DESIGN,INSILICO MOLECULAR DOCKING,SYNTHESIS AND EVALUATION OF ANTIULCER ACTIVITY OF BENZIMIDAZOLE DERIVATIVES

Dissertation Submitted to

The Tamilnadu Dr. M.G.R. Medical University, Chennai

In partial fulfillment for the award of the Degree of

MASTER OF PHARMACY

(Pharmacology)

OCTOBER -2016

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DEPARTMENT OF PHARMACOLOGY

KMCH COLLEGE OF PHARMACY

KOVAI ESTATE, KALAPPATTI ROAD,

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CERTIFICATE

This is to certify that the dissertation work entitled "**DESIGN,INSILICO MOLECULAR DOCKING,SYNTHESIS AND EVALUATION OF ANTIULCER ACTIVITY OF BENZIMIDAZOLE DERIVATIVES"** was carried out by **Reg No: 261425815** is a bonafide work carried out by the candidate to The Tamil Nadu Dr. M.G.R Medical University, Chennai, in partial fulfilment for the Degree of **Master of Pharmacy** in **Pharmacology** at the Department of Pharmacology, KMCH College of Pharmacy, Coimbatore, Tamilnadu during the academic year 2015-16.

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Date:

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Department of Pharmaceutical Chemistry

DECLARATION

I do hereby declare that the dissertation work entitled"**DESIGN,INSILICO MOLECULAR DOCKING,SYNTHESIS AND EVALUATION OF ANTIULCER ACTIVITY OF BENZIMIDAZOLE DERIVATIVES"** submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfilment for the Degree of **Master of Pharmacy** in **Pharmacology**, was carried out at the Department of Pharmacology, KMCH College of Pharmacy, Coimbatore, Tamil Nadu during the academic year 2015-16.

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EVALUATION CERTIFICATE

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Date:

Place:

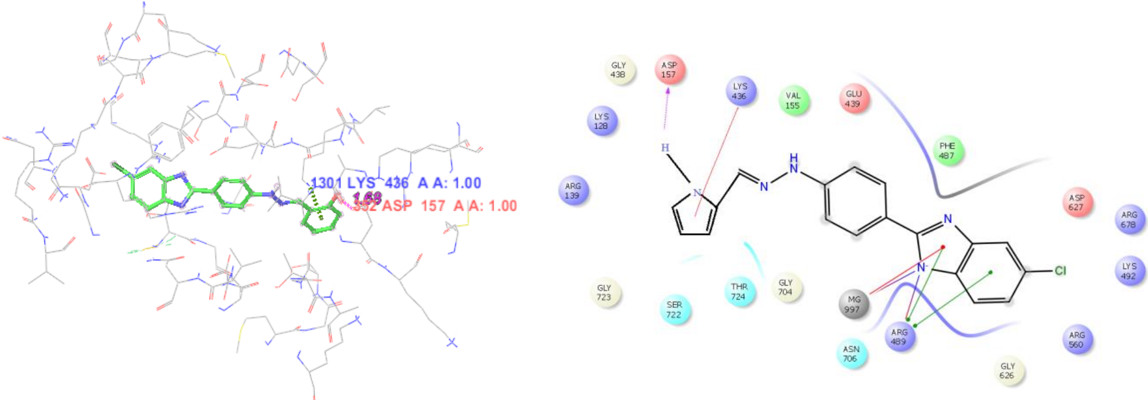
Internal Examiner **External Examiner**

Convener of Examination

Examination Centre: KMCH College of Pharmacy, Coimbatore

ABSTRACT

The present study was aimed to design some possible novel benzimidazole derivatives as H^+/K^+-ATPase receptor inhibitor, carry out their molecular docking study and finally to evaluate its antiulcer activity by Indomethacin induced and Pylorus ligation induced ulcer model. All the designed compounds were docked with the crystal structure of H^+/K^+-ATPase receptor (PDB ID:2ZBD) using Glide software version 10.1,Schrodinger,LLC,New York, NY, 2015 and a series of benzimidazole derivatives have been synthesized based on dock score and interactions. The structures of synthesized compounds were characterized by UV,FTIR,^{1}H NMR and mass spectral analysis. The synthesized compounds were evaluated for their *in vitro* antioxidant activity and *in vitro* cell viability assay .Acute toxicity study was carried out for the compound P-28(dock score:-8.67) based on OECD 423 Guidelines and the compound was found to be safe up to the dose of 1000mg/kg. After 6 days pretreatment with the test compound at the 50mg/kg (Low dose) ,100mg/kg (High dose) and the standard drug, pantoprazole was administered at the dose of (10mg/kg) ,on the 7th day toxicant was administered and after 4hrs animals were sacrificed , ulcer index , gastric volume, gastric pH, total acidity, free acidity was measured. *In vivo* antioxidant activity of treated group showed a increased level of SOD,CAT,GP_X, GSH, Total protein and an decreased level of LPO,MPO . Histopathological studies were carried out to further analyze the compound. Based on above results ,it was concluded that the compound P-28 posses significant *in vivo* antioxidant and antiulcer activity as that of the standard (pantoprazole) and can be used as a lead for the development of newer antiulcer agents.



Binding pose and ligand interaction of Compound P-28

INTRODUCTION

The stomach is a J-shaped dilated portion of the alimentary tract situated in the epigastric, umbilical and left hypochondriac regions of the abdominal cavity. The stomach is continous with the oesophagus at the cardiac sphintcter and with the duodenum at the pyloric sphincter. It has two curvatures. The lesser curvature is short, lies on the posterior surface of the stomach and is downward continuation of the posterior wall of the oesophagus. Just before the pyloric sphincter it cures upwards .Where the oesophagus joins the stomach the anterior region angles acutely upwards ,curves downwards forming the greater curvature and then slightly upwards towards the pyloric sphincter. The stomach is divided into three regions: the fundus, the body, the antrum. At the distal end of the pyloric antrum is the pyloric sphincter.[1]

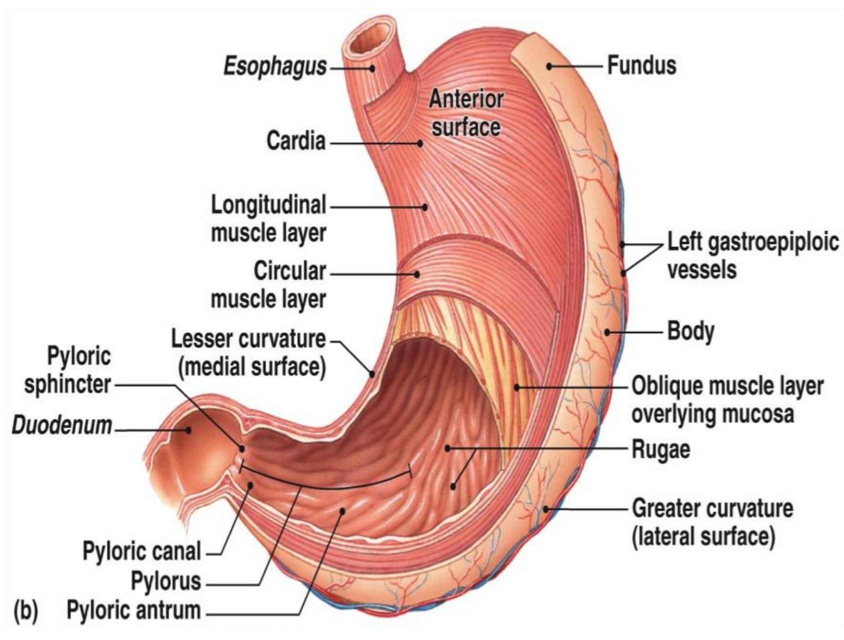


Figure1

GASTRIC GLANDS: [2]

Mucosa consist of 2 layers- superfacial and deep. Between the two layers is the lamina propria composed of network of fibrocollagenic tissue with a few lymphocytes, plasma cells , macrophages and eosinophils. The mucosa is externally bounded by muscularis mucosae. Gastric glands are simple or branched tubular glands

that emerge on the deeper part of the gastric foveola, inside the gastric areas and outlined by the folds of the mucosa.

There are three types of glands: cardiac glands, oxyntic glands and pyloric glands. The cardiac glands mainly contain mucus producing cells. The oxyntic glands is dominated by zymogen cells that produce pepsinogen. Parietal cell (oxyntic cell) within the glands secrete hydrochloric acid and intrinsic factor. The pyloric gland mucosa in the antrum synthesizes and releases the hormone gastrin from G cells.

Table 1: Gastric Glands and secretions

Source	**Substance secreted**	**Stimulus for release**	**Function**
Mucous neck cell	Mucus	Tonic secretion; increased with irritation of mucosa	Physical barrier between lumen and epithelium
	Bicarbonate	Secreted with mucus	Buffers gastric acid to prevent damage to epithelium
Parietal cells	Gastric acid (HCl)	Acetylcholine,gastrin, histamine	Activates pepsin;kills bacteria
	Intrinsic factor		Complexes with vitamin B12 to permit absorption
Enterochromaffin – like cell	Histamine	Acetylcholine, gastrin	Stimulates gastric acid secretion
Chief cells	Pepsin(ogen)	Acetylcholine,acid, secretin	Digests protein
	Gastric lipase		Digest fats
D cells	Somatostatin	Acids in the stomach	Inhibit gastric acid secretion
G cells	Gastrin	Acetylcholine,peptides,and amino acids	Stimulates gastric acid secretion

GASTRIC JUICE AND ITS FUNCTION: [2]

Gastric juice, thin, strongly acidic (pH varying from 1 to 3), almost colorless liquid secreted by the glands in the lining of the stomach. About 2 litres of gastric juice are secreted daily by specified secretory glands in the mucosa. It consist of :

- Water, mineral salt :secreted by gastric glands
- Mucus: secreted by globlet cells in the glands and on the stomach surface
- Hydrochloric acid, intrinsic factor: secreted by parietal cells in the gastic glands
- Inactive enzymes precursors: pepsinogens secreted by chief cells in the gland

Functions of gastric juice[2]

-Water: liquefies the food swallowed

-Hydrochloric acid:

- acidifies the food and stops the action of salivary amylase
- kills ingested microbes
- provides the acid environment

-Pepsinogens are activated to pepsins by hydrochloric acid and by pepsins already present in the stomach. They begin the digestion of proteins, breaking them into smaller molecules. pepsins act most effectively at pH 1.5to3.5.

-Intrinsic factor (a protein) is necessary for the absorption of vitamin b12 from the ileum.

-Mucus prevents mechanical injury to the stomach wall by lubricating the contents. It prevents chemical injury by acting as a barrier between the stomach wall and the corrosive gastric juice. Hydrochloric acid is present in potentially damaging concentrations and pepsins digest protein.

Gastric lipase: splits the triglycerides in fat molecules into fatty acid and monoglycerides.

REGULATION OF ACID SECRETION:[2]

The major function of gastric acid are to dissolve food fibres, act as bactericide against swallowed organisms, and transform pepsinogen to pepsin. The production of acid by the parietal cells requires the transport of hydrogen and chloride from the parietal cell to the stomach lumen. Acid is formed in the parietal cells, firstly through the hydrolysis of water. At a high rate of acid secretion, bicarbonates moves into the plasma, creating an' alkaline tide' in the venous blood, which also may result in a more alkaline urine.

Acid secretion by parietal cells is stimulated by acetylcholine (a neurotransmitter), gastrin (a hormone) and histamine (a biochemical mediator) and is inhibited by somatostatin (a hormone). The vagus nerve also liberate acetylcholine and stimulates the secretion of histamine. Histamine secretion is also stimulated by gastrin. Histamine receptors in the gastric mucosa are H2 receptors. Gastric lipase is secreated by glands in the fundus of the stomach and is most effective in an acid environment.

Parietal cell acid secretion is initiated by a variety of factors related to food ingestion. Regulation is via central, peripheral and cellular mechanisms.

Acid is produced by the carbonic anhydrase-mediated catalysis of CO_2 and H_2O to form H^+ and HCO_3^-. H^+ ions are then exchanged for K^+ by the H^+/K^+-ATPase pump and later coupled with Cl^- ions entering the parietal cell from the blood in exchange for HCO_3^-.

Most of the vagal fibers supplying the stomach are afferent and transmitt information to the brain regarding mechanical and chemical changes in the stomach. The efferent fibers are preganglionic neurons that do not directly stimulate the parietal cells, but rather synapse with postganglionic neurons in the wall of the stomach. These neurons contain neurotransmitters, such as acetylcholine, gastrin-releasing peptide (GRP), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), nitric oxide and substance P. Through these messengers, postganglionic neurons are able to maintain acid secretion directly by influencing the parietal cell, or indirectly by modulating the secretion of hormonal and paracrine ligands.

Sympathetic receptors of the stomach composed of unmyelinated nerve endings located within the smooth muscle layer. They recognize chemical stimuli more than mechanical stimulation and play a role in carring pain sensation associated with inflammatory states, such as gastritis.

The principal stimulants for acid secretion are histamine, gastrin and acetylcholine released from postganglionic enteric neurons. These increase intracellular levels of adenosine 3´,5´,-cyclic monophosphate (cAMP), inositol triphosphate (IP3), diacylglycerol and calcium. This sequence of events create H^+/K^+-ATPase rich tubulovesicles to fuse into the apical plasma membrane allowing the H^+/K^+-ATPase to secrete protons directly into the lumen of the canaliculus of the parietal cell and then into the lumen of the gastric gland.

Histamine: Histamine is produced in ECL cells located in the oxyntic mucosa which serves as the major paracrine stimulator of acid secretion. It is produced in ECL cells by decarboxylation of L-histidine by histidine decarboxylase (HDC). In the gut, H2 receptors on the parietal cell increase adenylate cyclase activity and produce cAMP. HDC promoter activity is upregulated by gastrin, *H. pylori* and PACAP. Targeted gene disruption of HDC and the H2 receptor show the key role of gastric acid secretion mediated by hormones such as gastrin or PACAP. However, functional antagonists of the H2 receptor only partially inhibits acid secretion stimulated by cholinergic agents. H2 receptors are also present in smooth muscle and cardiac myocyte. H3 agonists stimulate acid secretion indirectly by inhibition of somatostatin-induced histamine release. There are no approved drugs specifically targeting the H3 receptor.

Gastrin : It is the main stimulant of acid secretion during meal stimulation. It is produced in response to luminal amino acids derived from dietary intake. At first, gastrin is synthesized as a precursor molecule that is split post-translationally into acid-stimulatory peptides, of which gastrin-17 and gastrin-34 are the most abundant, and N-terminal fragments, of which progastrin 1-35 and progastrin 1-19 dominate. Gastrin is the most potent endogenous stimulant for gastric acid secretion by supporting the synthesis and release of histamine from ECL cells.

Gastrin be similar to cholecystokinin (CCK), as it hold an identical C-terminal pentapeptide sequence. Two main classes of gastrin/CCK receptors are characterized as : CCK-1 and CCK-2. CCK-1 receptors are specific for CCK whereas CCK-2 receptors identifies both CCK and gastrin. When CCK-2 receptors become activated in parietal and ECL cells, they lead to activation of phospholipase C and release of intracellular calcium. It is belived that gastrin will regulate the secretion of histamine by increasing the release of stored histamine and by increasing the activity and gene transcription of HDC. Gastrin also has a trophic effect on the oxyntic mucosa, particularly on ECL cells. A number of neoplasms are gastrin sensitive, including gastric carcinoids and cancers of the stomach, colon, pancreas and lung. In the stomach, gastrin regulates its effects primarily through the CCK-2 receptor. The stimulatory pathways for gastrin release are central and peripheral. Neural pathways to the G cells are both inhibitory and stimulatory. Peripheral pathways to the G cells are initiated by the presence of food in the stomach as signalled by mechanical distention, pH and the presence of amines and specific amino acids. When the pH of the gastric lumen falls below 3, a negative feedback mechanism involving calcitonin-gene related peptide inhibits gastrin release, while hydrogen ions may also protonate amino acids and reduce their uptake by the G cells. Luminal pH also stimulates sensory nerve cells, amplifying somatostatin release that acts as a paracrine agent to suppress gastrin secretion.

Acetylcholine: Acetylcholine from parasympathetic vagal efferents regulates basal acid secretion. It is discharged from postganglionic neurons of the enteric nervous system and directly stimulates acid secretion by binding to muscarinic (M3) receptors on parietal cells. Acetylcholine may also regulate acid secretion indirectly by inhibiting the release of somatostatin through activation of M2 and M4 receptors on D cells. The significance of acetylcholine in the PUDs has made it a target of anticholinergic drugs. However, the doses usually required to suppress acid secretion depends upon undesirable side effects, such as dry mouth, blurred vision and urinary retention.

Somatostatin: Somatostatin is the major physiological inhibitor of acid secretion. It is released in two forms. Somatostatin 14 is found mainly in the stomach, pancreas

and enteric neurons, while somatostatin 28 is present in the small intestine. Somatostatin exercise tonic inhibitory effects on parietal cells, however, the major effects are experienced by the inhibition of histamine release and gastrin release from ECL cells and G cells. The secretion of somatostatin is increased by gastric acid and by gastrin. It is repressed by cholinergic activation and increased by vasoactive intestinal peptide activation. The somatostatin analog octreotide has a theoretic potential in the treatment of acute ulcer bleeding.

Other Regulators of Acid Secretion: Ghrelin, a stimulant of acid secretion involving the vagus nerve and histamine release. Other neurotransmitters, such as the neuropeptide GRP have been linked with meal-stimulated acid secretion. GRP mediates its effects by gastrin release and it may also be an important neurotransmitter in the vagal-cholinergic pathway. CCK may also function as a physiologic inhibitor produced by the presence of nutrients in the intestine. Other inhibitors of acid secretion that regulate somatostatin release include glucagon-like peptide, CCK, VIP, leptin, amylin and EGF.

Phases:

The secretion of gastric juice is influenced by numerous stimuli that together ease the process of digestion. The main phases of gastric secretion are the Cephalic phase, the gastric phase and the intestinal phase.

Cephalic phase:

The sensory experiences of smelling ,seeing, tasting, chewing and swallowing food contribute to the cephalic phase of secretion .The cephalic phase of acid secretion is regulated by the vagus nerve through the myenteric plexus. Acetylcholine is released and stimulates the chief cells and parietal to secrete acid and pepsinogen respectively. The G cells in the antrum liberate gastrin into the blood stream, through which it travels to the gastric glands and stimulates the acid secretion.

Gastric phase:

The gastric phase of secretion starts with the entry of food in the stomach. The two major stimuli have a secretory effect:

1) distention of the stomach

2) the presence of digested protein

The vagus and enteric nerve plexuses are activated by distention and contribute to gastric secretion through a local reflex. Both neural reflexs are controlled by acetylcholine and can be blocked by atropine. As digestion continues, products of protein break down, which stimulates the release of gastrin from G cells in the antrum. Proteins in the stomach buffer the acid gastric juice and increase the gastric pH. Caffeine stimulates acid secretion, as does calcium.

Intestinal phase:

The process of movement of chyme from stomach into the duodenum begins the intestinal phase of secretion. This phase represents a slow down of the gastric secretory response and found to be hormonally mediated by a hormone called entero-oxyntin. Gastric inhibitory peptide reduces gastric motility and secretion of acid and pepsin when chyme enters the duodenum. The intestinal absorption of amino acids (protein breakdown products) also stimulates gastric seretion. The intestinal phase of gastric secretion is limited by the fact that acid chyme in the duodenum tends to inhibit both gastric acid secretion and gastric motility .Acid in the duodenum regulate the release of hormone that inhibit acid secretion while stimulating pepsinogen secretion. Cholecystokininpancreozymin, suppress gastrin-stimulated acid production.

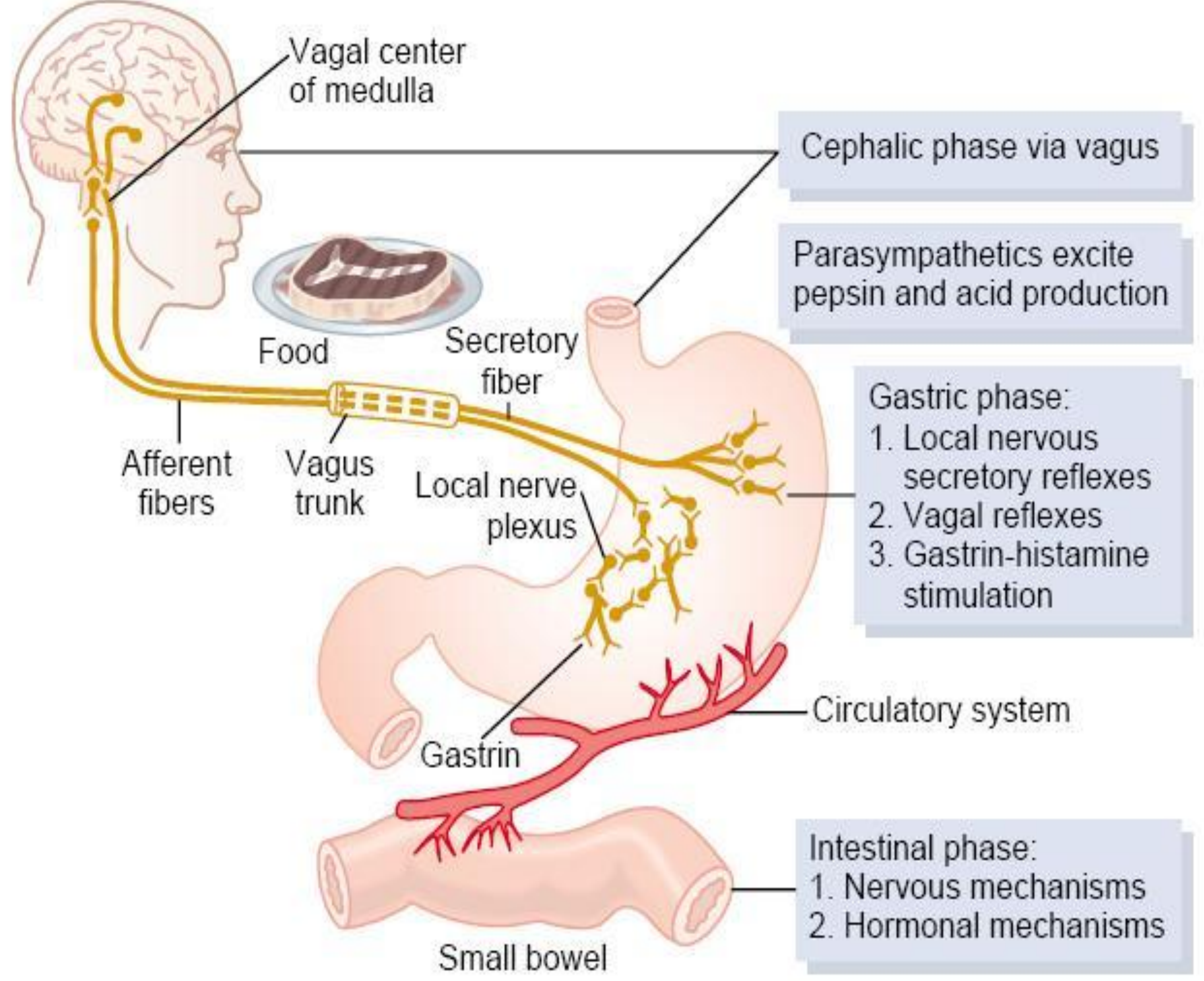


Figure 2: Regulation of acid secretion

Physiology of gastric secretion: [2]

The hydrogen ion concentration in parietal cell secretions is approximately 3 million fold higher than in blood, and chloride is secreted against both a concentration and electric gradient. Thus, the ability of the partietal cell to produce gastric acid is depends on active transport.

The key player in gastric acid secretion is a H^+/K^+ ATPase (proton pump) located in the cannalicular membrane. This ATPase is magnesium-dependent, and not inhibitable by ouabain. The energy for the transport of H^+ and K is obtained from adenosine tri phosphate (ATP). The transport function of H^+/K^+- ATPase is managed

by intracellular second messengers, either cAMP or calcium ions. The model for clarifying acid secretion is as follows:

- Hydrogen ions are produced within the parietal cells of stomach from dissociation of water. The hydroxyl ions formed by this process rapidly associate with carbon dioxide to form bicarbonate ion, a reaction mediated by carbonic anhydrase.

$$H_2O + CO_2 \xrightarrow{\text{Carbonic anhydrase}} H^+ + HCO_3^-$$

- Bicarbonate ions are transported out of the basolateral membrane in exchange for chloride ions. The flow of bicarbonate ions into blood results in a slight elevation of blood pH known as the alkaline tide. This process helps in the maintainance of intracellular pH in the parietal cell.
- Potassium and chloride ions are travelled into the lumen of the cannaliculus through conductance channels, and which is needed for secretion of acid.
- Hydrogen ion is transported out of the cell, into the lumen, in exchange for potassium through the action of the proton pump; thus potassium is effectively recycled.

Accumulation of osmotically active H^+ ion in the cannaliculus creates osmotic gradient across the membrane which will effect the outward diffusion of water , the resulting gastric juice is 15 mM KCl and 155 mM HCl with a small quantity of NaCl.

Acid secretion is regulated by paracrine (histamine), neuronal (acetylcholine) and endocrine (gastrin). Their respective receptors (H_2, M_3 and CCK2 receptors, respectively) are located on the basolateral membrane of parietal cells in the fundus and body of the stomach. The histamine (H2) receptor is a G-protein coupled receptor (GPCR) that stimulates the Gs-adenylcyclase-cyclic AMP-PKA pathway. Gastrin and Ach signal through GPCRs that associated with Gq-PLC-IP3-Ca^{2+} pathway in parietal cells. In parietal cells, the Ca^{2+} ion and cyclic AMP dependent pathways stimulates H^+/K^+-ATPase (the proton pump),which exchanges potassium and hydrogen ions across the parietal cell membrane.

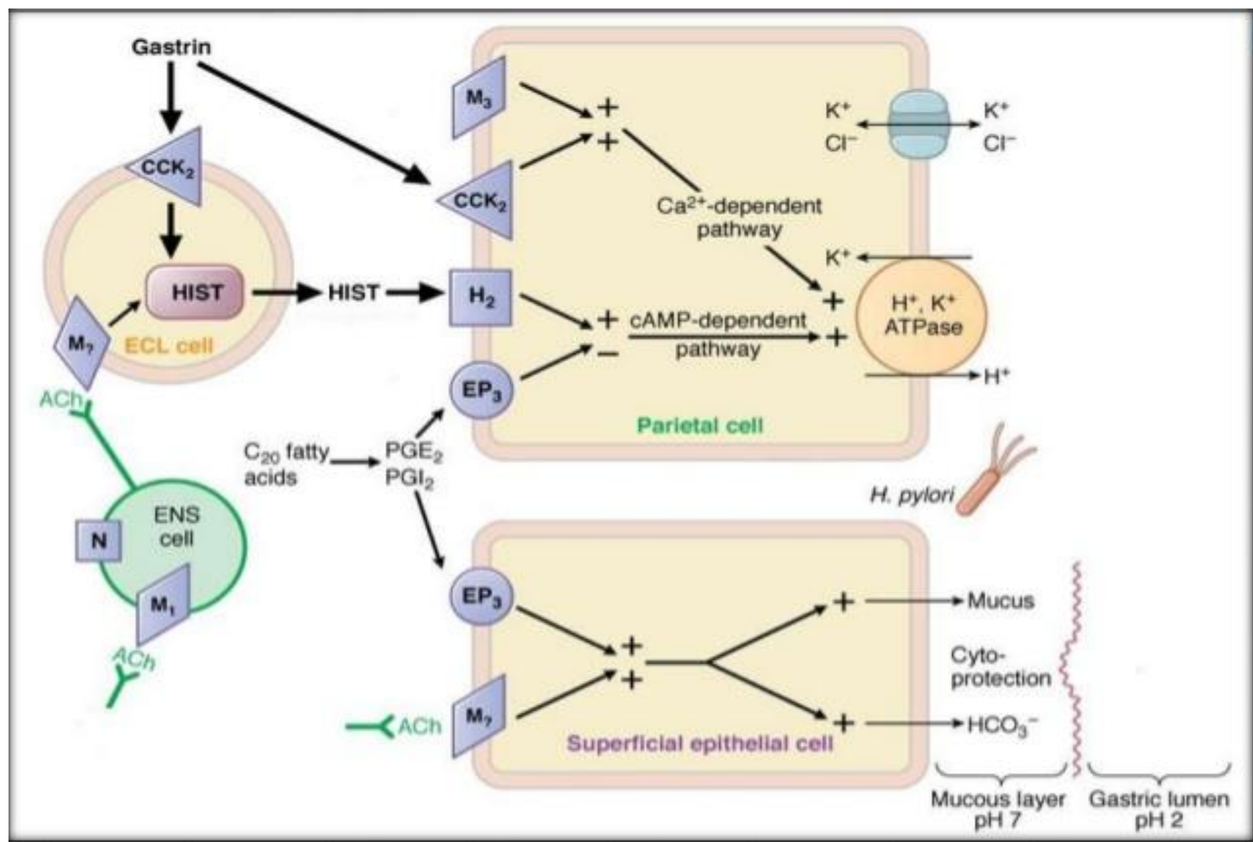


Figure.3

Peptic ulcer: [3]

Peptic ulcer disease (PUD) is characterised by excavation or inflamed lesions of the mucosa and underlying tissue of the upper GIT. Peptic ulcers can be acute or chronic and deep or superfacial. It is caused by disorder of the normal balance between the acidic effect of the gastric juice and protective effect of mucus on the gastric epithelial cells. It is viewed as an extension of the gastric erosions viewed in acute gastritis. The most common location for ulcers are the stomach and the first some centimeters of the duodenum. The pathophysiology of this GI disorder is occurs as a result of as an imbalance between mucosal defensive factors such as , prostaglandin, nitric oxide, peptides, bicarbonate, growth factors and injurious factors like smoking, acid, pepsin, , stress, alcohol etc. In healthy individuals, the digestive tract is layered with a mucous membrane that protect the layered tissue against the

harmful digestive acid; if the amount of acid is increased or the pH of the acid is significantly decreased or the mucus membrane layer becomes too thin or dry ,the gastric acid damage the tissue and ulceration occurs. Thus gastric ulcers are similar type of peptic ulcer which effects the stomach lining due to an disturbance between acid and gastric mucosa.

Classification: [11]

A peptic ulcer can be arise at various location:

- Stomach(called gastric ulcer)
- Duodenum(called duodenal ulcer)
- Esophagus(called esophageal ulcer)
- Meckel's diverticulum (called meckel's diverticulum ulcer)

Modified Johnson

- Type I: Ulcer along the body of the stomach, most often along the lesser curve at incisura angularis along the locus minoris resistantiae. Not associated with acid hypersecretion.
- Type II: Ulcer in the body in combination with duodenal ulcers. Associated with acid over secretion.
- Type III: In the pyloric channel within 3 cm of pylorus. Associated with acid over secretion.
- Type IV: Proximal gastroesophageal ulcer
- Type V: Can occur throughout the stomach. Associated with chronic use of NSAIDs (such as ibuprofen).

Gastric Ulcer

When a peptic ulcer occurs in stomach, it is called a gastric ulcer. The symptoms of gastric ulcers are more specific than symptoms of peptic ulcer.

Duodenal Ulcer

When a peptic ulcer occurs in duodenum, it is called a duodenal ulcer. This type of peptic ulcer occurs in the first part of the small intestine. Some of the symptoms of a

duodenal ulcer are interestingly quite reverse to those of gastric ulcers. Duodenal ulcers are the most commonly found in the Western world.

Lesser Known types of Ulcers

Esophageal Ulcer

It mainly occurs in the lower end of the esophagus. Esophageal ulcers are often related with a bad case of acid reflux, or GERD as it is commonly called (short for Gastro Esophageal Reflux Disease).

Bleeding Ulcer

Internal bleeding is caused by a peptic ulcer, is referred as bleeding ulcer - this is the most dangerous type of ulcer.

Refractory Ulcer

Refractory ulcers are simply peptic ulcers that have not healed after at least 3 months of treatment.

Stress Ulcer

Stress ulcers are a group of lesions (or lacerations) found in the esophagus, stomach or duodenum. These are normally only found in critically ill or severely stressed patients.

Macroscopic appearance[11]

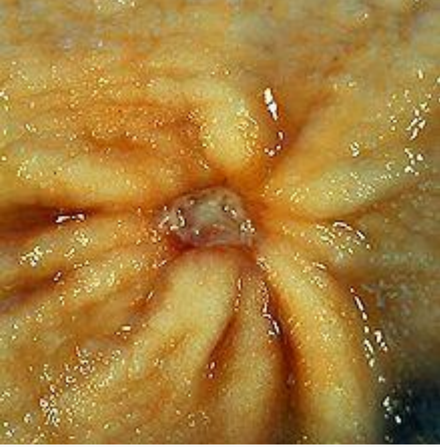


Figure.4:A benign gastric ulcer (from the antrum) of a gastrectomy specimen.

Gastric ulcers are most commonly found on the lesser curvature of the stomach. The ulcer is a round to oval parietal defect ("hole"), 2 to 4 cm diameter, with a smooth base and perpendicular borders. These borders are not elevated or irregular in the acute form of peptic ulcer, regular but with elevated borders and inflammatory surrounding in the chronic form. In the ulcerative form of gastric cancer the borders are irregular. Surrounding mucosa may present radial folds, as a consequence of the parietal scarring.

Microscopic appearance

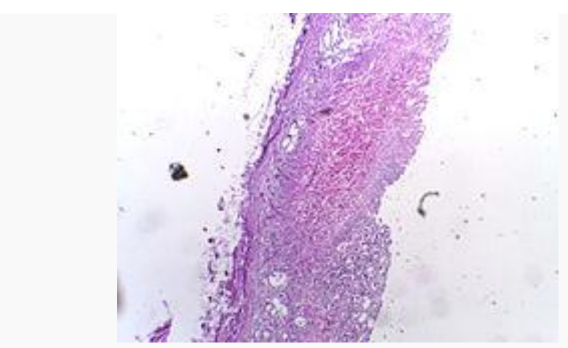


Figure.5:Micrograph showing erosive gastric ulcer. (H&E stain)

A gastric peptic ulcer is a mucosal defect which penetrates the muscularis mucosae and lamina propria, produced by acid-pepsin aggression. Ulcer margins are perpendicular and present chronic gastritis. During the active phase, the base of the ulcer shows 4 zones: inflammatory exudate, fibrinoid necrosis, granulation tissue and fibrous tissue.

Signs and symptoms[5]

- bloating and abdominal fullness.
- Abdominal pain
- waterbrash
- loss of appetite and weight loss
- melena (tarry, foul-smelling feces due to presence of oxidized iron from hemoglobin);
- nausea, and copious vomiting.

- hematemesis (vomiting of blood);this is due to bleeding caused by gastric ulcer, or from damage to the esophagus from severe/continuing vomiting.
- Rarely, an ulcer may lead to a gastric or duodenal perforation, which leads to acute peritonitis, extreme, stabbing pain, and requires immediate surgery.

Complications[5]

Gastrointestinal bleeding is the most common complication. Sudden large bleeding can be life-threatening. It occurs when the ulcer erodes one of the blood vessels, such as the gastroduodenal artery.

- Perforation (a hole in the wall of the gastrointestinal tract): If ulcer left untreated it often leads to catastrophic consequence. Spillage of stomach or intestinal content into the abdominal cavity occurs due to erosion of the gastro-intestinal wall by the ulcer. Acute peritonitis, initially chemical and later bacterial peritonitis due to perforforation at the anterior surface of the stomach. The first sign is sudden intense abdominal pain. Posterior wall perforation leads to bleeding due to involvement of gastroduodenal artery that lies posterior to the 1st part of duodenum.
- Penetration: It is a form of perforation in which the hole leads to and the ulcer continues into adjacent organs such as the liver and pancreas. Gastric outlet obstruction is the narrowing of pyloric canal by scarring and swelling of gastric antrum and duodenum due to peptic ulcers. Patient often presents with severe vomiting without bile.
- Obstruction: Pyloric stenosis occurs as a result of development of fibrous scar at or near to the pylorus. In the case of cured duodenal ulcer, it causes duodenal stenosis. Due to fibrosis and contraction healed ulcers along the lesser curvatures may produce ‘hour glass’ deformity.
- Malignant transformation: Cancer is included in the differential diagnosis (elucidated by biopsy), *Helicobacter pylori* as the etiological factor making it 3 to 6 times more likely to develop stomach cancer from the ulcer.

Pathophysiology:

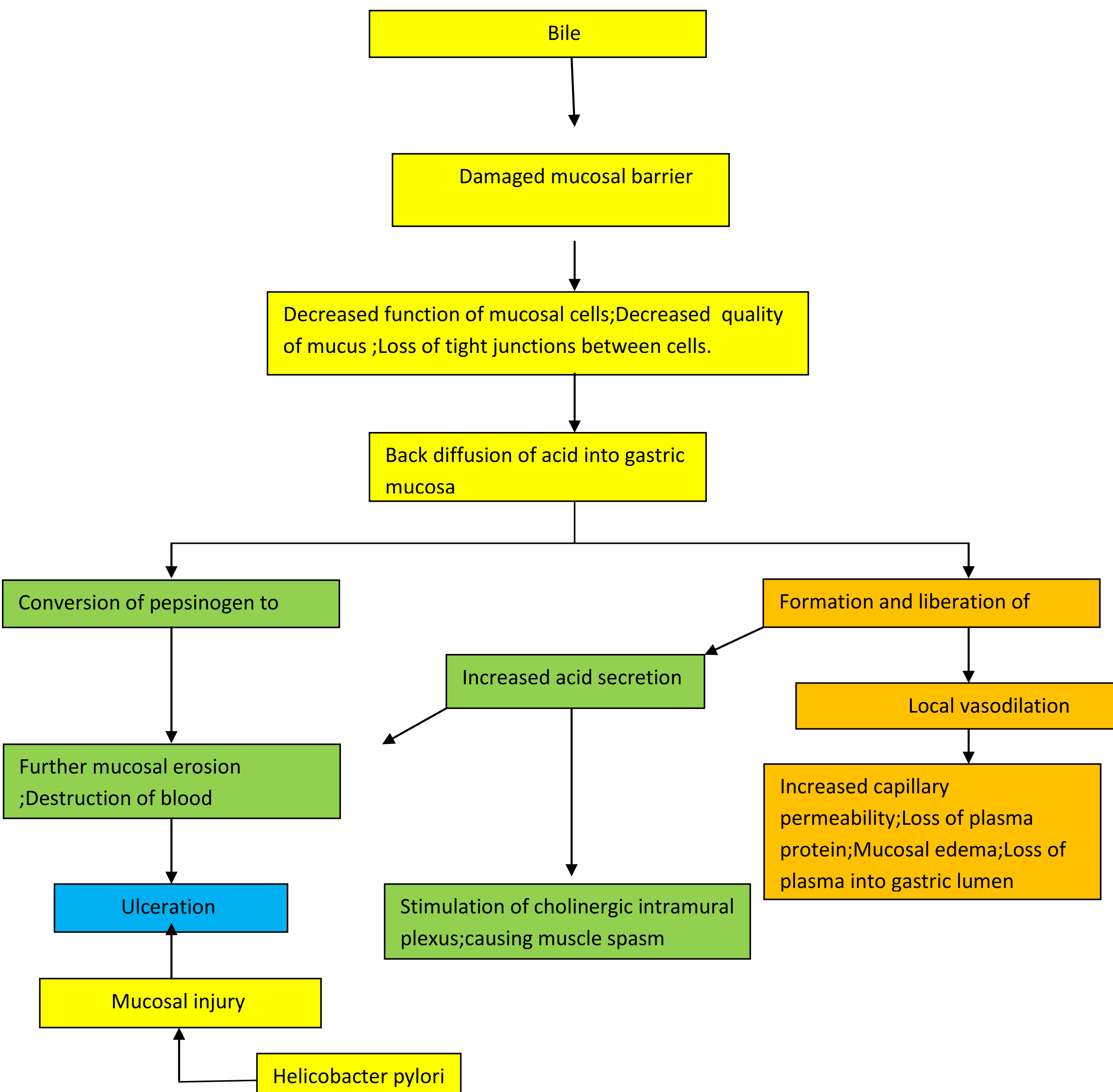


Figure 6: Pathophysiology of gastric ulcer formation[5]

FACTORS CAUSING PEPTIC ULCER: [8]

1. Helicobacter pylori gastritis:[6]

Helicobacter pylorus is a gram negative, motile, microaerophilic, flagellated and spiral shaped bacterium that inhibits various areas of the stomach and duodenum. It causes a chronic low level inflammation of the stomach lining and is strongly linked to the development of duodenal and gastric ulcers and stomach cancer. Type I strains of H.pylori have a pathogenic activity, which encodes the effector protein cytotoxin-associated gene A(cag A).After entry into the hostcell, cagA effects cell shape, raise cell motility, disturbs cell junctional activity and it causes gastric carcinomas and gastric ulcers. As a result of H.pylori increased expression of cytokines such as TNF-α and IL-1β in gastritis will occur. H.pylori-infected gastric mucosa shows infilitration of polymorphonuclear leukocytes, lymphocytes ,monocytes and plasma cell in the lamia propria and intraepithelial severe neutrophil infiltration. The immune system is unable to clear the infection, despite the appearance of antibodies. Thus, the bacterium can cause a chronic active gastritis (type B gastritis). Gastrin stimulates the production of gastric acid by parietal cells, in *H. pylori* colonization responses to increased gastrin, the increase in acid can contribute to the erosion of the mucosa and therefore ulcer formation.

2. NSAIDs: [5]

When ingested chronically, aspirin, indomethacin and other NSAIDs promote gastric ulcer formation. These drugs may injure the gastric mucosa by allowing back-diffusion of hydrogen into the mucosa. NSAIDs also inhibit the synthesis of prostaglandins, which are substances with a cytoprotective effect on the mucosa. Endogenous prostaglandins regulate the mucosal blood flow, epithelial cell proliferation, epithelial reconstitution, mucus and bicarbonate secretion.

3. Prostaglandins:

Prostaglandins are 20-carbon fatty acids, produced from arachidonic acid through the enzyme cyclooxygenase. Prostaglandins inhibit the leukocytes recruitment thereby it causes the decreased beneficial effects of these substances when the gastric mucosa is inflsmed.PGE_2 is a potent suppressor in release of PAF,

histamine and of TNF-α from peritoneal and intestinal mucosal mast cells. Prostaglandins also suppress the generation of reative oxygen metabolites by neutrophils

4. Genetic factors:

The lifetime prevalence of developing an ulcer in first degree relatives of ulcer patients is about threefold greater than in general population. This may be secondary to clustering of H.pylori within families. People with blood type O have an above-normal incidence of duodenal ulcers. Genetic influence appear to have greater role in duodenal ulcers are evidenced by their occurrence in families, monozygotic twins and association with HLA-B5 antigen.

5. Stress:

A stress ulcer is an acute form of peptic ulcer that tend to accompany severe illness , systemic trauma or neural injury. Mental stress may be associated with stress ulcer. Usually, multiple sites of ulceration are distributed within the stomach and duodenum. Decreased mucosal blood flow is an important contributing event in stress ulcer formation. Stress ulcers may be classified as ischemic or cushing ulcers.

6. Smoking :

Smokers have an increased risk of developing peptic ulcer disease .In addition, cigarette smoking delays ulcer healing and increases the risk and rapidity of relapse after the ulcer heals. Nicotine decreases biliary and pancreatic bicarbonate secretion. Smoking also accelerates the emptying of stomach acid into the duodenum. Reactive oxygen intermediates (ROI) generation and ROI-mediated gastric mucosal cell apoptosis are also considered to be important mechanism for aggravation of ulcer by cigarette smoke or nicotine. Smoking or smoke extracts impairs both spontaneous and drug-induced healing of ulcer. Smoke extracts also inhibit gastric mucosal cell proliferation by reducing ornithine decardoxylase activity, which synthesizes growth promoting polyamines.

7. Ethanol:

Ethanol-induced gastric lesions cause depletion of gastric mucus content, damaged mucosal blood flow and mucosal cell injury. It also decreases the secretion of bicarbonate (HCO_3^-) and mucus production, ethanol also produces the necrotic lesions in gastric mucosa. After the metabolism ethanol releases superoxide anion and hydro peroxy free radicals which causes raised lipid peroxidation. Membrane damage, cell death, exfoliation and epithelial erosion occurs due to increased lipid peroxide content and oxygen derived free radicals.

8. Associated disorders:

Peptic ulcer disease is more common in patients with hyperthyroidism, emphysema, rheumatoid arthritis, and alcoholic cirrhosis.

9. Advanced age:

Degenenaration of the pylorus permits bile reflux into the stomach, creating an environment that's favors ulcer formation.

10. Psychological factors:

Psychological stress, anxiety, fatigue and ulcer-type personality type are viewed as relatively minor influences to peptic ulcer disease.

11. Sex:

Duodenal ulcer is more commonly in man than women, though gastric ulcer is seen to be an equal extent in man and women.

12. Gastritis:

Some degree of gastritis is always present in the region of gastric ulcer, though it is not clear whether it is the cause or the effect of ulcer. Besides, the population distribution pattern of gastric ulcer is similar to that of chronic gastritis.

13. Mucus secretion:

Any condition that decreases the quantity or quality of normal protective mucus 'barrier' predisposes to the development of peptic ulcer.

Dietary factors like;

14. Alcohols:

A known mucosal irritant, alcohol causes marked irritation of gastric mucosa if ingested in large quantitites at concentration of 20% or greater .The only association between ethanol intake and ulcer disease exist in patients with portal cirrhosis.

15. Coffee:

Both regular and decaffeinated coffee contain peptides that stimulate release of gastrin, a hormone that triggers the flow of gastric juice. However, a direct link between coffee and peptic ulcer disease is not proven.

16. Dietary salt:

The evidence was found between an association of gastric ulcer and dietry salts. The occurrence of gastric ulcer may be linked to the amount of dietry salt consumption. The salt was shown to induce gastritis of experimental animals.

17. Effect of free radicals in gastric ulcer:

Oxygen free radicals are detrimental to the integrity of biological tissues and mediate their injury. The mechanism of damage involves lipid peroxidation, which destroys cell membranes with the release of intracellular components, such as lysosomal enzymes, leading to further tissue damage. The radicals also promote mucosal damage by causing degradation of the epithelial basement membrane components, complete alteration of the cell metabolism and DNA damage. The generation of the superoxide anion as a mechanism of damage is well established in different models of acute and chronic injury. The body has developed several endogenous antioxidant systems to deal with the production of reactive oxygen species. These systems can be divided into enzymatic and nonenzymatic groups. The enzymatic antioxidants include superoxide dismutase (SOD), which catalyzes the conversion of 02- to H2 Oa and Efo O, catalase, which then converts Ha Oa to Ha O and Oa and glutathione peroxidase, which reduces HaOa to HaO by reduced glutathione (GSH). Re-reduction of the oxidized form of glutathione (GSSG) is then catalyzed by glutathione reductase. These enzymes also require trace metal co-factors

for maximum efficiency, including selenium for glutathione peroxidase, copper, zinc or manganese for SOD and iron for catalase. The nonenzymatic antioxidants include the lipid soluble vitamins, vitamin E and vitamin A or provitamin A (beta-carotene), and the water-soluble vitamin C and GSH. Glutathione, which is synthesised intracellularly from cysteine, glycine, and glutamate, is capable of scavenging reactive oxygen species either directly or enzymatically via glutathione peroxidase. In addition, GSH is crucial to the maintenance of enzymes and other cellular components in a reduced state. The majority of GSH is synthesised in the liver. Its biologic role is believed to be a defence against dietary xenobiotics and lipid peroxidation . Changes in antioxidative molecule levels may be an important factor in ulcer generation.

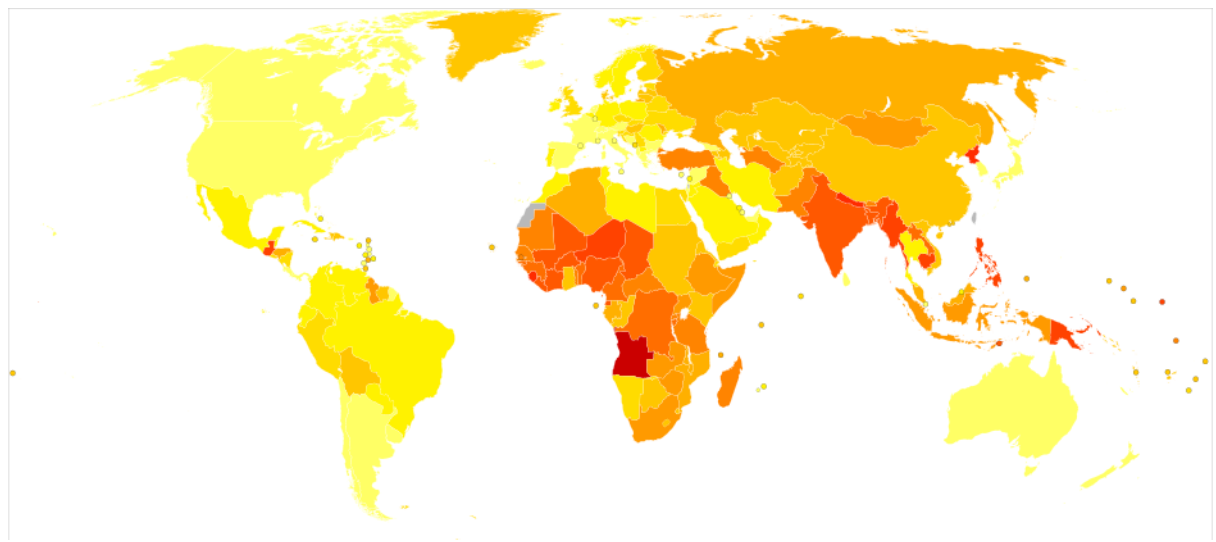
Epidemology: [11]

The lifetime risk for developing a peptic ulcer is approximately 10%.They resulted in 301,000 deaths in 2013 down from 327,000 deaths in 1990.In Western countries the percentage of people with *Helicobacter pylori* infections roughly matches age (i.e., 20% at age 20, 30% at age 30, 80% at age 80 etc.). Prevalence is higher in third world countries where it is estimated at about 70% of the population, whereas developed countries show a maximum of 40% ratio. Overall, *H. pylori* infections show a worldwide decrease, more so in developed countries. Transmission is by food, contaminated groundwater, and through human saliva (such as from kissing or sharing food utensils).

A minority of cases of *H. pylori* infection will eventually lead to an ulcer and a larger proportion of people will get non-specific discomfort, abdominal pain or gastritis. Peptic ulcer disease had a tremendous effect on morbidity and mortality until the last decades of the 20th century, when epidemiological trends started to point to an impressive fall in its incidence. The reason that the rates of peptic ulcer disease decreased is thought to be the development of new effective medication and acid suppressants and the discovery of the cause of the condition, *H. pylori*.

The incidence of duodenal ulcers has dropped significantly during the last 30 years, while the incidence of gastric ulcers has shown a small increase, mainly caused by the widespread use of NSAIDs. The drop in incidence is considered to be a cohort-

phenomenon independent of the progress in treatment of the disease. The cohort-phenomenon is probably explained by improved standards of living which has lowered the incidence of *H. pylori* infections.



Disability-adjusted life year for peptic ulcer disease per 100,000 inhabitants in 2004. no data less than 20 20–40 40–60 60–80 80–100 100–120 120–140 140–160 160–180 180–200 200–220 more than 220

Diagnosing Gastric Ulcers[11]

The diagnosis of gastric ulceration is initially based on a physical exam;

- through history, which determines risk factors; and the presence of characteristic symptoms, which are dominated by burning stomach pain.
- The presence of *H.pylori*, which can be detected by a blood test, stool test, or breath test.

  BLOOD TEST:

  The blood test determines antibody levels, although it is questionable whether a positive antibody test without confirming ulceration or inflammation via endoscopy is enough to warrant eradication therapy with antibiotics. A blood test is not always genuine for accurate peptic ulcer diagnosis because it cannot differentiate between past exposure to H. pylori and acute infection. Further, a false negative result is possible with a blood test

if the patient has recently taken certain drugs, such as antibiotics or proton pump inhibitors.

STOOL TEST:

The stool test involves examining the feces to find excessive numbers of H. pylori or antigens made against the bacteria.

BREATH TEST:

The breath test involves test for H. pylori proliferation via radioactive carbon dioxide or urea.

ESOPHAGOGASTRODUODENOSCOPY (EGD):

It is a form of endoscopy performed on patients in whom a peptic ulcer is suspected.. The endoscope allows for visual identification of the mucosal membranes of the gastrointestinal tract .By direct visual identification, the location and severity of an ulcer can be described.

BIOPSY:

Histological examination and staining are undertaken and a culture is grown in order to confirm H. pylori infection.

Prevention[12]

Prevention of gastric ulcers often involves ;

- Reducing NSAID use and use different medications or alternative approaches to relieve pain. If NSAIDs want to take long-term, H2 blockers or proton pump inhibitors is also be taken to prevent the development of peptic ulcers.
- Life style:

- The risks of peptic ulcers can be reduced by eating bland foods, dairy products and small portions during meals, but newer suggestions include eating foods rich in fiber, especially fruits and vegetables and whole grains which are vitamin-rich. They enhance the body’s ability to heal stomach irritation and prevent ulceration. Foods containing flavonoids, such as apples, cranberries, onions, garlic and tea may inhibit the growth of *H. pylori*
- Avoiding refined foods (white breads, pastas and sugar),eating less red meat and more cold-water fish, using healthy oils (olive oil or coconut oil), and

reducing trans-fatty acids are all common dietary suggestions for reducing the risk of peptic ulcer.

- Avoiding smoking is also an important approach because compounds in cigarettes obstruct with the protective lining of the stomach, making it more vulnerable to the development of ulcers. Smoking also increases stomach acid production.
- Reducing alcohol, coffee, and soda pop consumption can also help prevent ulcers as excessive use of these acidic beverages irritates and erodes the mucous lining of the stomach and intestines, leading to inflammation, ulceration and bleeding.
- Controlling stress and anxiety is also an important part of ulcer prevention.

The Pharmaceutical Treatment of Gastric Ulcers[4]

1) Proton-Pump Inhibitors

Proton-pump inhibitors are a group of drugs that shows noticeable and long-lasting decrease of stomach acid production. These drugs are widely sold in the world and are generally the most potent inhibitors of acid secretion, when compared to earlier class of inhibitors called H_2-receptor antagonists (H_2 blockers). Proton-pump inhibitors act by irreversibly blocking the hydrogen/potassium adenosine triphosphatase enzyme system (commonly called the gastric proton pump) of the stomach's parietal cells. The proton-pump is the final stage in stomach acid secretion, being directly responsible for secreting hydrogen ions into the gastric lumen and making it an ideal target for inhibiting acid secretion. Proton-pump inhibitors reduce gastric acid secretion by up to 99 %. Proton-pump inhibitors are given in an inactive form, which easily crosses cell membranes and enters into parietal cells, where they become activated by the acidic environment. The most commonly used proton-pump inhibitors include omeprazole , lansoprazole , dexlansoprazole , esomeprazole , pantoprazole and rabeprazole . The vast majority of these drugs are known as benzimidazole derivatives.

Adverse effects:

- hypochlorhydria (insufficient hydrochloric acid) can lead to a variety of side effects such as B-12 deficiency(which mimics symptoms of Alzheimer's)
- increased risk of bone fracture
- increased risk of certain heart arrhythmias and interstitial nephritis, low serum magnesium levels (hypomagnesemia), headaches (the most commonly reported adverse effect), nausea, diarrhea, abdominal pain, flatulence, constipation, fatigue and dizziness.
- stopping use can cause an increase in stomach acid production above that of normal levels that lasts for several weeks.

2) H_2 Blockers

H_2 blockers are also known as histamine blockers or H_2-receptor antagonists. It is a competitive antagonists of histamine at the parietal cell H_2-receptors in the stomach. H_2 blockers inhibit the secretion of hydrochloric acid by parietal cells by two mechanisms: histamine released by enterochromaffin-like cells in the stomach is blocked from binding on parietal cell H_2-receptors, which stimulate acid secretion; and as a consequence, other substances that promote acid secretion (such as gastrin and acetylcholine) have a reduced effect on parietal cells when the H_2- receptors are blocked. H_2 blockers are still commonly used for the treatment of dyspepsia, In the U.S., all four FDA-approved members of the H_2 blocker group (cimetidine, ranitidine, famotidine and nizatidine) are available over-the-counter in relatively low doses, or by prescription in larger doses. Cimetidine became the first "blockbuster drug".

Adverse effects:

- hypotension, gynecomastia in males, loss of libido, and impotence. (fewer adverse drug reactions)

3) Antibiotics

Antibiotics are used to kill bacteria such as *H. pylori*, but due to resistance and adaptability, more than one type is often suggested to be taken at the same time. Antibiotic regimens are different throughout the world, but in the U.S., antibiotics prescribed for treating *H. pylori* include amoxicillin, clarithromycin, metronidazole,

furazolidone and tetracycline. Antibiotics are usually prescribed for only two weeks at a time in order to avoid side effects.

Adverse effects:

- acquired resistance to antibiotic therapy
- serious allergic reactions (including anaphylaxis), nausea, upset stomach, diarrhea, sun sensitivity, disruption of the intestinal flora and fauna.
- Systemic overgrowth of pathogenic bacteria (such as *Clostridium difficile*) and yeast species (such as *Candida albicans*), and numerous potential interactions with other drugs.

4) Bismuth Salicylate

Medications that protect the lining of the stomach and small intestine from the damaging effects of acid are called cytoprotective agents and it include the medications sucralfate and misoprostol , and over-the-counter agents such as bismuth salicylate. Bismuth compounds may also directly kill *H. pylori*, but it cannot be used as a replacement for antibiotics. Bismuth salicylate, like all salicylates (especially aspirin), can cause serious bleeding problems when used alone in patients with bleeding ulcers. Bismuth quadruple therapy usually involves combining bismuth salicylate with a proton-pump inhibitor, tetracycline and metronidazole for 10-14 days. The main reasons to add bismuth salicylate to a treatment regimen for gastric ulcers is if the patient is still infected with *H. pylori* after a trial of triple therapy, or if the patient cannot take amoxicillin (a penicillin-like antibiotic) because of a penicillin allergy, or if the patient has been treated before with a macrolide antibiotic, such as clarithromycin.

Adverse effects:

- darkening of the stools and/or tongue, and a metallic taste in the mouth.

Summary of Common Pharmaceutical Protocols:

Proton-pump inhibitor / H_2-blocker + clarithromycin + metronidazole / amoxicillin

Proton-pump inhibitor / H_2-blocker + metronidazole + tetracycline / bismuth salicylate

Proton-pump inhibitor / H_2-blocker + furazolidone + tetracycline / bismuth salicylate

Antacids:

They neutralize existing stomach acid, which can provide relief of burning stomach pain, heartburn, and indigestion, but they are not used in the treatment for gastric ulcers. Antacids do not kill *H. pylori* and also not block the production of stomach acid. Commonly used antacids include aluminum hydroxide, magnesium hydroxide (Milk of Magnesia), aluminum hydroxide combined with magnesium hydroxide, calcium carbonate, and sodium bicarbonate. It is recommended to take antacids at least one hour before or two hours after taking other medications because antacids may block the medications from being absorbed and effectively utilized.

Adverse effects:

- constipation or diarrhea, depending on the main ingredients of the antacids.

$H^+/^{K+}$-ATPase:

The gastric H^+/K^+-ATPase is found in the parietal cell of the stomach and a small amount in the renal medulla. This enzyme can be seen in cytoplasmic tubular membranes in the resting state and also in the microvilli of the expanded secretory canaliculus in the stimulated state of the parietal cell. This morphological change is supposed to result from fusion of cytoplasmic vesicles with the rudimentary microvilli to form the elongated microvilli of the expanded secretory canaliculus. Once the enzyme reaches the canaliculus, the enzyme secrets acid by the exchange of cytoplasmic hydronium with extracellular K. The function of K efflux channel is to supply K to the luminal surface of the pump to allow H for K exchange.[9]

The H^+/K^+-ATPase is the proton pump responsible for gastric acid secretion. Using ATP hydrolysis as energy source, it catalyzes the electroneutral exchange of protons and K^+ ions across the cell membrane, generating an extracellular proton concentration that can be as much as a million times higher than the intracellular concentration. The H^+/K^+-ATPase consists of two subunits, a catalytic a subunit of about 110 kDa and a heavily glycosylated b subunit of about 35 kDa (with the sugar chains, the molecular weight of the b subunit is in the range of 60–80 kDa). The catalytic a subunit contains domains for ion and ATP binding, whereas the tightly bound b subunit is necessary for enzyme maturation and trafficking to the plasma membrane. The H^+/K^+-ATPase belongs to the large family of Ptype ATPases which also includes the Na^+/K^+-ATPase and the sarco-endoplasmic reticulum Ca^{2+}-Atpase.

P-type ATPases are characterized by an ATP hydrolysis –coupled ion transport cycle that proceeds through an intermediate, in which an aspartic acid becomes phosphorylated (EP) . The transport mechanism of P-type ATPases was originally proposed for the Na^+/K^+- ATPase . This mechanism appears to be conserved among P-type ATPases, although different family members transport different ions and with different stoichiometries. During the transport cycle, the Na^+/K^+-ATPase, like all the other P-type ATPases including H^+/K^+-ATPase, undergoes large conformational changes that have been characterized by a number of biochemical structure.[10]

Inspite of recently available drugs for peptic ulcer, the aim of the study is to design around 30 benzimidazole derivatives and docked with H^+/K^+-ATPase receptor using GLIDE (Grid-based Ligand Docking from Energetics) program (Glide, version 10.1 Schrodinger, LLC New York, 2015) and best compound are selected based on docking score ,*invitro* and *invivo* studies .

REVIEW OF LITERATURE

- **Vikash Kumar *et al.*.,[2014][17]:** Reported a series of benzimidazole derivatives and discussed the structural –activity relationship of the most potent compounds.
- **Neeraj Agarwal *et al.*.,[2013][18]**: Reported that QSAR study was made on novel series of biaryl imidazole derivatives acting as H/K-ATPase inhibitors .Also found that biaryl imidazoles can undergo hydrogen bonding and hydrophobic interaction. Docking studies of this compounds are also exhibited, which shows better interaction with receptors than already marketed drugs.

R
CH_3
N
CH_3
N
NH
X
H_3C
CH_3

- **Avinash patil *et al.*.,[2012][19]:** This investigation is concerned with synthesis of new substituted benzimidazole derivatives with the objective of discovering novel and potent antiulcer agent and they are characterized by spectral and elemental analysis. The synthesized compounds are screened for antiulcer activity and found that the benzimidazole derivatives linked with the piperazine moiety shows good antiulcer activity.

- **Kuldeep Kumar *et al..*,[2012][20]**: Reported some new 2-substituted benzimidazole derivatives were synthesized from microwave irradiation method by condensation of 2-nitro aniline with different carboxylic acids (aromatic, aliphatic and heterocyclic) and these compounds are identified by FT-IR and 1HNMR spectroscopic techniques.
- **Tribhuvan Singh *et al*..,[2012][21]:** Reported a series of novel pyrimidylthiomethyl benzimidazole and pyrimidylsulfinylmethyl benzimidazoles have been synthesized and evaluated for their antiulcer activity and concluded that sulfinyl derivative is more effective than thio analog.

- **Khan Farhan.R *et al..*,[2011][22]**:Reported a series of new substituted 2-(pyrimidinylsulfinyl)benzimidazoles derivatives synthesized and evaluation against antiulcer and antisecretory activity as a inhibition of H/K-ATPase by induction of gastric ulceration in male wistar rats. These series of new compounds are characterized by IR and elemental analysis and antiulcer activities of the compounds were determined by acetylsalicylic acid method-induced gastric ulcer.

- **Malladi Srinivas Reddy** ***et al..*****,[2011][23]:** Reported a series of six novel mercaptobenzimidazole derivatives is synthesized and the compounds are characterized by their analytical and spectral data. All the compounds are screened for their antiulcer and antimicrobial activity. 2-(1H-benzimidazole-2-sulfinyl)-N-(4-benzyloxy-phenyl)-acetamide, N-(4-benzyloxy-phenyl)-4-(1H-benzimidazole-2-sulfinyl) butyramide,N-(4-benzyloxy-phenyl)-4-(5-methoxy-1H-benzimidazole-2-sulfinyl) butyramide showed significant antiulcer activity.

- **GeraldDArtman III** ***et al..*****,[2010][24]**: Reported that 2-chloro-1,3-dimethyllimidazolinium chloride has been found to effectively and rapidly generate 2-aminobenzimidazoles from 1,2-diaminoarenes and isothiocyanates in moderate to good yields at room temperature in a one-pot operation.

- **O.Sandhya** ***et al..*****,[2009][25]:** Reported a series of nine new [1-benzyl-2-phenyl-sudstituted]-1H-5,6-substituted-benzo(d)imidazoles substituted benzaldehydes. All these compounds were characterized by IR, ^{1}H NMR and Mass spectroscopic data. Antioxidant activities of these compounds are evaluated by ferrous induced lipid peroxidation in rat brain homogenate. And it was found that compound having electron releasing group such a as dimethyl amino, methoxy and hydroxyl

substituent at position 1 and 2 of benzimidazoles increases the activities when compared to the benzimidazole having no substituent on the rings.

- **Swastika Ganguly** ***et al..*****,[2009][26]:** Reported about the synthesizing of 2-[5-substituted-1-H-benzo(d)imidazol-2-ylsulfinyl]methyl-3-substitutedquinazoline-4-(3H)-one derivatives and tested for antiulcer activity against pylorus ligation-induced, aspirin induced and ethanol induced ulcer in rat model and synthesized compounds were characterized by IR,MASS,^{1}H NMR and elemental analysis.

- **Xuan Guida** ***et al..*****,[2006][27]**: Reported a series of novel 1-substituted-2-aminobenzimidazole derivatives were synthesized and structures of synthesized compounds were confirmed by ^{1}H-NMR spectra and by elemental analysis. Acute toxicity studies of these compounds were done on mice via toxicity (logLD_{50}).QSAR analysis of these chemicals was done based on relationship between acute toxicity and the octanol/water partition coefficient (logP). Product was identified on basis of ^{1}H-NMR spectra.

R= $-CH_3$,-H,$-CH_2CH_2CH_3$

- **Junko Tanaka** ***et al..*****,[2004][28]:**Reported the effect of a new benzimidazole derivative,ME3407 on gastric acid secretion and gastric and duodenal ulcers in rats. And also found that it is effective against shay ulcers, water-immersion stress-ulcers acetyl salicylic acid and histamine induced erosion.ME3407 only active orally and have direct action on ulcers and acid secretion from the gastric membrane.

- **R.M. Shafik** ***et al..*****,[2004][29]:** Reported that inorder to establish new antiulcer agents a series of 2-(2-substituted amino) -1H- benzimidazoles,1 ,3-disubstituted-3,4-dihydropyrimido [1,6-a] benzimidazoles, 3-substituted-3,4 dihydropyrimidol [1,6-a] benzimidazol-1(2H)-thiones and 3-substituted-1,2,3,4-tetra hydropyrimido [1,6-a] benzimidazoles were synthesized and they are evaluated for the gastric antisecretory activity using *invivo* pylorus ligated rat method.Omeprazole was used as reference and the result indicates that the test compound exhibit anti secretoryactivity.

- **Dow Kwon *et al.*.,[2001][30]**: Reported that the inhibitory effects of IY-81149(2-[[-4-methoxy-3-methyl)-2-pyridinyl]-methyl-sulfinyl]-5-(1H-pyrol-1-yl)-1H-benzimidazole,a newly developed proton pump inhibitor on gastric acid secretion was investigated on *invitro* and *invivo* and this compound shows 2-3 times stronger inhibitory activity than omeprazole in pylorus-ligated rats.
- **Sung Yun Cho *et al.*.,[2001][31]:** Reported that a series of benzimidazole derivatives containing oxycyclic pyridine was prepared and evaluated for their H^+/K^+ ATPase inhibitory activity and several of the synthesized compound shows potent antisecretory pylorus-ligated rats when administered intraduodenally and also found that their inhibitory activity is similar to omeprazole.

- **Young-Kuk Cung *et al.*.,[1998][32]:** Reported about the study to determine the effect of a newly synthesized proton pump inhibitor ,2-dimethylamino-4,5-dihydrothiazolo[4,5:3,4] pyridol[1,2-a] benzimidazole (YJA2O379-2)on gastric H^+/K^+- ATPase activity ,acid secretion and experimental gastroduodenal lesions or ulcers in rat and found that YJA20379-2 has potent antisecretory and antiulcer effects.

- **Hyae Gyeong Cheon *et al.*.,[1996][33]:** Reported the *invitro* effect of various benzimidazole derivatives on gastric H^+/K^{+-}ATP ase activity .The result showed that the effect of substituents on the benzimidazole ring were not significant. Replacement of sulfoxide connecting the two ring system to sulfide result in the inactive compound, its suggesting the importance of sulfoxide group in the inhibition. And also compound with 5 or 6 membered oxacyclic substituents attached to the pyridine ring displayed the most effective inhibitory activity. Among the series AU-47 shows better inhibitory activity and is a relevant candidate for the development of antiulcer agent.

- **Shin-ichi Yamada *et al.*.,[1995][34]:** Reported a series of 2-[(cycloalka[b] pyridinyl)sulfinyl]-1H-benzimidazoles was synthesized and tested for antisecretory activity against pentagastrin-induced gastric acid secretion in rats. A novel benzimidazole derivative containing a cyclohepta[b]pyridine moiety was found to be the most potent among the series, which include five to eight membered cycloalka [b] pyridine ring system.2-[(6,7,8,9-tetrahydro-5H-cyclohepta[b]pyridin-9-yl) sulfinyl]-1H-benzimidazole analogs with various substituents on the aromatic ring shows superior properties to omeprazole.

- **Okabe *et al.*.,[1992][35]**: Reported about the novel imidazole derivative wherein R^1 is hydrogen or an alkyl group having 1-6 carbon atoms ,R^2 is an alkyl group having 2-6 carbon atoms substituted with an alkoxy group having 1-4 carbon atoms,R^3,R^4,R^5,R^6 independently is hydrogen, halogen, an alkyl group having 1-6 carbon atoms or a fluorine substituted alkoxy group having 1-6 carbon atom and this imidazole derivative have antiulcer activity.

- **BernhardKohl *etal* .,[1992][36]:**Reported [(pyridylmethyl)sulfinyl]benzimidazoles are a class of highly potent antisecretory (H^+/K^+)-ATPase inhibitor. The aim of the study was to identify compounds with high H^+/K^+-ATPase inhibitory activity in stimulated gastric gland possessing acidic pH . And also reported that the introduction of 3-methoxy group inhibitors possessing high potency similar lansoprazole and omeprazole.

- **Briving *et al* .,[1992][37]:** Reported that benzimidazole derivative containing comounds and ther use as antiulcer agent and the aim of the study to invent novel compounds which inhibit exogenously or endogenously stimulate gastric acid secretion and thus useful as antiulcer agent.

- **Minoru Uchida *et al.*.,[1990][38]**: Reported a series of 4-substituted 8-[(-2-benzimidazolyl) sulfinylmethyl]-1,2,3,4-tetrahydroquinolines were synthesized and examined for ATPase inhibitory and antisecretory activities. Compounds tested were potent H^+/K^+ATPase inhibitors and showed good antisecretory activity. The compounds like 4-(N-allyl-N-methylamino)-1-ethyl-8-[(5-fluro-6-methoxy-2-benzimidazolyl)sulfinylmethyl]-1-ethyl-1,2,3,4-tetrahydroquinoline have potent activity..The structural activity relationship was also studied.

- **John C. Sih *et al.*,[1990][39]:** Reported about the synthesis of N-substituted benzimidazole(H^+-K^+)ATPase or proton –pump inhibitors. And these compounds are synthesized to function as prodrug of the parent N-H compound and evaluated their ability in inhibiting gastric (H^+-K^+)-ATP ase and gastric acid secretion. The N-(acyloxy) alkyl-substituted benzimidazoles showed improved chemical stability in solid state and in aqueous solution compared to their parent N-H compounds. The N-ethoxy -1-ethyl-substituted benzimidazoles shows equal activity as N-H compound in shay rat (10mg/kg).

- **Susumu Okabe *et al.*,[1988][40]:** Reported the effect of a newly synthesized benzimidazole derivative NCe-1300-B on H^+/K^+ ATPase in the hog gastric mucosa and on the basal gastric acid secretion and necrotizing agent-induced gastric lesion in rats. It inhibit the gastric secretion on basis of concentraction-dependent manner. The antisecretory effect in a dose of 100mg/kg persist for upto 72hr.The effects of N-1300-B is almost similar to that of established proton pump inhibitor.

- **Adelstein *et al.*,[1988][41]:** Reported about the invention of 2-[(imidazo[1,2-a]pyridine-3-ylmethyl)sulfinyl]-1H-benzimidazoles that are useful in treatment of ulcers. And reported that this invention relates to 2-[(imidazo[1,2-a]pyridine-3-ylmethyl)sulfinyl]-1H-benzimidazoles that inhibit (H^+/K^+)-ATPase obtained from gastric mucosa and thus inhibit acid secretion by parietal cells of the stomach.

- **Enzo CEREDA *et al.*,[1987][42]:** Reported a series of sulfinyl benzimidazoles and tested for antisecretory activity. In the initial screening two compounds are tested for antiulcer activity. The new compounds showed pharmacological properties differ from omeprazole, and they proved to be weak antisecretory and specific anti-ulcer activity .And some structural requirements for optimum activity were elucidated.

- **G.Raynaud *et al.*,[1976][43]:** Reported a new compound 7110MD 1-(2-benzoyl ethyl)2-(cinnamyl piperazinyl 1-methyl)benzimidazole dimaleate belongs to a series of benzimidazole derivative which possess gastric anti secretory and antiulcer properties. In pylorus ligated rats for 4hrs, it decreases the volume and acid concentration of gastric secretion at doses well below LD_{50}.

CH_2-CH_2-CO-C_6H_5

N

H_2
C

N — N — CH_2-CH=C — C_6H_5
H

N
H

AIM AND OBJECTIVES

Peptic ulcer disease (PUD) is characterised by inflamed lesions or excavation of the mucosa and underlying tissue of the upper gastrointestinal tract. The ulcers are the result of damage to the mucus membrane that normally protects the oesophagus, stomach and duodenum from gastric acid and pepsin.

Treatment of ulcer disease is prominently focused on reduction of aggressive factors and strengthening mucosal defense of stomach and duodenum . These are all treated by blocking acid secretion through proton pump inhibitors such as benzimidazole derivatives.

These derivatives potently inhibit gastric proton pump by converting into active metabolite, that is, thiophilic cyclic sulphenamides. This transformation takes place in the luminal compartment of secreting parietal cell. The benzimidazole contains a benzene ring fused to an imidazole ring. Almost all benzimidazole derivatives with their two ring systems bear functional substituents and this leads to modification of physico-chemical, metabolic and pharmacokinetic properties of drugs.

- ❖ Benzimidazole is a versatile nucleus with various biological activities, and many antiulcer drugs have the benzimidazole moiety.

Omeprazole

Pantoprazole

Licensed compounds available in the market

- ❖ Benzimidazole derivatives plays important role in medicinal field with so many Pharmacological activities such as antimicrobial, antiviral, antiulcer, antidiabetic,

anticancer, antifungal, antihistaminics.

- ❖ The enzyme H^+/K^+ ATP ase,is responsible for gastric acid production was selected for the study .The gastric H^+/K^+ ATPase is the member of the P_2 type ATPase family and undergo cycle of phosphorylation and dephosphorylation coupled to the outward and inward transport of hydrogen and potassium ions in the secretory canaliculus of the parietal cells.
- ❖ Over activity of this enzyme causes hyperacidity by producing more of hydrochloric acid inside the stomach. This enzyme, therefore was found to be a good target for designing compounds to treat hyperacidity.
- ❖ The design of proton pump inhibitors is focused on achieving long lasting and rapid inhibition of acid secretion. The PPIs decrease the amount of acid secretion by inhibiting the function of the pump and are useful to treat ulcer.
- ❖ After introduction of the first PPI, the apparent drawbacks of irreversible proton pump inhibitors, mainly because of their prolonged acid suppression, are becoming a cause of concern and also the main side effects of available proton pump inhibitors are confusion, delirium, dementia, cardiovascular risk, kidney injuries etc.
- ❖ The emphasis has been put on the molecular modulation specifically modifying different substituents and its positions in the aromatic system to overcome these drawbacks.
- ❖ Hence the aim and objective of study was to design the molecules with docking software and synthesis, characterize, novel benzimidazole derivatives

as antiulcer agents by H^+/K^+ATP ase inhibition and to evaluate its *invivo* and *invitro* anti ulcer activity.

PLAN OF STUDY

Phase I

Designing of compounds: Compounds were designed for the H+/K^+ATP ase-receptor antagonist.

Phase II

Docking analysis: Docking studies of designed compounds were carried out using GLIDE (Grid-based Ligand Docking with Energetics) module version 4.5, Schrodinger, LLC, New York , NY, 2015. The designed compounds were used for docking on H^+/K^+-ATPase receptor antagonist (PDBID:2ZBD)

Phase III

Synthesis

Step I

Cl, NO_2, NH_2 —— Zn / NaoH,EtOH ——> Cl, NH_2, NH_2

4-Chloro-2-nitrobenzene 4-Chlorobenzene-1,2-diamine

Step II

Cl NH2 NH2 + COOH NH2 4N HCl → Cl N N H NH2

4-Chlorobenzene-1,2-diamine

Para amino benzoic acid

4-(5-chloro-1H-benzo[d]imidazol-2-yl) benzenamine

Step III

Cl N N H NH2 → NH2.NH2.H2O Ethanol, △ 2hr → Cl N N H NHNH2

4-(5-Chloro-1H-benzo[d]imidazol-2-yl)benzenamine

1-(4-(5-chloro-1H-benzo[d]imidazol-2-yl) phenylHydrazine

Step IV

Cl N N H NHNH2 + O=C(R) R' → Glacial acetic acid Ethanol,30min △ → Cl N N H NH N=C H R'

1-(4-(5-1H-benzo[d]imidazol-2-yl)phenyl)hydrazine

(Z)-2-benzylidene-1-(4-(5-chloro-1h-benzo[d]imidazol-2-yl)phenyl)hydrazine derivatives

R=H,CH3
R'=Cl,CH3,OCH3,OH etc

Phase IV

Characterization: All the newly synthesized compounds were characterized by melting point determination, solubility property, TLC analysis and their structures were elucidated by IR spectroscopy, H^1NMR spectroscopy and mass spectroscopy.

Phase V

Biological Evaluation:

- ***Invitro* antioxidant activity:** All the synthesized compounds were evaluated for in *vitro* antioxidant activity by DPPH and ABTS methods.
- ***Invitro* cell viability assay:** Evaluation of cell viability assay was conducted in AGS (human epithelial gastric cell) cell lines.
- **Acute toxicity study:** Toxicity study of the selected compound (P-28) was conducted on female adult Wistar rats (150-200g) following the OECD 423 guidelines.
- **Antiulcer study:** Evaluation of antiulcer activity of P-28 was done by Pyloric Ligation induced and Indomethacin induced ulcer model.
- ***Invivo* antioxidant activity:** Estimation of catalase, superoxide dismutase, glutathione peroxidase, reduced glutathione, Protein estimation by Lowry's method, Myeloperoxidation was carried out on stomach tissue.

METHODOLOGY

METHODOLOGY PROTOCOL:

The experimental work was conducted under the following sub headings:

METHODOLOGY PART I:

- A library of novel molecules were designed for the H^+/K^+ ATPase receptor.
- Docking studies of the designed molecules were carried out using glide software version 10.1 ,Schrodinger, LLC, New York,NY,2015
- Synthesis of targeted compounds as H^+/K^+ ATPase antagonist
- Physicochemical studies and characterization of synthesized compounds
- Spectral studies characterization of synthesized compounds

METHODOLOGY PART II:

- Biological evaluation:
- Evaluation of in vitro cell viability assay in AGS (human epithelial gastric cell)cell lines
- Evaluation of *invitro* antioxidant activity
- Evaluation of acute toxicity study following OECD 423 Guidelines
- Evaluation of antiulcer study: By 1)Indomethacin induced ulcer

  2)Pyloric Ligation induced ulcer

- Evaluation of *invivo* antioxidant activity :Estimation of catalase, superoxide dismutase, glutathione peroxidase, reduced glutathione, Lipid peroxidation, Myeloperoxidation, protein by lowry's method will be carried out
- Statistical analysis.
- Histopathological study: Histolopathology of stomach was carried out to further analyze the effect of the test compound.

5.1. DESIGNING OF THE NOVEL MOLECULES OF H^+/K^+-ATP ase RECEPTOR

Novel drug molecules were designed and their docking studies have been carried out on H^+/K^+ ATP ase using the Glide software to study on their dock score and interacting residues. The details of the designed molecule are given in the table: 2

STRUCTURES OF DESIGNED COMPOUNDS

Compound Code	R	R_1
P1	Cl	
P2	Cl	
P3	Cl	
P4	Cl	
P5	Cl	

P6	Cl	
P7	Cl	
P8	Cl	
P9	Cl	
P10	Cl	
P11	Cl	
P12	Cl	

P13	Cl	
P14	Cl	
P15	Cl	
P16	Cl	
P17	Cl	
P18	Cl	

P19	Cl	
P20	Cl	
P21	Cl	
P22	Cl	
P23	Cl	
P24	Cl	

P25	Cl	
P26	Cl	
P27	Cl	
P28	Cl	
P29	Cl	
P30	Cl	

5.2. DOCKING STUDIES

Docking procedures aims to estimate the experimental binding modes and affinities of small molecules within the binding site of particular receptor targets and is presently widely used as a standard computational tool in drug design for virtual screening studies and lead compound optimisation to find novel biologically active molecules. Glide docking uses the assumption of a

rigid receptor, although scaling of vander waals radii of nonpolar atom, which decrease penalties for close contacts, can be used to model a light "give" in the receptor and/or ligand. Docking studies of designed compounds were carried out using GLIDE (Grid-based Ligand Docking with Energetics) module version 10.1, Schrodinger, LLC, New York, NY, 2015. The software package is running on multi-processor Linux PC. GLIDE has previously been validated and applied successfully to predict the binding orientation of many ligands.

5.2. a. DOCKING METHODOLOGY

The steps involved in the docking are as follows:

Ligand structure: The chemical structure of each ligand was drawn using build module.

Ligand preparation: In order to prepare high quality, all atom 3D structures for large numbers of drug-like molecules, starting with the 3D structures in SD Maestro format, Lig Prep was used. Lig Prep produced a single, low energy, 3D structure with corrected chiralities for each successfully processed input structure.

Preparation of protein: The typical structure file from the PDB is not suitable for immediate use in molecular modeling calculations. A typical PDB structure file consists of heavy atoms and may include a co-crystallized ligand, water molecules, metal ions and cofactors. Some structures are multimeric, and may need to be reduced to a single unit. Because of the limited resolution of X-ray experiments, it can be difficult to distinguish between NH and O, and the placement of these groups must be checked. PDB structures may be missing information on connectivity, which must be assigned, along with bond orders and formal charges. This was done using the Protein Preparation Wizard.

Receptor grid generation: Receptor grid generation requires a "prepared" structure: an all atom structure with appropriate bond orders and formal charges. Glide searches for favourable interactions between one or more ligand molecules and a receptor molecule, usually a protein. The shape and properties of the receptor are represented on a grid by several different sets of field that provide progressively more accurate scoring of the ligand poses. The options in each tab of the Receptor Grid Generation panel allow defining the receptor structure by excluding any co-crystallized ligand that may be present, determine the position and size of the active site as it

will be represented by receptor grids, and set up Glide constraints. A grid area was generated around the binding site of the receptor.

 Ligand docking: This is carried out using GLIDE DOCK. Glide searches for favourable interactions between one or more ligand molecule and a receptor molecule, usually a protein. Each ligand acts a single molecule, while the receptor may include more than one molecule, e.g., a protein and a cofactor. Glide was run in rigid or flexible docking modes; the latter automatically generated conformations for each input ligand. The combination of position and orientation of a ligand relative to the receptor, along with its conformation in flexible docking, is referred to as a ligand pose. The ligand poses that the Glide generates pass through a series of hierarchical filter that evaluate the ligand's interaction with the receptor. The initial filters test the spatial fit of the ligand to the defined active site and examines the complimentarily of ligand-receptor interactions using a grid-based method patterned after the empirical Chem Score function. Poses that passed these initial screens enter the final stage of the algorithm, which involves evaluation and minimization of a grid approximation to the OPLS-AA non bonded ligand-receptor interaction energy. Final scoring is then carried out on the energy-minimized poses.

Glide Extra-Precision Mode (XP) – The extra-precision (XP) mode of Glide combines a powerful sampling protocol with the use of a custom scoring function designed to identify ligand poses that would be expected to have unfavourable energies, based on well-known principles of physical chemistry. The presumption is that only active compounds will have available poses that avoid these penalties and also receive favourable scores for appropriate hydrophobic contact between the protein and the ligand, hydrogen-bonding interactions, and so on. The chief purposes of the XP method are to weed out false positives and to provide a better correlation between good poses and good scores. Extra-precision mode is a refinement tool designed for use only on good ligand poses. Finally, the minimized poses are re-scored using Schrodinger's proprietary *Glide Score* scoring function. Glide Score is based on Chem Score, but includes a steric-clash term and adds buried polar terms devised by Schrodinger to penalize electrostatic mismatches:

$$\text{Glide Score} = 0.065*\text{vdW} + 0.130*\text{Coul} + \text{Lipo} + \text{Hbond} + \text{Metal} + \text{BuryP} + \text{RotB} + \text{Site}$$

Table 3:Components of the Glide Score (G-Score)

Component	Description
vdW	Vander Waals energy term is calculated with reduced net ionic charges on groups with formal charges, such as metals, carboxylates and guanidiniums.
Coul	Coulomb energy term is calculated with reduced net ionic charges on groups with formal charges, such as metals, carboxylates and guanidiniums.
Lipo	Lipophilic contact term rewards favourable hydrophobic interactions.
H-bond	Hydrogen-bonding term is separated into differently weighed components that depend on whether the donor and acceptor are neutral, one is neutral and the other is charged, or both are charged.
Metal	Metal-binding term use only for the interactions with anionic acceptor atoms is included. If the net metal charge in the apoprotein is positive, the preference for anionic ligands is included; if the net charge is zero the preference is suppressed.
BuryP	Penalty for buried polar groups.
RotB	Penalty for freezing rotatable bonds.

Site	Polar interactions in the active site. Polar but non-hydrogen- bonding atoms in a hydrophobic region are rewarded.

5.2. b. DOCKING PROCEDURE

Docking studies of compounds P-01 to P30 were performed using a membrane protein, Hydrogen/Pottassium ATP-ase receptor (PDB ID: 2ZBD) obtained from the RCSB Protein Data Bank, http://www.rcsb.org which is represented fig: 7

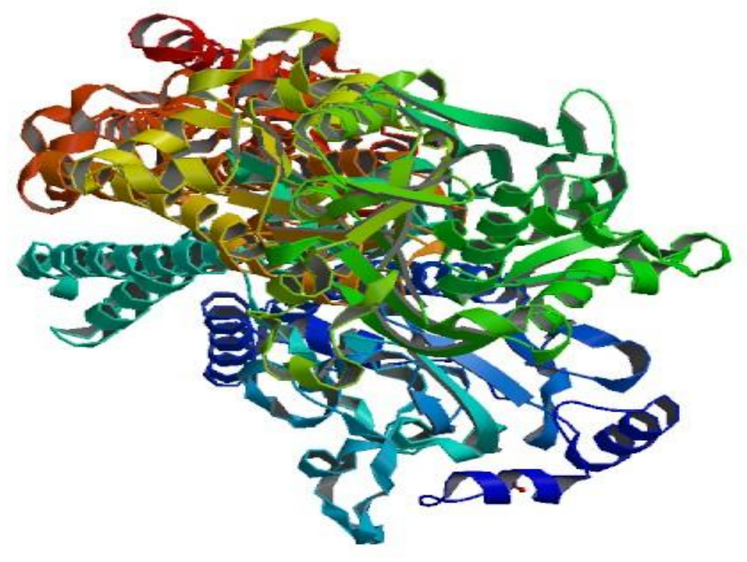
The computational modeling studies relied upon the GLIDE (Grid-based Ligand Docking from Energetics) program (Glide, version 10.1 Schrodinger, LLC New York, 2015) for the docking simulations. These simulations were performed using the X-ray crystal structure of the (PDB ID: 2ZBD). All the water molecules in the crystal structure were deleted, bond orders were assigned, hydrogen's were added and the protein was then further refined for the docking studies by processing it using Schrodinger's protein preparation wizard. This procedure minimizes the protein to 0.30 Å RMSD using OPLS-2001 force field. Ligands were prepared using build panel in maestro. Further the ligands were prepared for docking using LigPrep tool and were energy minimized using MMFF Force Field. Glide Grid generation panel has been used to generate receptor grid for docking. Default XP (Extra Precision) docking protocol was used to dock the library ligands. Molecular docking were performed for 30 compounds using the GLIDE program (Version 10.1, Schrodinger, LLC, New York,) to understand the interaction of ligands with H/K-ATPase receptor.

The Maestro user interface was employed to set up and execute the docking protocol and also for analysis of the docking results. H/K ATPase receptor (PDB ID: 2ZBD) was selected for docking studies and was prepared for docking through protein preparation wizard, energy minimization has been carried out using OPLS-2001 force field. Structures of benzimidazole derivatives were sketched using built panel and prepared for docking through LigPrep module (energy minimised using MMFF force field). GLIDE grid generation wizard has been used to

define the docking space. Docking was performed using XP (Extra Precision mode) docking protocol. The series of compound that are designed for molecular docking study are present in the table: 2

Fig: 7 MOLECULAR DESCRIPTION OF HYDROGEN/POTTASSIUM ATP-ase RECEPTOR

(PDB ID-2ZBD)



Structure weight	111868.54
Polymer	1
Type	Protein
Length	995
Chain	A
Organism	Homo sapiens
Resolution	2.40Å

5.3. SCHEME OF SYNTHESIS

STEP 1:

Cl, NO_2, NH_2 — Zn, NaoH,EtOH → Cl, NH_2, NH_2

4-Chloro-2-nitrobenzene 4-Chlorobenzene-1,2-diamine

STEP 2:

4-Chlorobenzene-1,2-diamine + Para amino benzoic acid —(4N HCl)→ 4-(5-chloro-1H-benzo[d]imidazol-2-yl) benzenamine

STEP 3:

NH2.NH2.H2O

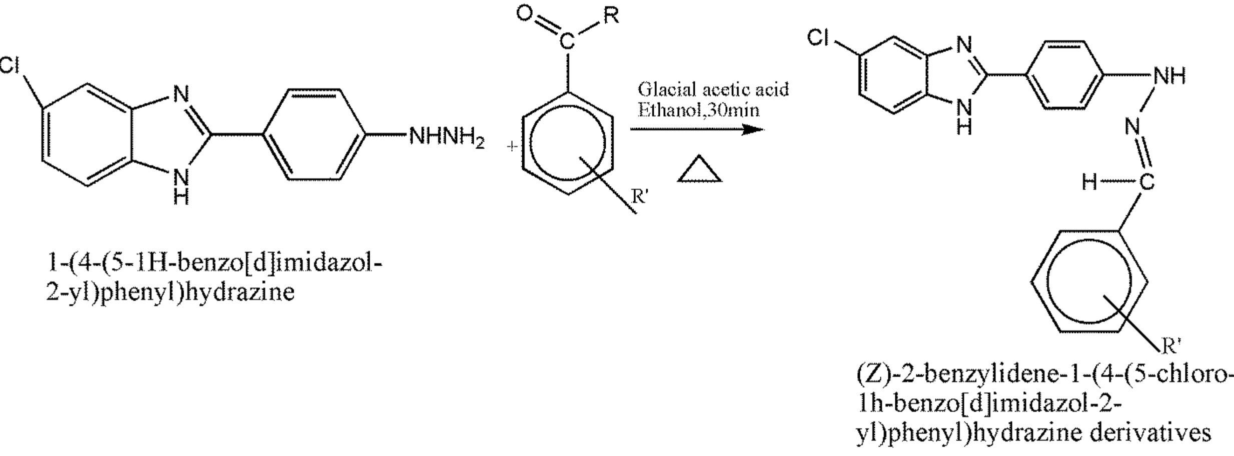
Ethanol,

△ 2hr

4-(5-chloro-1H-benzo[d]imidazol-2-yl)benzenamine

1-(4-(5-chloro-1H-benzo[d]imidazol-2-yl)phenyl Hydrazine

STEP 4:



R=H,CH3

R'=Cl,CH3,OCH3,OH etc

5.3.1 STEP I: SYNTHESIS OF 4-CHLOROBENZENE-1,2-DIAMINE FROM 4-CHLORO-2-NITRO BENZENE

Zn

NaoH,EtOH

4-Chloro-2-nitro benzene 4-Chlorobenzene-1,2-diamine

PROCEDURE:

10g of 4-chloro-2-nitrobenzene , 4.8ml of 20% solution of sodium hydroxide ,24ml of 95% ethanol are placed in round bottom flask connected to reflux condenser. The mixture is stirred vigorously and heated on a steam bath until the solution boils gently. The steam is turned off and 16g zinc dust in portion are added frequently enough to keep the solution boiling. After addition of zinc dust, reflux for 1hr until colour changes from deep red to nearly colourless. The hot mixture is filtered by suction and zinc residue is returned to flask and extracted with two 50ml portion of hot ethanol. To the combined filterates, added 2-3g of sodium hydro sulfite and the solution is concentrated under reduced pressure on steam bath .Then cool in ice bath and yellow crystals are collected, washed with ice water.

5.3.2. STEP II:SYNTHESIS OF 4-(5-CHLORO-1H-BENZO[d]IMIDAZOL-2-YL)BENZENAMINE FROM 4-CHLOROBENZENE-1,2-DIAMINE

4N HCl

4-Chlorobenzene-1,2-diamine Para amino benzoic acid 4-(5-chloro-1H-benzo[d]imidazol-2-yl) benzenamine

PROCEDURE:

4g of 4-(5-Chloro-1H-benzo[d]imidazol-2-yl) benzenamine was condensed with paraamino benzoic acid in 50ml of 4N HCl and stirr for 4hr with magnetic stirrer at 80^0c.The product was precipitated by adding sodium hydroxide. Filtered and washed with cold water, recrystallized from water and ethanol.

5.3.3 STEPIII: SYNTHESIS OF 1-(4-(5-CHLORO-1H-BENZO[d] IMIDAZOL-2-YL)PHENYL)HYDRAZINE FROM 4-(5-CHLORO-1H-BENZO[D]IMIDAZOL-2-YL)BENZENAMINE

NH2.NH2.H2O

Ethanol,

△ 2hr

4-(5-chloro-1H-benzo[d]imidazol-2-yl)benzenamine 1-(4-(5-Chloro-1H-benzo[d]imidazol -2-yl) phenyl) hydrazine

PROCEDURE :

To a solution of 0.1 mol of 1-(4-(5-Chloro-1H-benzo[d]imidazol-2-yl) phenyl) hydrazine in 100ml of water and equimolar amount of Hydrazine hydrate .The reaction mixture was made alkaline by adding 2g sodium hydroxide pellets and few ml of ethanol was added to give a clear solution .Then it was heated under reflux for 2hr in a round bottom flask and cooled in ice. The crystallized product was filtered under suction and recrystallized from ethanol.

5.3.4 STEP IV: SYNTHESIS OF BENZIMIDAZOLE DERIVATIVES FROM 1-(4-(5-Chloro-1H-BENZO[d]IMIDAZOL-2-YL)PHENYL)HYDRAZINE

Glacial acetic acid
Ethanol,30min

1-(4-(5-1H-benzo[d]imidazol-2-yl)phenyl)hydrazine

(Z)-2-benzylidene-1-(4-(5-chloro-1h-benzo[d]imidazol-2-yl)phenyl)hydrazine derivatives

R=H,CH3
R'=Cl,CH3,OCH3,OH etc

PROCEDURE:

To a solution of 1-(4-(5-Chloro-1H-benzo[d]imidazol-2-yl) phenyl) hydrazine (0.1 mol) in 20ml of ethanol and added slowly to an ethanolic solution of 0.1 mole of carbonyl compound (Aromatic aldehyde and ketone) in ethanol was added. Then the reaction mixture was acidified with 4ml of glacial acetic acid. Then reflux for half an hour .Cool, filter the precipitate and recrystallized from ethanol.

5.4. PHYSICOCHEMICAL STUDIES AND CHARACTERIZATION OF SYNTHESIZED COMPOUNDS

5.4. a. Melting point

Melting points of the synthesized compounds were determined in a one end fused capillary tube method by using THERMONIC MODEL-C-LMP-1, Campbell, melting point apparatus and were uncorrected.

5.4. b. Solubility

Solubility of synthesized compounds was checked in the following solvent:

Ethanol

Methanol

Hexane

Chloroform

Water

Dimethyl sulphoxide(DMSO)

Dimethyl formamide(DMF)

5.4.c. Thin layer chromatography

Determination of R_f value is an important technique to identify the formation of synthesized compounds and to determine the purity of the compound. R_f value is the characteristic property for a compound. Thin layer chromatographic analysis was carried out for all the synthesized compounds by using silica gel G (0.5 mm thickness) coated over glass plate (12 x 20 cm) as stationary phase and CHLOROFORM:BENZENE(6:4)as mobile phase. The spots were visualized by using iodine vapours.

$$\text{Rf value} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

5.5. SPECTRAL STUDIES AND CHARACTERIZATION OF SYNTHESIZED COMPOUNDS

5.5. a. Ultra Violet spectral analysis

The maximum absorbance or λ_{max} of the synthesized compounds were determined in the region of 200-400nm in the concentration of 0.01% w/v in ethanol by using Shimadzu UV Pharma Spec 1700 UV-Visible spectrophotometer.

5.5. b. Infrared spectral analysis

The structures of the synthesized compounds were elucidated by JASCO FT-IR 4100 infrared spectrophotometer in KBr pellets. The IR value was measured in cm^{-1}.

5.5. c. Nuclear Magnetic Resonance spectroscopy

The structures of the synthesized compounds were elucidated by Bruker AVANCE 500 MHz NMR Spectrometer using DMSO (dimethyl sulphoxide) as internal standard at Sophisticated Analytical Instrument Facility (SAIF) IIT MADRAS, Chennai.

5.5. d. Mass spectroscopy

The structures of the synthesized compounds were elucidated by JEOL GC MATE II GC-MS at Sophisticated Analytical Instrument Facility (SAIF) IIT MADRAS, Chennai. The mass spectroscopy values were measured in m/e ratio.

5.6. BIOLOGICAL EVALUATION OF THE SYNTHESIZED COMPOUNDS

5.6.1. ESTIMATION OF *IN VITRO* ANTIOXIDANT ACTIVITY

5.6.1. a.DPPH Radical scavenging activity

Principle[44]

The DPPH assay method is based on the reduction of DPPH (1,1-diphenyl-2-picrylhydrazyl), which is a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple colour). When an antioxidant reacts with DPPH, it becomes paired off in the presence of hydrogen donor (eg: a free radical scavenging antioxidant) and get reduced to DPPH, as a consequence of which the absorbance get decreased. Radical react with DPPH-H form with resulting decolourisation (yellow colour) with respect to the number of electrons captured. More the decolourisation more is the reducing ability.

$$\underset{\text{Purple colour}}{\text{DPPH}} + \underset{\text{Antioxidant}}{\text{H-A}} \longrightarrow \underset{\text{Yellow colour}}{\text{DPPH-H}} + \text{A}$$

Reagents

Diphenyl-2-picrylhydrazyl (DPPH)

Ethanol

Procedure [45, 46]

The free radical scavenging activity of the chemical substance was measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH radical method. The DPPH solution of 5.91 mg (0.3 mM) in 50 ml methanol was prepared and 1 ml of this solution was added to test compounds (P-28, P-12, P-24) and standard ascorbic acid solution at different concentrations. After 30mins, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\%\ \text{Inhibition} = \frac{\text{Acontrol} - \text{Atest}}{\text{Acontrol}} \times 100$$

where ‘$A_{control}$’ is the absorbance of the control reaction mixture and ‘A_{test}’ is the absorbance in the presence of test substance. The mean values were obtained from triplicate experiments.

ABTS Radical scavenging activity

Principle [47]

The spectrophotometric method using synthetic radical of ABTS (2,2-azinobis (3-ethylbenzoline-6- sulfonic acid) diammonium salt is used to calculate the total antioxidant capacity of a substance. The colourless ABTS molecule is converted to a blue-green coloured radical, $ABTS^{+\bullet}$ which has an absorption maxima at wavelengths 645 nm, 734 nm and 815 nm as well as more commonly used maximum at 415 nm. Oxidation of ABTS will commence immediately, but the absorbance is maximal and stable until more than 6 hrs has elapsed. The maximum of absorbance is achieved after 12-16 hrs. Addition of antioxidants to the preformed free radical cation reduces ABTS to an extend and on a time scale depending order based on the antioxidant

activity, concentration of the antioxidant and duration of the reaction. Decrease in absorbance after addition of antioxidants is directly proportional to the number of $ABTS^{+\bullet}$ radicals.

1e-(potassium persulphate)

ABTS ⇌ ABTS+. Free radical cation

1e-(antioxidant)

Reagents

4.9 mM potassium persulphate

14 mM 2,2-azinobis (3-ethylbenzoline-6-sulphonic acid) (ABTS)

Procedure[48, 49]

ABTS radical cation ($ABTS^{+\cdot}$) was produced by reacting 5 ml of 14 mM ABTS solution with 5 ml of 4.9 mM potassium persulphate solution and the mixture was allowed to stand in dark place at room temperature for 12-16 hrs before use. After the time duration, the mixture was diluted with water to yield an absorbance of 0.7 ± 0.02 at 734 nm. Different concentrations of test compounds (S-16, S-22, S-25, S-26) and standard ascorbic acid were added and then the volume was made to 2ml with ABTS radical solution. The blank solution was prepared by mixing 950 µl of ABTS solution and 50µl methanol. After 6 minutes, the absorbance was read at 734 nm and the percentage inhibition was calculated. The experiment was performed in triplicate. The scavenging activity of ABTS radical was calculated by using the following equation:

$$\% \text{ Inhibition} = \frac{\text{Acontrol} - \text{Atest}}{\text{Acontrol}} \times 100$$

where 'A $_{control}$' is the absorbance of the control reaction mixture and 'A $_{test}$' is the sample absorbance in the presence of test substance. The mean values were obtained from triplicate experiments

5.6.2 DETERMINATION OF INVITRO CELL VIABILITY ASSAY ON CULTURED HUMAN EPITHELIAL GASTRIC CELLS(AGS)

Cell culture: [50, 51]

Human epithelial gastric cells (AGS) were grown in Eagles Minimum Essentials Medium (EMEM) containing 10% Fetal Bovine Serum (FBS).Cells were maintained at 37^0c, 5% CO_2, 95% air and 100% relative humidity. Cells were subcultured ever third day by trypsinization with Trypsin-EDTA solution and medium changed daily.

Principle:

This Colorimetric assay is based on the capacity of mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide(MTT) into a insoluble, colored formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

Procedure:

The monolayer cell culture was trypsinized to make single cell suspensions and viable cells were counted using a hemocytometer and the cell count was adjusted to $2x10^4$ cells per ml using EMEM containing 10% Fetal Bovine Serum. To each well of 96 well microtiter plates, 0.1ml of diluted cell suspension approximately 20,000cells per well was added. After 24 hours, when the monolayer formed the supernatant was flicked off and 100µl of different test compounds at serial concentration was added to the cells in microtitre plates and kept for incubation at 37^0c in 5% CO_2 incubator for 48 hours. After 48 hours, the sample solution in wells was flicked off and 15µl of MTT dye (5mg/ml) in phosphate buffered saline was added to each well. The plates were gently

shaken and incubated for 4 hours at 37^0c in 5% CO_2 incubator. The supernatant was removed, 100µl of DMSO was added, and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured at 570nm using micro plate reader. The medium without samples were served as control and triplicates were maintained for all concentrations. The percentage cell viability was then calculated by using equation,

% Cell viability = Absorbance of treated cells / Absorbance of control x 100

5.6.3ACUTE TOXICITY STUDY PROTOCOL

Name of the study	Acute Toxicity
Guideline followed	OECD 423- Acute toxic class method [52]
Animals	Healthy young adult Wistar rats, nulliparous, non pregnant
Body weight	150-200 g
Sex	Female
Administration of dose and volume	5,50,300, 2000 mg/kg body weight in single dose in 1 ml
Number of group and animals	1 rat each was used to study LD50 value. Rats were dosed in sequence and observed for 48 h. Animals were observed for 14 days to study the presence or absence of any toxicity sign
Route of administration	Oral by using rat oral feeding needle
Vehicle	Carboxy methyl cellulose (0.5 %)
Room temperature	22 ± 3^0c
Humidity	40-60 %

Light	12 h :12 h (light-dark cycle)
Feed	Standard laboratory food pellets with water *ad Libitium*
Initial once observations	First 30 min and periodically for 24 h
Special attention	First 1-4 h after drug administration
Long term observation	Upto 14 days
Direct observation parameters	Tremors, convulsion, salivation, diarrhea, lethargy, sleep and coma
Additional observation parameters	Skin and fur, eyes and mucous membrane, respiratory, circulatory, autonomic and CNS, somatomotor activity and behavioral pattern

STUDY PROCEDURE

Acute oral toxicity study was performed as per the Organization for Economic Co-operation and Development (OECD) guideline 423 method. Protocols for the study were approved by the Institutional Animal Ethical Committee (IAEC) for animal care (Proposal Number:KMCRET/M.Pharm/07/2015-16). The animals were fasted overnight prior to dosing. Following the period of fasting, rats were weighed and test compound was administered in a single dose to a single animal in sequence by gavage using a oral feeding needle. The test compound was suspended in 0.5% carboxy methyl cellulose (CMC) and a volume not exceeding 1ml/100 g body weight was administered. After the administration of test compound (P-28), food was withheld for 2 hrs. Rats were observed during the first 30 mins followed by periodic observation during the first 24 hrs, with special attention given during first 4 hrs and daily

thereafter for a total duration of 48 hrs. 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.

5.6.4. PHARMACOLOGICAL EVALUATION OF ANTIULCER ACTIVITY

5.6.4.a 1)INDOMETHACIN INDUCED ULCER MODEL:

Table 4: Experimental design for Indomethacin induced ulcer

Group	NUMBER OF ANIMALS	TREATMENT
Group I	6	Normal control rats
Group II	6	Ulcer control rats receving Indomethacin(toxicant,25mg/kg bw) p.o
Group III	6	Rats receving Standard drug (Pantoprazole ,10mg/kg bw) p.o
Group IV	6	Rats receving Low dose of test drug(50mg/kg bw) + Indomethacin (25mg/kg bw) p.o
Group V	6	Rats receving High dose of test drug(100mg/kg bw) + Indomethacin (25mg/kg bw) p.o

EXPERIMENTAL PROCEDURE:

5.6.4. b. ULCER INDUCTION:

Rats were administered with a single oral dose of Indomethacin (25 mg/kg body weight). They were deprived of food but had free access to water 24 h prior to ulcer induction. Various degrees of ulceration have manifested 4 h after Indomethacin administration.

PRINCIPLE: [53]

Indomenthacin is a synthetic Non-steroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic activity. It is a potent inhibitor of postaglandins synthesis that are important mediators of the inflammatory response. The anti-inflammatory action of Indomethacin is due to inhibition of vasodilator prostaglandin E2 and prostaglandin I2 synthesized from arachidonic acid through cyclo-oxygenase pathway by inhibiting cyclo-oxygenase I (COX-I) and cyclo-oxygenase-II (COX-II). Deficiency of COX-I is of pivotal importance in anti-inflammatory response of NSAIDs.

5.6.4. c. PROCEDURE: [54,55]

Species : wistar Albino rats

Weight : 100-150g

Gender : Either sex

30 Animals were taken and divided into 5 groups. Animals were pretreated with test drug and toxicant for 7 days. The animals were fasted for 48hrs prior the experiment, but had free access to water. On the 7th day all the animals excluding the control group were orally given Indomethacin 25mg/kg .Four hours later these animals were sacrificed and stomach was cut opened through its greater curvature .ulcer score and ulcer index were calculated.

5.6.4.d.2)PYLORUS LIGATION INDUCED ULCER:

Table 5: Experimental design for pylorus ligation induced ulcer

GROUPS	NUMBER OF ANIMALS	TREATMENT
Group I	6	Normal control rats
Group II	6	Ulcer control rats
Group III	6	Rats receiving Standard drug(Pantoprazole 10mg/kg bw) p.o
Group IV	6	Rats receiving Low dose of test drug(50mg/kg bw) p.o
Group V	6	Rats receiving High dose of test drug(100mg/kg bw) p.o

5.6.4. e. EXPERIMENTAL PROCEDURE:

PRINCIPLE: [56]

Pylorus ligation caused ultra structural changes in the gastrin cells of the pyloric gland area and in the histamine-storing ECL and A-like cells of the oxyntic gland area. The size of the gastrin cells seemed unaffected. In pylorus ligation the number of cytoplasmic granules reduced. It appears unlikely that the ultra structural changes of the ECL and A-like cells reflect an increased rate of histamine mobilization. The acid response to pylorus ligation reflects neuronal reflex mechanisms exclusively.

PROCEDURE: [57,58]

Species : wistar Albino rats

Weight : 100-150g

Gender : Either sex

30 animals were taken and divided into 5 groups. Animals were pretreated with drugs for 7 days. The animals were fasted for 48hrs prior the experiment, but had free access to water. After the fasting period, the animals were given the test drug samples p.o, 1hr prior the ligation .Thereafter, the rats were anaesthetized with anesthetic ether. Then pyloric ligation was carried out. Four hours later these animals were sacrificed and stomach was cut opened through its greater curvature .Its contents emptied into graduated test tubes, volume was recorded, pH was measured by pH meter, ulcer score and ulcer index were calculated.

5.6.5. BIOCHEMICAL PARAMETERS:

The stomach was carefully excised keeping esophagus closed and opened along greater curvature and luminal contents were removed. The gastric contents were collected in a test tube and centrifuged. The gastric contents were analyzed for pH, total and free acidity.

5.6.5.1. MEASUREMENT OF GASTRIC JUICE VOLUME AND pH GASTRIC JUICE:

Gastric juice was collected from each animal and centrifuged at 3000 rpm for 10 min. The volume of supernatant was measured and expressed as ml/100g body weight. The pH of the supernatant was measured using digital Ph meter after diluting of 1ml of gastric juice with 1ml of distilled water.

5.6.5.2. DETERMINATION OF TOTAL ACIDITY:

An aliquot of 1ml gastric juice was diluted with 1ml of distilled water was taken into a 50ml conical flask and added two drops of phenolphthalein indicator into it and titrated with 0.01N NaOH until a permanent pink colour was developed. The total acidity is expressed as mEq/L by the following formula:

$$\text{Acidity} = \frac{\text{volume of NaOH} \times \text{N} \times 100\text{mEq/L}}{0.1}$$

5.6.5.3.DETERMINATION OF FREE ACIDITY:

An aliquot of 1ml of gastric juice was titrated with 0.01N NaOH using Toper's reagent as indicator until a canary yellow colour was developed. The free acidity is expressed as mEq/L by the following formula:

$$\text{Acidity} \quad = \quad = \quad \frac{\text{volume of NaOH} \times N \times 100\text{mEq/L}}{0.1}$$

5.6.5.4.ULCER INDEX:

The stomachs were opened along the greater curvature, rinsed with saline to remove gastric contents and blood clots and examined by a 10X magnifier lens to assess the formation of ulcer. The numbers of ulcer was counted .Scoring of ulcer are made as follows:

Normal coloured stomach …………… 0

Red coloration…………………………0.5

Spot ulcer………………………………1

Hemorrhagic streak……………………1.5

Deep ulcer……………………………...2

Perforation……………………………...3

Mean ulcer score for each animal will be expressed as ulcer index.

$$\text{Ulcer index(UI)} \quad = \quad UN+US+UPX10^{-1}$$

Where,

UI=Ulcer Index , UN=Average number of ulcer per animal; US=Average number of severity score; UP=Percentage of animals with ulcer.

5.6.5.5. PERCENTAGE INHIBITION:

Percentage inhibition of ulceration was calculated as below:

$$\% \text{ inhibition of ulceration} = \frac{(\text{Ulcer index}_{control} - \text{Ulcer index}_{test}) \times 100}{\text{Ulcer index}_{control}}$$

5.6.6.EVALUATION OF *IN VIVO* ANTIOXIDANT PROPERTY

5.6.6. a. PREPARATION OF TISSUE HOMOGENATE

The stomach was opened and washed with normal saline scoring the ulcers. The mucosa was scraped from the glandular part of the stomach, suspended in 5.0 ml of cooled 0.15M Kcl-10Mm potassium phosphate buffer (pH 7.4)containing 0.1% Triton X -100 and centrifuged at 10000 rpm for 15min at 5^0c.

5.6.6. b. Estimation of Catalase (CAT)

Principle

The normal antioxidant activity of the enzyme catalase is due to the accelerated decomposition of hydrogen peroxide to water and oxygen. This method is based on the rate of decomposition of hydrogen peroxide by the enzyme catalase which is measured spectrophotometrically at 570 nm, since hydrogen peroxide has absorbance on this range.[59]

Requirements

Dichromate acetic acid reagent (5 % potassium dichromate + glacial acetic acid were mixed at 1:3 ratio (v/v)

0.01 M phosphate buffer (pH 7.0)

0.2 M hydrogen peroxide

Assay procedure

To 1 ml of the tissue homogenate, 4 ml of hydrogen peroxide and 5 ml of phosphate buffer were added and mixed well. From this, 1 ml of the solution was taken and mixed with dichromate acetic acid reagent and allowed to incubate for 30mins at room temperature. The absorbance was measured at 570 nm. The activity of catalase was expressed as µmole of H_2O_2 consumed/min/mg protein.

5.6.6.c Estimation of Superoxide Dismutase (SOD)

Principle

Pyrogallol autoxidizes rapidly in aqueous solution, where the reaction will be faster at higher pH, and leads to the formation of several intermediate products. Thus the solution first becomes yellow-brown with a spectrum showing a shoulder between 400 and 425nm. Molecular oxygen, carrying two unpaired electrons with parallel spins, has a preference for univalent reduction because spin restrictions arise when reduction with electron pairs is attempted. The recently discovered enzyme superoxide dismutase rapidly dismutases univalently reduced oxygen $O_2.^-$ i.e., the superoxide anion radical ($2O_2.^- + 2H^+ \longrightarrow O_2 + H_2O_2$). The enzyme has proven to be a useful probe for studying the participation of the radical in reactions involving oxygen such as autoxidations. Thus O_2^-. has been shown to be involved in the autoxidation of e.g. sulphite, adrenalin and 6-hydroxydopamine.

Requirements

Pyrogallol

Tris- HCL (pH 8.2)

0.1 Mm EDTA

Assay procedure

This method might be used for determination of antioxidant activity of a sample, and it was described by McCord and Fridovich. The main purpose of this method that was estimated 5% of tissue homogenate after adding 75mM, 30mM, and 2mM from Tris-HCL (pH 8.2), EDTA, and

pyrogallol respectively. Then, the absorbance was measured at 420nm. The percentage of inhibition was calculated depending on that the ability of enzyme to inhibit of oxidation. So, any changes might be happened on the absorbance, it will give a clear picture on the ability of enzyme activity to prevent oxidation.[60]

5.6.6. d. Estimation of Glutathione Peroxidase (GPx)

Principle

This assay is based on the reduction of hydrogen peroxide (H_2O_2), by glutathione peroxidase through simultaneous oxidation of reduced glutathione (GSH) to form oxidized glutathione (GSSG). GSSG is again reduced by glutathione reductase (GR) and β-nicotinamide adenine dinucleotide phosphate (NADPH) forming $NADP^+$which results in the reduced absorbance at 340 nm and recycling the GSH. The decrease in absorbance at 340 nm is directly proportional to the GPx concentration.[61]

Requirements

0.32 M phosphate buffer, pH 7.0

0.8 mM EDTA and 10 mM sodium azide

3 mM reduced glutathione

2.5 mM H_2O_2 and 10 % TCA

0.3 M disodium hydrogen phosphate

DTNB solution (40 mg of DTNB in 100 ml of 1% sodium citrate)

Reduced glutathione

Assay procedure

To 0.1 ml of the tissue homogenate, 0.2 ml of EDTA, sodium azide and hydrogen peroxide were added and mixed. To this, 0.4 ml of phosphate buffer was added and allowed to incubate at room temperature. The reaction was arrested by the addition of 0.5 ml of TCA. The reaction mixture was centrifuged at 2000 rpm and the supernatant was collected. To 0.5 ml of the supernatant, 4 ml

of disodium hydrogen phosphate and 0.5 ml of DTNB were added and the colour developed was read immediately at 420 nm. The activity of glutathione peroxidase was expressed as μmoles of glutathione oxidized/min/mg.

5.6.6.e. Estimation of Reduced Glutathione (GSH)

Principle

DTNB is a disulphide compound, which is reduced by sulfhydryl groups in GSH. This reduction leads to the formation of yellow colour, which is measured at 412 nm.

Requirements

5 % TCA

0.6 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 0.2 M sodium phosphate

0.2 M phosphate buffer, pH 8.0

Assay procedure

To 1 ml of the homogenate, 1 ml of the TCA solution was added and centrifuged. The supernatant was collected and the precipitate formed was removed. To 0.5 ml of supernatant 2 ml of DTNB was added and the volume was made up to 3 ml with phosphate buffer. Then the absorbance was read at 412 nm. The amount of glutathione was expressed as μ g/mg protein.[62]

5.6.6.f. Determination of Lipid Peroxidation

Principle

This assay is based on the reaction between thiobarbituric acid with malonyldialdehyde which is formed as a result of polyunsaturated fatty acid oxidation. This reaction leads to the formation of pink coloured TBA-MDA complex which is measured at 532 nm.[63]

Requirements

Thiobarbituric acid (0.37%)

0.25N HCl and TCA (15%)

Assay procedure

To 0.1 ml of the sample, 2 ml of TBA-TCA-HCl reagent (ratio of 1:1:1) was added, mixed and kept in a water bath for 15 min. Afterwards the solution was cooled and supernatant was removed and the absorbance was measured at 535 nm against reference blank. The level of lipid peroxidase was given as µmoles of MDA formed/min/mg protein.

5.6.6.g. ESTIMATION OF PROTEINS:

Reagents

- Alkaline copper reagent
- Solution A: 2% sodium carbonate in 0.1 N NaOH.
- Solution B: 0.5% copper sulphate in 1% sodium potassium tartarate
- Solution C: 50 ml of solution A was mixed with 1 ml of solution B just before use.
- Folin's phenol reagent (commercial reagent, 1:2 dilutions) Bovine serum albumin(BSA).

Principle: [64]

This method involves two steps;

Step: 1-protein binds with copper in alkaline medium and reduces it into cu^{++}.

Step: 2- the cu^{++} formed catalyses the oxidation reaction of aromatic amino acids by reducing phosphomolybdotungstate to heteropolymolybdanum, which leads to the formation of blue colour and absorbance was measured at 640 nm.

Procedure:

0.1 ml homogenate was made up to 1 ml with distilled water and to this; 5 ml of alkaline solution was added, mixed well and allowed to stand for 10 min. Then a volume of 0.5ml Folin's reagent was added, mixed well and incubated at room temperature for another 10 min. The blue colour developed was measured at 660 nm against blank. Bovine serum albumin (1 mg/ml) served as the standard and form the standard graph obtained; the amount of protein in the sample was calculated and expressed as mg/100 mg tissue.

5.6.6.h. ESTIMATION OF MYELOPEROXIDASE(MPO)

Reagents:

Phosphate buffer(0.01M)

H_2O_2 (0.3mM)

Principle:

Myeloperoxidase activity is a measure of leukocyte infiltration. It oxidizes tyrosine to tyrosyl radical using hydrogen peroxide as an oxidizing agent. The enzyme involves in the nitration of tyrosine residues, which cause variety of inflammatory disorders.[65]

Procedure:

Pieces of inflamed tissues were taken. The tissues was then rinsed with saline, blotted dry, weighed and excised. Minced tissue was homogenized in phosphate buffer (pH 7.4), using remi tissue homogenizer. The homogenate was centrifuged at 10000 rpm for 20min at 4^0C. supernatant was discarded, myeloperoxidase activity was measured by DTNB method as follows:

0.1ml of tissue homogenate was treated with 0.1ml of TNB and 1ml of sodium acetate buffer followed by addition of about 0.8ml of NaCl solution and 35µl of hydrogen peroxide.The change in absorbance was measured spectrophotometrically at 490nm. One unit of MPO activity is defined as the change in absorbance per minute by 1.0 at room temperature, in the final reaction.

5.6.7. STATISTICAL ANALYSIS

All the Statistical analysis was performed by using Graph pad prism 5.0. All the data were expressed as mean ± SEM. Groups of data were expressed with one way analysis of variance (ANOVA) followed by Tukey test. P value <0.05 was considered as significant.

5.6.8. HISTOPATHOLOGICAL STUDY

All group rat stomach were dissected out about 3 to5 mm and fixed with 10% neutral formalin solution. Dehydration process was initiated through a graded alcohol series and embedded in paraffin. Sections of about 3 µm were cut out and stained with hematoxylin and eosin. Histopathological evaluation was carried out under light microscope.

DISCUSSION

Peptic ulcer disease refers to painful sores or ulcers in the lining of the stomach or first part of the small intestine, called the duodenum. It is caused by disturbance of the normal balance between the corrosive effect of gastric juice and the protective effect of mucus on the gastric epithelial cells. The pathophysiology of this gastro-intestinal disorder is viewed as an imbalance between mucosal defensive factors such as bicarbonate, prostaglandin, nitric oxide, peptides, growth factors and injurious factors like acid, pepsin, smoking, stress, alcohol etc.

In silico Molecular modeling studies will lead to generation of a potent molecule in less time with reduction in the usage of chemicals and animals and also it will save money. It is a process of In-silico- chemico-biological approach of drug designing. The recent growth in molecular sciences and the advances in genomics and proteomics have generated several potential new drug targets, leading to changes in the paradigms of antiulcer drug discovery toward molecularly targeted therapeutics.

The titled compounds were designed and the docking studies of the 30 designed compounds were carried out by using Glide software with Hydrogen-Potassium ATP-ase receptor (H^+/K^+-ATP-ase)(PDB ID:2ZBD). Based on the docking score and interaction the targeted compounds was synthesized from 4-chloro-2-nitroaniline as a starting material, which is then converted to 4-(5-chloro-1H-benzo[d]imidazol-2-yl)benzenamine and finally to 1-(4-(5-chloro-1H-benzo[d]imidazol-2-yl)phenyl hydrazine which reacted with glacial acetic acid ,alcohol and aldehydes and ketones to get the targeted benzimidazoles derivatives .The structure of synthesized compounds were characterized by physical and spectral analysis.

Melting point

Melting point of the synthesized compounds were determined in a one end fused capillary tube method by using THERMONIC MODEL-C-LMP-1, Campbell melting point apparatus and are uncorrected. Melting point of compound P-28 was found to be 140-143^{0}c.

Solubility

Solubility of the synthesized compounds was checked out in the solvent like ethanol, methanol, hexane, chloroform, water, dimethylsulphoxide(DMSO),dimethylformamide (DMF).The compounds were found to be soluble in ethanol and DMSO.

Thin layer chromatography

Thin layer chromatographic analysis was carried out for all synthesized compounds by using silica gel G(0.5mm thickness) coated over glass plate(12 x 20 cm)as stationary phase, chloroform:benzene(6:4) as mobile phase and the spot was visualized by iodine vapours. Determination of R_f value is an important technique to identify the formation of synthesized compounds and to determine the purity of the compound . R_f value is the characteristic for each of the compound.R_f value of compound P-28 was found to be 0.75.

Infrared spectral analysis

The structures of the synthesized compounds were elucidated by JASCO FT-IR 4100 infrared spectrophotometer in KBr pellets. The IR value is measured in cm^{-1} .The spectra's showed the relevant functional group frequency for the designed compounds. 3452cm^{-1}: Amine NH stretching, 2853cm^{-1}: Aliphatic CH-Stretching,1629cm^{-1}:C=N Stretching,936cm^{-1}:Aromatic CH Bending, which confirms the chemical structure of the compound synthesized.

Nuclear Magnetic Resonance spectroscopy

The structures of the synthesized compounds were elucidated by Bruker AVANCE 500 MHz NMR spectrometer using DMSO (dimethyl sulphoxide) as internal standard at Sophisticated Analytical Instrument Facility(SAIF)IIT MADRAS,Chennai. The NMR spectra's have showed a characteristic proton resonance shift for the entire synthesized compounds.

Mass spectroscopy

The structures of the synthesized compounds were to be elucidated by JEOL GC MATE II GC-MS at Sophisticated Analytical Instrument Facility (SAIF) IIT MADRAS, Chennai.The mass spectroscopic values are measured in m/e ratio and the molecular ion peak corresponds to the molecular weight of the compound. The Mass Spectral analysis of the

synthesized compounds P-28 was performed, and the mass spectrum of the compound was in agreement with its molecular weight.

Based on the IC_{50} value , the docking scores, spectral and physical analysis compound P-28 was selected for further animal studies against Indomethacin and pylorus ligation induced gastric ulcer on Wistar rats.

Pharmacological studies

In vitro antioxidant studies such as ABTS and DPPH were carried out and the synthesized compounds exhibited slightly lower free radical scavenging activity than that of the standard ascorbic acid. Further ,*in vitro* cell viability assay of the synthesized compounds were carried out in AGS(human epithelial gastric cell) cell lines using cultured MTT assay. Cell line facilitate examination of cell types for their physiological and pathological processes without the use of animal models. Further,it could be used for studying the effect of various compounds on specific cell types. Based on the results of *in vitro* cell viability assay, concentraction of synthesized compounds for studying antiulcer activity was determined. A significant reduction in cell damage was observed when the AGS cells were pretreated with compound P-28 compared to control.

Treatment of ulcer diseases is prominently focused on reduction of aggressive factors and strengthening mucosal defense of stomach and duodenum. These are all treated by blocking acid secretion through proton pump inhibitors such as benzimidazole derivatives. Almost all benzimidazole derivatives with their two ring systems bear different functional substituents and this leads to essential modification of the physico-chemical, metabolic and pharmacokinetic properties of these drugs. Tissue selectivity of this type of antiulcer drugs is based on both their pH dependent accumulation, as weak bases in the acidic compartment of secreting parietal cell, and the subsequent acid-induced rearrangement of the parent compound to the pharmacologically active principle . The enzyme H^+/K^+ ATPase is responsible for gastric acid production and is located in the secretary membranes of the parietal cell . Gastric acid secretion is regulated by interaction of basolateral parietal cell receptors with their physiological stimulants gastrin, acetylcholine, and histamine. The irreversible inhibition of the H^+ /K^+ -ATPase, a means of controlling gastric pH has attracted considerable attention in recent years

with the discovery of the benzimidazole class of antisecretory agents. Synthesis and utility of novel substituted benzimidazole derivatives is evaluated by their ability to inhibit gastric H^+ /K^+-ATPase and by blocking the gastric acid secretion .[91]

Indomethacin induced ulcer

In the present study ,ulcer control group showed signs of elevated gastric volume, decreased gastric P_H ,increased total acidity and free acidity and ulcer index followed by oral administration of Indomethacin .Ulcer induction in rats by Indomethacin is related with inhibition of cyclooxygenase that prevents prostaglandin biosynthesis.[92] Compound P-28 was found to be produce a significant($P<0.001$) reduction in ulcer index in treated groups . It was also found to have significant ($P<0.001$) increase in the pH and decrease in the acid volume and total acidity of gastric fluid in pylorus-ligation model, thus proving its anti-ulcer activity when compared to that of ulcer control group. Hence the test compound and standard drug has significant antiulcer activity against ulcer control group.

Pylorus ligation indced ulcer

In the present study, ulcer control group showed signs of elevated gastric volume, decreased gastric P_H, increased total acidity and free acidity and ulcer index. Ligation of the pylorus induces ulcers that serve as a useful model for investigating the efficacy of drugs on gastric secretions. These ulcers result from autodigestion of the gastric mucosa leading to a breakdown of the gastric mucosal barrier. So, basically an increase in acid-pepsin accumulation due to pylorus obstruction may cause subsequent mucosal digestion. The model is useful for evaluating the effects of anti-secretory drugs that reduce secretion of gastric aggressive factors such as acid and pepsin. The model is also useful for assessing the cytoprotective effects of drugs that increase secretion of mucus.[93] Compound P-28 was found to be produce an significant ($P<0.001$) reduction in ulcer index in treated groups . It was also found to have significant ($P<0.001$) increase in the pH and decrease in the acid volume and total acidity of gastric fluid in pylorus-ligation model, thus proving its anti-ulcer activity when compared to that of ulcer control group. Hence the test compound and standard drug has significant antiulcer activity against ulcer control group.

Oxidative stress plays an important role in the pathogenesis of various diseases including gastric ulcer, with antioxidants being reported to play a significant role in the protection of gastric mucosa against various necrotic agents. Reactive oxygen species are involved in the pathogenesis of pylorus ligation-induced and Indomethacin induced gastric mucosal injury in vivo. As compared to normal rats, pylorus-ligation and Indomethacin administration was found to increase lipid peroxidation and decrease SOD, catalase and reduced glutathione, GP_X ,total protein ,increased MPO in the control groups, thus leading to oxidative stress. Preventive antioxidants, such as superoxide dismutase (SOD) and catalase (CAT) enzymes are the first line of defence against reactive oxygen species. Reduced glutathione (GSH) is a major low molecular weight scavenger of free radicals in the cytoplasm and an important inhibitor of free radical mediated lipid peroxidation . Administration of Compound P-28 in both the models resulted in a significant increase in the SOD, catalase , reduced glutathione levels, GP_X ,total protein, reduced MPO as compared to the control animals, which suggests its efficacy in preventing free radical induced damage. Lipid peroxidation is a free radical mediated process, which has been implicated in a variety of disease states. It involves the formation and propagation of lipid radicals, the uptake of oxygen and rearrangement of double bonds in unsaturated lipids which eventually results in destruction of membrane lipids. The control groups reported an increase in lipid peroxidation. The study has revealed a significant decrease in lipid peroxidation by Compound P-28 in both the experimental models, which suggests its protective effect. [93,94]

Thus the compound P-28 confirmed the free radical scavenging potential and it has potential to prevent the macro and microvascular complications

Histopathological study

The control group of rats pylorus ligated model showed histopathological changes of the gastric mucosa characterised by loss of glandular architecture, oedema and infiltration by inflammatory cells. Few glands show reactive atypia. Laminapropria shows scattered chronic inflammatory infiltrates and neutrophils. Submucosa shows congested vessels and chronic inflammation. The rats treated with Compound P-28 (50mg/kg) and pantoprazole (10mg/kg) showed minimum ulceration and inflammatory infilterates. However, Compound P-28 (100 mg/kg) and pantoprazole(10mg/kg) showed significant intact and normal mucosa .

Histopathology of the stomach of the control animals of indomethacin induced ulcer model showed few scattered chronic inflammatory infiltrates in lamiapropia region. Submucosa shows significant pathological changes. Gastric section from untreated rats shows ulceration of the mucosal cells associated with inflammatory changes and necrosis. Animals treated with Compound P-28 (100 mg/kg) and pantoprazole(10mg/kg) showed gastric mucosa with intact lining epithelium. The mucosal layer and submucosal layer shows mild lymphocytic and neutrophilic infiltrates. The muscular and serosal layers appeared within normal limits.

RESULTS

DOCKING ANALYSIS

DOCK SCORE:

Docking studies of compounds P-01 to P-30 were performed using a nuclear protein Hydrogen Potassium ATP-ase receptor (PDB ID: 2ZBD) obtained from the RCSB Protein Data Bank,http://www.rcsb.org. Experiments were performed using the program GLIDE (Grid-based Ligand Docking from Energetics) (Maestro version 10.1, Schrodinger , LLC New York) and their Dock score are given in the below table:6

Table 6:Dock score of designed compounds

Compound code	Dock score	Glide score	Lipophilic Evd W	phobEn	phobEn HB	PhobEn Pair HB	HBond
P-01	-3.33	-3.33	-2.42	0	0	0	-0.31
P-02	-4.05	-4.05	-1.35	0	0	0	-0.26
P-03	-3.07	-3.07	-1.58	0	0	0	-0.85
P-04	-6.47	-6.47	-1.21	0	0	0	-1.39
P-05	-4.63	-4.63	-1.36	0	0	0	-1.33
P-06	-7.69	-7.69	-2.88	0	0	0	-0.84
P-07	-6.61	-6.61	-3.08	0	0	0	-0.9
P-08	-7.59	-7.59	-1.45	0	0	0	-1.52
P-09	-4.23	-4.23	-1.9	0	0	0	-0.21
P-10	-4.03	-4.03	-2.01	0	0	0	-0.56
P-11	-5.16	-5.16	-1.81	0	0	0	-0.46
P-12	-7.76	-7.76	-2.46	0	0	0	-1.59
P-13	-5.88	-5.88	-1.36	0	0	0	-0.61

P-14	-5.67	-5.67	-2.32	0	0	0	-0.64
P-15	- 5.89	- 5.89	-1.06	0	0	0	-0.61
P-16	-6.56	-6.56	-2.37	0	0	0	-0.15
P-17	-5.54	-5.54	-1.08	0	0	0	-1.94
P-18	-5.3	-5.3	-2.31	0	0	0	-1.86
P-19	-6.31	-6.31	-1.52	0	0	0	-1.47
P-20	-7.66	-7.66	-1.34	0	-1.5	0	-2.56
P-21	-3.49	-3.49	-1.45	0	0	0	-0.77
P-22	-4.12	-4.12	-2.7	0	0	0	-0.64
P-23	-6.54	-6.54	-1.76	0	0	0	-0.45
P-24	-6.04	-6.04	-1.53	0	0	0	-1.15
P-25	-6.84	-6.84	-1.11	0	0	0	-0.24
P-26	-7.77	-7.77	-1.4	0	0	0	-0.35
P-27	-5.03	-5.03	-2.62	0	0	0	-0.64
P-28	-8.67	-8.67	-1.41	0	0	0	-0.95
P-29	-6.57	-6.57	-2.35	0	0	0	-1.37
P-30	-6.09	-6.09	-2.18	0	0	0	-1.01

Based on the ease of reaction, availability and cost of chemicals ,synthesis of compounds P-6,P-12,P-20,P-26 and P-28 were carried out and their physicochemical and spectral characterization have been carried out.

The glide score for the synthesized compounds are given in the below table:

Table 7:**Glide score of synthesized compounds**

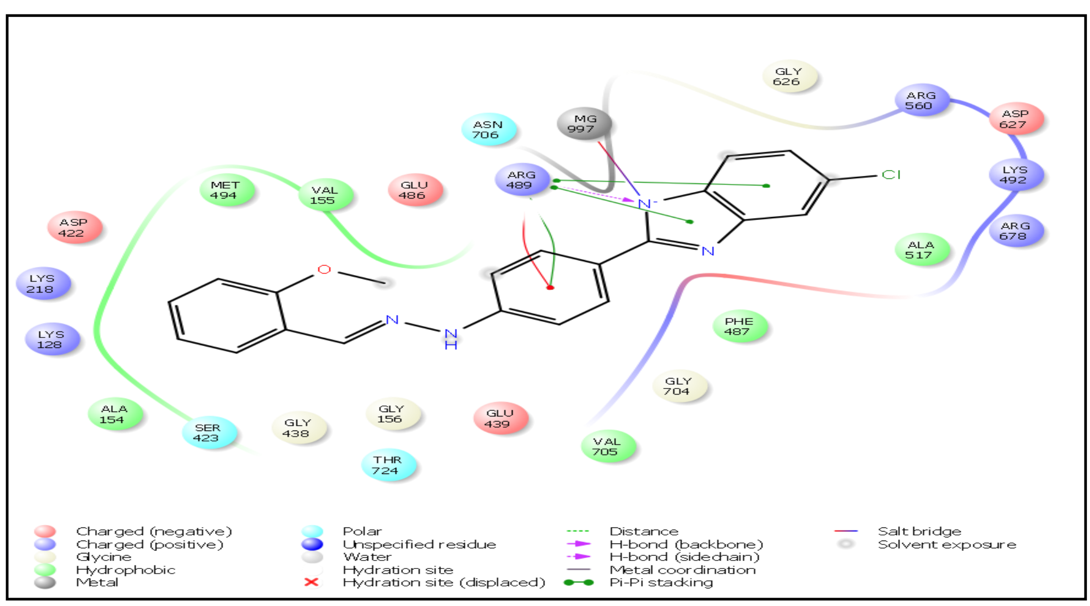
SI NO.	Compound code	structures	Chemical name	Glide score
1	P-6		(E)-2-(2-methoxybenzylidene)-1-(5-chloro-*1H*-benzo[d]imidazol-2-yl)phenyl)hydrazine	-7.69
2	P-12		2-(3-nitrobenzyldene)-1-(4-(5-chloro-*1H*-benzo[d]imidazol-2-yl)phenyl)hydrazine	-7.76
3	P-26		2-((3H-indol-2-yl)methylene)-1-(4-(5-chloro-*1H*-benzo[d]imidazol-2-yl)phenyl)hydrazine	-7.77
4	P-28		2-((*1H*-pyrrol-2-yl)methylene)-1-(4-(5-chloro-*1H*-benzo[d]imidazol-2-yl)phenyl)hydrazine	-8.67

DRUG-RECEPTOR INTERACTION

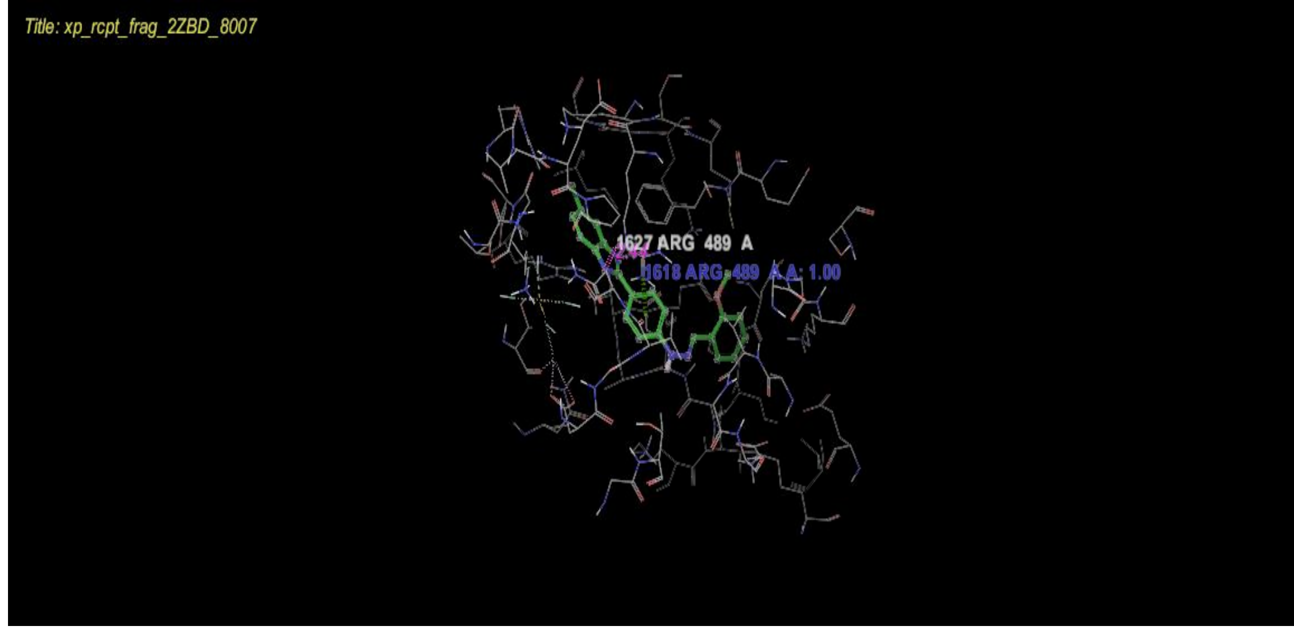
Interaction of the designed compounds with the receptor H^+/K^+ATP-ase was observed and studied.Its details were given in below fig: 8,9,10,11 and table:8

P-6

Figure 8: Snapshots of Drug-Receptor interaction

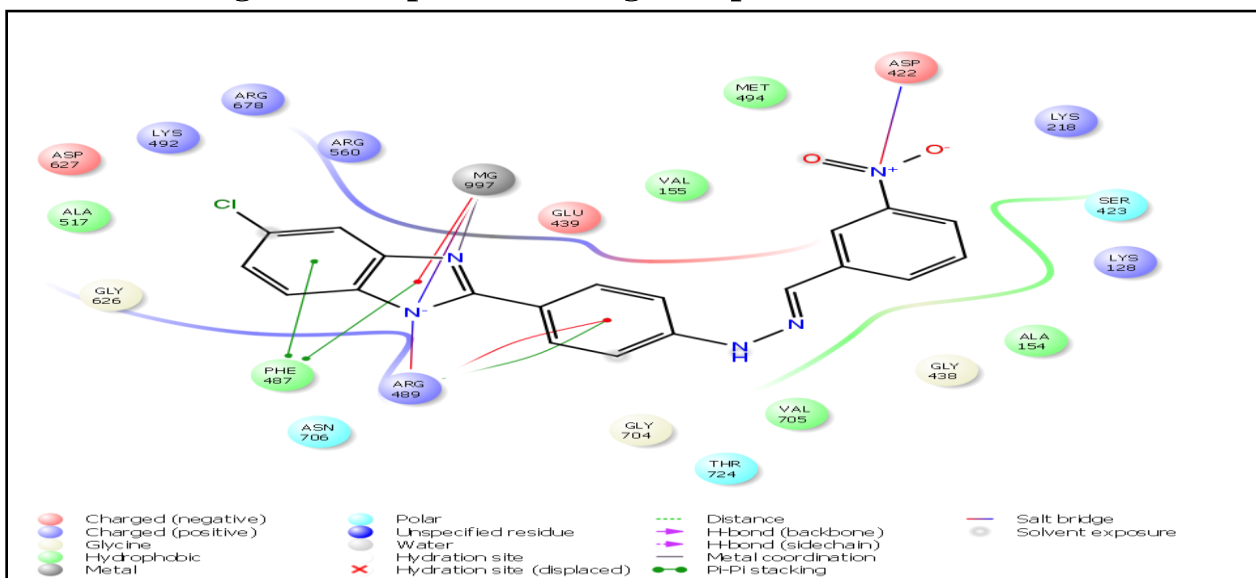


Predicted binding mode of compound P-6 into the catalytic site of 2ZBD

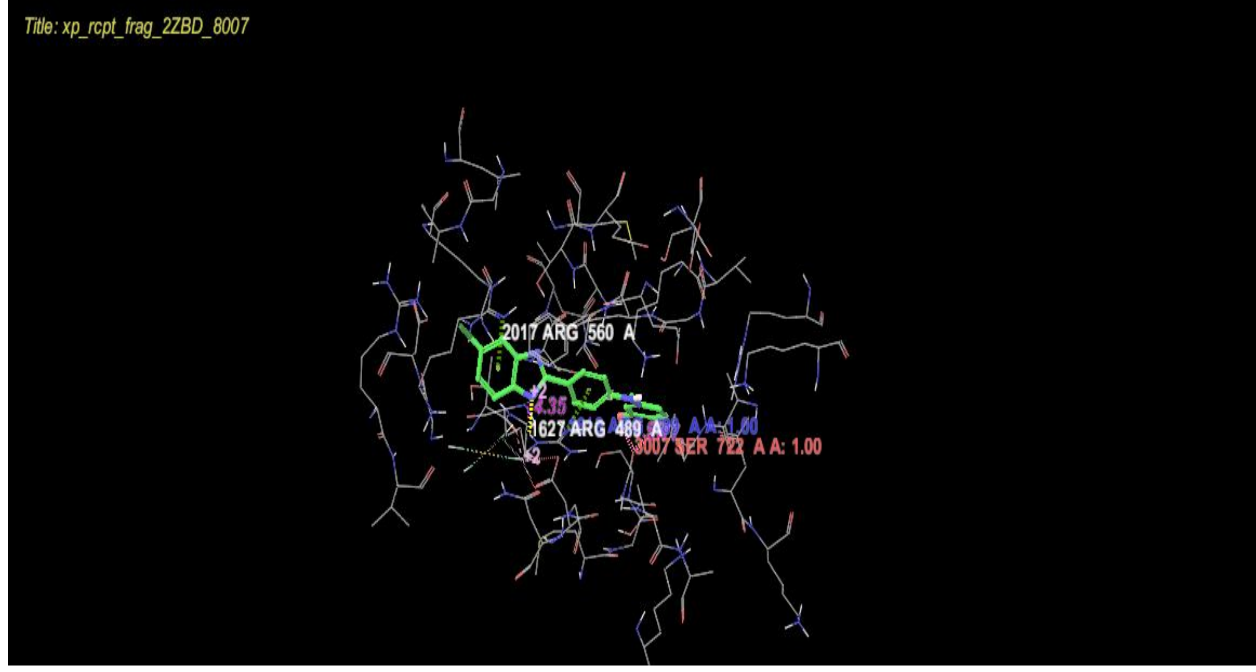


P-12

Figure 9:Snapshots of Drug-Receptor interaction

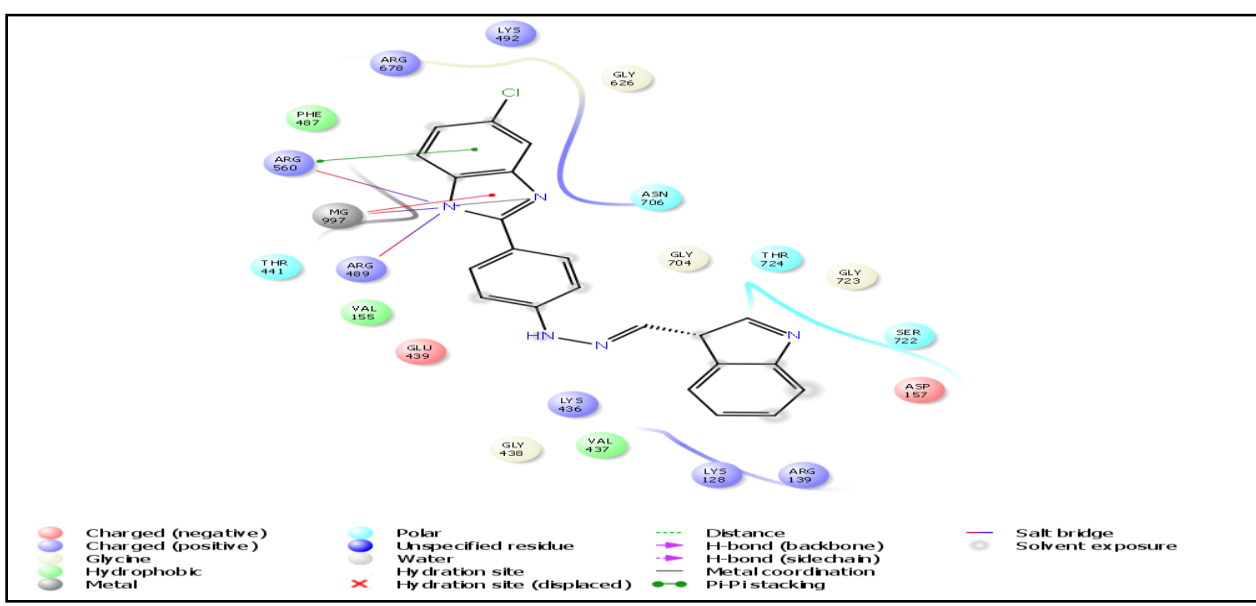


Predicted binding mode of compound P-12 into the catalytic site of 2ZBD

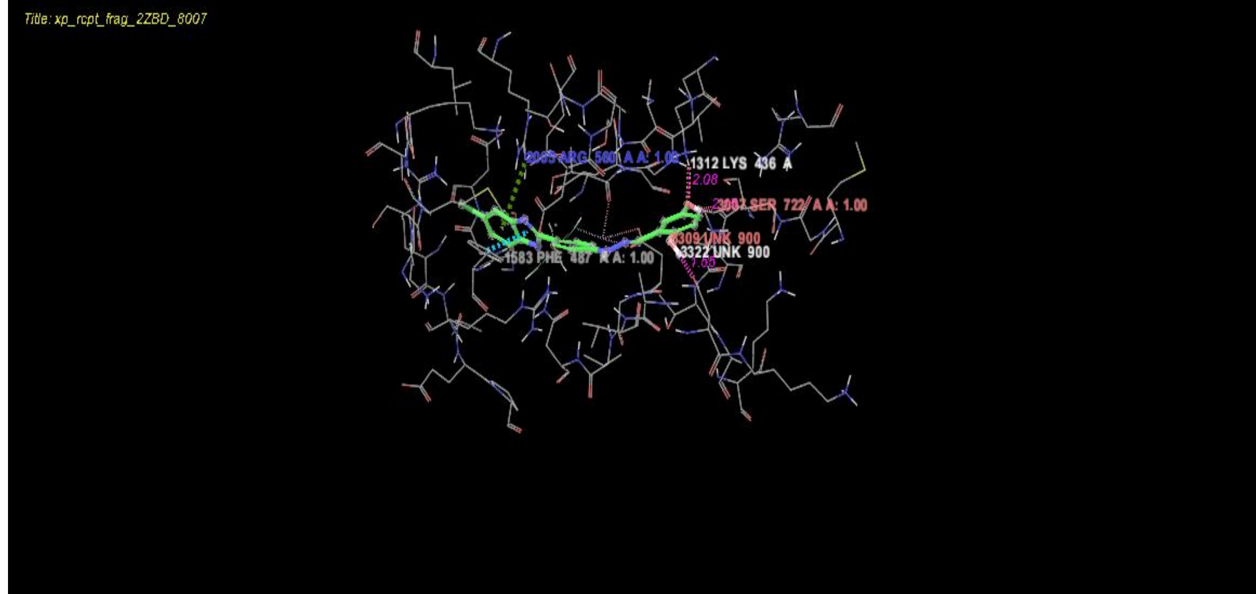


P-26

Figure 10:Snapshots of Drug-Receptor interaction

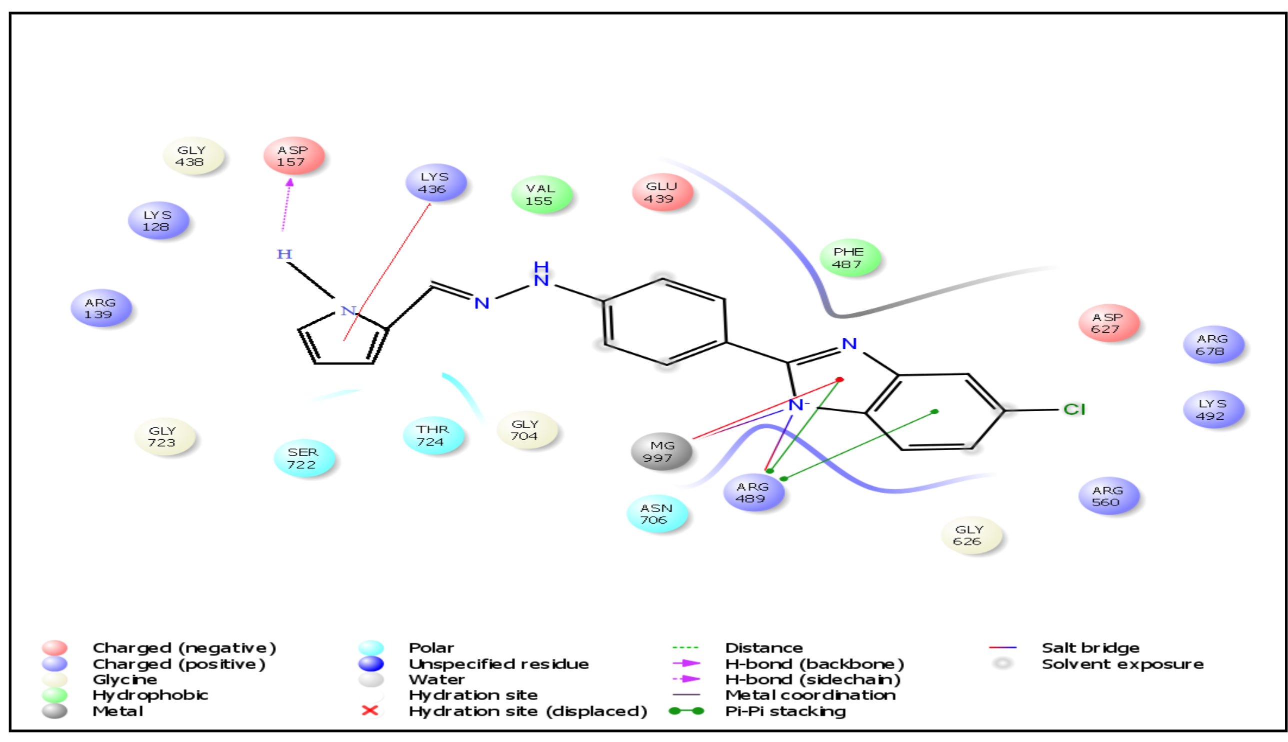


Predicted binding mode of compound P-26 into the catalytic site of 2ZBD



P-28

Figure 11:Snapshots of Drug-Receptor interaction



Predicted binding mode of Compound P-28 into the catalytic site of 2ZBD

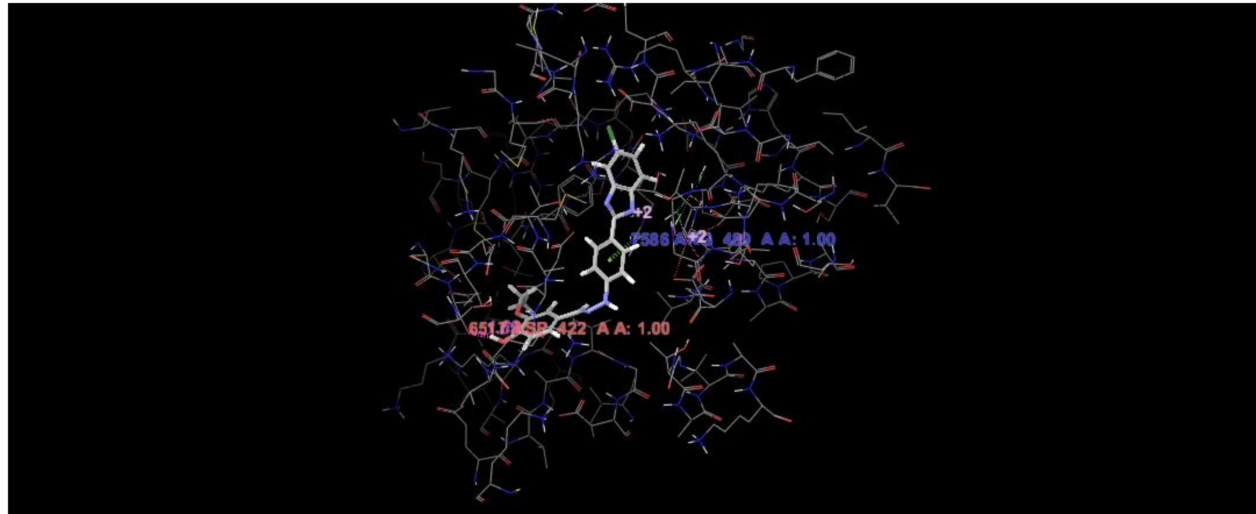


Table 8: Drug-Receptor interaction

Sl No.	Code	Compound Name	Receptor	Interacting residues
1	P-06	(E)-2-(2-methoxybenzylidene)-1-(5-chloro-*1H*-benzo[d]imidazol-2-yl)phenyl)hydrazine	H^+/K^+ATP-ase receptor (PDB ID:2ZBD)	a)Pi-Pi stacking with ARG 489 b)Hydrogen bond(side chain)with ARG 489.
2	P-12	2-(3-nitrobenzydene)-1-(4-(5-chloro-*1H*-benzo[d]imidazol-2-yl)phenyl)hydrazine		a)Pi-Pi stacking with PHE 487 b)Salt bridge with ARG 489 and ASP422
3	P-26	2-((3H-indol-2-yl)methylene)-1-(4-(5-chloro-*1H*-benzo[d]imidazol-2-yl)phenyl)hydrazine		a)Pi-Pi stacking with ARG560 b)Salt bridge with ARG 489, ARG 560 and MG 997
4	p-28	2-((*1H*-pyrrol-2-yl)methlene)-1-(4-(5-chloro-*1H*-benzo[d]imidazol-2-yl)phenyl)hydrazine		a)Pi-Pi stacking with ARG 489 b)Hydrogen bond (side chain)ASP 157 c)Salt bridge with ARG 489 and MG 997

SYNTHESIS AND CHARACTERISATION OF DOCKED COMPOUNDS

SYNTHESIS OF SUITABLE DOCKED COMPOUNDS

Suitable benzimidazole derivatives were synthesized and their structures were as represented below:

P-06

(E)-2-(2-methoxybenzylidene)-1-(5-chloro-*1H*-benzo[d]imidazol-2-yl)phenyl)hydrazine

P-12

2-(3-nitrobenzydene)-1-(4-(5-chloro-*1H*-benzo[d]imidazol-2-yl)phenyl)hydrazine

P-26

2-((3H-indol-2-yl)methylene)-1-(4-(5-chloro-*1H*-benzo[d]imidazol-2-yl)phenyl)hydrazine

P28

2-((*1H*-pyrrol-2-yl)methlene)-1-(4-(5-chloro-*1H*-benzo[d]imidazol-2-yl)phenyl)hydrazine

CHARACTERISATION OF SYNTHESIZED COMPOUNDS

a)Melting point

Melting points of the synthesized compounds were determined in a one end fused capillary tube method by using melting point apparatus and are as provided in the below table:9

b)Solubility

Solubility of the synthesized compounds was checked in various solvents and the compounds were found to be soluble in ethanol and DMSO .The results of solubility are as provided in the below table:9

c)Thin layer chromatography

Thin layer chromatographic analysis was carried out for all synthesized compounds by using silica gel G as stationary phase and Chloroform:Benzene (6:4)as mobile phase. The spot was visualized by iodine vapours. R_f value is a characteristic for each of the compound and are as provided in the below table:9

$$\text{Rf value} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Table 9: Physical data of synthesized compounds

Sl. No.	Code	Molecular formula	Molecular Weight (g/mol)	Melting Point range (^{0}c)	Solubility	R_f^*	Percentage yield
1	P-06	$C_{15}H_{13}ClN_4O$	300.74	160-165	Ethanol/ DMSO	0.71	53.1%
2	P-12	$C_{14}H_{10}ClN_5O_2$	315.71	170-172	Ethanol/ DMSO	0.72	56.89%
3	P-26	$C_{16}H_{12}ClN_5$	309.75	190-195	Ethanol/ DMSO	0.77	60.75%
4	P-28	$C_{12}H_{10}ClN_5$	259.69	140-143	Ethanol/ DMSO	0.75	64.66%

*Mobile phase-Chloroform:Benzene(6:4)

Detection – Iodine vapours

SPECTRAL ANALYSIS OF SYNTHESIZED COMPOUNDS

a)Ultra Violet spectral analysis

The maximum absorbance or λ_{max} of synthesized compounds were determined in the region of 200-400nm by using Shimadzu UV PharmaSpec 1700 UV-Visible spectrophotometer and the results are as provided in the below table: 10

b) Infrared spectral analysis

The structures of the synthesized compounds were elucidated by JASCO FT-IR 4100 infrared spectrophotometer in KBr pellets. The results are as provided in the below table:11

c)Nuclear Magnetic Resonance spectral analysis

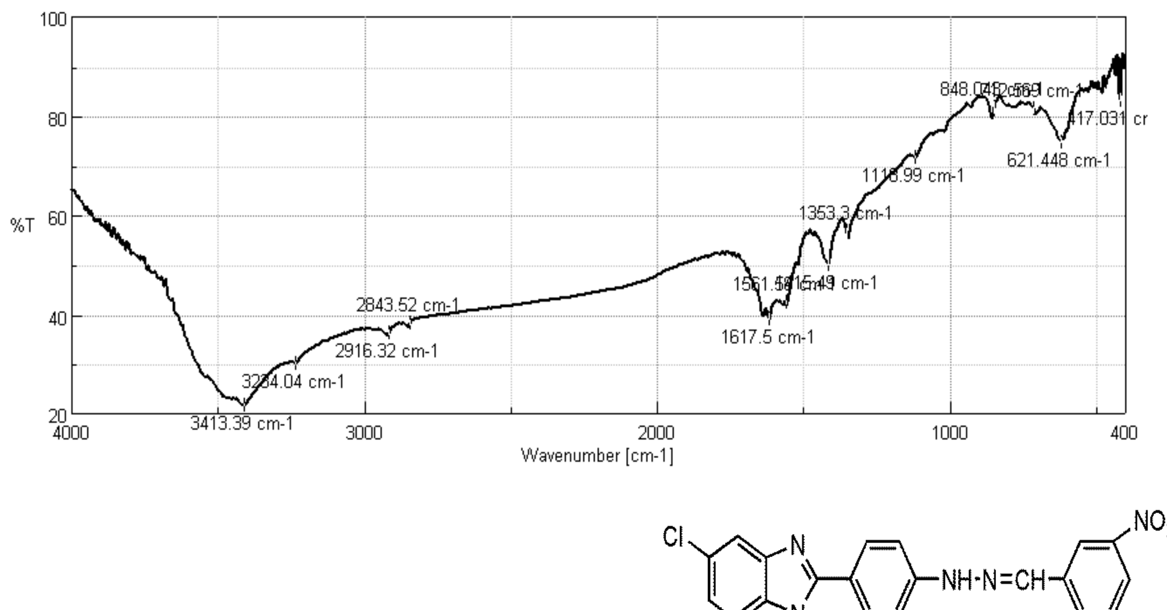
The structures of the synthesized compounds were elucidated by Bruker AVANCE 500 MHz NMR Spectrophotometer using DMSO (dimethyl sulphoxide) as internal standard. The results are as provided in the below table:12

d)Mass spectroscopy spectral analysis

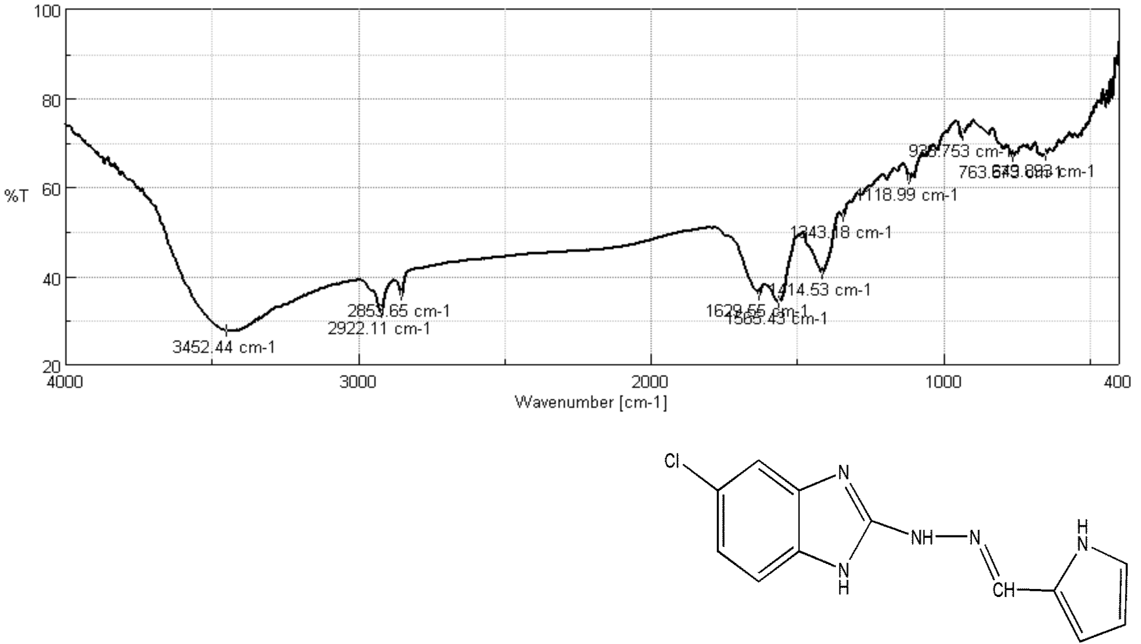
The structures of the synthesized compounds were to be elucidated by JEOL GC MATE II GC-MS. The results are as provided in the below table:13

SPECTRAL ANALYSIS

IR SPECTRA OF P-12

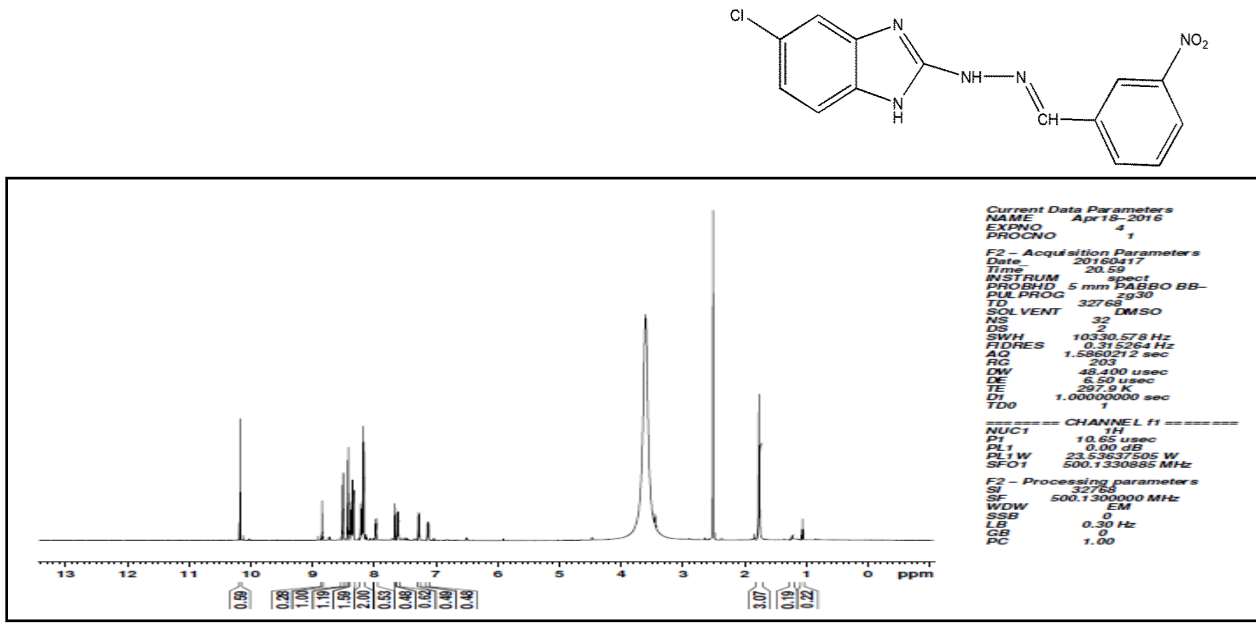


IR SPECTRA OF P-28

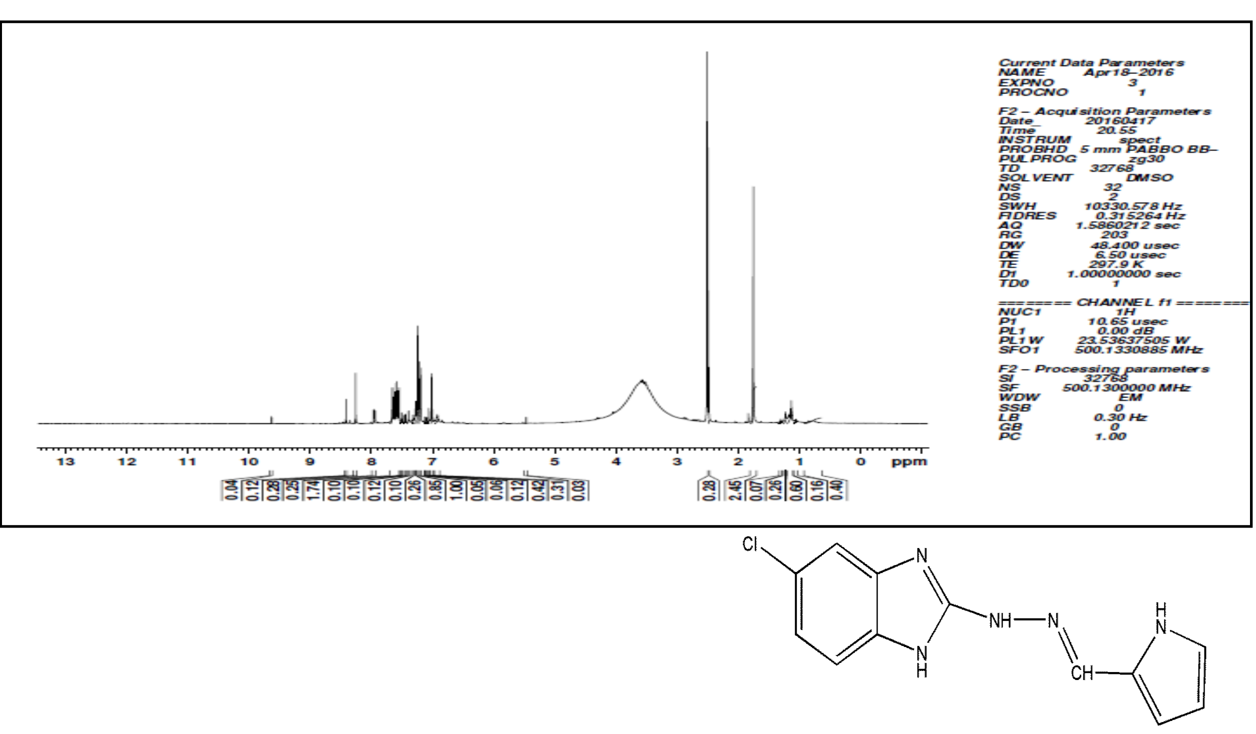


NMR SPECTRA

NMR SPECTRA OF P-12

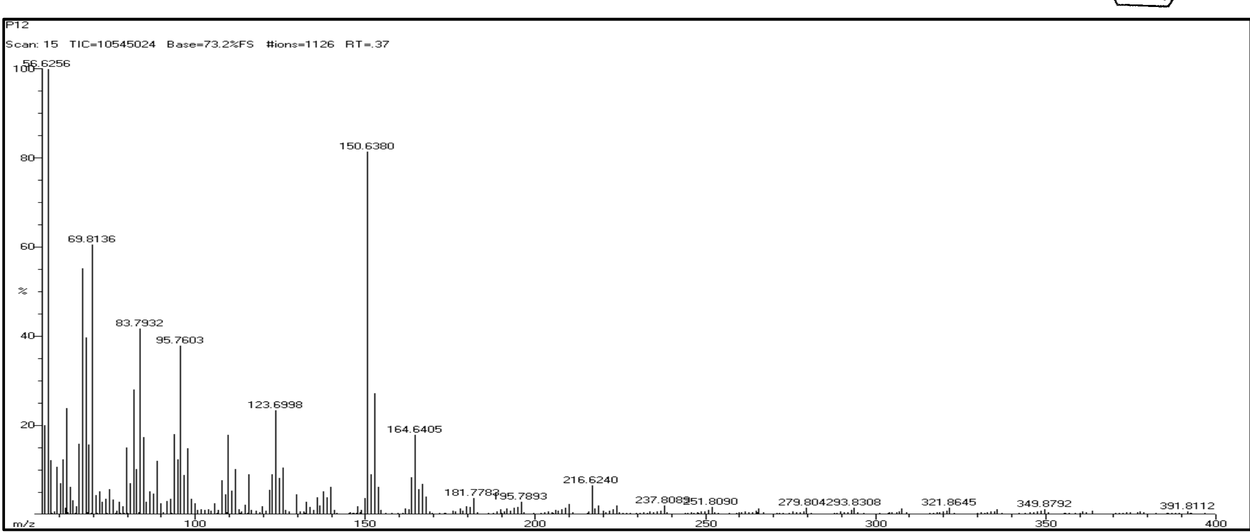


NMR SPECTRA OF P-28



MASS SPECTRA

MASS SPECTRA OF P-12



MASS SPECTRA OF P-28

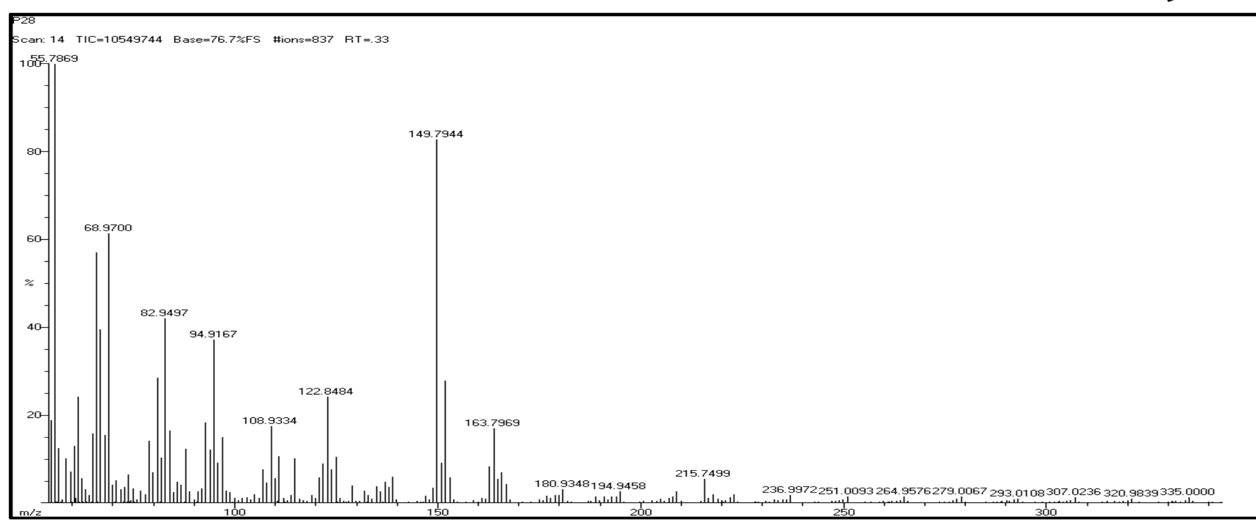


Table 10:UV-Visible spectral data of synthesized compounds

Sl.No.	Code	Structures	λ_{max}(nm)
1	P-06		309.5
2	P-12		312.5
3	P-26		358.5
4	P-28		325.5

Table 11: Infrared spectral data of synthesized compounds

Sl.No.	Code	Structures	IR(cm^{-1})
1	P-28		3452:Amine NH stretching 2853:Aliphatic CH-Stretching 1629:C=N Stretching 936:Aromatic CH Bending
2	P-12		3413: Amine NH stretching 3234:Aromatic-CH-Stretching 2843:Aliphatic CH-Stretching 1617:C=N-Stretching 1353:Aromatic-NO2-Stretching 848 :Aromatic-CH-Bending

Table 12:NMR spectral data of synthesized compounds

SI No.	Code	Structures	Functional Groups	δvalues (in ppm)
1	P-28		(1H,NH-N=),Singlet (1H,Ar-H),Multiplet (1H,CyclicNH),Singlet (1H,Alicyclic-H,CH),Singlet	9.63 7.19-8.14 3.6 2.48
2	P-12		(1H,NH-N=),Singlet (1H,Ar-H),Multiplet (1H,Cyclic NH),Singlet (1H,Alicyclic-H,CH),Singlet	10.17 7.1-8.4 3.6 2.5

Table 13:Mass spectral data of synthesized compounds

SI. No.	Code	Structures	Molecular mass(m/z,amu)	Base peak	Molecular ion peak
1	P-28		335.5	55.7869	335
2	P-12		392.5	56.6256	391.81

***IN VITRO* ANTIOXIDANT ACTIVITY**

DPPH RADICAL SCAVENGING ACTIVITY

Table 14:DPPH radical scavenging activity of Ascorbic acid(standard)

Sl No.	Sample	Concentraction (µg/ml)	% Inhibition (%)	IC_{50} (µM)
1	Ascorbic acid	0.5	2.74	10.12
2		1	3.37	
3		1.5	5.08	
4		2	6.66	
5		2.5	7.60	
6		5	20.98	
7		10	50.36	

Table 15: DPPH radical scavenging activity of compound P-06

Sl No.	Sample	Concentraction (µg/ml)	%Inhibition (%)	IC_{50} (µM)
1	P-06	50	46.39	105.7
2		100	48.22	
3		200	52.91	
4		300	56.57	
5		400	60.06	
6		500	62.98	

Table 16:DPPH radical scavenging activity of compound P-12

Sl No.	Sample	Concentraction (µg/ml)	% Inhibition (%)	IC_{50} (µM)
1	P-12	50	53.34	47.58
2		100	61.23	
3		200	62.38	
4		300	68.73	
5		400	76.75	
6		500	88.89	

Table 17:DPPH radical scavenging activity of compound P-26

Sl No.	Sample	Concentraction (µg/ml)	% Inhibition (%)	IC_{50} (µM)
1	P-26	50	45.78	87.06
2		100	50	
3		200	57.45	
4		300	63.86	
5		400	75.28	
6		500	84.62	

Table 18:DPPH radical scavenging activity of compound P-28

Sl No.	Sample	Concentraction (µg/ml)	% Inhibition (%)	IC_{50} (µM)
1	P-28	50	60.78	43.03
2		100	72.43	
3		200	84.58	
4		300	92.65	
5		400	109.54	
6		500	130.45	

Graphical representation

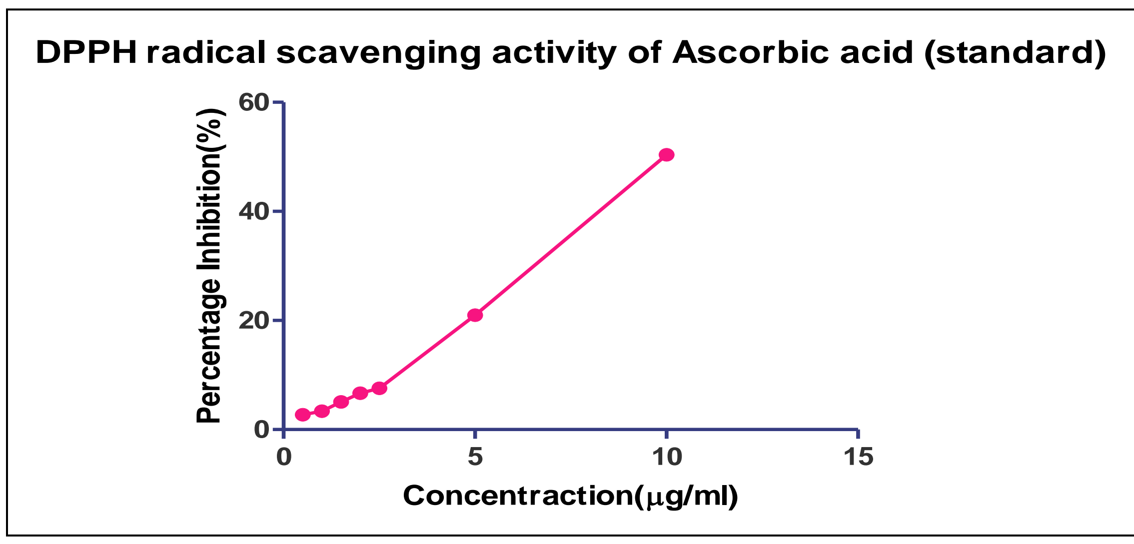


Figure 12

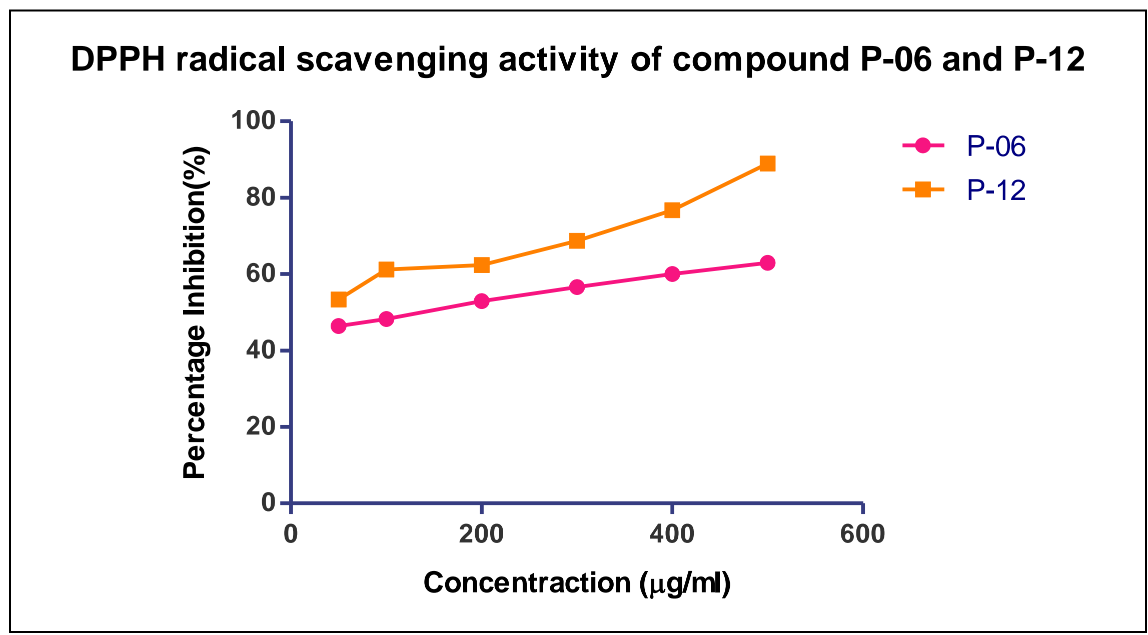


Figure 13

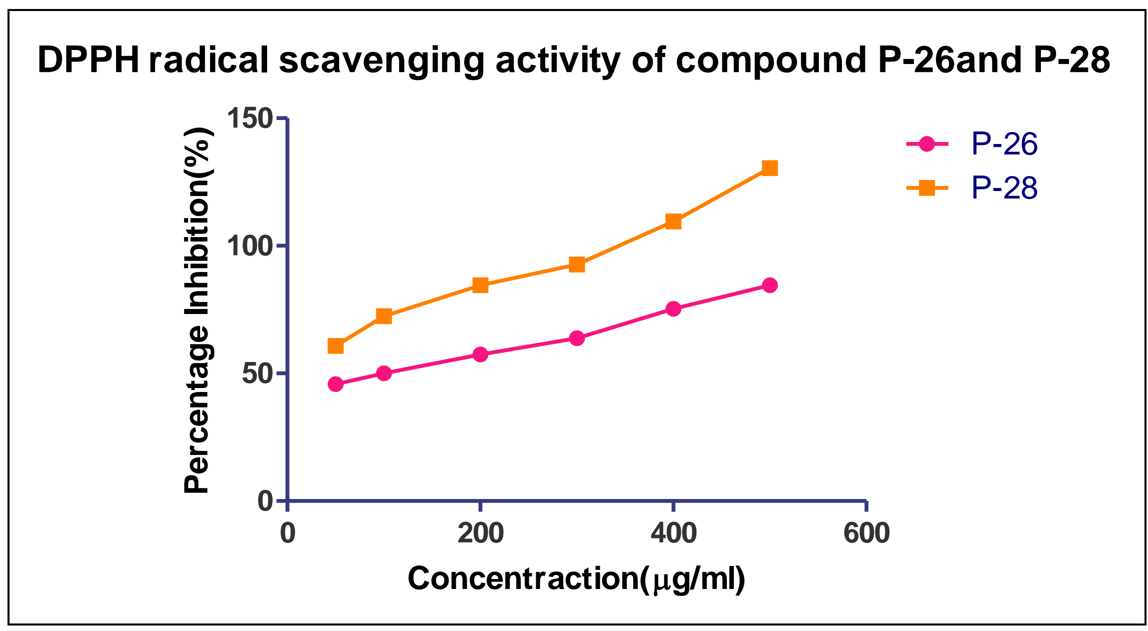


Figure 14

ABTS radical scavenging activity

Table 19:ABTS radical scavenging activity of Ascorbic acid(standard)

Sl No.	Sample	Concentraction (µg/ml)	% Inhibition (%)	IC_{50} (µM)
1	Ascorbic acid	0.2	26.79	0.937
2		0.4	32.17	
3		0.6	34.94	
4		0.8	38.64	
5		1	43.49	
6		1.5	63.04	
7		2	80.22	

Table 20:ABTS radical scavenging activity of Compound P-06

Sl No.	Sample	Concentraction (µg/ml)	% Inhibition (%)	IC_{50} (µM)
1	P-06	50	42.45	83.88
2		100	48.10	
3		150	58.17	
4		200	73.84	
5		250	82.95	

Table 21:ABTS radical scavenging activity of compound P-12

Sl No.	Sample	Concentraction (µg/ml)	% Inhibition (%)	IC_{50} (µM)
1	P-12	50	46.78	58.27
2		100	64.34	
3		150	73.02	
4		200	82.32	
5		250	88.98	

Table 22:ABTS radical scavenging activity of compound P-26

Sl No.	Sample	Concentraction (µg/ml)	% Inhibition (%)	IC_{50} (µM)
1	P-26	50	42.54	74.39
2		100	56.67	
3		150	62.21	
4		200	67.82	
5		250	87.62	

Table 23:ABTS radical scavenging activity of compound P-28

Sl No.	Sample	Concentraction (µg/ml)	% Inhibition (%)	IC_{50} (µM)
1	P-28	50	57.89	42.71
2		100	68.98	
3		150	79.89	
4		200	88.65	
5		250	97.76	

Graphical representation

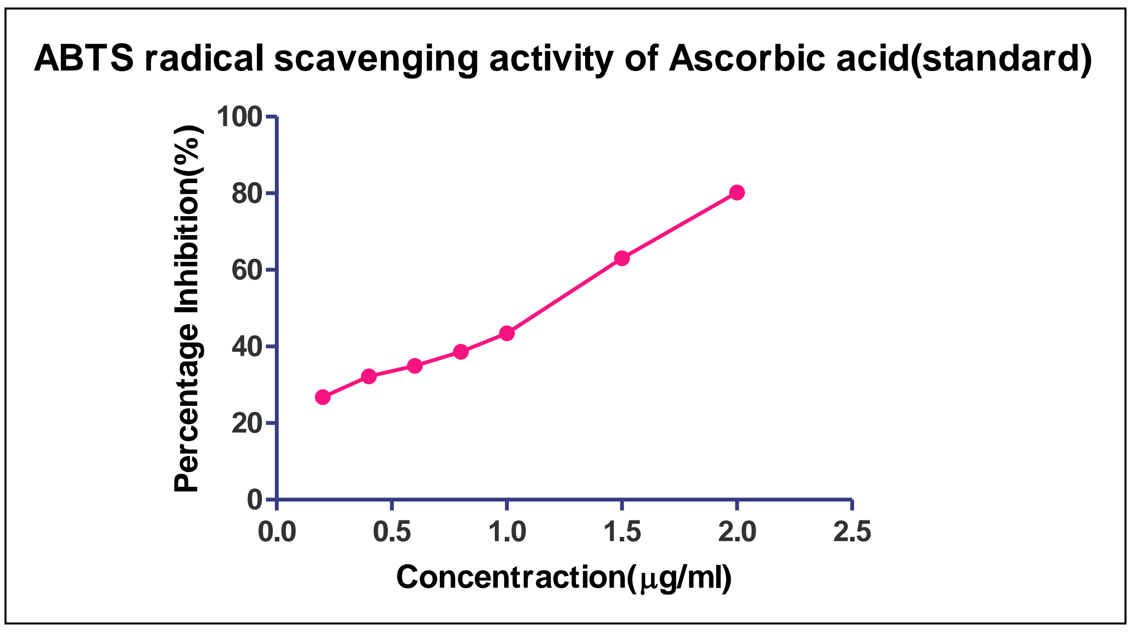


Figure 15

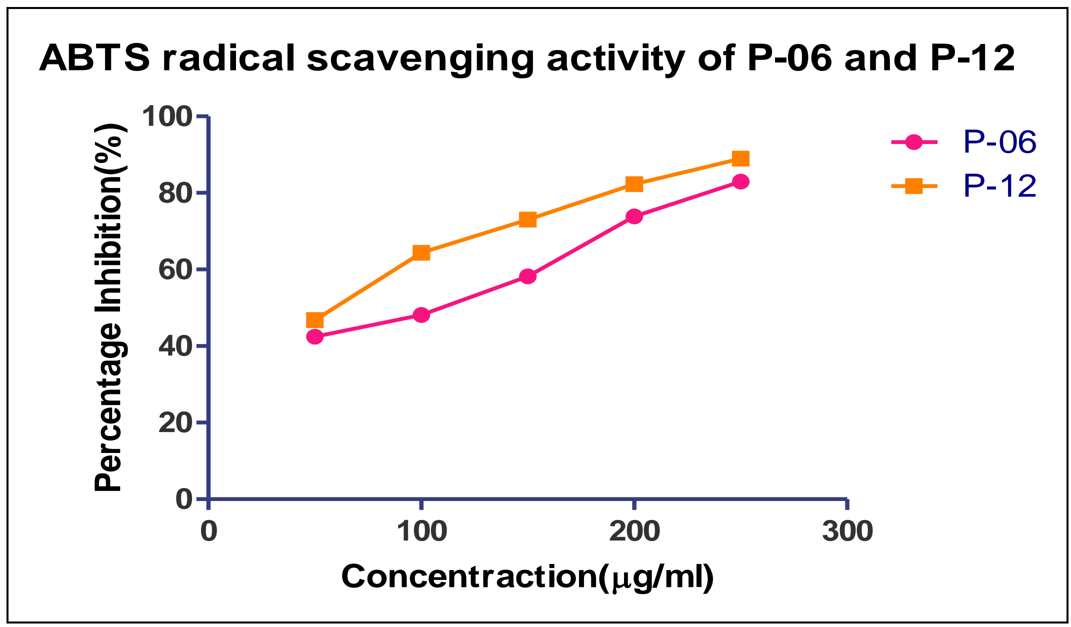


Figure 16

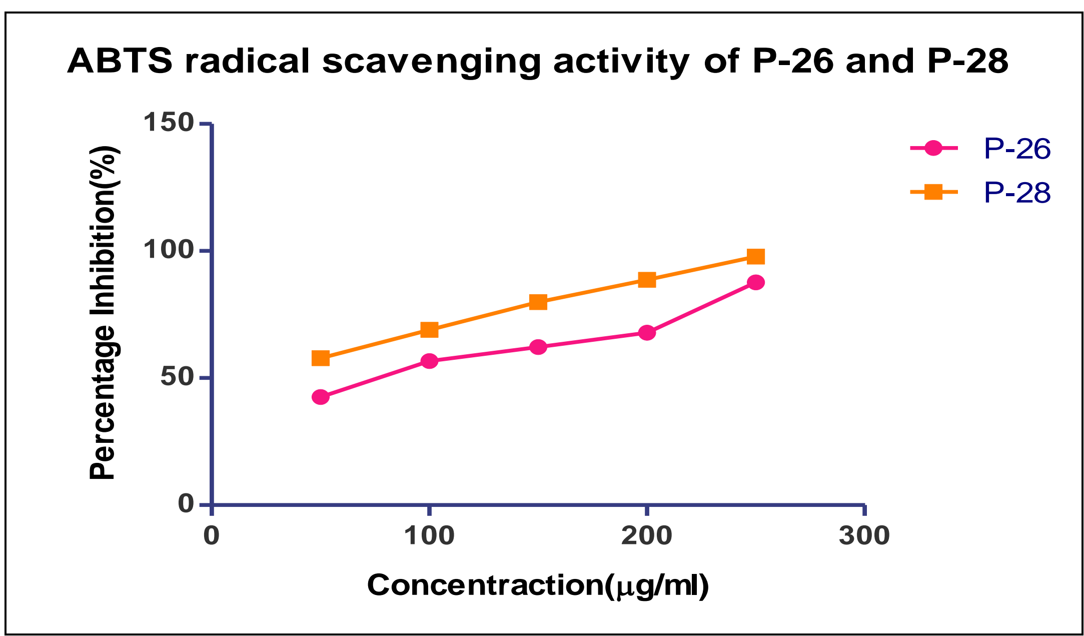


Figure 17

***IN VITRO* CELL VIABILITY ASSAY: (Table:24)**

SI No.	Sample	Concentraction (µM)	%Viability
1	P-28	0.25	97.64
2		2.5	95.44
3		25	94.49
4		50	91.39
5		100	88.34
1	P-12	0.25	93.33
2		2.5	89.56
3		25	85.45
4		50	83.34
5		100	79.83
1	P-26	0.25	76.34
2		2.5	74.45
3		25	68.89
4		50	64.34
5		100	55.70

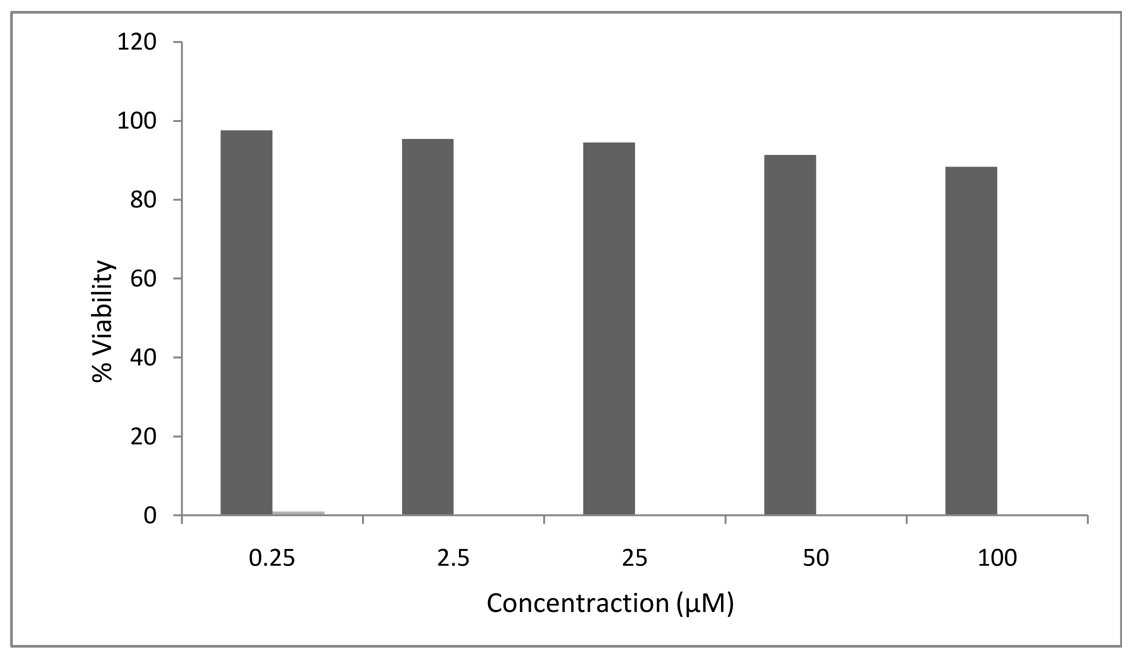
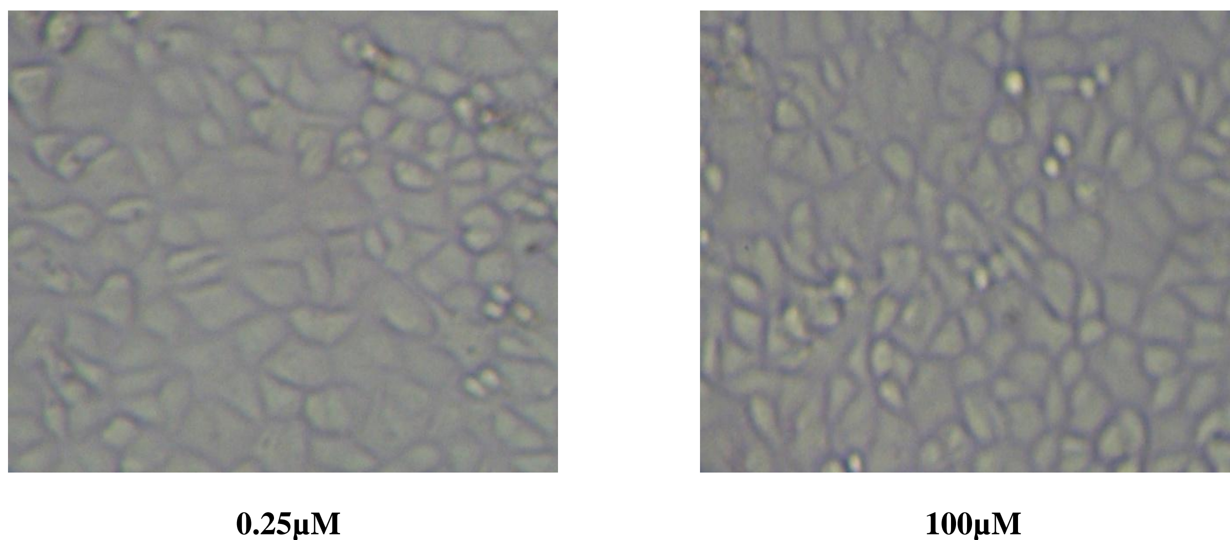


Figure 18:*In vitro* cell viability assay of compound P-28

Figure19 : *IN VITRO* CELL VIABILITY ASSAY OF COMPOUND P-28

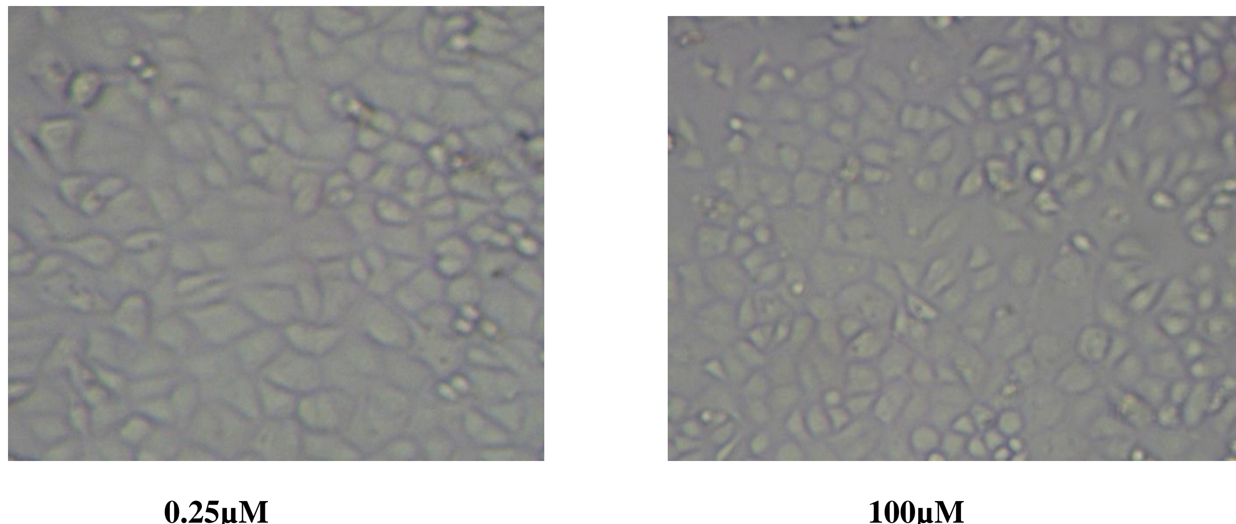
Representative photomicrograph shows morphological changes of AGS (Human Epithelial Gastric Cells)cells



0.25µM **100µM**

Figure 20:*In vitro* cell viability assay of compound P-12

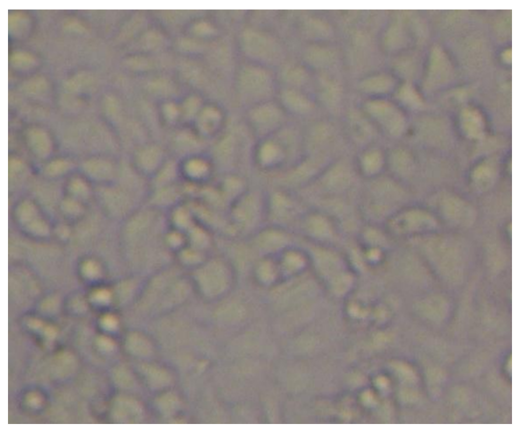
Representative photomicrograph shows morphological changes of AGS (Human Epithelial Gastric Cells)cells

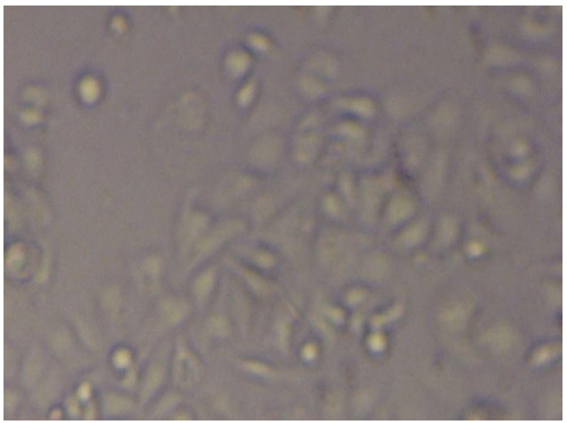


0.25µM **100µM**

Figure 21:*In vitro* cell viability assay of compound P-26

Representative photomicrograph shows morphological changes of AGS (Human Epithelial Gastric Cells)cells





0.25µM **100µM**

ACUTE ORAL TOXICITY STUDY

Table 25: Toxicological sign evaluation of compound P-28 on rats

Sl.No.	Responses	Compound P-28					
		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	Tremor	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 reflux	Present	Present	Present	Present	Present	Present
12	Writhing	Absent	Absent	Absent	Absent	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

EVALUATION OF ANTIULCER ACTIVITY

Table 26:Effect of compound P-28 on Indomethacin induced ulcer

GROUPS	ULCER INDEX(UI)	PERCENTAGE (%) INHIBITION
Normal control	00.00 ±00.00	-
Ulcer control (Indomethacin)	28.67±0.7601[a]	-
Low dose(Test compound)+Indomethacin	11.03±0.03[b,c]	61.39±0.90[b,c]
High dose(Test compound)+Indomethacin	6.85±0.08[b,c]	76.69±0.57[b,c]
Standard(pantoprazole)	3.83±0.34[b]	86.62±1.16[b]

All data are expressed as Mean± SEM(n=6).**Statistical comparison:** One way analysis of variance (ANNOVA) followed by Tukey test. [a]***P<0.001,ulcer control was compared with normal control, [b***]P<0.001 standard (10mg/kg) and drug treated groups were compared with ulcer control, [c***]P<0.001,drug treated groups compared with standard.

Graphical representation:

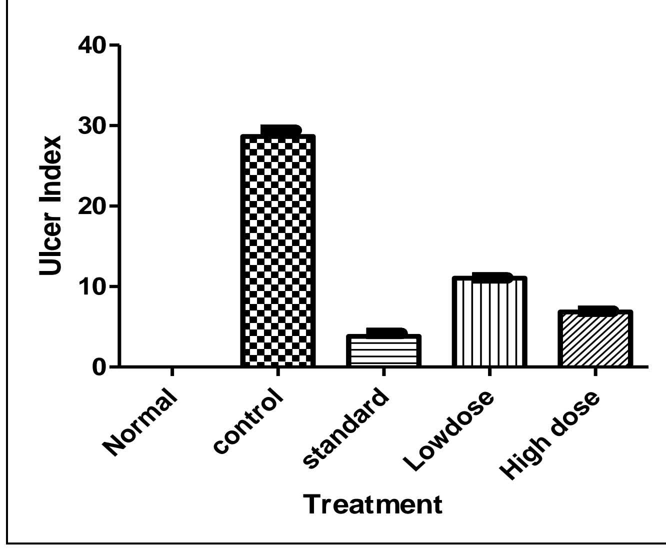


Figure 22

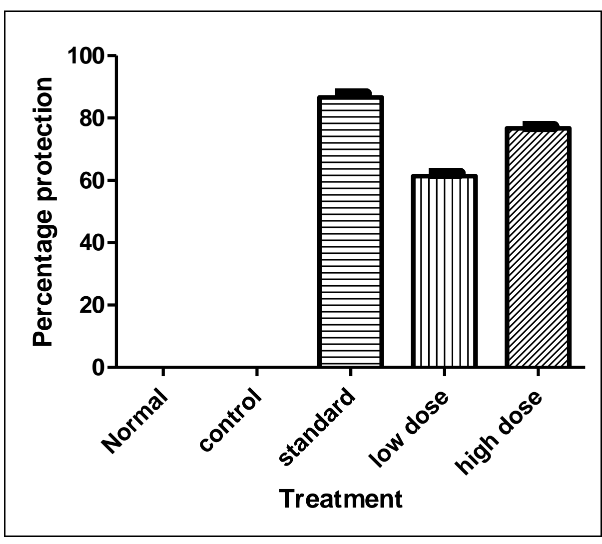
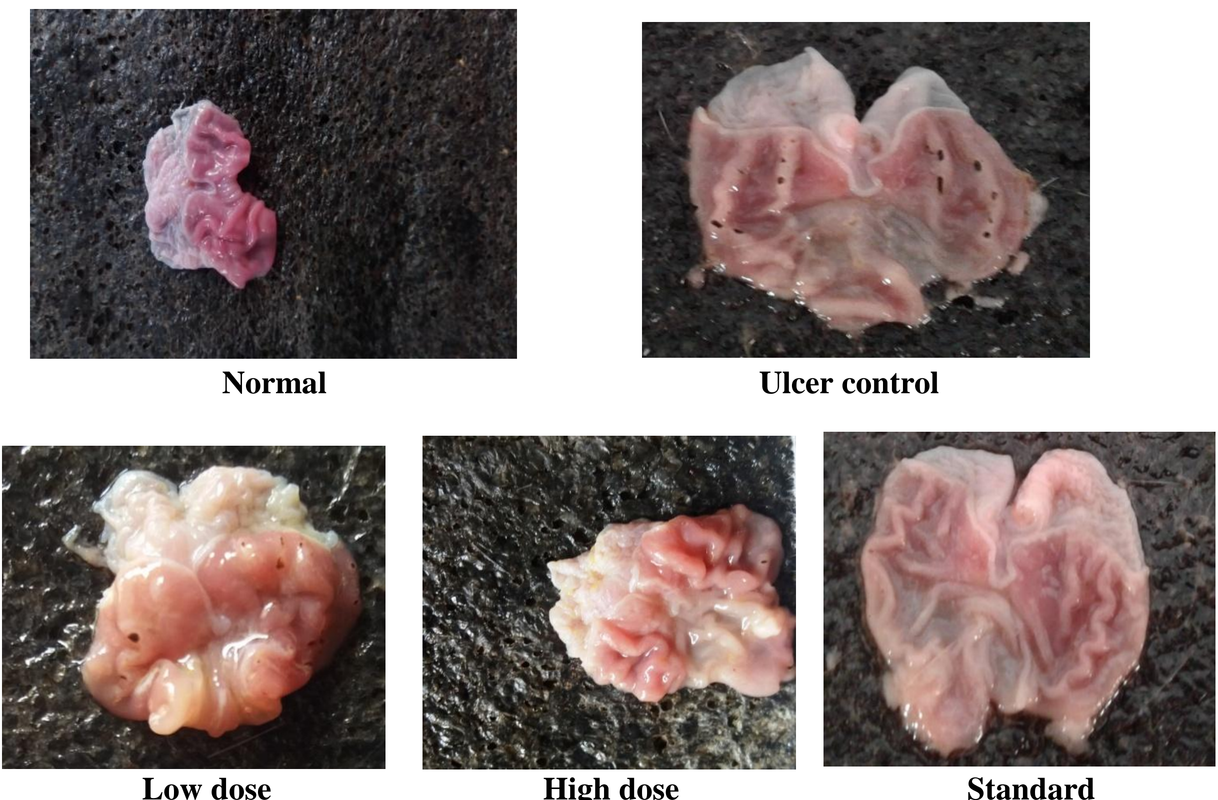


Figure 23

INDOMETHACIN INDUCED ULCER:



Normal **Ulcer control**

Low dose **High dose** **Standard**

Table 27: Effect of compound P-28 on pylorus ligation induced ulcer

Group	ULCER INDEX(UI)	PERCENTAGE INHIBITION(%)
Normal control	00.00±00.00	-
Ulcer control	19.00±0.73[a]	-
Low dose+ pylorus ligation	6.76±0.05[b,c]	65.11±1.25[b,c]
High dose+pylorus ligation	5.06±0.03[b,d]	73.41±1.38[b,c]
Standard(pantoprazole)	3.33±0.02[b]	82.55±0.91[b]

All data are expressed as Mean± SEM(n=6).**Statistical comparison**: One way analysis of variance(ANNOVA) followed by Tukey test. [a]***P<0.001, ulcer control was compared with normal control , [b]***P<0.001 standard (10mg/kg) and drug treated groups were compared with ulcer control, [c***]P<0.001,[d***]P<.01 ,drug treated groups compared with standard.

Graphical representation:

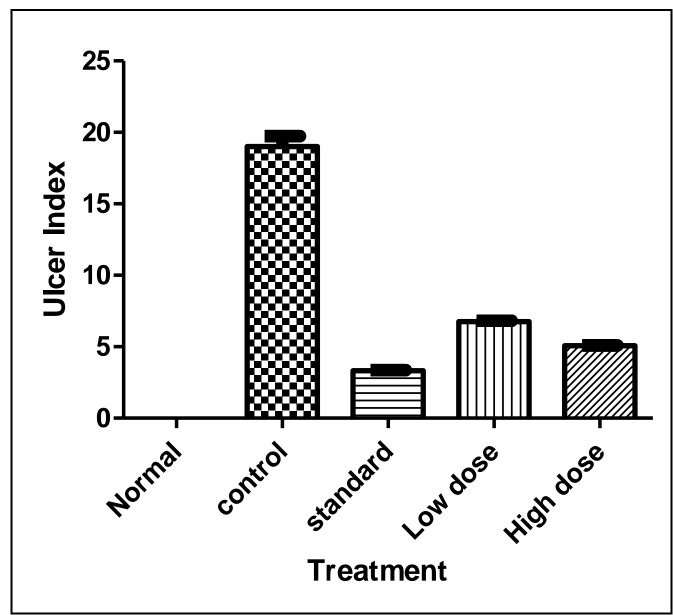


Figure 24

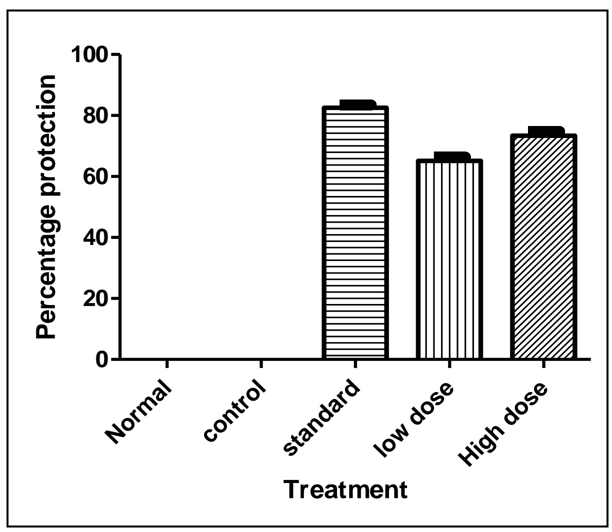
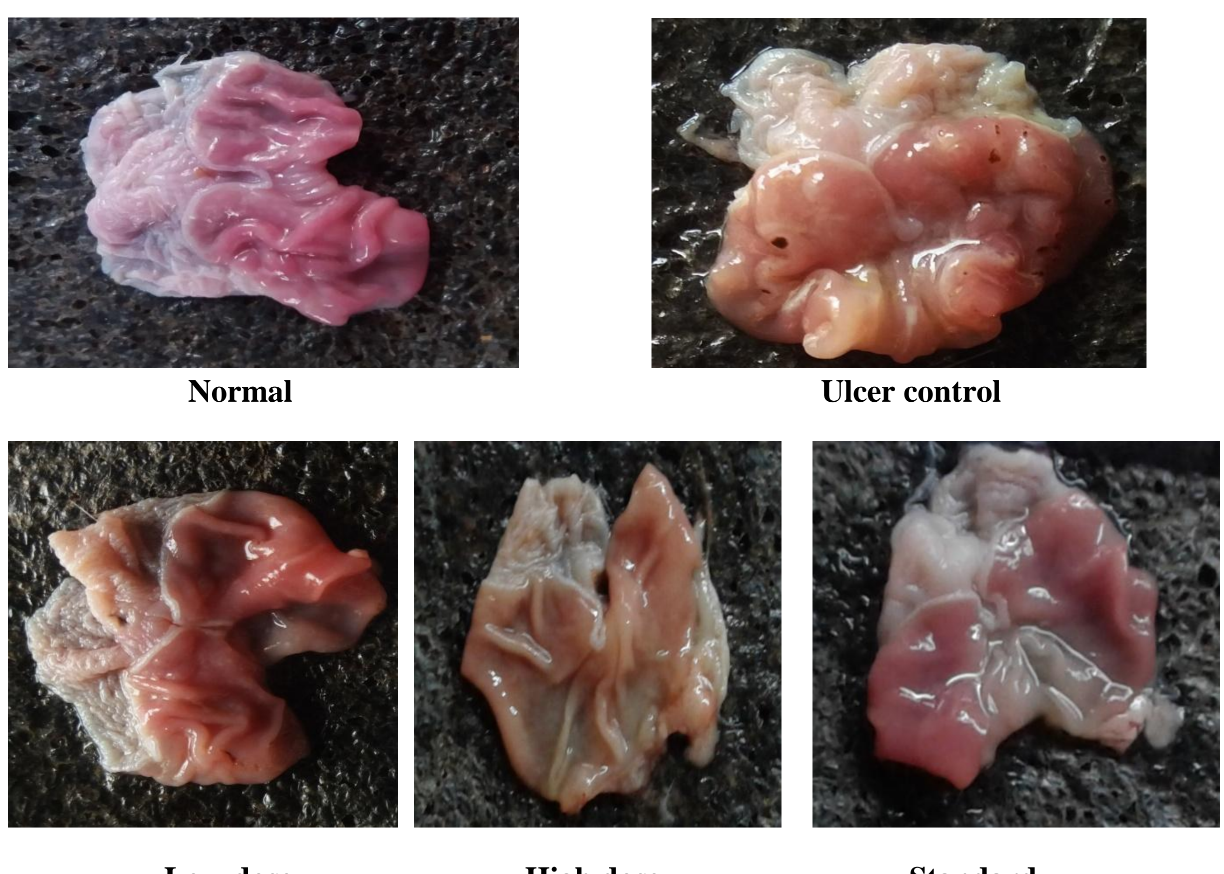


Figure 25

PYLORUS LIGATION INDUCED ULCER:



Normal **Ulcer control**

Low dose **High dose** **Standard**

Table 28: Effect of compound P-28 on Gastric secretion, Total and Free acidity pH of gastric juice using pylorus ligation induced ulcer

Group	Gastric volume (ml/100g)	pH of gastric juice	Total acidity	Free acidity
Normal control	1.28±0.03	2.67±0.006	54.21±0.68	29.82±0.54
Ulcer control	2.58±0.009[a]	1.43±0.10[a]	87.37±0.72[a]	57.32±0.53[a]
Low dose	1.83±0.004[b,d]	1.85±0.006[b,d]	67.40±0.51[b,d]	38.81±1.03[b,d]
High dose	1.63±0.01[b,d]	2.49±0.007[b,c]	60.44±0.88[b,d]	33.89±0.76[b,e]
Standard	1.30±0.006[b]	2.55±0.006[b]	56.58±0.50[b]	30.32±1.15[b]

All data are expressed as Mean± SEM(n=6).**Statistical comparison:** One way analysis of variance(ANNOVA) followed by Tukey test. [a]***P<0.001,ulcer control was compared with normal control, [b***]P<0.001, standard (10mg/kg) and drug treated groups were compared with ulcer control, [c*]P<0.05,[d***]P<0.001,[e**]p<0.01 , ns: non significant, drug treated groups compared with standard

Graphical representation:

Gastric volume

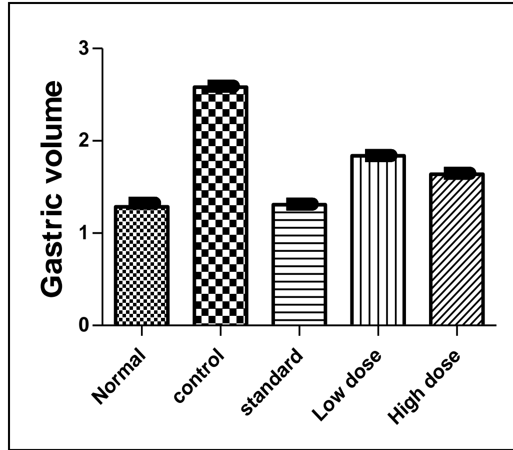


Figure 26:Effect of compound P-28 on gastric volume

pH of gastric juice

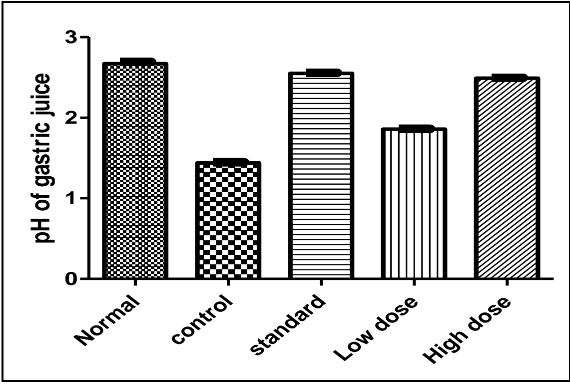


Figure 27: Effect of compound P-28 on p^H of gastric juice

Total acidity

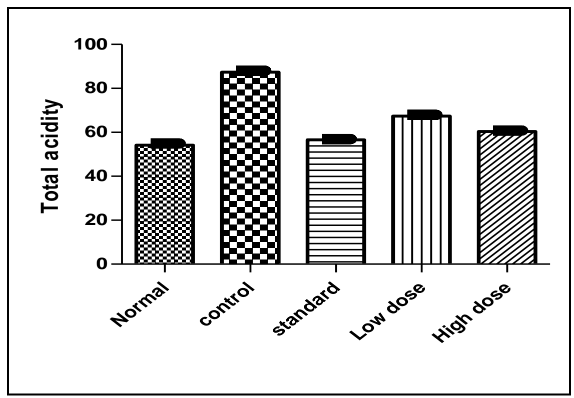


Figure 28: Effect of compound P-28 on total acidity

Free acidity

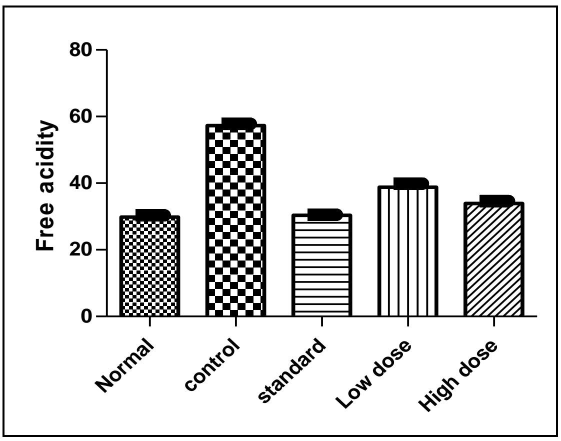


Figure 29:Effect of compound P-28 on free acidity

GRAPHICAL REPRESENTATION:

EFFECT OF COMPOUND P-28 ON TOTAL PROTEIN AND ANTIOXIDANT LEVELS IN INDOMETHACIN INDUCED ULCER MODEL

Figure 30: ESTIMATION OF TOTAL PROTEIN:

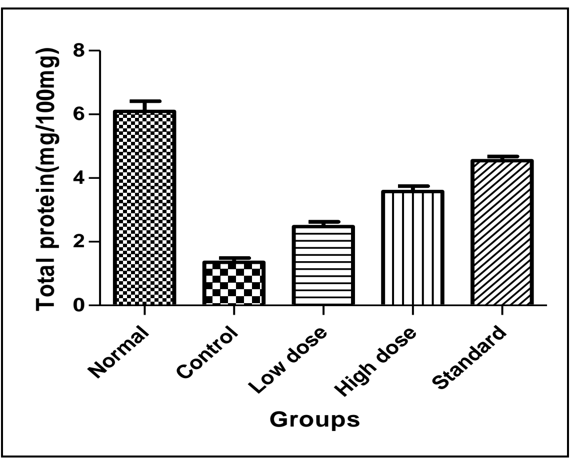


Figure 31: ESTIMATION OF SOD:

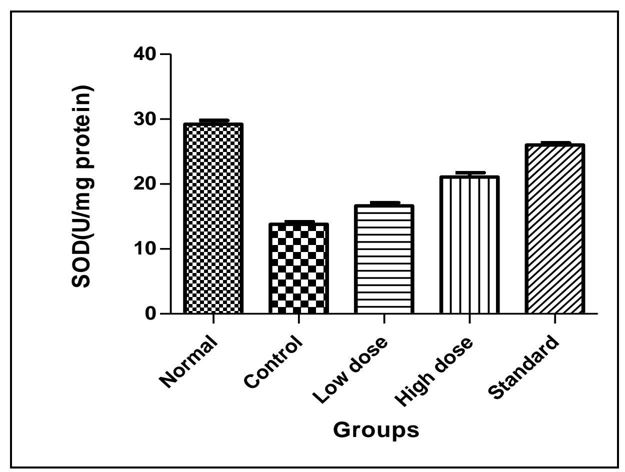


Figure 32: ESTIMATION OF CATALASE:

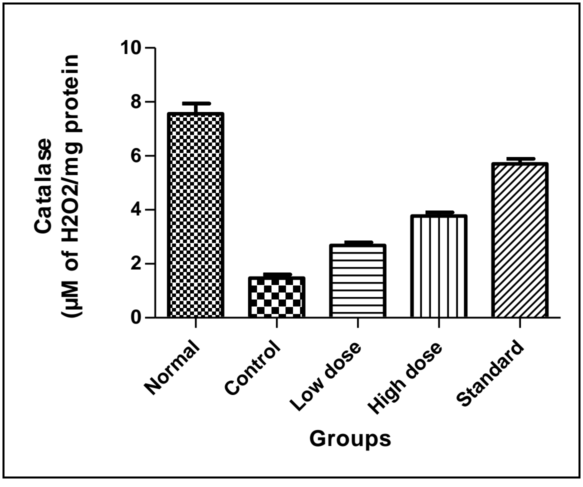


Figure 33: ESTIMATION OF GP_X:

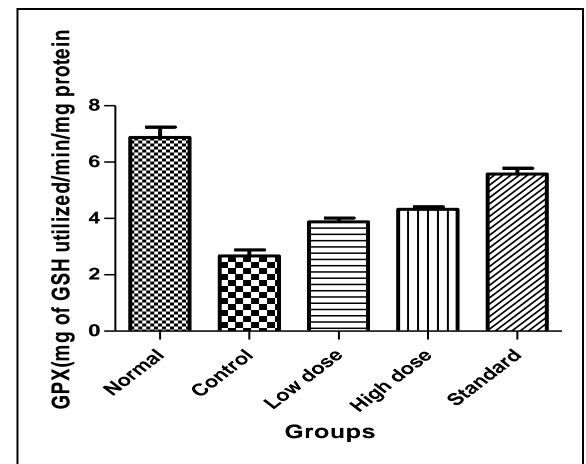


Figure 34: ESTIMATION OF GSH:

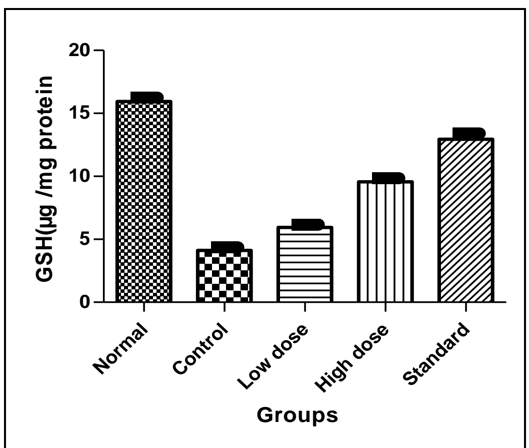


Figure 36: ESTIMATION OF LPO:

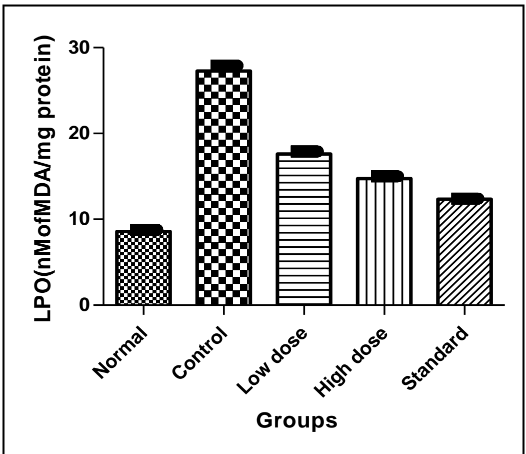
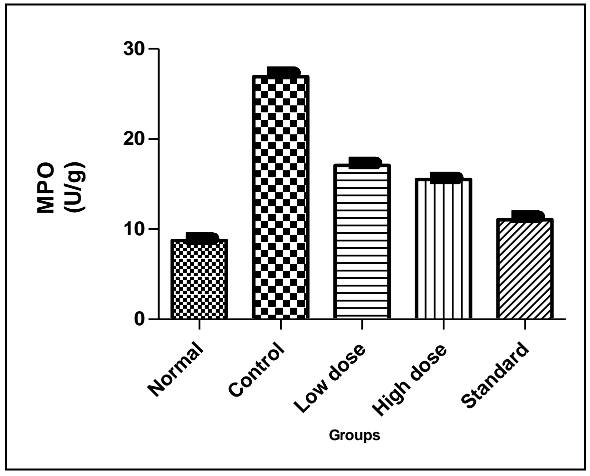


Figure 35: ESTIMATION OF MPO:



EFFECT OF COMPOUND P-28 OF TOTAL PROTEIN AND ANTIOXIDANT LEVELS IN PYLORUS LIGATION INDUCED ULCER MODEL:

Figure 37: ESTIMATION OF TOTAL PROTEIN:

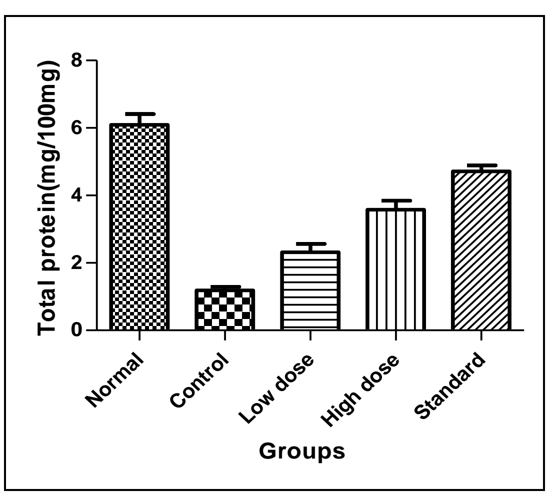


Figure 38: ESTIMATION OF SOD:

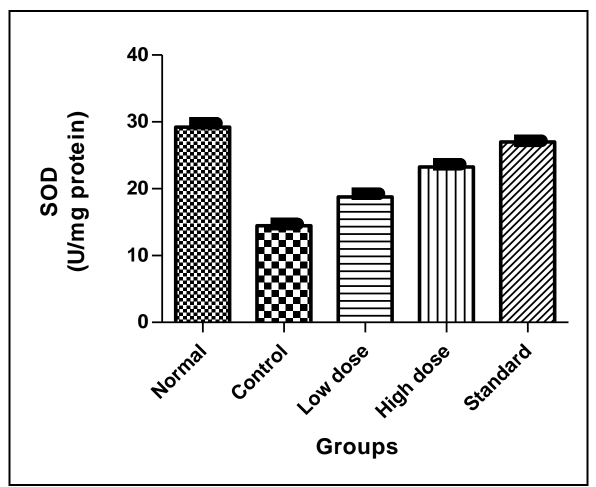


Figure 39: ESTIMATION OF CATALASE:

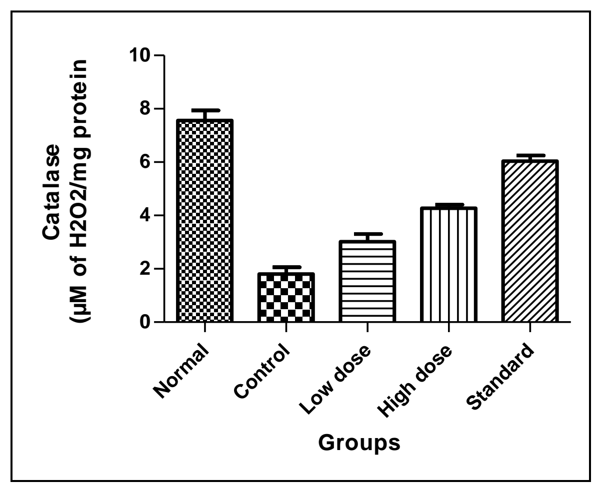


Figure 40: ESTIMATION OF GP_X:

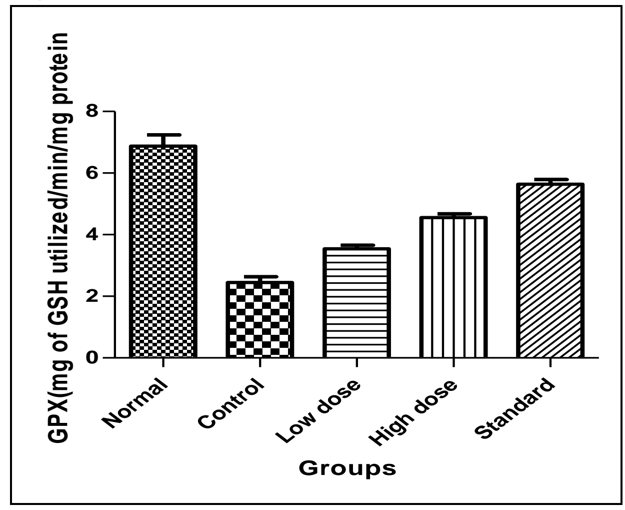


Figure 41: ESTIMATION OF LPO:

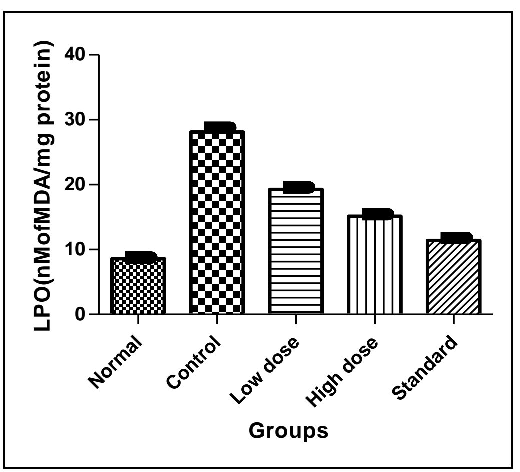


Figure 42: ESTIMATION OF MPO:

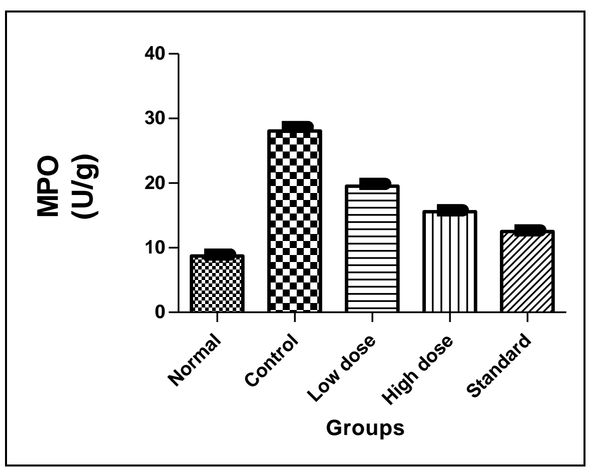
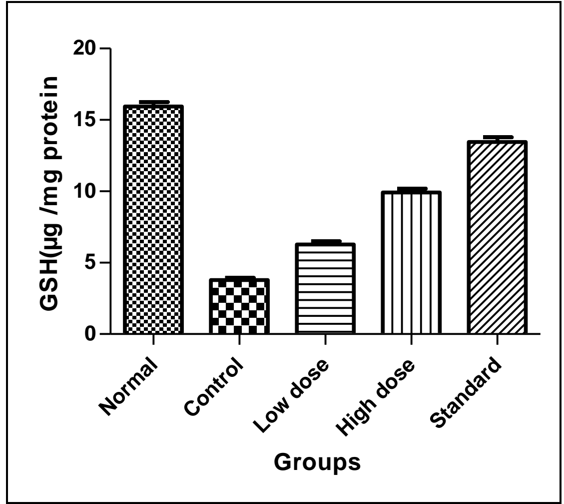


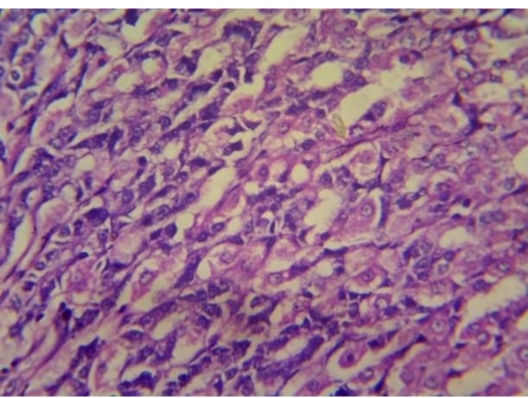
Figure 43: ESTIMATION OF GSH:



HISTOPATHOLOGICAL STUDIES:

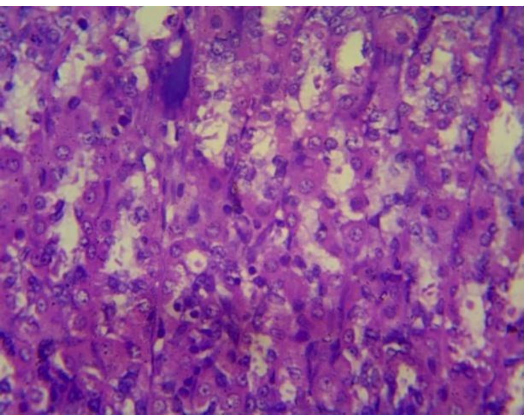
INDOMETHACIN INDUCED ULCER:

Figure 44: GROUP I:(NORMAL)



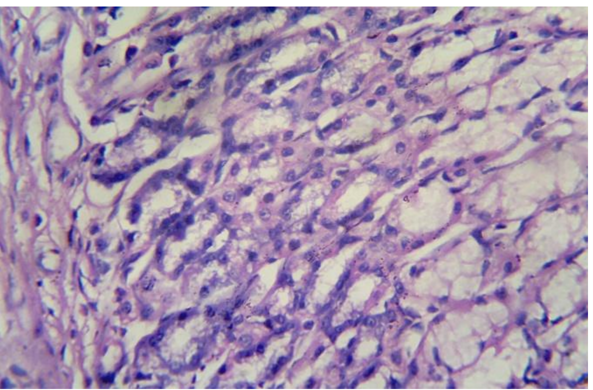
Section from stomach shows normal epithelium. Submucosa shows no significant pathology. There is no malignancy/granuloma seen.

Figure 45:GROUP II:(ULCER CONTROL:INDOMETHACIN ONLY)



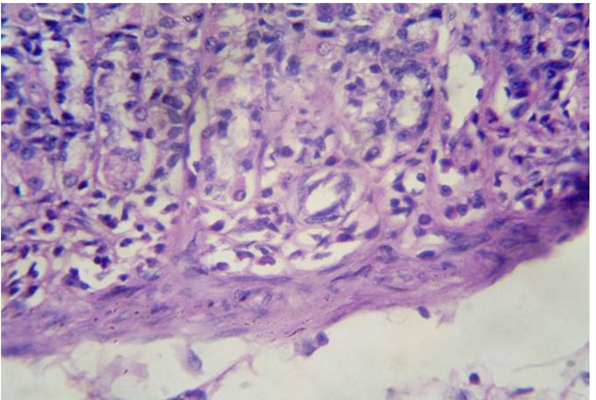
Laminapropria shows few scattered chronic inflammatory infiltrates. Submucosa shows significant pathological changes. Gastric section form untreated rats shows ulceration of the mucosal cells associated with inflammatory changes and necrosis.

Figure 46: GROUP III:(STANDARD:PANTOPRAZOLE)



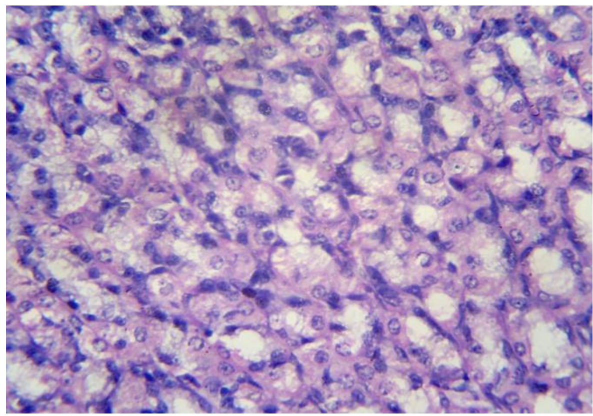
Section from stomach shows normal epithelium. Laminapropria shows mild inflammatory infiltrates. Blood vessel shows congestion. There is no malignancy/granuloma seen.

Figure 47: GROUP IV: (LOW DOSE, 50mg/kg)



Section from stomach shows normal epithelium. Laminapropria shows mild lymphocytic infiltrates. Submucosa shows mild lymphocytic and neutrophilic infiltrates. Muscular layer shows no significant pathology.

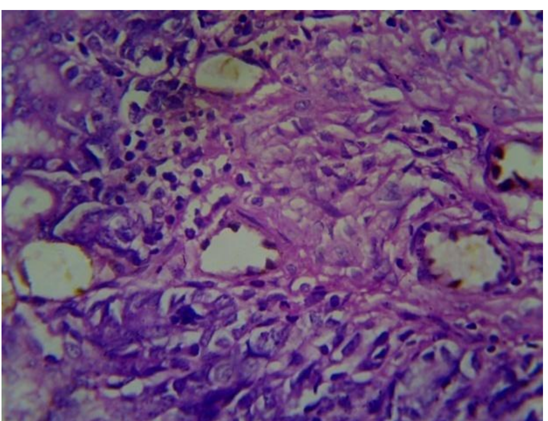
Figure 48: GROUP V: (HIGH DOSE,100mg/kg)



Section from stomach shows normal epithelium. Laminapropria and sub mucosa shows mild lymphoplasmacytic infiltrates. There is no malignancy/granuloma seen.

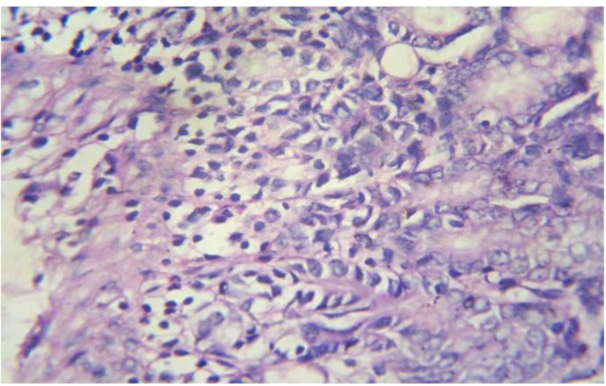
PYLORUS LIGATION INDUCED ULCER:

Figure 49: GROUP I :(ULCER CONTROL)



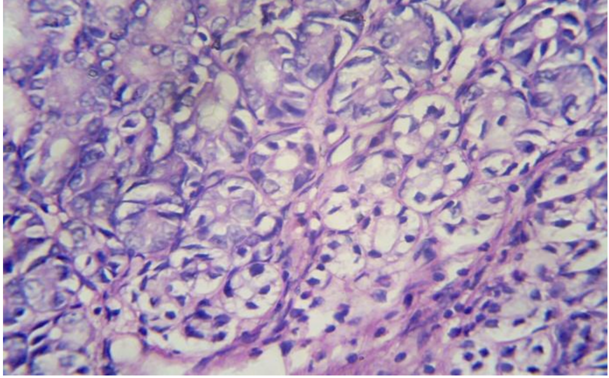
Few glands show reactive atypia. Laminapropria shows scattered chronic inflammatory infiltrates and neutrophils. Submucosa shows congested vessels and chronic inflammation.

Figure 50: GROUP II: (STANDARD:PANTOPRAZOLE)



Section from stomach shows normal epithelium. Laminapropria shows mild inflammatory infiltrates. Submucosa shows inflammation. There is no malignancy/granuloma seen.

Figure 51: GROUP III: (LOW DOSE,50mg/kg)



Section from stomach shows normal epithelium. Laminapropria shows mild lymphocytic infiltrates. Submucosa shows mild lymphocytic and neutrophilic infiltrates. Muscular layer shows no significant pathology.

Figure 52: GROUP IV: (HIGH DOSE,100mg/kg)

Section from stomach shows normal epithelium. Laminapropria shows mild lymphocytic infiltrates. Submucosa shows no significant pathology. There is no malignancy/granuloma seen

CONCLUSIONS

- A total of 30 compounds were designed for studying on its binding affinity with the H^+/K^+-ATP ase receptor.
- The docking analysis of the designed compounds with H^+/K^+-ATPase receptor was studied using the Glide software version 10.1, Schrodinger, LLC, New York, NY, 2015.
- Based on dock score and interaction, 4 of the best compounds were selected and synthesized.
- The structures of the synthesized compounds were characterized by UV, IR, NMR and Mass spectral data. The data's of the analytical spectra's were correlating with the structures of the synthesized compounds.
- The melting point and R_f of the synthesized compounds were checked to determine the physical properties and purity of compounds.
- In vitro antioxidant studies such as DPPH and ABTS radical scavenging activity of the synthesized compounds was carried out. It was revealed that the compounds have a slightly lower free radical scavenging activity than that of standard (ascorbic acid).
- *In vitro* cell viability assay was carried out using AGS (human epithelial gastric cells) inorder to confirm the capability of synthesized compounds to sensitize the target cell mainly the gastric cell.
- The antiulcer activity of the compound P-28 was carried out using two models Indomethacin induced and Pylorus ligation induced ulcer .Based on the results, compound P-28 exhibited as antiulcer activity as that of standard pantoprazole. These action may be possibly due to the inhibition of H^+/K^+-ATP ase enzyme responsible for the gastric acid secretion.
- So further studies can be carried out to test the potency of the compound and can be used as a lead for the development of newer antiulcer agents.

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