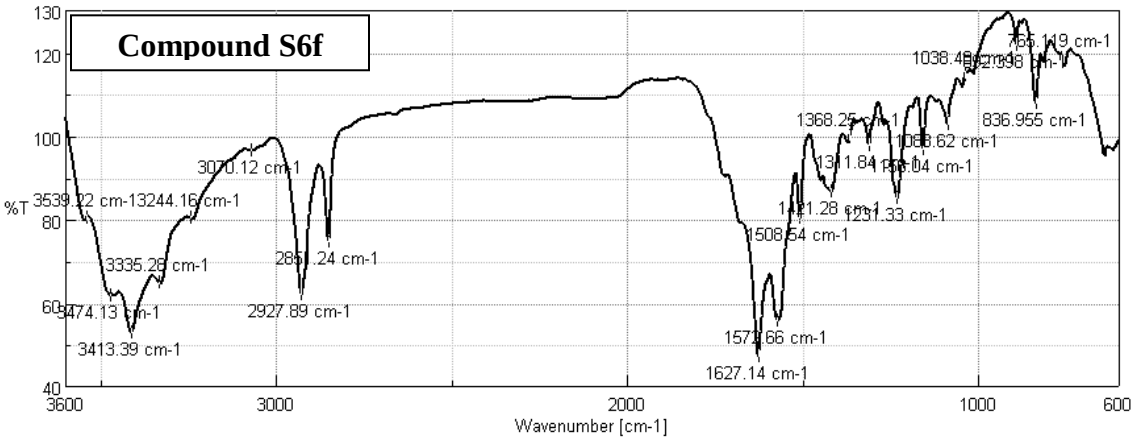
3465.46 cm-1
2927.89 cm-1
2851.24 cm-1
2041.28 cm-1
1627.14 cm-1
1573.63 cm-1
1485.74 cm-1
1244.83 cm-1
1088.62 cm-1
1047.16 cm-1
892.88 cm-1
759.334 cm-1
642.179 cm



6.2.3 NMR spectra of compounds S5i and S5m

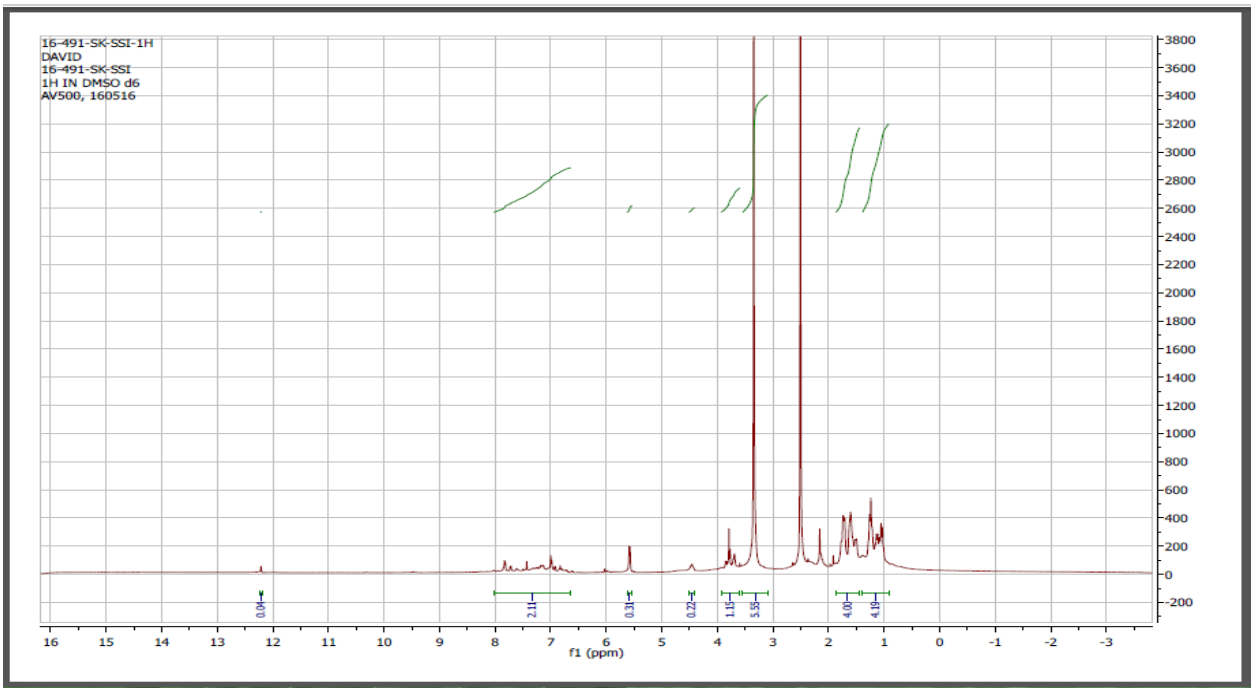


Figure 6.1 NMR Spectra of compound S5i

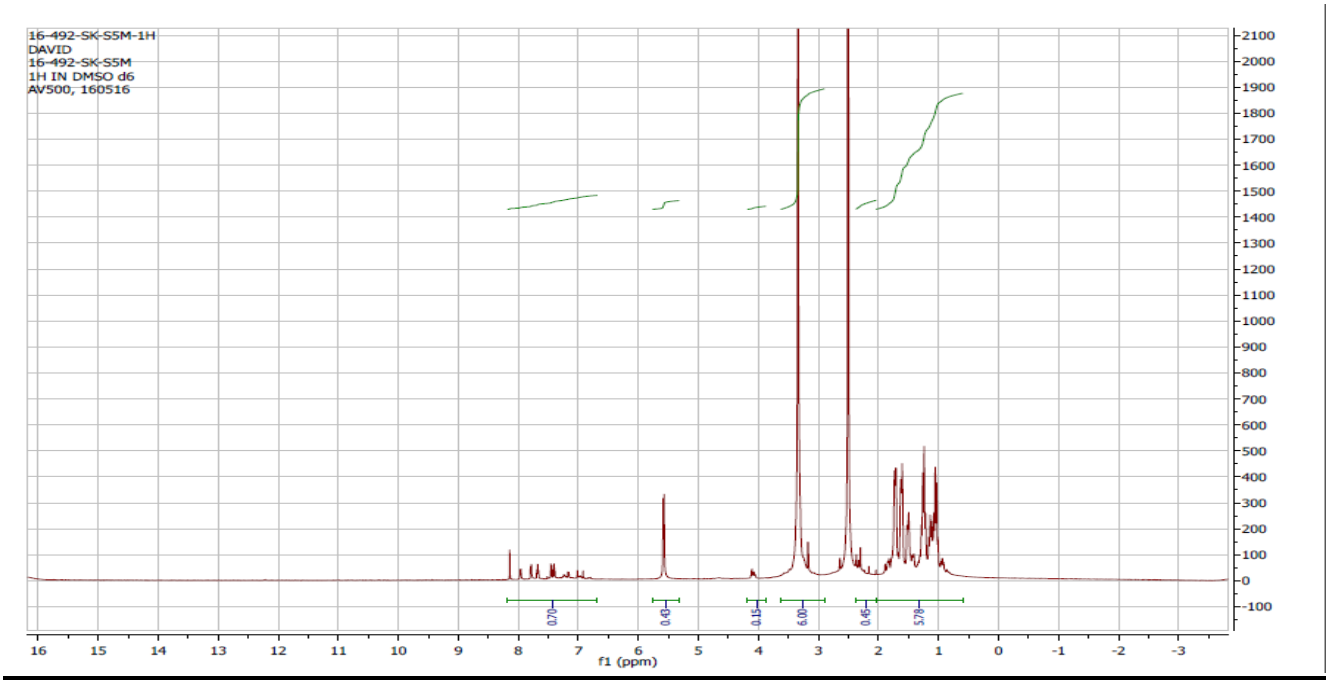


Figure 6.2 NMR spectra of compound S5m

6.2.4 Mass spectra of compounds S5i and S5m

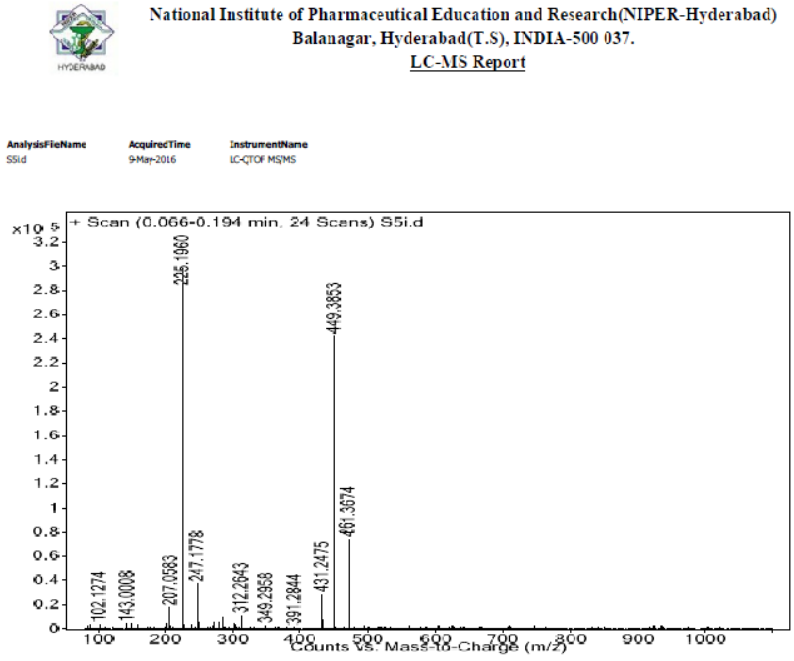


Figure 6.3 Mass spectra of compound S5i

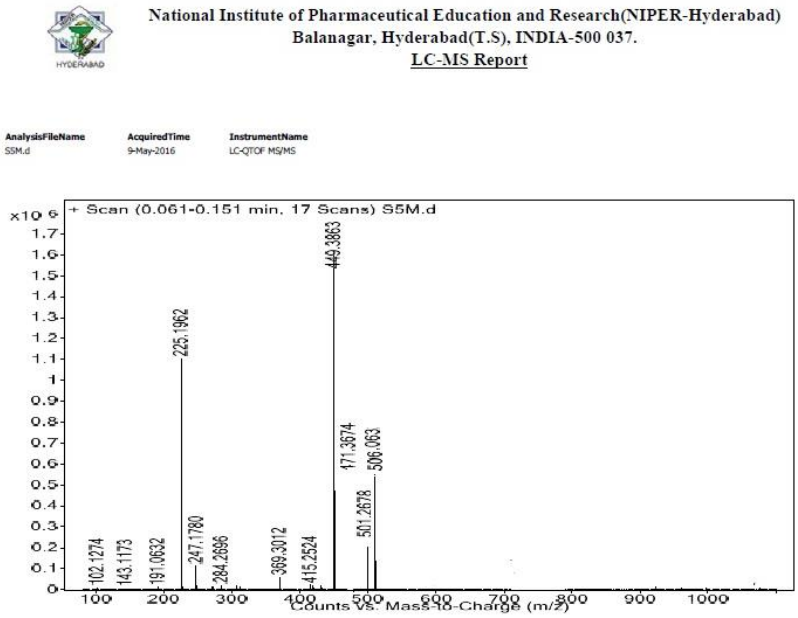


Figure 6.4 Mass spectra of compound S5m

CHAPTER 7

RESULT AND DISCUSSION (PHARMACOLOGICAL ACTIVITY)

7.1. *In vitro* antioxidant activity.

Free radicals are key signalling molecules that play an important role in the progression of inflammatory disorders. An enhanced ROS generation by polymorphonuclear neutrophils at the site of inflammation causes endothelial dysfunction and tissue injury. These reactive radicals and oxidants may injure cells and tissue directly via oxidative degradation of essential cellular components as well as injure cells indirectly by altering the protease/antiprotease balance that normally exist within the tissue interstitium. The Reactive oxygen metabolites may also initiate and/or amplify inflammation via the up regulation of several genes involved in the inflammatory response such as those code for pro inflammatory cytokines and adhesion molecules. Thus maintaining adequate anti-oxidant status may provide an useful approach in attenuating inflammation.

Previous studies suggest that the compounds with quinolin-2-one nucleus posess remarkable anti-oxidant activity.Also,the anti-oxidant potential evaluation is inevitable as quinolin-2-one is isosteric to coumarins, which are well known for its anti-oxidant property. The anti-oxidant activity of all the synthesised compounds was evaluated by using DPPH and ABTS assay. The IC_{50} values were calculated using Graphpad prism5 software and compared with that of ascorbic acid and quercetin.**T**heIC_{50} value is defined as the concentration of antioxidant required for 50% scavenging of radicals and lower IC_{50} value corresponds to a higher antioxidant activity. The antioxidant activity of the compound was measured in terms of hydrogen donating or radical scavenging ability, using the DPPH method. The mechanism of ABTS assay is based upon the electron donating radical scavenging reaction by antioxidants which prevent the formation of coloured ABTS radical. The IC_{50} values of the synthesised compounds were compared with that of ascorbic acid and quercetin.The *in vitro* anti-oxidant activity of synthesised compounds in ABTS and DPPH radical scavenging assay as tabulated in Table 7.2 and Table 7.4. The results demonstrate that the tested compounds serve as free radical scavenger/inhibitor, acting possibly as primary antioxidants

7.1.1 DPPH radical scavenging assay:

Table 7.1 DPPH Radical scavenging activity of ascorbic acid and quercetin by DPPH method

Ascorbic acid		IC_{50} (µg/mL)	Quercetin		IC_{50} (µg/mL)
Concentration (µg/mL)	% Inhibition		Concentration (µg/mL)	% inhibition	
0.5	2.74	10.12	5	20.33	10.38
1	3.32		10	44.50	
1.5	5.08		15	66.08	
2	6.66		20	86.82	
2.5	7.60		25	96.69	
5	20.98		30	97.84	
10	50.36				

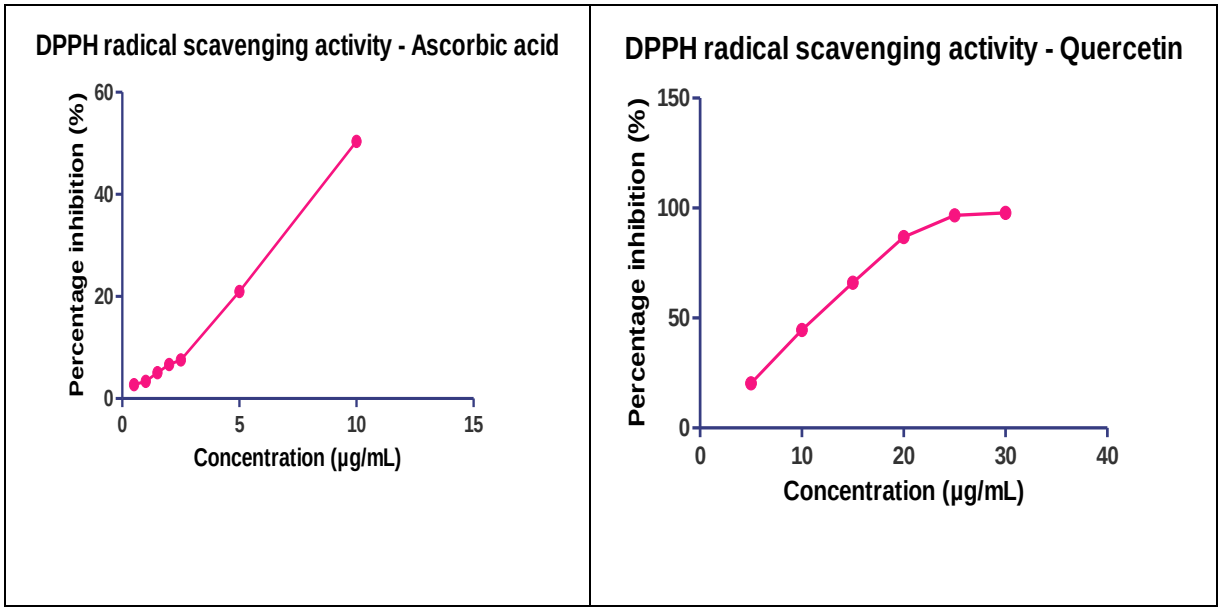


Figure 7.1DPPH radical scavenging activity of ascorbic acid and quercetin.

Table 7.2DPPH radical scavenging activity of synthesised compounds

Cpd code	Percentage inhibition(%)						IC_{50} (µg/mL)
	Concentration(µg/mL)						
	33.33	**66.66**	**133.33**	**200**	**266.66**	**333.33**	
S5a	1.37	2.02	382	5.44	6.84	7.85	>1000
S5b	2.08	2.12	2.44	2.94	3.03	3.47	>1000
S5c	1.37	3.17	3.82	6.92	8.30	11.80	>1000
S5d	1.52	1.68	3.59	7.06	9.92	18.68	>1000
S5e	1.03	1.06	1.63	2.78	5.24	8.44	>1000
S5f	7.71	9.75	14.08	15.83	17.43	32.5	>1000
S5g	2.53	17.67	29.30	42.43	53.56	66.85	231.3
S5h	20.10	21.6	33.81	43.33	51.11	52.82	283.8
S5i	29.80	54.80	78.02	88.7	91.80	93.25	58.26
S5j	14.75	20.14	30.53	48.08	62.02	68.02	208.7
S5k	7.26	8.88	21.4	30.65	32.31	34.11	619.8
S5l	25.94	39.25	44.43	54.96	63.95	68.25	151.8
S5m	28.55	36.03	52.94	62.00	65.52	67.55	118.9
S5n	7.90	9.50	39.60	44.07	52.00	71.61	214.9
S5o	5.69	7.62	10.67	22.64	25.21	32.11	667.4
S6a	1.67	4.36	25.81	40.42	59.86	63.61	235.8
S6b	7.5	11.86	21.08	32.05	54.11	63.87	258.9
S6c	18.79	40.29	74.96	78.62	91.01	97.71	79.33
S6d	7.89	14.01	18.83	31.11	34.41	45.04	447.2
S6e	12.20	18.40	28.78	38.91	53.65	55.07	272.5
S6f	21.78	31.83	51.00	67.15	77.82	83.58	112.8

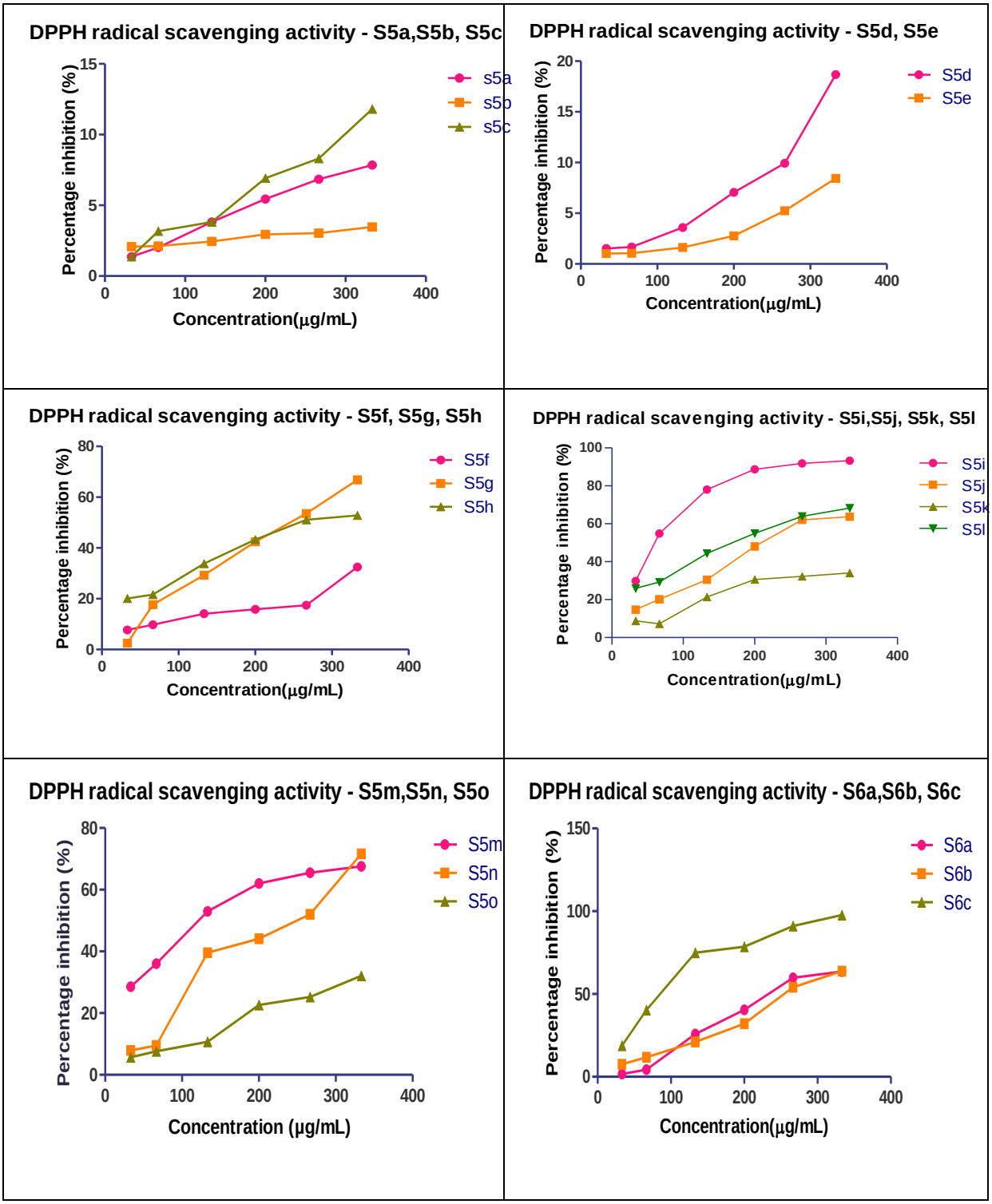


Figure7.2 DPPH radical scavenging activity of synthesised compounds S5a-o; S6a-e

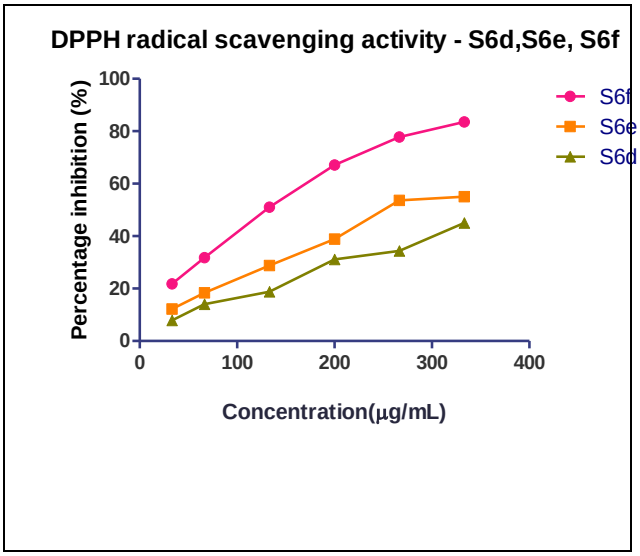


Figure7.2a DPPH radical scavenging activity of synthesised compounds S6d-f

DPPH is the most extensively used assay to detect antioxidant activity.Its violet colour disappears in the presence of substance which can donate a hydrogen depending on the antioxidant activity[92]. This assay is being used as preliminary test which provides information on the reactivity of the test compounds with a stable free radical since odd electron of DPPH gives strong absorption band at 517 nm (violet colour) and when it is quenched by the test compound, there is a decrease in absorbance. The IC_{50} values for ascorbic acid and quercetin were found to be 10.12 µg/mL and 10.38 µg/mL respectively Among the synthesised compounds compound S5i, S6c, S6f, and S5m showed more significant activity with IC_{50} values 58.26 µg/mL,79.33 µg/mL,112.8 µg/mL, and 118.9 µg/mL respectively.

7.1.2 ABTS radical scavenging assay:

Table 7.3ABTS radical scavenging activity of ascorbic acid and quercetin.

Ascorbic acid		IC_{50} (µg/mL)	Quercetin		IC_{50} (µg/mL)
Concentration (µg/mL)	(%) Inhibition		Concentration (µg/mL)	(%) inhibition	
0.2	26.79	0.93	0.25	62.50	0.19
0.4	32.17		0.50	69.15	
0.6	34.94		0.75	79.37	
0.8	38.64		1.0	86.34	
1.0	43.49		1.25	99.47	
1.5	63.04		1.50	99.57	
2.0	80.22				

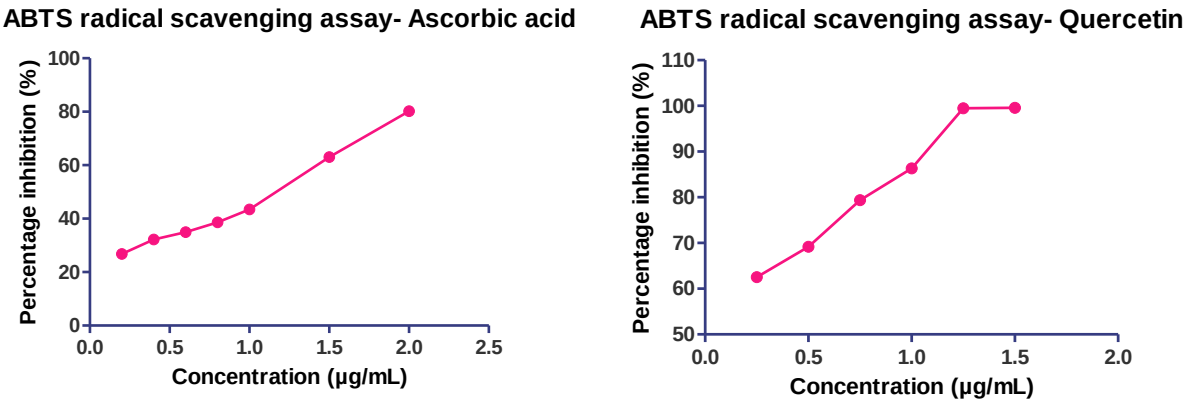


Figure 7.3ABTS Radical scavenging activity of ascorbic acid and quercetin

Table 7.4 ABTS Radical scavenging activity of synthesised compounds

Compound code	Percentage inhibition(%)					IC_{50} (µg/mL)
	Concentration(µg/mL)					
	0.5	1	2.5	5	10	
S5a	28.23	38.06	39.93	56.85	57.24	4.15
S5b	31.75	32.35	33.20	38.40	46.96	12.19
S5c	27.63	32.95	34.04	39.84	60.61	7.14
S5d	24.05	26.83	30.04	52.04	56.71	6.19
S5e	20.5	20.76	24.28	32.86	51.34	13.66
S5f	16.33	18.51	22.03	29.88	39.33	19.73
S5g	22.01	23.29	31.73	33.60	52.93	14.08
S5h	22.69	30.69	34.86	38.15	54.35	12.09
S5i	10.43	10.74	19.52	26.52	46.02	14.06
S5j	16.89	17.89	26.92	31.26	46.86	16.10
S5k	17.10	24.60	27.00	33.64	44.15	21.13
S5l	1961	21.84	26.20	27.54	32.81	17.78
S5m	19.99	20.76	38.40	44.16	63.74	5.49
S5n	25.36	28.10	33.88	33.35	47.76	14.65
S5o	20.78	28.26	28.84	29.92	30.33	16.10
S5a	24.97	25.54	27.81	34.05	39.49	16.18
S5b	30.59	31.06	35.45	38.33	39.99	19.79
S5c	21.55	22.45	24.15	27.08	38.28	13.30
S5d	21.76	25.66	38.77	42.22	46.22	12.09
S5e	22.64	26.44	27.98	31.20	40.50	13.13
S5f	23.61	27.10	28.81	40.54	51.54	11.49

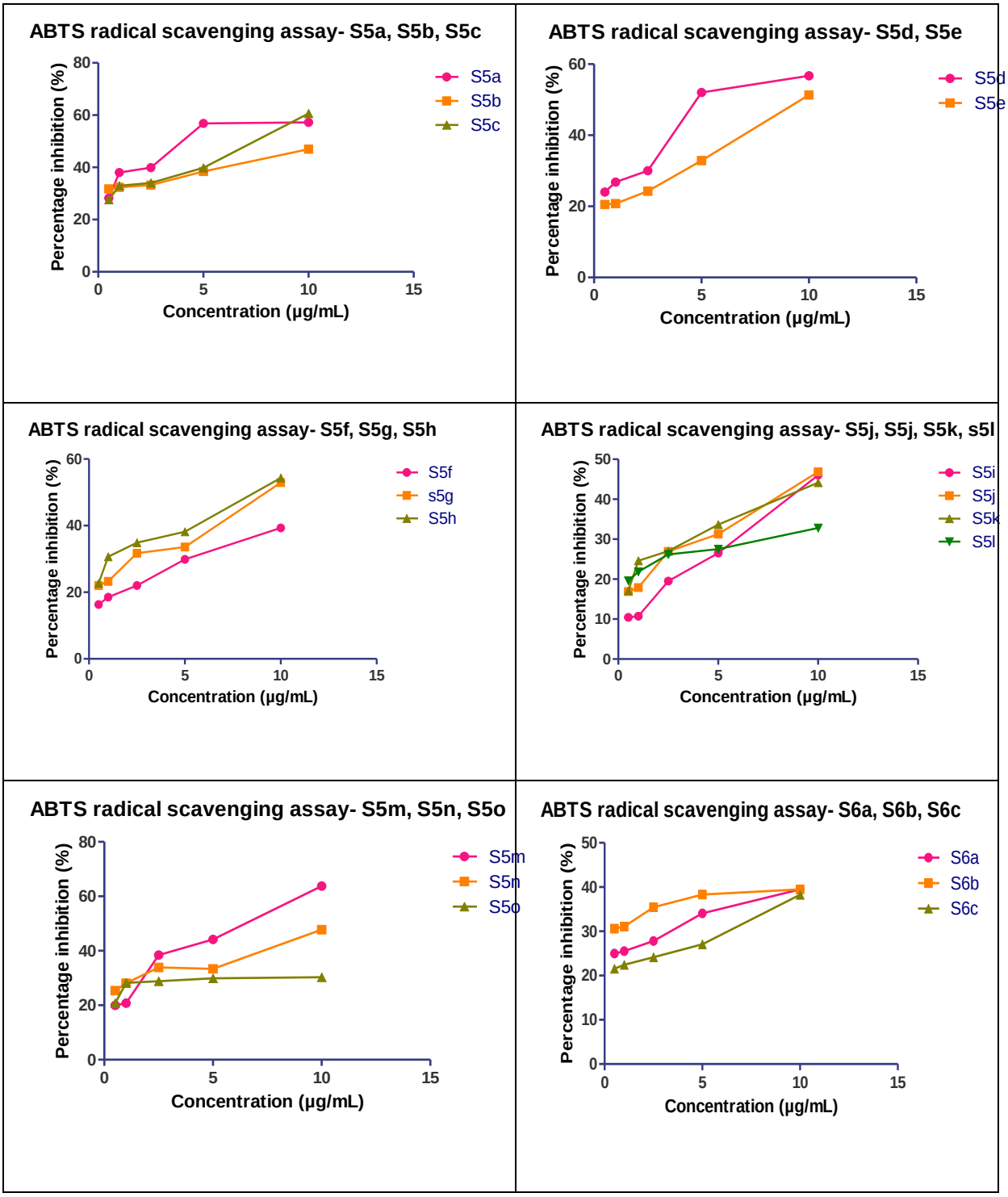


Figure7.4 ABTS Radical scavenging activity of synthesised compounds S5a-o; S6a-e

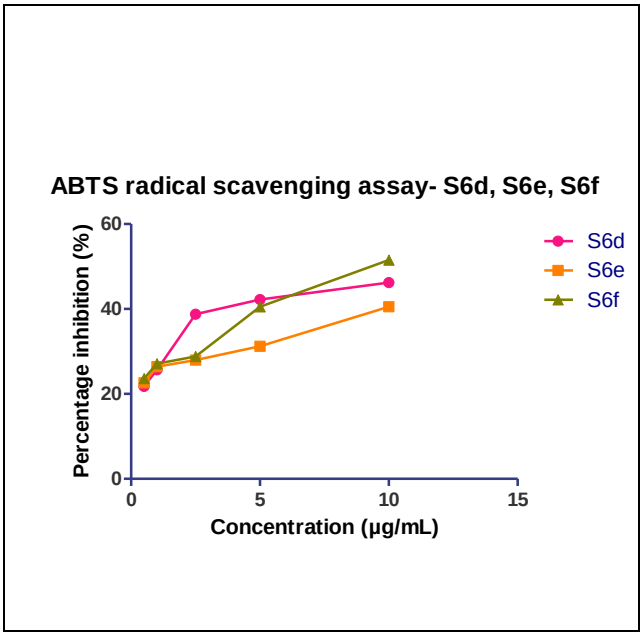


Figure7.4a ABTS Radical scavenging activity of synthesised compounds S6d-f

The decolourisation of ABTS radical is an unambiguous way to measure antioxidant activity. In this assay, ABTS is converted to its radical cation by addition of sodium per sulphate. The radical cation is blue in colour and absorbs light at 734 nm. The ABTS radical reactions involve electron transfer and take place at a much faster rate compared to DPPH radicals.[93] IC_{50} values of standard compounds ascorbic acid and quercetin was found to be 0.93 µg/mL and 0.19µg/mL respectively. Compounds S5a, S5m and S5c were found to posess considerable antioxidant activity with IC_{50} values 4.15 µg/mL,5.49 µg/mL, 7.14µg/mL respectively.

After docking the designed library of molecules onto the binding pocket of COX2, based upon the ligand interactions with the amino acid residues and SAR from previous studies as well as the *in vitro* anti-oxidant activity,compounds S5i and S5m were selected for *in vivo* pharmacological evaluation to assess the anti-inflammatory and analgesic potential.

7.2 Acute oral toxicity study.

Administration of test compound 5,50,300 and 2000 mg/kg per oral caused no behavioural, loco motor, toxic manifestations and mortality upto 14 days. Hence, it may be inferred that the compound was relatively non-toxic Even though there was no mortality, the animals showed abdominal writhing responses within one hour of test drugs administration.Further testing was also performed at lower doses and the animals showed relatively less writhing

manifestations at a dose of 1000mg/kg. Thus therefore, for the further studies, $1/16^{th}$ (125 mg/kg) and $1/32^{th}$ (62.5 mg/kg) of LD_{50} was selected. .

Table 7.5 Effect of test compounds on acute toxicity in rats.

	Response	Head		Body		Tail	
		Before	After	Before	After	Before	After
1	Alertness	Normal	Normal	Normal	Normal	Normal	Normal
2	Grooming	Absent	Absent	Absent	Absent	Absent	Absent
3	Touch response	Absent	Absent	Absent	Absent	Absent	Absent
4	Torch response	Normal	Normal	Normal	Normal	Normal	Normal
5	Pain response	Normal	Normal	Normal	Normal	Normal	Normal
6	Tremors	Absent	Absent	Absent	Absent	Absent	Absent
7	Convulsion	Absent	Absent	Absent	Absent	Absent	Absent
8	Righting reflux	Normal	Normal	Normal	Normal	Normal	Normal
9	Gripping strength	Normal	Normal	Normal	Normal	Normal	Normal
10	Pinna reflux	Present	Present	Present	Present	Present	Present
11	Corneal reflex	Present	Present	Present	Present	Present	Present
12	Writhing	Absent	Absent	Absent	Present	Absent	Absent
13	Pupils	Normal	Normal	Normal	Normal	Normal	Normal
14	Urination	Normal	Normal	Normal	Normal	Normal	Normal
15	Salivation	Normal	Normal	Normal	Normal	Normal	Normal
16	Skin colour	Normal	Normal	Normal	Normal	Normal	Normal
17	Lacrimation	Normal	Normal	Normal	Normal	Normal	Normal

7.3 Screening of anti-inflammatory activity

7.3.1 Carrageenan induced paw edema in rats

Effects of compound and diclofenac on carrageenan induced paw edema in rats are shown in Table 7.6 .Oral administration of compound at doses 62.5 and 125 mg/kg significantly suppressed the paw edema at fourth hour after carrageenan injection in rats.Diclofenac at a dose of 20mg/kg significantly suppressed paw edema at 3 and 4h after carrageenan administration.The inhibition of carrageenan induced inflammation in rats is an established model to screen compounds for potential anti-inflammatory activity.

Sub plantar injection of carrageenan into the rat paw produces plasma extravasation and the inflammation characterized by increased tissue water and plasma protein exudation with neutrophil extravasation and metabolism of arachidonic acid by both cyclooxygenase and lipoxygenase enzyme pathways. The development of carrageenan induced paw edema is

biphasic;the first phase occurs within one hour administration of carrageenan and is attributed to the release of cytoplasmic enzymes, histamine and serotonin, from mast cells[94][95] .The second phase begins after 1h and remains through 3h which is mediated by an increased release of prostaglandins in the inflammatory area and continuity between the two phases is provided by kinins.

Effects of compounds S5i and S5m (62.5 and 125 mg/kg) and diclofenac (20 mg/kg) are shown in table 7.6 Diclofenac at a dose of 20mg/kg significantly supressed more than 50% paw edema from 3h. In the control group, paw edema volume was maximum at the 4h.

Some NSAIDs strongly inhibit the second phase of carrageenan induced paw edema, but some others are effective against both phases.In the present study, the compounds **S5i** and **S5m** at doses 62.5 and 125 mg/kg seems effective only in the second phase as the paw edema volume was supressed more than 50% in 4h. So this compounds might be blocking prostaglandin and /or bradykinin release rather than histamine and/or serotonin. Diclofenac also has shown similar effect only at second phase.

Table 7.6 *In vivo* anti-inflammatory activity of the compounds S5i and S5m by carrageenan induced paw edema in rats.

Groups	Treatment	Mean paw edema volume (mL) and percentage inhibition (%)				
		1h	2h	3h	4h	5h
I	Carrageenan 1%w/v (0.1mL)p.o	0.23±0.00	0.29±0.005	0.42±0.02	0.46±0.018	0.17±0.01
II	Diclofenac 20mg/kg i.p	0.18±0.003*	0.17±0.004***	0.12±0.001***	0.07±0.003***	0.01±0.002***
		21.7	41.37	71.42	84.89	94.11
III	Compound **S5i**(62.5mg/kg) p.o	0.22±0.002^{ns}	0.25±0.004***	0.20±0.015**	0.19±0.01***	0.14±0.01^{ns}
		4.37	13.7	52.38	58.69	17.64
IV	Compound **S5i**(125mg/kg) p.o	0.21±0.003^{ns}	0.24±0.007***	0.19±0.002***	0.16±0.05***	0.14±0.004^{ns}
		8.69	17.24	54.70	65.21	17.64
V	Compound **S5m**(62.5mg/kg)p.o	0.21±0.006^{ns}	0.20±0.002***	0.18±0.002***	0.15±0.01***	0.16±0.0097^{ns}
		8.69	31.03	57.14	67.39	5.86
VI	Compound **S5m**(125mg/kg) p.o	0.19±0.011*	0.18±0.006***	0.14±0.01***	0.08±0.003***	0.15±0.013^{ns}
		17.39	39.93	66.60	82.60	11.71

Values are mean ±SEM of 6 animals in a group.*p<0.05, of ANOVA followed by Dunnets test compared with control.

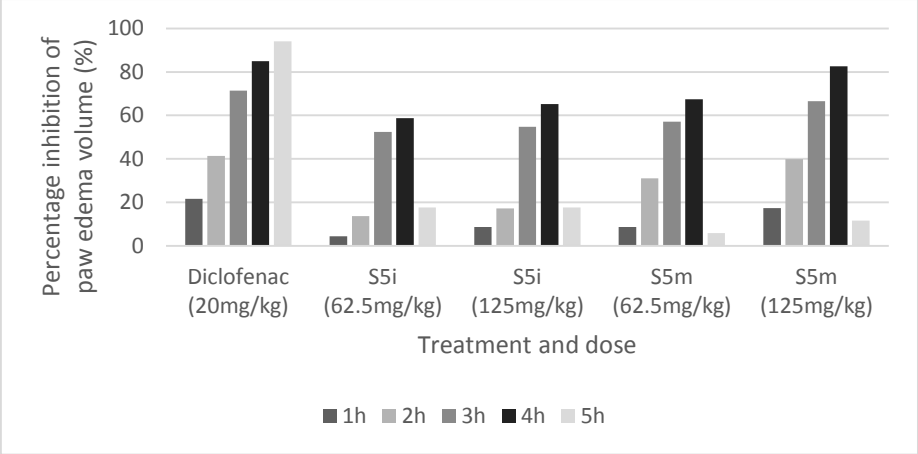


Figure 7.5 *In vivo* anti-inflammatory activity of the compounds S5i and S5m by carrageenan induced paw edema in rats

7.3.2 Cotton pellet-induced granuloma in rats[96][97]

Effects of compound and Diclofenac on cotton pellet-induced paw granuloma in rats are shown in Table 7.7 .Oral administration of compound at doses 62.5 mg/kg and125 mg/kg relatively inhibited the cotton pellet induced granuloma in rats. Compound **S5m** showed comparatively more significant inhibition of inflammatory response.

Table 7.7 *In vivo* anti-inflammatory activity by of the compoundsS5i and S5m cotton pellet- induced granuloma in rats.

Groups	Treatment and Dose	Weight of dry cotton pellet granuloma(mg) (Mean±SEM)	% Inhibition of granuloma formation
I	1% w/v CMC p.o	101±0.002	-
II	Diclofenac (20mg/kgi.p)	43.2±0.001**	57.2
III	Compound **S5i**(62.5mg/kg)p.o	65.1±0.002ns	35.54
IV	Compound **S5i**(125mg/kg)p.o	55.3±0.011*	45.24
V	Compound **S5m**(62.5mg/kg)p.o	64.2±0.001ns	36.40
VI	Compound **S5m**(125mg/kg)p.o	51.4±0.003*	49.10

Values are mean ±SEM of 6 animals in a group.*p<0.05, of ANOVA followed by Dunnets test compared with control.

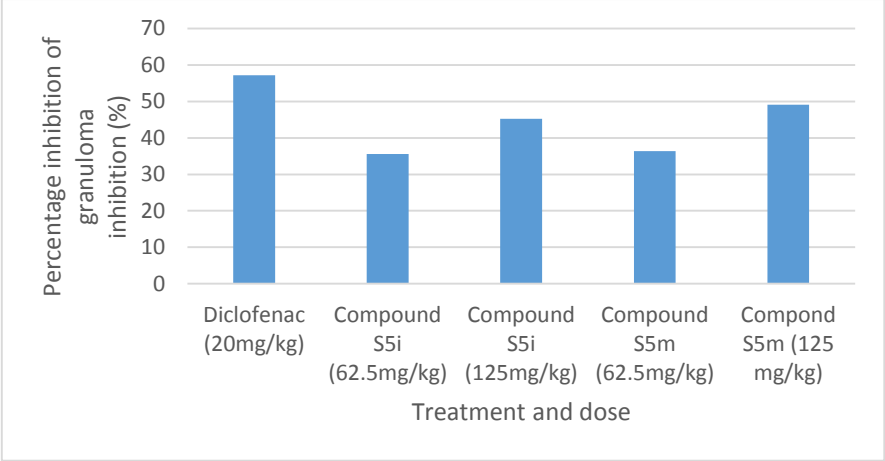


Figure 7.6 *In vivo* anti-inflammatory activity by of the compounds S5i and S5m cotton pellet- induced granuloma in rats.

The anti-inflammatory activity of the compounds can be justified by the docking study of the ligands with COX-2 enzyme binding pocket.[98] The ligand interaction and the binding poses of compound S5i and S5mare given in figure 7.7 and 7.8. The ligand S5i in the COX-2 binding pocket is stabilised within the binding site through H-bond interactions with residues Met 522, Val 523, Gly 526 and electronic interactions with residues His 90, Thr 94, Val 349, Ser 353. The ligand S5m in the COX-2 is stabilised within the binding site through H-bond interactions with residues Met 522, Val 523, Ala 527, Ser 353 and electronic interactions with residues His90, Ser 353, Arg 513, Ala 516, Gly 52.

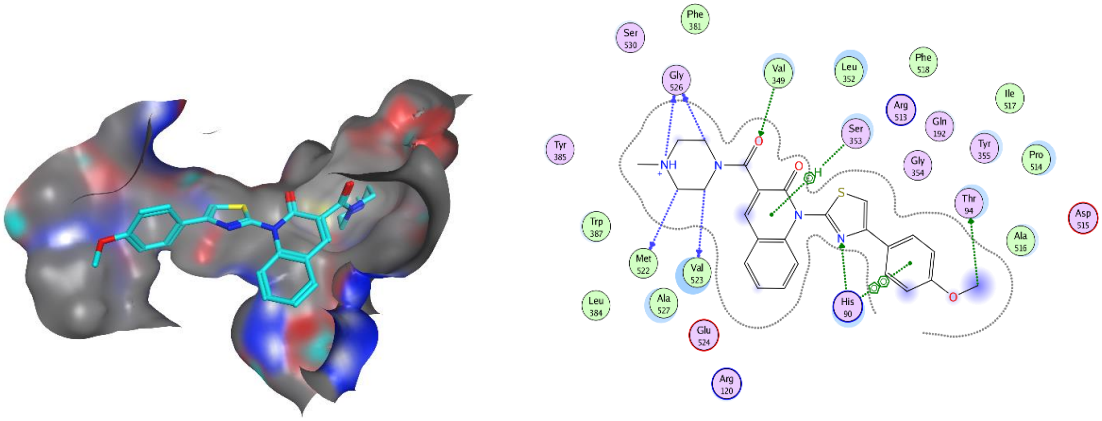


Figure 7.7 Binding poses and ligand interactions of compounds S5i

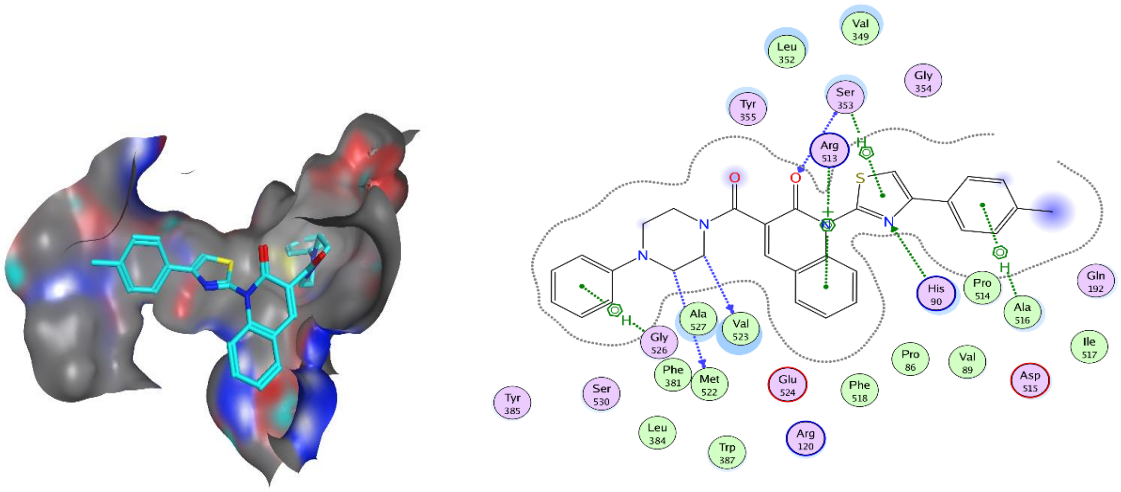


Figure 7.8 Binding poses and ligand interactions of compounds S5m

7.4 Screening of analgesic activity:

7.4.1 Eddy's hot plate [99]

The paws of mice and rats are very sensitive to heat at temperatures which are not damaging the skin. The responses are jumping, withdrawal of the paws and licking of the paws. The time until these responses occur is prolonged after administration of centrally acting analgesics, whereas peripheral analgesics of the acetylsalicylic acid or phenyl-acetic acid type do not generally affect these responses. Both of the compounds **S5i** and **S5m** (62.5 and 125 mg/kg) showed no significant prolongation of pain response reaction time and hence it drives us to the assumption that the titled compounds does not interrupt the central pain pathways.

Table 7.8 *In vivo* analgesic activity of the compounds S5i and S5m by hot plate method in mice.

Groups	Treatment and Dose	Reaction time (sec) at different time intervals				
		0 min	**15 min**	**30 min**	**45min**	**90 min**
I	Morphine (5mg/kg i.p)	2.33±0.210	3.33±0.21***	6.01±0.365***	9.33±0.55***	11.00±0.36***
II	Compound **S5i** (62.5mg/kg)p.o	1.67±0.33	3.0±0.001^{ns}	3.33±0.88^{ns}	3.33±0.66^{ns}	4.23±0.33*
III	Compound **S5i** (125mg/kg)p.o	2.33±0.33	3.02±0.001^{ns}	4.2±0.015**	4.66±0.33***	4.3±0.33***
IV	Compound **S5m** (62.5mg/kg)p.o	2.01±0.02	3.02±0.51^{ns}	3.12±0.21^{ns}	3.66±0.33*	4.0±0.57*
V	Compound **S5m** (125mg/kg)p.o	2.33±0.33	3.00±0.57^{ns}	4.13±0.012^{ns}	4.66±0.33*	4.66±0.667*

Values are mean ±SEM of 6 animals in a group.*p<0.05 compared with reaction time before drug treatment. Data were analyzed by using One-way ANOVA followed by Dunnett's test

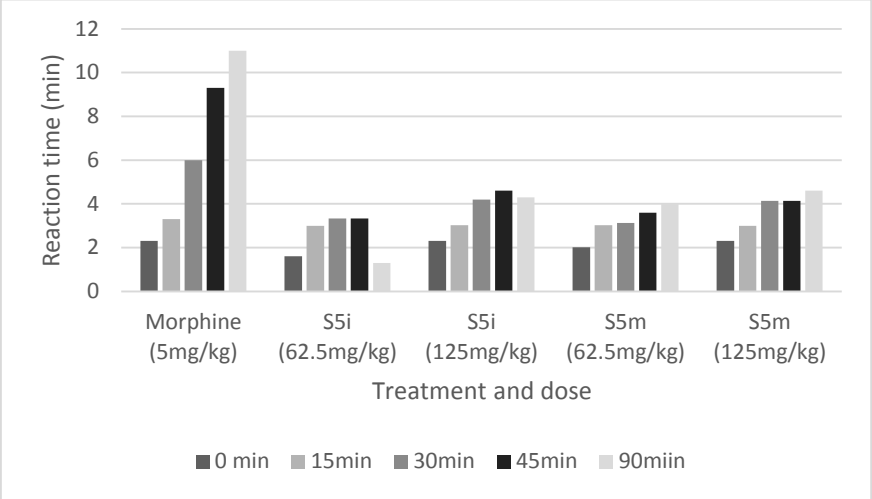


Figure 7.9 *In vivo* analgesic activity of the compounds S5i and S5m by hot plate method in mice.

7.4.2 Acetic acid induced writhing test in mice

Acetic acid induced writhing test is a model of visceral or peritoneal pain in animals. Intra peritoneal administration of acetic acid irritates serous membranes and provoke a stereotyped behaviour known as writhing which involves turning movements of the body and extension of hind limbs. These behaviours are considered as reflexive and are evidence of peritoneo-visceral or visceral pain associated with visceral chemoreceptors.

The effect of compounds **S5i** and **S5m** (62.5 and 125 mg/kg,p.o) and Diclofenac sodium (20mg/kg i.p) were evaluated by acetic acid induced writhing responses in mice. The number of writhings of each animal within 15 min after acetic acid injection was cumulatively counted immediately and the percentage protection was calculated using the following ratio.

Percentage of protection = (Control mean – treated mean) / (control mean) x 100

The effects of compound and Diclofenac on writhing test are shown in Table 7.9. The compound at respective doses significantly inhibited the writhing response of mice caused by intraperitoneal injection of acetic acid. Since the abdominal constriction induced by acetic acid involves the process of release of arachidonic acid metabolites via cyclooxygenase (COX) and prostaglandin biosynthesis.So the compound may act by inhibiting the release of arachidonic acid which adds on evidence deriving the conclusion that the titled compounds act by blocking the COX pathway.

Table 7.9 *In vivo* analgesic activity of the compounds S5i and S5m by acetic acid Induced writhing in mice.

Groups	Treatment and Dose	Number of writhing (Mean±SEM)	Percentage protection(%)
I	1% w/v CMC	56.25±1.109	
II	Diclofenac(20mg/kg) i.p	32.00±0.912***	43.11
III	Compound **S5i**(62.5mg/kg)p.o	46.00±2.160**	18.22
IV	Compound **S5i**(125mg/kg)p.o	35.00±2.09***	37.77
V	Compound **S5m**(62.5mg/kg)p.o	42.50±1.708***	24.44
VI	Compound **S5m**(125mg/kg)p.o	33.50±1.080***	40.17

Values are mean ±SEM of 6 animals in a group.*p<0.05 compared with the control. Data were analyzed by using One-way ANOVA followed by Dunnett's test

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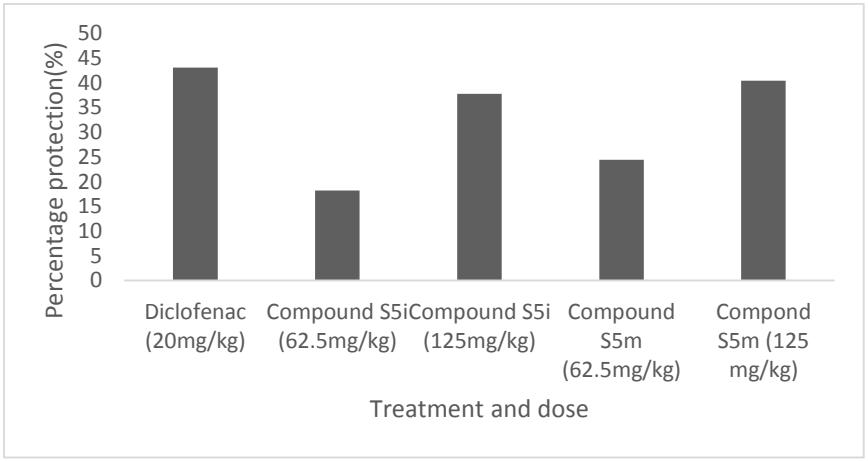

Figure 7.10 *In vivo* analgesic activity of the compounds S5i and S5m by acetic acid Induced writhing method in mice

7.5 Ulcerogenic effect[100]

NSAIDs are often accompanied by side effects in which the gastric irritation is more common and prominent. The gastric mucosa protects itself from gastric acid with a layer of mucus,the secretion of which is stimulated by certain prostaglandins. The non selective inhibition of COX enzymes are primary cause for gastric irritation as it blocks COX1 which is essential for the prostaglandin synthesis

The ulcerogenic potential of the titled compounds were studied after observation of stomach of fasted rats after 5h of administration of compounds and compared with indomethacin(10 mg/kg p.o). The presence of mucosal erosion, ulcers and perforations were investigated. No signs of ulceration were observed after 5h of drug administration of the compounds **S5i** and

S5m at the doses which it displayed anti-inflammatory and analgesic activity (62.5 and 125mg/kg) thus indicating the safety of the drug. On the other hand indomethacin (10 mg/kg) showed 3 spot ulcers under microscopical evaluation (10X). This lead us the assumptions that the titled compounds might be selective inhibitors of COX-2.Further enzyme inhibition assays are essential to confirm the selectivity of the compounds.

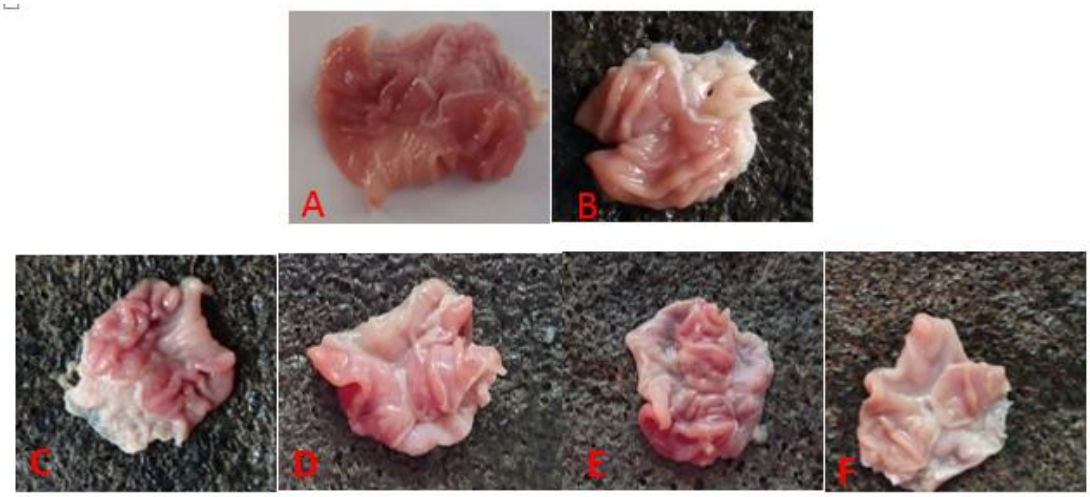

Figure 7.11 Ulcerogenic potential studies. A. Normal**; B.** Indomethacin (10mg/kg.p.o); **C.** Compound **S5i** (62.5 mg/kg) p.o; **D.** Compound **S5i** (125 mg/kg) p.o; **E.** Compound **S5m** (62.5 mg/kg) p.o**; F.** Compound **S5m** (125 mg/kg) p.o.

7.6 ANTI BACTERIAL STUDIES

The structure activity relationships of compounds based on nalidixic acid have led to a large group of synthetic antibacterial agents known collectively as quinolones. The 4-quinolones such as ciprofloxacin, ofloxacin, lomefloxacin, enoxacin are established synthetic antibacterial agents as DNA gyrase inhibitors . The benzopyrone ring in compounds of coumarinsand flavones is isosteric to quinolones and has shown DNA gyrase inhibition. The quinolin-2-ones, also called carbostyrils or 1-aza coumarins, are isosteric to coumarins and isomeric to 4-quinolones and could become a potential candidate for antibacterial activity.[101]

All the compounds were screened for antibacterial activity against various strains of pathogenic bacteria by using disc diffusion method. The compounds **(S6f and S5j)** which showed considerably higher percentage zone of inhibition were selected for determination of Minimum inhibitory concentration(MIC). The zone of inhibition and percentage growth of inhibition of all the synthesised compounds against various strains of bacteria are shown in Table 7.10

Based upon the zone of inhibition two compounds were selected for determining MIC. Minimum inhibitory concentration is the lowest concentration that prevents visible

growth of an organism. Compound S5j and S6f were found to have considerable anti bacterial activity with MIC of 25µg/mL against *Pseudomonas aeuroginosa and* 50µg/mL against *Bacillus subtilis*.

Table 7.10 Antibacteralstudies : zone of inhibition and percentage growth of inhibition

Cpd code	Zone of inhibition(mm) and percentage growth inhibition(%)						
	E.coli	***k.Pneumoniae***	***S.Paratyphi***	***V.cholorae***	***P.aeuroginosa***	***S.aureus***	***B.subtilis***
S5a	-	-	-	-	-	-	-
S5b	-	-	-	-	-	-	-
S5c	-	-	-	-	-	-	-
S5d	-	-	-	-	-	-	-
S5e	7(41.1)	9(23.04)	-	-	-	-	-
S5f	-	11(28.2)	-	-	-	-	-
S5g	-	7(17.90)	-	-	-	-	-
S5h	-		-	-	-	-	-
S5i	-	10(25.6)	-	-	-	-	-
S5j	-	-	-	-	21(70.0)	11(45.83)	-
S5k	-	-	-	-	-	7(29.16)	-
S5l	-	-	-	-	-	-	-
S5m	-	-	-	-	10(33.33)	10(41.66)	-
S5n	7(41.1)	10(25.6)	-	-	-	-	9(64.28)
S5o	9(52.9)		-	-	12(40)	-	-
S6a	-	11(28.2)	-	15(45.54)	-	-	-
S6b	-	11(28.2)	-	19(57.57)	-	-	-
S6c	-	10(25.6)	-	-	-	-	-
S6d	-	10(25.6)	-	-	-	-	-
S6e	-		-	20(60.60)	10(33.33)	-	5(35.71)
S6f	-	12(30.76)	-	-	13(43.33)	-	13(92.85)
Cpf	17	39	18	33	30	24	14

- : No inhibition; **Cpf**: Ciprofloxacin

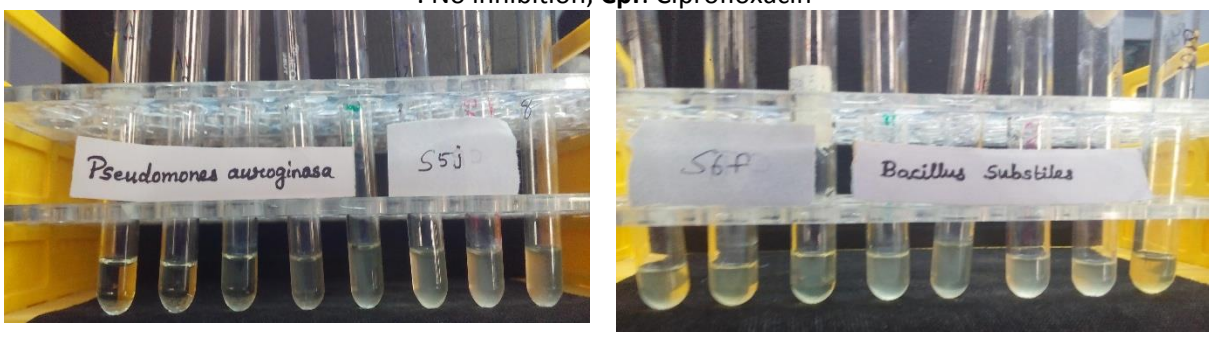

Figure 7.12 MIC of compounds S5j; S6f

CHAPTER 9

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