EVALUATION OF PYRIMETHAMINE AS APOPTOSIS INDUCING AGENT ON CHEMICALLY INDUCED GASTRIC CANCER

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BY

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CERTIFICATE

This is to certify that the dissertation work entitled "EVALUATION OF PYRIMETHAMINE AS APOPTOSIS INDUCING AGENT ON CHEMICALLY INDUCED GASTRIC CANCER" submitted by Ms. TRISHA MANISHKUMAR BHATT with Regn. No. (17MPH211) in partial fulfillment for the award of Master of Pharmacy in "Department of Pharmacology" is a bonafide research work carried out by the candidate at the Department of Pharmacology, Institute of Pharmacy, Nirma University under the guidance of Dr. JIGNA SHAH, Professor, Department of Pharmacology, Institute of Pharmacy, Nirma University. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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CERTIFICATE OF ORIGINALITY OF WORK

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I hereby declare that the dissertation entitled "EVALUATION OF PYRIMETHAMINE AS APOPTOSIS INDUCING AGENT ON CHEMICALLY INDUCED GASTRIC CANCER" is based on the original work carried out by me under the guidance of Dr. JIGNA SHAH, Professor, Department of Pharmacology, Institute of Pharmacy, Nirma University. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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Dedicated to my father Late Shri ManishKumar Bhatt

If it is destined for you, no matter what you will achieve it.

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List of content

CHAPTER-1

ABSTRACT

Evaluation of Pyrimethamine as Apoptosis inducing agent on chemically induced Gastric cancer

1.ABSTRACT

Background:

Gastric cancer is one of the leading death modalities amongst all type of cancers. The morbidity and mortality associated with gastric carcinogenesis is ranked second highest amongst all malignant neoplasms. Anti-malarial agents are being evaluated for their potential role in various cancers which are proved to have promising therapeutic effects. Pyrimethamine, belonging to the anti-folate and anti-protozoal class of agent has been suggested to exert anti-tumour effects by inducing apoptotic cancer cell death in gastric cancer via acting on several pathway and mechanisms.

Materials and Methods:

In-silico docking studies: The molecular docking was performed using GOLD suite software to check the activity of various class of anti-malarial agents and the standard chemotherapeutic agent on target protein caspase-3, which is an effector caspase to induce apoptosis.

In-vitro study*:* The *in-vitro* study was carried out on AGS cell lines. The cell viability assay (MTT assay) was performed to evaluate the IC_{50} of pyrimethamine.

In-vivo: Gastric cancer was induced chemically by 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) in Wistar rats. MNNG was given in a dose of 100mg/kg for 21 days (3 weeks) daily by oral route. The treatment with pyrimethamine was initiated from 4^{th} week by administering different doses of pyrimethamine alone and in combination with doxorubicin (standard drug). Pyrimethamine was given orally for 30 days (4 weeks) daily orally and doxorubicin (1mg/kg) twice a week for 2 weeks via i.p route. At the end of 8 weeks, the animals were sacrificed and the stomach tissues were isolated from different groups for the evaluation of various parameters. Physical parameters (body weight, food and water intake), tumour parameters (tumour volume, tumour burden and % tumour

incidence), oxidative stress parameters (MDA, GSH and SOD), oncogenic marker (CEA), inflammatory markers (IL-6, TNF- α, IFN- γ, IL-1 β, NF- ϰB), histopathological studies, immunohistochemistry (BCL-2) and mRNA expression of caspase-3 was carried out via RT-PCR.

Results:

***In-silico* studies**

The docking results depicted the binding of pyrimethamine on caspase-3 with gold score of 42.43 which was found to be lower than chloroquine, sulfadoxine and quinine but the important and reported amino-acids which are necessary to be present to inhibit the caspase-3 were present in pyrimethamine as well as in doxorubicin.

***In-vitro* studies**

The MTT assay results depicted that the IC_{50} of pyrimethamine and doxorubicin was found to be 91.1µM and 4.5 µM respectively after 24 hours.

***In-vivo* studies**

Tumourogenic parameters and biomarker study

In-vivo studies depicted 100% tumour incidence of gastric cancer in disease control animal group while it was reduced by 100% in animals treated with pyrimethamine and combination with doxorubicin groups. There was significant reduction ($p<0.001$) in tumour volume, tumour burden and % tumour incidence in animals treated with pyrimethamine and combination compared to disease control group. Tumour biomarker carcinoembryonic antigen (CEA) were significantly elevated ($p<0.001$) in disease control group to that of normal control animals. Pyrimethamine and combination treated group of animals showed significant ($p<0.001$) decrease in the CEA levels. Histopathological studies revealed highly differentiated squamous cell carcinoma with dysplastic and hyperplastic changes in the mucosa, submucosa and serosa of the squamous epithelium in disease control animals. Treatment with pyrimethamine and combination significantly reduced the dysplastic changes, tumour invasion in squamous epithelium.

Oxidative stress parameters studies

There was significant ($p<0.001$) increase in the disease control group which showed higher levels of MDA, reduced levels of SOD and GSH in comparison to the normal control groups. Treatment with pyrimethamine and combination showed significant ($p<0.001$) decrease in the MDA levels and increase in SOD and GSH levels.

Inflammatory markers evaluation

The disease control group showed significant ($p<0.001$) increase in the inflammatory levels as compared to the normal control group. Animals treated with pyrimethamine and combination depicted significant ($p<0.001$) reduction in levels of inflammatory markers.

Apoptotic marker

In-vivo apoptosis was evaluated by immune-histochemical staining of BCL-2 expression which showed reduced expression in disease control animal group to that of in normal control. There was increase in BCL-2 expression in animal groups treated with pyrimethamine and combination.

mRNA expression

mRNA expression of TP53 was checked through RT-PCR. The results showed that disease control group has reduced expression of TP53 in comparison to normal group. The animal treated with pyrimethamine and combination showed increased expression of TP53.

Conclusion:

In-vitro and *in-vivo* studies of pyrimethamine in gastric cancer showed potential anti-cancer effects of pyrimethamine. However, no significant effects were obtained on the minimum dose of pyrimethamine as well as when given in combination with standard (50mg/kg pyrimethamine and 50mg/kg in combination with 1mg/kg doxorubicin). Highly significant effects were only obtained at the highest dose of pyrimethamine and combination (75mg/kg pyrimethamine and in combination with 1mg/kg doxorubicin). From these studies, we can say that pyrimethamine might work through inducing apoptosis and inhibition of cancer cell proliferation as well a effect in gastric cancer.

CHAPTER-2

INTRODUCTION

2. INTRODUCTION

Gastric cancer is one of the leading death modalities amongst all type of cancers. The deaths occurring due to gastric cancer are specifically focused in the countries including China, East Asia and Japan. Carcinogenesis of gastric cancer is a sedate process which comprises of complex, multifactorial and multivariate pathological changes. Pathologically gastric cancer is divided into adenocarcinoma, intestinal metaplasia, atrophic and chronic superficial gastritis. Moreover, stomach cancer also includes several etiological factors like excessive ingestion of nitrate and salt, infection with *Helicobacter pylori (H. pylori)*. The deadliest part of the disease is it remains deceptive and asymptomatic at an early stage (Chen, et al., 2018).

According to Global Cancer Statistics 2018 (GLOBOCON) Gastric Carcinoma (GC) is the fourth most common malignancy worldwide (989,600 new cases per year in 2008) and remains the second cause of death (738,000 deaths annually) of all malignancies worldwide. (GLOBOCON website accessed on 08/03/19). Many Patients are diagnosed with gastric cancer in the late stage which is specifically due to insufficient standardized screening system. There is a balanced decrease worldwide in the incidence of gastric cancer, but on a contrary note India will witness an increase in the years of 2015-2020 in the incidence of gastric cancer. The numbers illustrate a marked increase from 34,000 to 50,000 new cases annually in India in the year of 2020. Regional variation is observed in the occurrence of gastric cancer in India which can be stated from the data available from National Cancer Registries (NCR), population based tumor registries and hospital based cancer registries but the major disadvantage of these registries is only 7% of Indian population is covered by these various registries. The highest incidence of gastric cancer in India is reported in the district of Aizwal which is in the state of Mizoram as per NCRP (64.2/100,000 population) which is followed by other southern states where Tamil Nadu where the incidence rate is 12.2/100,000 population in men and the AAR (age adjusted rate of women in the state of Bangalore is the highest incidence of AAR 5.5 (Indian Council of Medical Research). Gujarat has the lowest incidence rate of 1.1/100,000 population in men and up to 0.5/100,000 population in women (Murugesan, Servarayan, et al., 2018). The later stage of the disease leads to the poor prognosis and the average 5-year survival rate after the surgical resection is only 20-30% in contrast to the patients

diagnosed at early stage of gastric cancer is 90%. Thus, it is necessary to manifest more desirable method for gastric carcinogenesis in routine (Chen, et al., 2018).

Survival rates depicts the average population suffering from the same type of the cancer as well as stage still alive for a time period which is usually 5 years after the cancer has been diagnosed. Average survival rate doesn't illustrate the increased life spam of the patients rather it provides a clarity regarding how successful the treatment will be. According to the statistics given by American Cancer Society there will be 27,510 new cases of gastric cancer in the year 2019 in United states where as 11,140 people will die because of gastric cancer. The ratio of deaths in women to men will be 6800 and 4340. The average of being diagnosed with gastric cancer is 68 years which states that it is affected in the latter stage of the life. Nearly 6 out of 10 people are diagnosed by gastric cancer after the age of 65 years. The risk of developing gastric cancer in men are higher than women as 1 out of 95 men are affected by GC whereas 1 out of 154 women are diagnosed (American Cancer society website).

Surgical resection of the gastric tumor is the primary option for the treatment and cure of gastric cancer. The surgical resection is one of the effective modality in the complete remedy of gastric carcinogenesis (Swan, & Miner., 2006). Gastric cancer is referred as locoregional disease where initial objective is to excise the primary tumour (Weledji, 2017).

Radiotherapy is a palliative treatment which cannot ameliorate the survival but can control 70% rates of locoregional tumours. Thus, it is used for uncontrolled gastric bleeding and tumours which are unresctable. (Henning, et al., 2000). Radiotherapy as an adjuvant therapy in gastric cancer is assessed in many stage II and stage III trials of gastric cancer. Preoperative radiotherapy can be considered as good treatment option as it won't delay the postoperative recovery as well as target area of the tumour is easy to identify. The disadvantage of radiotherapy is no pathological staging is available (Hartgrink, et al., 2009).

Chemoradioatherapy is also one of the other treatment modality where significantly improved survival rates are observed after surgery (Fiorica, et al., 2007). Chemoradioatherapy refers to chemo plus radiotherapy which is proved to be a beneficial approach of treatment since 1980s. Fluorouracil combined with radiotherapy has proved

to be effective modality as it improved local control and survival (Moertel, et al., 1982; Schein and Gastrointestinal Tumor Study Group,1982; Klaassen, et al., 1985; Gastrointestinal Tumor Study Group,1990). However, there are evidences of renal toxicity after treatment with chemoradioatherapy (Jansen, et al., 2007).

Chemotherapy and perioperative adjuvant chemotherapy is considered as multimodality treatment regimen after surgical resection to improve the survival of gastric cancer (Orditura, et al., 2014; Proserpio, et al., 2014). Since past three decades, chemotherapy is used as one of the effective treatment regimen and according to NCCN 5-Fu, cisplatin, and epirubicin are first line therapeutics for the treatment of gastric cancer (Ajani, et al., 2013). Despite of that, novel chemotherapeutic agents like oral fluoropyrimidines (capecitabine and S-1) and taxanes (paclitaxel and docetaxel) as well as irinotecan and oxaliplatin are emerging in the recent years (Yuan, et al., 2014).

Due to the advancement in the medicines as well as novel technologies there are new targets which are emerging as new treatment types.

Currently available molecular targets for the treatment of advance gastric cancer includes an anti-vascular endothelial growth factor receptor 2 monoclonal antibody ramucirumab and trastuzumab, an anti-human epidermal growth factor 2 (HER2) are the only two molecular targeted agents which have been buoyantly developed and available in the market (Takahari, 2017).

Due to the prolong use of the above mentioned chemotherapeutic agents there is development of resistance which leads to frequent relapses as well as several side effects. Thus, newer drug and agents are required which acts as novel targets. Recently antimalarial are being investigated for their potent anti-cancer effects acting on several different pathways. This is being evaluated in the current investigation for their potent anti-cancer effect.

Anti-malarial are class of agents which are being evaluated for their efficacy and potency as anti-cancer agents. Since past two decades, various studies have been performed and recognised of antimalarial having antitumor actions. The molecular analysis of the these drugs indicates that anti-malarial acts through various pathway including apoptosis

(intrinsic pathway which involves Bcl-2, Bcl-XL, Bak/Bax, release of cytochrome c which leads to the activation of caspase-9 mediating the apoptosis (Van et al., 2013).

Pyrimethamine (2,4-diamino-5-p-chlorophenyl-6-ethyl-pyrimidine) belonging to the group of anti-folate drugs which works by blocking of dihydrofolate reductase enzyme. This enzymatic reaction is necessary for the synthesis of folic acid; a cofactor necessitate for DNA synthesis. Pyrimethamine is used in the treatment of infections caused by protozoan parasites like Plasmodium falciparum and Toxoplasma gondii (Katlama, et al., 2002). In addition to the potent antiprotozoal activity, pyrimethamine may have a potential immunomodulating action including the induction of peripheral blood lymphocyte apoptosis (Bygbjerg, 1985; Viora, et al., 1996; van der Werff Ten Bosch, et al, 2002). The upstream caspases, this drug induces apoptosis via activation of lymphocytes (Pierdominici, et al., 2005). Though there are evidences of pyrimethamine acting on both the intrinsic and extrinsic pathway. It may be driven by caspase-8 cascade which acts on mitochondria; leading to the membrane depolarization mitochondrial driven by caspase cascade (Kroemer and Susin, 1997). Recently, the efficacy of pyrimethamine was checked on ovarian cancer, which proved to be very potent as it exhibited anti-cancer effects via inducing cellular apoptosis and inhibition of growth of intratumoral micro vessels.

However, there are no evidences of any studies carried out on gastric cancer, thus the objective of this study:

1) To investigate the pharmacological effect of pyrimethamine in N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) induced gastric cancer.
2) To study the possible mechanism of action of pyrimethamine as anti-cancer drug in gastric cancer.

CHAPTER-3

REVIEW

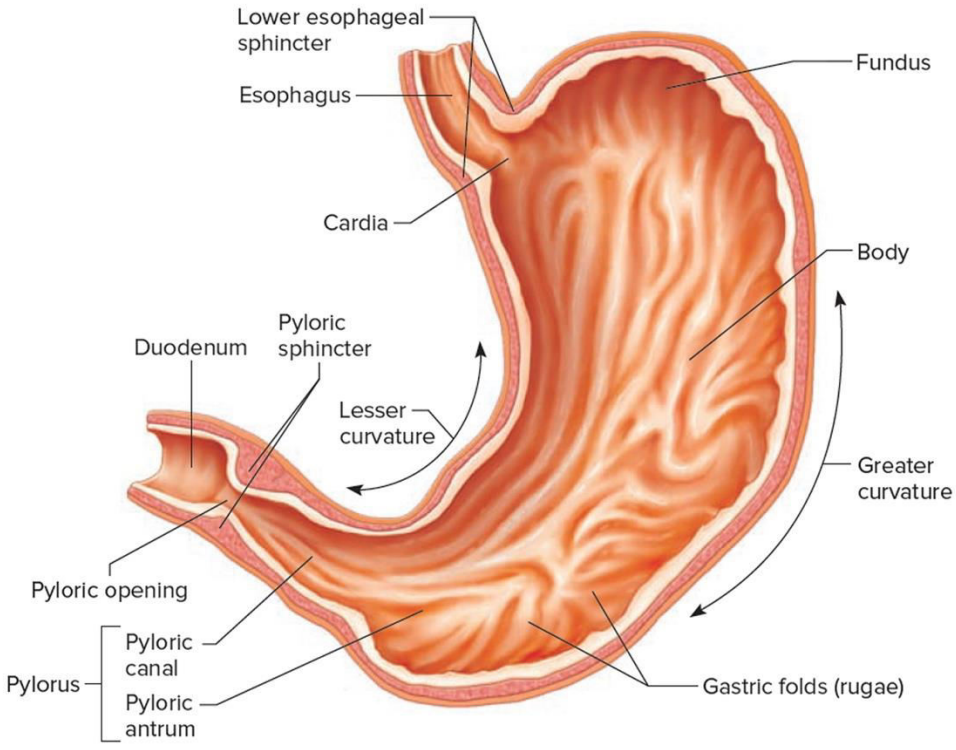
OF

LITERATURE

3. REVIEW OF LITERATURE

3.1 ANATOMY AND PHYSIOLOGY OF STOMACH

Stomach is a hollow shaped muscular organ located in between the oesophagus and small intestine. Primarily, the organ is connected to esophagus which is attached to the cardia portion where the food enters in the stomach followed by fundus which is having a shape of dome, distended as well as superior portion of the organ. Body portion is followed by fundus and it is the largest portion of the stomach. Pylorus, which is joined to the duodenum and empties the food into it or the upper portion of the small intestine. Stomach acts as a temporary storage of the food where the digestion begins, it initiates chemical and mechanical digestion of the food. Stomach is divided into four main parts.



Cardia, body and fundus are divided as the upper portion of the stomach which relaxes as soon as the food enters and the holding capacity is also increased as the quantity of the food is elevated. Rhythmic contraction is observed in the lower part of the stomach where mechanical digestion takes place which mixes the food with juices secreted. This digestion is referred as chemical digestion. Here, the food is broken down in the smaller molecular forms. The food is mixed with the chemical and mixed food is called as

"chyme". The mixing waves are initiated within 20 seconds after the digestion and as the intensity of the waves are increased, it reaches to the lower portion of the organ. Pyloric sphincter allows the passing of the liquefied chyme to pass to the small intestine with each wave. The partially churned food is passed to the duodenum with regulated quantities. For the chemical digestion stomach secretes natural juices from the fundus part including secretion of hydrochloric acid and pepsin from the enzyme. In the secretion of the intrinsic factor, parietal cell helps the digestion in absorption of vitamin B_{12} which is then passed to small intestine. Cobalamin or vitamin B12 has a crucial role in the synthesis of red blood cells as well as plays an important role in neurological functions. The average time in processing the food to the small intestine from stomach is 2-4 hours, which also depends upon the type of the food consumed by stomach fats such as triglycerides consumes highest time for digestion, proteins less and carbohydrates the least time to get digested and it not capable for absorbing the nutrients. The substances which can be absorbed in the stomach includes water in response to dehydration, water soluble vitamins, medicine like aspirin, caffeine and few amino acids. Stomach protects the body from various bacteria entering in the body by having the acidic environment where bacteria can't sustain and thus protect it from various infections. (Chaudhry and Bhimji, 2018)

3.2 EPIDEMIOLOGY AND ITS TREND

Gastric cancer is one the leading death modalities worldwide. The incidences of stomach cancer have been decreased in the developed countries despite of that, it ranks fifth most common cancer and third most common cause of death worldwide (Riquelme, et al., 2015). There is marked geographic variation with nearly two thirds of all cases occur in developing countries in Eastern Europe, South America, and Asia, with 42% of cases in China alone. Gastric cancer is one of the most common cancer types, commonly diagnosed in advanced stages where curative treatment is ineffective.

According to Global Cancer Statistics 2018 (GLOBOCON), Gastric carcinoma (GC) is the fourth most common malignancy worldwide (989,600 new cases per year in 2008) and remains the second cause of death (738,000 deaths annually) of all malignancies worldwide. (GLOBOCON, 2018). There is tenfold variation in the incidence of Gastric cancer worldwide. The incidence of gastric cancer is recorded highest in the countries of

south east Asia including south Korea, Japan and China. So far, India has a low incidence of gastric cancer than other countries like Australia, USA, Africa and Philippines. In India regional variation is observed in the occurrence of gastric cancer with India which can be stated from the data available from National Cancer Registries (NCR), population based tumor registries and hospital based cancer registries but the major disadvantage of these registries is only 7% of Indian population is covered by these various registries. The highest incidence of gastric cancer in India is reported in the district of Aizwal which is in the state of Mizoram as per NCRP (64.2/100,000 population) which is followed by other southern states where Tamil Nadu where the incidence rate is 12.2/100,000 population in men and the AAR (age adjusted rate for women in the state of Bangalore is the highest incidence of AAR 5.5 (Indian Council of Medical Research). Gujarat has the lowest incidence rate of 1.1/100,000 population in men and up to 0.5/100,000 population in women. There is significant decrease in the incidence of gastric cancer worldwide, but on a contrary note, India will note an increase in the years of 2015-2020 in the incidence of gastric cancer. The numbers illustrate a marked increase from 34,000 to 50,000 new cases annually in India in the year of 2020 due to the poor sanitation, eating patterns as well as other factors including genetic factors (Murugesan, et al., 2018).

3.3AGE INCIDENCE AND AGE ADJUSTED RATE (AAR)

There is an excruciating escalation in the incidence of gastric cancer worldwide after the age of 50 years and in the age groups pf late 60's and 80's. These criteria were decided on the basis of 687 patients' data availed from International Gastric Cancer Congress 2015, where 102 (14.4%) patients were under the age of 40 years, 585 (85.1%) were above 70 years of age. In younger patients the male-female ratio was 1.5:1 which magnified to 3:1 above 40 years and in the patients of age 70 years it was found to be 5.6:1 which signified that in the advancement of the age, there is remarked increase in the male predisposition to develop the disease. Cases of distal gastric cancer were found to be equal in both sexes whereas incidence was 70.58% in the age group of 40 years and 66.03% in the patients of above 70 years.

There is a huge regional variation in the age-adjusted incidence and mortality in the rates of gastric cancer as well as the relative proportion in the population based cancer

registries in India. The reports from the Urban registries in India depict the AAR of gastric cancer incidence to deviate from 3.0-13.2, while the scenario form the world reports stated of 4.1-95.6. The highest AAR is observed in the state of Mizoram from years of 2010-2015 with increasing in the number of incidence of gastric cancer. (Murugesan, et al., 2018)

3.4 AGE, SEX AND SITE DISTRIBUTION

The peak incidence in the rate of stomach cancers is observed at the age of between 60-80 years. There are rare cases of gastric cancer in the patients of age less than 30 years. The average aging range for cancer is 35-55 years in India in the region of south India and 45-55 in the region of North part of India. In gastric cancer male predominance is observed across the globe having two to four times higher rates in than in females where the development of the cancer can be both either the proximal or the distal region. The ascendancy of the distal gastric cancer in developing countries is among the black and people of lower socio-economic groups. Whereas, proximal tumors are more customary in the developed countries amongst the white and in the people of higher socio-economic classes. One of the paramount cause of distal tumors is dietary habit and infection with *Helicobacter pylori* bacteria (*H. pylori*) which are also very common in Japan in comparison to proximal tumors which is a matter of worry across the rest of the world. (Nagini, 2012)

3.5 GEOGRAPHIC DISTRIBUTION

Due to the changes in patterns like food habits, dietary pattern, reduced *H. pylori* infection and storage of food, there is a perpetual decline in the incidence as well as mortality of gastric cancer. There is a marked geographic variation observed in the incidence of the gastric cancer where the highest incidence of the disease is perceived in the Eastern Asia and Europe, North and South America. The highest rates of esophageal/gastric cardia cancer are recorded in Linxian of China across the world. The rates of Gastric cancer is higher in India in the north-eastern and southern states. The statistics of the year 2010 stated by national representative survey depicted that there were 5,56,400 deaths due to cancer in India where gastric cancer has the mortality rate of 12.6% and was found to be the second most common fatal cancer (Nagini, 2012).

Amongst different ethnic people living in the similar regions, consequential variations are noted; Americans-Africans, native Americans are likely to be influenced more than Caucasians in the States. However, to classify gastric cancer on the basis of geographical variation, gastric cancer cannot be classified solely on the racial differences. Taking an example of native countries Chinese and Japanese, staying in Singapore have higher rates than their counterparts in Hawaii. Migration also reduces the incidences of gastric cancer. People who migrated from an area of high incidence to low incidence the rates were significantly reduced (Nagini, 2012).

3.6 RISK FACTORS

Multifactorial etiologies are responsible for the incidence of gastric cancer which is genetically heterogeneous and related to environmental and genetic factors. The strong factors which are contributing to the development of gastric cancer are H. pylori infection, inherited genetic factors, lifestyle factors, dietary factors (Cheng, Lin, & Tu, 2016). Risk factors also subdivided amongst the countries having high incidence of the distal and proximal gastric tumours. Previous gastric surgeries, gastric adenocarcinomas and gastritis are most of the common causes of gastric cancer across the globe. (Murugesan, et al., 2018)

3.6.1 HEREDITARY AND GENETIC FACTORS

Hereditary of gastric cancer is very rare, alternation in the genes in sporadic gastric cancer had been reported very frequently. Genetically predisposition can be considered if out of any two members in the family or relatives, any one which had tumor incidence under the age of 50 years. Genetic factors tend to play a vital role in affecting gastric carcinogenesis by affecting immune and inflammatory responses specifically to the *H. pylori* infection. Prolonged *H. pylori* infection elevates the vulnerability to stomach cancer whereas, few high pervasive genes has been distinguished which are responsible for the development of cancer development (Cheng, et al., 2016).

Interleukin 1Beta gene (IL-1B) has been identified as a crucial gene which contributes to the initiation and amplification of inflammatory response. Polymorphism of IL-1B and antagonist of interleukin 1 receptor (IL-1RN) are closely related with the risk of gastric cancer. Mucin-1 gene is also associated with reproducible relationship in single

nucleotide polymorphisms, cell surface associated gene (MUC1) gene, PLCE1 and prostate stem cell antigen gene (PSCA) are also related to the risk of gastric cancer. These above observed genetic variation are studied mainly from Korean, Japanese and Chinese populations (Cheng, et al., 2016).

3.6.2 *HELICOBACTER PYLORI* INFECTION

World health Organization has characterized *H. pylori*, a gram negative bacterium as class 1 carcinogen for gastric cancer in the year 1994. Gastric mucosa is colonized by *H. pylori* in 50% of total human population and several epidemiological studies reflects that infection with this negative bacterium leads to the development of gastric cancer. Two main mechanisms depict the oncogenic effects of *H. pylori* infection 1) inflammatory response to the *H. pylori* infection on gastric mucosa and 2) epigenetic changes on the epithelial cells on gastric mucosa. CagA- cytotoxin associated gene is linked pathologically with the colonization of H. pylori. CagA acts as an inhibitor of p53 function similar to human papillomavirus and other DNA (oncogenic) viruses (Peek et al., 2006). A bacterial toxin VacA is secreted by vacA gene which implicates multiple functional and structural alterations in the epithelial cells. *H. pylori* infection with vacA/cagA mutations are correlated with high risks of gastric cancer (Buti, et al., 2011).

In India, the predominance of *H. pylori* infection is considered as major risk factor as the *H. pylori* positivity is between 80-90% and also reported to expand in 0.1-3% of the patients. Endoscopic biopsy of 141 patients with gastric cancer showed presence of *H. pylori* in adjacent gastric glands and carcinomatous area in 39.7% patients. GE junction tumors showed positive effects in 20 patients (33.3%) out of total 60 patients. Several studies also showed that there is no co-relation between *H. pylori* infection and gastric cancer, 1314 patients from Kashmir showed no association between *H. pylori* infection and gastric cancer. Various studies from North India and Mizoram also failed to shows any significant association between gastric cancer and H. pylori infection (Murugesan, et al., 2018).

3.6.3 RELATION BETWEEN LIFESTYLE AND DIETARY HABITS ASSOCIATED WITH GASTRIC CANCER IN INDIA

Dietary patterns and lifestyle varies in India according to the difference in the states. The dietary habit of every state is different which play a vital role in the prognosis and development of gastric cancer. The major factors associated with gastric cancer are high intake of salt in various food items, salted, smoked and preserved food. Increased consumption of rice and chilli and food cooked at high temperatures are one of the main reasons for the development of gastric cancer in Kerala. A study was carried out by International Gastric Cancer Congress in 2011 across India to identify the possible reasons of gastric cancer. Dietary pattern and lifestyle stated that 84% of cases were due to use of ethanol, 98% were non-vegetarians, 83% of tobacco users and the least of 37% was observed in the people chewing tobacco. Consumption of pulses tends to have a protective effect on incidence of gastric cancer as it reduced to 55%. In India, tobacco is consumed in various forms including smoking hukka, beedi and cigarettes (Murugesan, et al., 2018).

3.6.4 OCCUPATION AND INCIDENCE OF GASTRIC CANCER

There is a positive correlation between incidence of gastric cancer and occupations including farming, mining, fishing, refining as well as workers associated with processing of rubber, asbestos and timber. A significant increase in the rates of gastric cancer is observed in the people exposed to occupational works like high temperature and dusty environment, wood processing plant operators and areas with elevated temperatures (Nagini, 2012).

3.6.5 GENETIC POLYMORPHISMS AND EPIGENETIC CHANGES

Genetic polymorphism plays an important role in the development of gastric cancer as well as other gastric problems including gastric ulcers and chronic gastritis. A case control study was carried out in South India correlating genetic polymorphisms with lifestyle, diet and socio-economic factors in the population. Genetic polymorphism was assayed based upon selection of 5 single nucleotides. The SNP's selected or the

assessment of the polymorphisms are -TGFβ C-509T, TGFβ T869C, XRCC1 Arg194Trp, IkBα C642T and IL4C-590T (Pavithra, et al., 2018).

3.7 CLASSIFICATION OF GASTRIC CANCER

Various methods are used for the classification of Gastric cancer like the major classification is done as 1) Sporadic Gastric cancer 2) Early onset gastric cancer 3) Gastric stump cancer 4) Hereditary diffuse gastric cancer (Sitarz, et al., 2018).

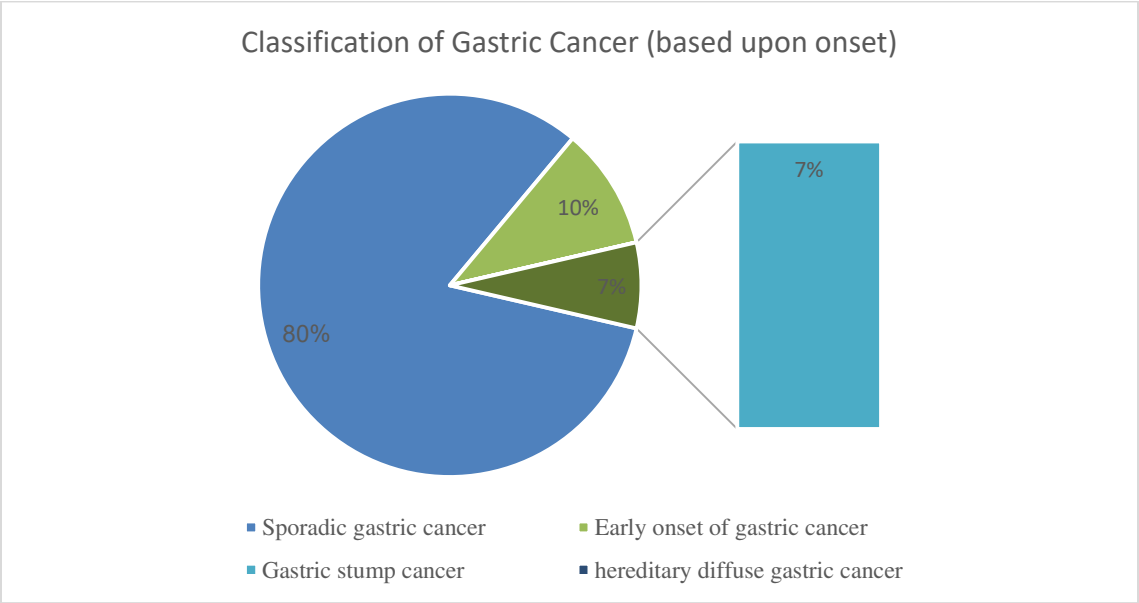


FIG 1.

Table 1: Classification of Gastric cancer

Type of cancer	Prevalent Age	Causative Factor	Prevalent Sex
1) Sporadic Gastric Cancer (SGC)	45 years or in the range of 60-80 years	Coincidence of environmental factors	Males are affected twice than females.
2) Early Onset of Gastric Cancer (EOGC)	Before the age of 45 years	Genetic factors causing diffuse and multifocal type of carcinoma	Females are more affected.
3) Gastric Stump Cancer (GSC)	Can Occur at any age	Relapse after 5 years of surgery of peptic ulcer as well as gastrectomy. Epstein Bar virus infection increases the risk 4-7 fold after 15 years of	Males are more prone than female

		gastrectomy surgery.	
4) Hereditary Diffuse Gastric Cancer (HDGC)	Can Occur at any age	Inherited syndromes and mutated CDH1 gene which codes for E-cadherin	Prevalence of sex is not available.

3.7.1 PATHOLOGICAL CLASSIFICATION

Several pathological classification of gastric cancer are available. According to World Health Organization (WHO) has classified Gastric cancer as gastric adenocarcinoma, signet ring-cell carcinoma and undifferentiated carcinoma. However, this is not widely used, there are other several classifications, discussed as below.

Lauren classification, which focuses on the two major types of Gastric cancer 1) Diffuse type 2) Intestinal type which is based on macroscopic and microscopic difference. Diffuse type of gastric tumors arises from normal gastric mucosa whereas, chronic atrophic gastritis and intestinal metaplasia is related with the development of intestinal gastric cancer. Both of them have different prevalence across the countries and continents. Intestinal type is more common in European countries; diffuse type is more prevalent in younger patients. To classify the extent of surgical resection it is categorized on the Lauren's histological subtype of gastric cancers (Sitarz, Robert, et al., 2018). There is other classification apart from WHO and Lauren which is Goseki and Ming classification.

3.7.2 LAUREN CLASSIFICATION OF GASTRIC CANCER

Amongst all the classification types, Lauren classification (established in 1965) of gastric cancer has been used and studied as the most common classification for gastric adenocarcinoma. Gastric cancer is divided into two types 1) diffuse and 2) intestinal type. The other classification of indeterminate type was added for the uncommon histological classification. Signet cell carcinoma is comprehended under diffuse type of carcinoma. The most common type of carcinoma is intestinal trailed by diffuse and rarest are the indeterminate type. The prevalence and co-relation with the other factors are also important in the pathogenesis of the gastric cancer for e.g. intestinal type is equated with intestinal metaplasia of gastric mucosa and presence of *H. pylori* and many studies demonstrated that the incidence of the diffuse type of gastric cancer is more common in

younger female patients which finally leads to the development of diffuse adenocarcinoma or distinct intestinal tumour development. There are controversies for the use of Lauren classification in prognostic relevance because Lauren's pathohistological subtypes are not related to the patients' outcome thus few of the investigator stated that Lauren classification is better to use for independent prognostic factor. Here in few of the cases diffuse type of adenocarcinoma was correlated with the worse outcome and because of the correlation was not verified with the other patient cohorts, significance of the Lauren's classification is not considered generally (Berlth, Felix, et al., 2014).

3.7.3 WORLD HEALTH ORGANIZATION CLASSIFICATION

World health classification of gastric cancer was issued in the year 2010 and it is considered as one of the most detailed pathohistological classification system amongst all other. The reason for widely acceptance of WHO classification as the detailed one because it not only includes the adenocarcinoma of the stomach type but also other types of stomach tumours with lower frequency. The gastric adenocarcinoma is classified into several subgroups comprising of tubular, mucinous, papillary and mixed type of carcinoma which can be contrasted to the indeterminate type of Lauren gastric cancer classification. The most common type of gastric cancer in WHO classification is tubular adenocarcinoma followed by papillary and mucinous. The prognosis of the signet cell carcinoma is controversial because only 10% of the gastric cancers accounts of signet cell carcinoma. Papillary adenocarcinoma has a poor prognosis as it has a tendency for the metastasis and elder age at the diagnosis located in the upper third part of the stomach. Thus, apart from the most common types of gastric malignancies, WHO classification is more widely accepted for the study of gastric cancer. Japanese classification system has an identical system of classification similar to given by WHO. There are differences given by Japanese classification of adenocarcinoma which includes subtypes i.e. tubular adenocarcinoma is sub-classified in to well-differentiated and moderately differentiated adenocarcinoma which is based in difference in lymph node metastasis, submucosal invasion rate and size of the lesions (Berlth, Felix, et al., 2014).

3.7.4 GOSEKI AND MING CLASSIFICATION

Goseki classification was introduced by Goseki and group of researchers which described the histopathological classification in 1992. Gastric cancer was divided into four groups based upon the patterns of metastasis, interrelation of subtypes and local growth. There is not much difference between the Goseki classification to that of Lauren's and WHO classification. An extortionate difference is observed with respect to the production of mucus highly interdependent on prognostic significance though it was later subsequently debated for independent prognostic significance. The Ming classification of gastric cancer is established on the lesions with their pattern of growth. The growth pattern of lesion was categorized into two main types i.e. infiltrating growth pattern which is found to be less frequent and expanding growth pattern. The Ming classification is an interlink between specific characteristics which arises from intestinal metaplasia for the expanding growth pattern in contrary to that infiltrating begins from individual cells. Thus, Ming classification is simple for clinical use as well as can be used as a correlation between the pre-existing classification types but not prominent for the independent prognosis (Berlth, et al., 2014).

3.8 PATHOPHYSIOLOGY

Any neoplasm extending from the region of gastro-esophageal junction and pylorus can be referred as gastric cancer. Adenocarcinoma is the most common epithelial type of tumors of gastric cancer comprising of 95%. However, adenosquamous, squamous and undifferentiated are the most uncommon and rare type of tumors. The poorly differentiated diffuse-type is characterized by thickening of the stomach wall formed without discrete mass and infiltration. The well differentiated intestinal type of tumors appears to have neoplastic cells which forms gland-like tubular structures. The intestinal type of tumors are more common in older people living in high-risk region, it is also more commonly occurring type of tumor in men. Environmental factors including obesity, dietary habits and *H. pylori* infection leads to the development of precancerous lesion forming intestinal metaplasia and gastric atrophy. In endemic areas, diffuse type of lesions are major and more common in women and younger patients. It is associated with specific blood group A which describes genetic susceptibility. Following a cascade of precancerous lesions, the invasive gastric carcinoma develops via stepwise changes in the

histology of gastric mucosa leading to the development of atrophic gastritis. This is characterized with the loss of parietal cell mass, dysplasia and intestinal metaplasia which leads to the development of carcinoma. These changes are more common in intestinal type of gastric cancer which is similar to colorectal type of cancer. (Nagini, 2012)

The genetic factors play a vital role in the carcinogenesis of the gastric cancer which is due to the anomalous expression of the gene which leads to malignant phenotype. In gastric cancer, activation of β- catenin (17–27% is found in intestinal type) and K-ras (0-18% activation is found, leading to oncogenic activation. Whereas, in 10% cases, amplification of c-met genes and c-erbB2, p53 mutations are reported in both intestinal and diffuse type of tumours with 0-21% and 36-43% respectively. In gastric adenomas, the mutations in APC genes are reported commonly rather than carcinoma whereas, somatic mutations of E-cadherin are specifically identified in sporadic diffuse type of gastric cancer (33-50%). In diffuse, type Microsatellite instability is observed in 5-10% total and 15-40% in gastric carcinoma. In human neoplasia epigenetic changes like alterations in the promoter CpG island and hyper methylation. The epigenetic changes (Ling, et al., 2010).

3.9 DIAGNOSIS AND ITS PROBLEMS

Diagnosis of the gastric cancer is done with the help of the traditional techniques which can be described as follows (Takahashi, et al., 2013):

1) Upper Gastrointestinal Endoscopy
2) ^{8}F-Fluorodeoxyglucose (FDG)-Positron Emission Tomography (PET)
3) Staging Laparoscopy (SL)

Gastric cancers can grow and spread to the distant organs depending upon various pathways, where tumors are also spread to the nodes of the lymph which are involved in the mechanism of protection.

The stages of gastric cancer can be classified as follows:

Stage 0

Stage 0 refers to the initial stage where the cancer is just restricted to the inner lining of stomach and not invaded further, which can be treated through surgical methods where radiation and chemotherapy is not needed. The surgery of gastric cancer refers either to subtotal gastrectomy (removal of a part of stomach) or total gastrectomy (removal of the entire stomach). Endoscopic resection can treat the small stage 0 cancer where, an endoscope is passed down the throat, this is only possible when it is identified in the initial/early stage.

Stage I

Stage I is sub-classified into Stage IA and IB:

The *stage IA* commonly refers to the patients which have resected their cancer either by total or subtotal gastrectomy including removal of the nearby lymph nodes.

The *stage IB* cancers may also be resected with the help of surgery only. Chemotherapy and chemoradiation (chemo plus radiation therapy) is helpful to reduce the size of the tumor to make the surgery easy for the removal. After the surgical resection, the further treatment is either chemotherapy alone or in combination with the radiation is recommended. If the cancer has spread to the lymph nodes, treatment with either chemo alone, chemoradiation or both combination is recommended. Depending upon the patient health either of the above treatment regimen is followed.

Stage II

The stage II treatment are more or less relevant to the stage I where the patients have been under the surgical resection and removed part of stomach, the omentum and the nearby nodes of lymph followed by which chemoradiation and chemotherapy alone or in combination is started.

Stage III

The patients suffering from stage III gastric cancer requires only surgical resection depending upon their medical and health condition. Chemotherapy and chemoradiation is decided based upon the surgical prospects for e.g. patients who didn't undergo chemotherapy before surgery may be prescribed for it. Depending upon the tumour growth and surgical outcomes, the patients who didn't get chemotherapy prior to surgery may only require chemoradiation after surgery.

Stage IV

When the cancer has spread to the distant organs it is called as metastasis where the cure is usually not possible. Gastric bypass or subtotal gastrectomy may be helpful to control the bleeding as well as to keep intestines/stomach from being obstructed. Chemotherapy combinations are used according to the health and medical condition of the patient.

The excision of the tumour is done from the circumferential and longitudinal resection margin along with the organ resection and assisted nodes of lymph. To legitimize the nutritional intake, it is necessary to restore the biliary and intestinal continuity safely (Weledji, 2017). Laparoscopy is one of the essential staging modality distinguishing the unresectable disease. Laparoscopic surgery is being used since 1991 and it is one of the oldest method to treat gastric cancer. Mostly laparoscopic techniques are used for early and distal gastric cancer (Shehzad et al., 2007; Shiraishi, et al., 2006). Laparoscopy has emerged as an essential staging modality prior to gastric resection, identifying unresectable disease in a significant number of patients deemed resectable by current radiographic and endoscopic modalities. The diagnostic yield of laparoscopy has been improved by the addition of laparoscopic ultrasound and peritoneal cytology (Swan, & Miner, 2006). Surgical aspect is only opted to resect the primary lesions and to clear distal and proximal margins of adenocarcinoma of the stomach. For significant omentum; D2 lymphadenectomy is suggested in for pertinent staging as well as for better long term survival of the patients (Zilberstein, et al., 2004; Zia, et al., 2010). Post-operative complications are still one of the major problems associated with gastric cancer as the extent of surgery for gastric cancer is heterogeneous rate (Hartgrink et al. 2004; Yasuhiro 2007). After the surgical resection, there are chances of relapses. Predominantly the

relapses occur at the distant site within first 3 years followed by surgery. The chances of relapses are 51% in first year, 79% in second year and 92% in the third year. Moreover, 51% patients were found having elevated tumour markers and 72% were observed to be symptomatic at the time of relapse (Moorcraft, et al., 2016). Certainly there are advances in the surgical methods like robot assisted lymph node dissection and laparoscopic total gastrectomy (LTG) (Degiuli, et al., 2016).

The following classification is based upon the histological features, genotypes and phenotypes

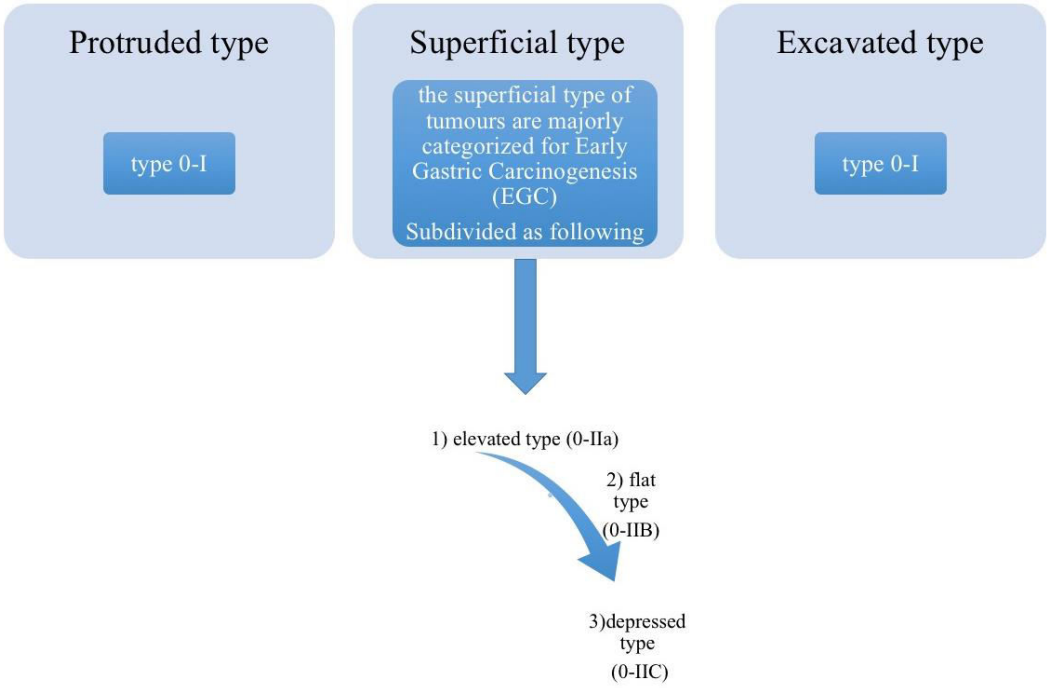


FIG 2.

3.10 TREATMENT APPROACHES

After the detection of gastric cancer, the prognosis and the treatment/recovery depends upon the stage of the disease is reached to. The chance of recovery depends upon two factors: 1) the general health of the patient 2) the cancer stage whether, it is spread to the distinct organ i.e. to the lymph nodes and other organs of the body or it is just limited to the stomach itself (www.cancer.gov).

A. SURGERY

The treatment regimen and options plays a major role in the cure of gastric carcinogenesis. Treatment includes various prospects including surgery, chemotherapy, adjuvant therapy as well as the novel targets and molecular pathway.

There are options available for the adjuvant and neo-adjuvant therapies after the surgical resections. Depending upon the health and condition of the patients the follow up therapies are prescribed. Surgery is highly effective after local resection which tends to be relapsed in the following years. Thus, preoperative and postoperative chemotherapy, radiotherapy or combination of both can be assessed for the treatment.

After the surgical procedure, there are escalated incidences of locoregional failures. So, radiotherapy is considered one of the major modality as a curative treatment for gastric carcinogenesis (Jansen, et al., 2005; Smalley, et al., 2002).

B. CHEMOTHERAPY

Chemotherapy and perioperative adjuvant chemotherapy is considered as multimodality treatment regimen after surgical resection to improve the survival of gastric cancer (Orditura, et al., 2014; Proserpio, et al., 2014).

1. Doxorubicin

It belongs to the class of anthracycline is used as fluorouracil based combinations. Anthracylines are used as second line of chemotherapeutic agent in the treatment of gastric cancer. The FAM (fluorouracil-doxorubicin-mitomycin C) was widely used in the late 1980s (Macdonald, et al., 1980). Doxorubicin exerts antitumor effects by acting on cell cycle which induces autophagy, apoptosis and necrosis. Doxorubicin blocks the enzyme topoisomerase-II which is responsible for cell division and growth. It is also used in combination with other drugs to treat several malignancies. Doxorubicin has poor prognosis when given as monotherapy, only 17% of the patients produced responses whereas when given in combination the % responses increased to 36% (Tacar, et al., 2013). Recent development suggests that in the treatment of advance gastric cancer via Notch-1 targeting siRNA has proved to be potential target for the treatment of advance stage (Zhou, et al., 2018).

There are several mechanisms by which resistance occurs and the therapeutic effects as well as potency of the chemotherapeutic agent is reduced.

2. 5-Flurouracil (5-FU)

It is an analogue of uracil with a fluorine atom substituted at the 5-carbon position of the pyrimidine ring in place of hydrogen. 5-FU has potential anti-cancer activity with specific pharmacologic and biochemical properties. Heidelberger et al. have synthesised 5-fluorinated pyrimidines and it became successful in the treatment of solid tumours including breast, gastric, pancreatic, colorectal and head and neck squamous cell carcinomas (Papanastasopoulos and Stebbing, 2014). 5-FU works by four mechanisms primarily to act as a potential anti-cancer drug. Initially there is incorporation of fluorouridine triphosphate into the RNA which hinders the synthesis of RNA and normal functioning; followed by it, fluorodeoxyuridine monophosphate inhibits thymidylate synthase, which leads to the depletion of thymidine 5′ monophosphate and thymidine 5′ triphosphate and the accumulation of deoxyuridine monophosphate and deoxyuridine triphosphate; DNA replication and stability is affected by incorporation of fluorodeoxyuridine triphosphate and deoxyuridine triphosphate into DNA. Genotoxic stress can activate pathways of programmed cell death (Longley, et al., 2003; Mojardín, et al., 2013). 5-FU resembles the structure of uracil which gets incorporated into structure of DNA and RNA. This is further associated in interference with nucleoside metabolism followed by cell death (Wilson, et al. 2014). Despite of its cytotoxic action and widespread use, it has now been limited to use because of development of resistance in several cancers including breast, colon and gastric cancer which identifies a need to develop new drugs and anti-cancer agents. One of the most important mechanism for resistance development to 5-FU is increased expression of thymidylate synthase (TS) which can also be acquired from 5-FU treatment (Van et al. 1999, Longley et al. 2003). Another enzyme involved in 5-FU catabolism is dihydropyrimidine dehydrogenase (DPD) which is first step involved in 5-FU conversion. Intrinsic over expression of DPD and higher levels of DPD mRNA is also involved in 5-FU resistance (Longley and Johnston 2005, Takebe et al. 2001). Other mechanisms associated with resistance development are variable number of tandem repeats (VNTRs) and single nucleotide polymorphism (SNPs) of MTHFR, DPYD, UPMS and TYMS genes (Panczyk 2014). Increased activity of deoxyuridine triphosphate (Grem 2005), efflux out of tumor cell

components (Longley and Johnston 2005), methylation of MLH1 gene (Arnold et al. 2003), over expression of Mcl-1 protein (Shi et al. 2002), BCL-2 (Violette et al. 2002) and Bcl-XL protein (Liu et al. 1999, Arnold et al. 2003) is also contributing to 5-FU resistance. Thus, new approaches are needed to overcome resistance for future therapy. As like 5-FU, **capecitabine** is also FP analogue and a prodrug which gets converted into 5-FU after administration. It has same mechanism of action for resistance development to that of 5-FU (Vallböhmer et al. 2007).

3. Cisplatin (cis-diammine-dichloro-platinum)

Cisplatin was used first time in 1960s as an anticancer drug (Florea and Büsselberg, 2011). Cisplatin works by displacement reaction which bind to DNA, RNA and other protein molecules stably. DNA damage is induced by cisplatin which leads to successive cellular damages comprising of inhibition of DNA synthesis, suppression of RNA transcription, interference with cell cycle and induces apoptosis. By binding covalently with the DNA to form DNA-protein and DNA-DNA inter and intra-strands crosslinking cisplatin, distorts the function of DNA; it also induces DNA damage by activating cell cycle checkpoints which causes cell cycle arrest. Followed by it damaged DNA causes activation of MAPK and P53 pathway. Every pathway activates significant genes and mechanisms as P53 promotes apoptosis by inhibition of anti-apoptotic Bcl-2 leading to caspase activation (Siddik, 2003; Gumulec, et al., 2014). Multiple platinum derivatives are used clinically as a chemotherapy regimen amongst which, oxiplatin and carboplatin have received approval for clinical use worldwide. However, there are primary or acquired resistance to cisplatin which leads to poor survival and relapse of the cancer.

There are several mechanisms under which the resistance may occurs including epithelial-mesenchymal transition (EMT) and overexpression of HER2 (Huang, et al., 2016) as well as expression of P-glycoprotein, multi-drug resistance associated protein and lung resistance protein (Hu, et al., 2009). Epithelial-mesenchymal transition (EMT) is a process in which epithelial cells lose their polarity and cell-cell adhesion takes place by which the characteristics of mesenchymal cells are acquired. Other processes like attaining spindle-cell shape, intercellular separation, loss of polarity and formation of pseudopodia (Kodera, et al., 2009; Di Lauro, et al., 2009; Gottesman, et al., 2002). During the EMT, few of the epithelial cell markers including Claudin1, E-cadherin and zonula

occluden-1 (ZO1) are decreased whereas, fibronectin and vimentin are elevated. Upregulation of the transcription factors including Slug, ZEB1 and ZEB2, Twist and Snail is also observed. These factors play a major role in cancer biological behaviour such as metastasis and acquisition of stem cell-like properties, migration and invasion (Nobili, et al., 2006; Saglam, et al., 2008; Alexander, et al., 1999).

5. Epirubicin

A synthase derivative which is a potent anticancer agent and used clinically against varied solid tumours. It has cytotoxic and anti-proliferative activity in cancer cells (Song, et al., 2015). Epirubicin works by forming a complex with DNA by intercalation of its planar rings between base pairs of nucleotides which causes inhibition of protein and nucleic acid (DNA and RNA) synthesis. This intercalation leads to DNA cleavage by topoisomerase-II which causes cytocidal activity. Epirubicin also hinders activity of DNA helicase, which prevents the enzymatic separation of double-stranded DNA leading to damages in replication and transcription. By generation of cytotoxic free radicals, epirubicin is involved in oxidation/reduction reactions (Conte, et al., 2000). Certainly there are evidences of resistance to epirubicin treatment via various mechanism in gastric cancer. Several mechanisms are involved in the resistance process including GAS1 (growth arrest specific-1) and PTEN (phosphate and tensin analogue) (Zhao, et al., 2009) and overexpression of ABCB1 efflux pump also known as P-glycoprotein (Felipe, et al., 2018).

The subsequent line of therapy after 5-FU based therapy can be treated with other chemotherapeutic agents including irinotecan, docetaxel and paclitaxel is a line of therapy which significantly improves the survival (Takashima, et al., 2014). Several trials (phase III) were conducted to compare and contrast the superiority of first and second line therapies. The median overall survival rates were compared after the completion of the clinical trial. The results depicted that treatment strategy for first-line as well as second-line chemotherapy is important for overall survival in advance gastric cancer (AGC) patients (Takahari, 2017).

6. Irinotecan

It is used as second line as well as third line monotherapy for treatment of gastric cancer. It gets converted to its active metabolite SN-38. Irinotecan is a semisynthetic analogue of camptothecin which acts through inhibition of DNA topoisomerase I, an enzyme responsible for relaxation of supercoiled DNA strand (Xu, amd Villalona-Calero 2002). Inhibition of this enzyme leads to inhibition of DNA replication and subsequent cellular damage and death (Xu, amd Villalona-Calero 2002). Resistance can develop due to decrease in topoisomerase I expression, decrease in intratumor level of SN-38, suppression of apoptosis or enhancement of DNA repair and cell cycle alterations. Level of SN-38 can be decreased by several efflux mechanisms like active transport out of cells by multidrug resistance protein (MRP) and antigen binding cassette (ABC) transporter protein (Thomas and Coley 2003, Longley and Johnston 2005). Several studies have shown that, liver metabolic enzyme levels can change sensitivity of the drug to tumor cells. Such genetic variations in liver enzyme glucuronidase mediated clearance of SN-38 can lead to resistance development in cancer cells (Cummings et al. 2002). Also level of Topo1 gene responsible for regulation of topoisomerase I, if decreased, can contribute to intrinsic resistance to irinotecan (McLeod and Keith 1996; Smith et al. 2013). Thus, there's need to develop reversal of resistance to these cytotoxic agents for more beneficial effects in gastric cancer patients.

Docetaxel belonging to the class of taxane discovered in 1990s. The main mechanism through which docetaxel works is by inhibition of microtubule depolymerisation which leads to the formation of stable microtubule bundles which are non-functional. This destroys the mitosis of cancer cells and helps to attenuate anti-tumour effects. In comparison to the paclitaxel, it has broader anti-tumour spectrum with high potency (Li, et al., 2019). Resistance to the docetaxel is one of the prominent problem in all type of tumours including gastric cancer (Corcoran, et al., 2012; Okada, et al., 2013). Up regulation of forkhead box protein M1 (FOXM1) is responsible for the alteration of microtubule. This protects the tumour cells from docetaxel mediated apoptosis (Li, et al., 2014).

The anti-HER2 monoclonal antibody is often the first line therapy for AGC patients after identification of HER2 positive patients (Bang, et al., 2010). One of the novel target of

anti-HER2 monoclonal antibody ado-trastuzumab emtansine (T-DM1) which comsists of combination of trastuzumab linked to emtansine (DM1) which belongs to the class of microtubule inhibitors (Kang, et al. 2016). Bevacizumab is a vascular endothelial growth factor (VEGF) which has a potential activity in other cancers including breast, colorectal, ovarian, cervical and non-small lung cancer. It is often given in the combination with capecitabine with cisplatin or 5-FU with cisplatin (Cidon, et al., 2013). Ramucirumab and Nimotuzumab, a recombinant human monoclonal immunoglobin G1 antibody against human VEGF receptor 2 (VEGFR2) plays a critical role in formation of new blood vessels termed as angiogenesis which is involved in growth of tumour and metastases. It binds to the extracellular domain of VEGFR2 and inhibits the interaction with the ligands including VEGF-A, VEGF-C and VEGF-D. Thus, it inhibits the signalling and angiogenesis causing antitumor effect (Fuchs, et al., 2014; Wilke, et al., 2014). The other targets include mammalian target of rapamycin inhibitor including everolimus which is a potent target of rapamycin inhibitor which is used as second or third line therapy in AGC (Ohtsu, et al., 2013), signal transducer and activator of transcription 3 inhibitor (STAT3), immune checkpoint inhibitors, EGFR inhibitors and poly (ADP-ribose) polymerase inhibitor (PARP) works on the genetic aberrations of BRACA1 and BRACA2 genes. Olaparib, a PARP inhibitor which induces cell death by acting on the DNA repair pathway, often given as a drug of second line treatment in patients with AGC (Ledermann, et al., 2012).

Regardless of all the advances in the neoadjuvant therapy, the prognosis and overall patient survival has failed in overall/relapse-free survival of the adjuvant therapies.

3.11 APOPTOSIS

Apoptosis term was first used by Kerr et al., 1972 to illustrate morphologically distinct form of cell death. Apoptosis can be defined as “programmed” cell death that involves elimination of cells which is genetically governed. Apoptosis can be transpiring as a normal process in aging which can be a homeostatic mechanism to conserve cell population in tissues. It is also defined as defence mechanism in response to immune reactions as well as in response to noxious agents and cell injuries. Broad range of conditions and stimuli which may be pathological and physiological triggers apoptosis. Cancer chemotherapy and irradiation may also be responsible for DNA damage in some

cells which lead to apoptotic death though p-53 mediated pathway. The process of apoptosis may be dependent or independent or same and other pathway. Corticosteroids may lead to death in cells (e.g. thymocytes) which may be stimulated by other cellular pathways (Elmore, 2007). Apoptosis and necrosis are two separate terms which can be distinguished via different factors. These two processes can occur independently as well as simultaneously in response to same type and degree of stimuli which defines and segregates as apoptosis or necrosis. Injurious stimuli including radiation, heat, cytotoxic anti-cancer drugs or hypoxia may result in apoptosis, the same stimuli in higher doses results in necrosis as well. In apoptosis it is often characterized as energy dependent process which causes activation of a group of cysteine proteases called as "caspases" and complex cascade events which links to final cellular death (Elmore, 2007).

3.11.1 MECHANISM OF APOPTOSIS

The mechanism of apoptosis is regulated as cellular suicide mediated by various cell events including nuclear condensation, membrane blebbing, cell shrinkage and fragmentation of DNA. As described earlier, caspases, a cysteine proteases family are the regulator of apoptosis which is centrally mediated. Caspase-2, -8, -9, -10, -11 and -12 are termed as initiator caspases and they are responsible to initiate pro-apoptotic signals. After the initiation signals these caspases activates the downstream effector caspases and cleaves to execute the cellular proteins. The effector caspases are caspase-3, -6 and -7 which cleaves to specific Asp residues. Caspase -8 and -10 are activated by activation of Fas and TNFR by FasL and TNF respectively. DNA damage impels the expression of P-53 induced protein with a death domain (PIDD) which binds to receptor interaction protein associated with a death domain (RAIDD) and caspase-2 and causes it activation, whereas damaged mitochondria releases cytochrome C and leads to the activation of caspase-9. Multiple pro-apoptotic molecules are released from mitochondria including AIF, Smac/Diablo, Endo G and HtrA2. To inhibit the caspases Smac/Diablo binds to XIAP. Caspase-1 activation leads to release of pro inflammatory and pro-apoptotic stimuli of caspase-11 which in the end triggers caspase-3. Under ER stress conditions caspase-12 and -7 are activated.

Forkhead family of transcription factors (FoxO) promotes apoptosis via stimulating pro-apoptotic molecules including Bim and FasL.

3.12 ANTI-MALARIAL AGENTS AS ANTICANCER

Artemisinin are natural derivatives of sesquiterpene lactones consisting of 1,2,4-trioxane ring system. It is isolated from Artemisia plants. The cornerstone of this scaffold shows the potential to act against malaria (Krishna, et al., 2008). There are several derivatives of the artemisinin including artesunate (ART), artemether, arteether and dihydroartemisinin (DHA). These class of compounds are well established from safety perspectives. Several *in-vivo* studies are also carried out which also proves the synergism of the drug along with other standard anticancer drugs (Zhang, et al., 2012). There are reported clinical trials of the single agent which shows modest improvement in the patients with non-small cell lung cancer. There is vast preclinical literature on anticancer properties of artemisinins and its use in cancer. The identified issue with these agents is their short half-life (Li and Hickman, 2013) and variability between patients in terms of drug exposure (Newton, et al. 2006 ; Byakika-Kibwika, et al., 2012).

Recently the 4-aminoquinolines which includes chloroquine and its analogue ferroquine were evaluated for antitumor activity. Chloroquine acts on multiple pathway including via inhibiting the EGFR pathway, activation of caspase-3 (Kim, et al., 2010), modulating the Bcl-2/Bax ratio for the induction of apoptosis. It inhibits the tumour growth of several cancers including glioblastoma, liver cancer, breast cancer, colon cancer, mammary adenocarcinoma and hepatocarcinoma (Verbaanderd, et al., 2017). Ferroquine acts by inhibiting the AKT kinase pathway, autophagy disrupts the lysosomal function leading to impair tumour growth in prostate cancer *in-vivo.* It inhibited the LNCaP-d (lymph node carcinoma of the prostate cells) pathway in xenograft model of mice which proves the therapeutic efficacy of the drug. There are reports of significant toxicity at high doses which limits the efficacy of leading to no optimal use clinically (Kondratskyi, et al., 2017). Chloroquine is effective in treatment of cancer cells with primary resistance and restores the sensitivity to Transtuzumab in HER-2 positive breast cancer (Cufí, et al., 2013).

Mefloquine, a quinolone derivative of the antimalarial class of agents illustrated a potent in-vitro and in-vivo anticancer effects by acting and inhibiting several pathways including PI3K, AKT, mTOR, ERK, AMPK, autophagy, ROS/Oxidative stress and drug efflux pumps. This class of drugs are also combined with the chemotherapeutic agents and has

potent *in-vitro and in-vivo* effects. Though, there are no evidences of clinical trials which proves the efficacy of the drug in human (Mereddy and Ronayne, 2018).

Quinine is a cinchona alkaloid derivative that belongs to the class of aryl amino alcohol group of drugs, which is a basic compound (Hellgren, et al., 2014). It is the first successful anti-malarial agent use to treat infectious disease (Jacoby & Youngson, 2004). Quinine induces cell death and apoptosis in breast cancer in prostate cancer via acting on NF-κB pathway (Qureshi, et al., 2018). There are reports that quinine acts through protein kinase (AKT) pathway by activation of pro-apoptotic protein BCL-2 as well as inhibition of lipopolysaccharide (LPS)-induced activation of AKT. It acts via inhibition of cell proliferation and migration in lung adenocarcinoma (Liu, et al., 2016).

Quinidine, the other derivative of cinchona alkaloid inhibits the proliferation malignant mesothelioma cells and rat glioma cells via inducing apoptosis in a dose dependent manner. It showed specific anti-apoptotic mechanisms by inhibition of chromatin condensation without causing necrosis (Ru, et al., 2015). Quinidine also showed activity by reversing the resistance of MDR-1 gene via inhibition of the efflux pump in hepatocellular carcinoma (Shen, et al., 1991). A pilot study was carried out to evaluate the combination of quinidine and epirubicin to reverse the resistance of P-glycoprotein responsible for mediating resistance in advance breast cancer (Jones, et al., 1990).

Proguanil, an anti-malarial agent acts via inhibition of dehydrofolate reductase. It showed inhibition of mitochondrial complex and induction of apoptosis in cancer stem like cells of pancreatic cancer (Fiorillo, et al., 2016).

Primaquine, an 8-aminoquinoline is approved for the treatment of vivax malaria as well as a prophylaxis (Fernando, et al., 2011). It has showed effect on vinblastine resistant cancer cells by inhibition of cellular proliferation and microtubule formation (Choi, et al., 2016). Anti-tumour effects of primaquine were also observed on human breast cancer cell lines via inhibition of cell migration and proliferation (Gakhar, et al., 2007).

There are other classes anti-malarial including sulphonamide and sulfones which are not reported to have any anti-cancer effects.

Pyrimethamine (PYR) belonging to the class of di-aminopyrimidine is a folate antagonist

used in the treatment of malaria which is also effective in treatment of resistant malaria (Gelband, et al., 2004). PYR (2,4-diamino-5-p-chlorophenyl-6-ethyl-pyrimidine) works via blocking the enzyme dihydrofolate reductase, which is important for the synthesis of folic acid as well as a cofactor responsible for the synthesis of DNA. It is also used in the treatment of infectious disease caused by protozoan parasites including Toxoplasma gondii and plasmodium falciparum. PYR may also exert its antiprotozoal effects by exerting immunomodulating activities via induction of peripheral blood lymphocyte apoptosis (Bygbjerg, 1985; Bygbjerg et al., 1986; Viora, et al., 1996).

Recently, pyrimethamine has shown prominent effects as anti-cancer agent in several cancers including breast cancer, small cell lung carcinoma, adrenocortical cancer, melanoma, prostate cancer and invasive pituitary adenomas. Thus there are prominent reports of PYR as potent anti-cancer drug. The class of anti-malarial were screened to evaluated for anti-cancer properties where, PYR was found to act through blocking S-phase when checked on different human cancer cell lines (Van, et al., 2013).

Pyrimethamine significantly induced apoptosis on the ovarian cell lines via causing cell cycle arrest, nuclear DNA damage, growth inhibition. The *in-vivo* results were also promising which showed increase in the survival rate of tumour bearing mice (Liu, et al., 2019). There are very recent reports which suggets the efficacy of this antiprotozoal drug against non-small cell lung cancers (NSCLCs) and small cell lung cancers (SCLCs) by depicting prominent results in both *in-vitro* and *in-vivo* experimentation. It was confirmed by the studies that it has potent effect on mitochondrial pathway which induces apoptosis, inhibits cell proliferation via arrest of G_1 phase as well as it suppresses tumour growth in xenograft animal model of NSCLCs and SCLCs (Lin, et al., 2018).

Effects of pyrimethamine was checked on human metastatic melanoma cancer. The *in-vitro* and *in-vivo* experiments were carried out to check the efficacy of the drug. Human metastatic melanoma cell lines were taken and different concentration of pyrimethamine were used to confirm its activity including caspase and cathepsin B activity and cell cycle analysis which confirmed that pyrimethamine is a potent apoptosis inducer, which can inhibit the growth of the of the cells and induces mitochondrial modifications. The data from *in-vivo* studies showed potent effects by reducing the tumour growth in SCID mouse model as well as it was checked on rats with different doses (Giammarioli, et al., 2008).

Pyrimethamine was also found to inhibit the STAT3 pathway, the reports suggest that it has a prominent effect on inhibition of phosphorylation of STAT3 pathway. This pathway is activated via cytokine signalling in haematopoietic cells which plays a dual role in inflammation of tumour and stimulation of pro-oncogenic inflammatory pathways comprising of nuclear factor κB (NF-κB), interlukin-6 and GP130-Janus kinase (JAK) pathway. Thus inhibition of STAT3 has a potent anti-tumour immune responses (Gattinoni, et al., 2009). The efficacy of pyrimethamine acting on STAT3 pathway was checked on breast cancer model. The *in-vitro* and *in-vivo* studies were performed, where pyrimethamine inhibited the STAT3 phosphorylation leading to anti-tumour effects (Khan, et al., 2018). There are reports which states that pyrimethamine has enhancing effects when used with other e.g. in the treatment of invasive pituitary adenomas, it has a potent effect when used with temozolomide by inducing toxicity (Chen, et al., 2009; Dai, Congxin, et al., 2013). It also acts by suppressing the telomerase activity in PC-3 prostate cancer cell lines (Khorramizadeh, et al., 2007).

Recently reports suggested that pyrimethamine showed a dual role as it inhibits the tumor growth via inhibiting the proliferation. The metastasis is reduced via acting on dihydrofolate reductase pathway (DHFR) in lung cancer cell lines as well as in-vivo studies in C57BL/6J mice model (Liu, et al., 2019). Pyrimethamine, proved to be an anti-tumor agent via inhibition of the tumour cell proliferation in melanoma *in-vitro* and *in-vitro* studies (Tommasino et al., 2016). Anti-tumour effects of pyrimethamine were observed *in-vitro* and *in-vitro* via acting on cathepsin B-dependent and caspase dependent apoptotic pathway in pituitary adenomas (Dai, et al., 2013). Pyrimethamine has shown potent effect in various cancers including breast cancer, small cell lung carcinoma, adrenocortical cancer, melanoma, prostate cancer and invasive pituitary adenomas.

Our research suggested that pyrimethamine may acts as a potent anticancer agent in gastric cancer.

CHAPTER-5

PRELIMNARY STUDY

5. PRELIMNARY STUDY

MNNG (N-methyl-N′-methyl-N-nitrosoguanidine) is a chemical which is commonly used for the induction of gastric carcinogenesis.

The reported dose of MNNG in various articles is 200mg/kg, p.o. for 14 days daily. However, when we administered the reported dose to our experimental animals, we obtained 70% mortality within 2 days. In lieu of this, a pilot study was carried out to fix the dose of the compound used for induction of the gastric cancer.

MNNG is insoluble in water and the reports are suggestive of its solubility in dimethyl sulfoxide, acetone (Popescu et al., 1980) and corn oil. We tested the solubility of MNNG in corn oil and found that the drug was not completely soluble in corn oil. Thus, DMSO was taken as a choice of solvent. Toxicity study of DMSO was also referred and evaluated. The highest dose of DMSO which can be given to laboratory rats is 9ml/kg/day (Noel, et al., 1975).

The toxicity of DMSO (dose as per body weight) was also checked by keeping vehicle control group and no mortality was obtained with no ulceration or lesions in stomach.

Based on our preliminary study with 200 mg/kg dose of MNNG, we selected three doses of MNNG for induction of gastric cancer: 1) 50mg/kg 2) 100mg/kg and 3) 150mg/kg.

The first dose chosen for the induction of gastric cancer was 50 mg/kg, p.o. for 30 days daily. No mortality was observed with the dose of 50 mg/kg. The animals were sacrificed on 30^{th} day and stomach tissue were fixed in 10% formalin to evaluate for histopathology studies. No solid tumors were obtained in the dose of 50mg/kg. In histopathological evaluation, dysplasia stage was confirmed. Also there was presence of cells of an abnormal type within the tissue and it signifies a stage preceding the development of cancer.

In the second group of 100 mg/kg dose, the animals were administered with 100 mg/kg, p.o. daily for 21 days. The animals were sacrificed after 21 days and solid tumors were obtained in glandular part as well as fore-stomach of gastric tissue. The tissues were collected and fixed in 10% formalin for histopathological evaluation. Lesions in the

gastric region were also observed with severe ulceration. Squamous cell carcinoma was confirmed in the histopathology studies with inflammation and other related parameters.

The third dose selected for the induction of gastric cancer was 150 mg/kg, p.o. The animals were administered with 150 mg/kg orally for 14 days. But we observed 40% mortality (2 animals died out of 6) on first day itself. So, further induction was terminated due to the mortality.

Thus, in the pilot study, 100 mg/kg dose was fixed for the further induction of gastric cancer. The animals were dosed at 100 mg/kg orally for period of 21 days.

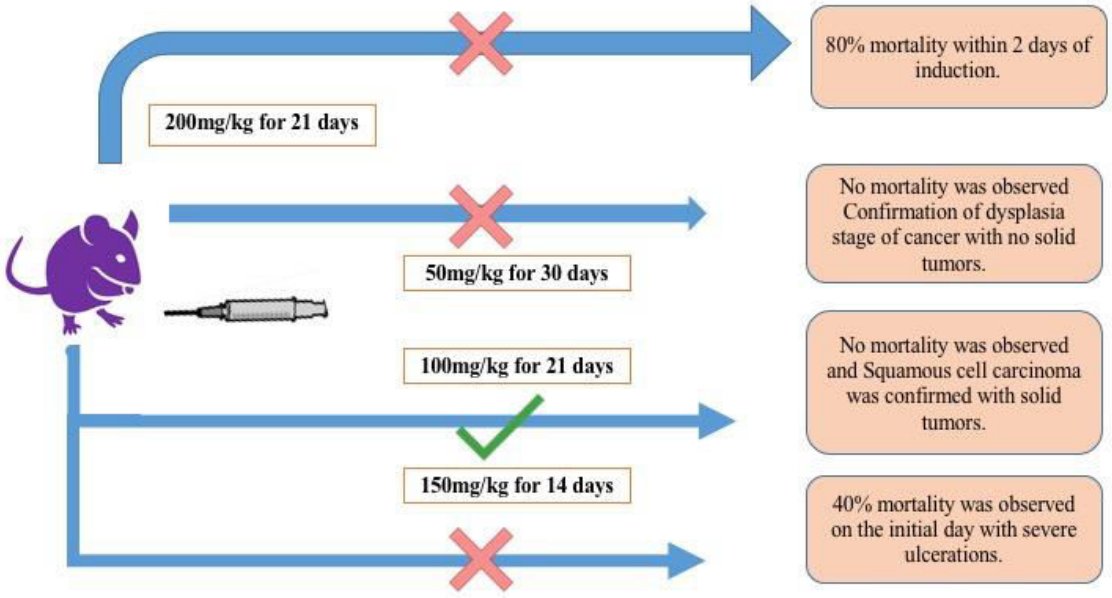


FIG. 5.1 PICTORIAL REPRESENTATION OF PRELIMNARY STUDY

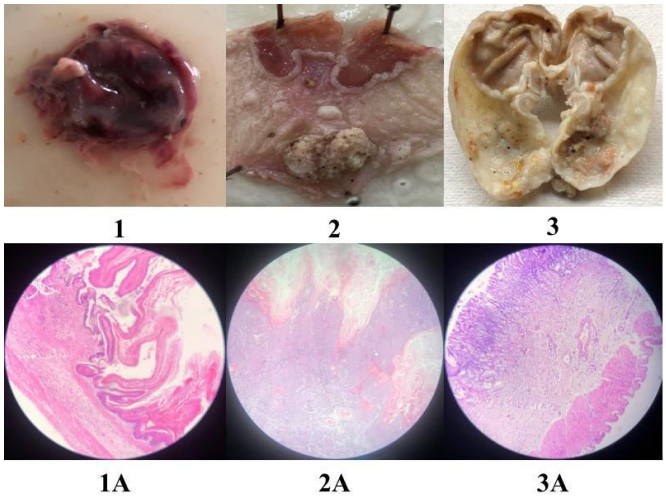


FIG 1; 1A- 200mg/kg MNNG dose
FIG 2; 2A- 100mg/kg MNNG dose
FIG 3; 3A- 200mg/kg MNNG dose

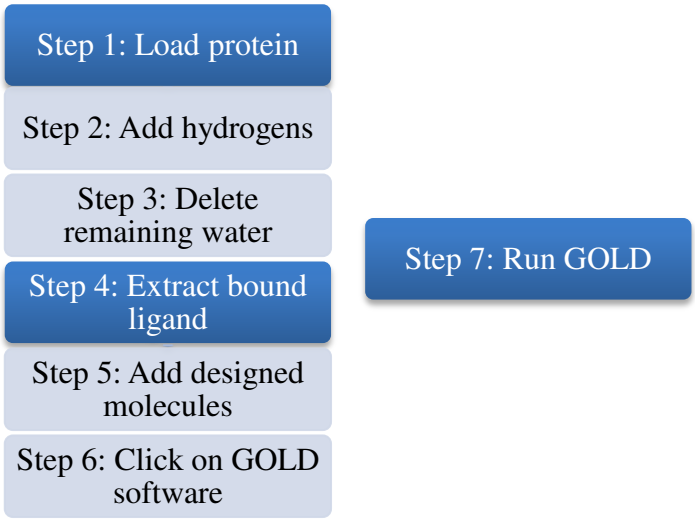
FIG. 5.2 HISTOPATHOLOGY OF DIFFERENT DOSES OF N-methyl-N′-methyl-N-nitrosoguanidine (MNNG)

CHAPTER-6
MATERIALS AND METHODS

6. MATERIALS AND METHODS

6.1 IN SILICO STUDY:

The in-silico studies were performed by molecular docking method. Molecular docking studies of antimalarial agents and doxorubicin was performed using GOLD 5.2 software. This software works by genetic algorithm, which finds the best fit populated, based on a predefined fitness function. The very first step in performing the molecular docking is to download a protein structure of your target from RCSB Protein Data Bank (PDB). The co-crystal structure of caspasase-3 were downloaded from PDB. The protein structure was prepared by adding hydrogens, delete remaining water molecules and co-crystal ligand was extracted from protein structure. Multiple steps for performing docking studies using GOLD is mentioned in below figure.



6.2 IN-VITRO STUDY:

6.2.1 MTT Assay

Principle:

MTT assay is a colorimetric method for measuring the activity of enzymes in living cells that reduce MTT to formazan dyes, giving a purple colour. It is commonly used to determine cytotoxicity of potential medicinal agents and toxic materials, since these types of materials are expected to stimulate or inhibit cell viability and growth.

Reagents:

- AGS cell line
- Thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich, catalog number: M5655)
- Dimethyl sulfoxide (DMSO) (procured from sigma-aldrich)
- Phosphate Buffer Solution (PBS)
- General chemicals (Sigma-Aldrich)

Equipment:

- 96 well plate
- Laminar Air Flow (LAF)
- Tissue roll
- Incubator

Procedure:

5000 cells in 200 µl media per well in a 96 well plate were plated. 6 empty wells were left empty for blank controls. Cells were incubated at (37 °C, 5% CO2) overnight to allow the cells to attach to the wells. Pyrimethamine (PYR) was added in different concentrations. Cells were incubated (37 °C, 5% CO2) for 24 hours to observe the effect of PYR. After that 2 ml of MTT solution was prepared for 96 well plate at 5 mg/ml in PBS. Then 20 µl of MTT dye solution was added to each well. The plate was then incubated for (37 °C, 5% CO2) for 2 h to allow the MTT to be metabolized. After that media was dumped off. In Final step 200µl DMSO was added in formazan (MTT metabolic product). The same procedure was repeated for 48 hours' time point. Optical density was read at 560 nm.

6.3 IN VIVO STUDIES

6.3.1 Ethics committee approval

Institutional animal ethics committee approved the experimental protocol according to guidance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), held under Ministry of Environment, Forest and Climate change, Government of India. (IP/PCOL/MPH/23/2018/011).

6.3.2 Drugs and chemicals

Pyrimethamine was procured from local chemical vendor, Ahmedabad. Doxorubicin was procured from industrial sources. N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) was purchased from Tokyo Chemical Industries co. Ltd.

6.3.3 INDUCTION OF GASTRIC CANCER

Adult female wistar rats of 6-8 weeks and weighing between 250-500 gms were chosen for the study and maintained under well-controlled conditions of temperature (25° ± 2° C), humidity (55 ± 5%) and 12h/12h light-dark cycle. Standard laboratory rat chow and UV filtered water was provided ad libitum. Wistar rats were taken and divided into 10 groups. Upper Gastrointestinal cancer was induced by administering N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) 100mg/kg body weight once daily by oral gavage for three weeks. Doxorubicin (1mg/kg, i.p) was administered twice a week from 4^{th} to 6^{th} week. Pyrimethamine was given in three different doses (50mg/kg, 60mg/kg and 75mg/kg by oral route) alone and was also co-administered along with Doxorubicin from 4^{th} week.

6.3.4 TREATMENT PROTOCOL

The rats were divided randomly into ten groups:

GROUP-1: NC- Normal Control (treated with DMSO as a vehicle used for PYR)

GROUP-2: DC- Disease Control

GROUP-3: PC- Pyrimethamine Control (60mg/kg)

GROUP-4: DD- Disease treated with Doxorubicin (1mg/kg)

GROUP-5: DP50- Disease treated with 50mg/kg PYR

GROUP-6: DP50+S Disease treated with combination of PYR (50mg/kg) with Doxorubicin (1mg/kg)

GROUP-7: DP60- Disease treated with 60mg/kg PYR

GROUP-8: DP60+S Disease treated with combination of PYR (60mg/kg) with Doxorubicin (1mg/kg)

GROUP-9: DP75- Disease treated with 75mg/kg PYR

GROUP-10: DP75+S Disease treated with combination of PYR (75mg/kg) with Doxorubicin (1mg/kg)

6.3.5 Blood sample collection and tissue preparation

a) Blood collection

Blood samples were collected in clean and dry centrifuge tubes from the retro orbital plexuses under light ether anaesthesia at the end of experimental period i.e. 7 weeks, and were allowed to clot for 30 min at room temperature. Thereafter, it was centrifuged at 4000 rpm for 20 min for serum separation and the separated serum was stored at –20ºC until the biochemical analyses were carried out. CEA levels were analysed from serum by using Elisa kits (Krishgen, Mumbai, India).

b) Tissue preparation:

After blood collection and serum separation, animals were sacrificed and stomach was removed from each animal. Tumour volume, tumour burden and tumour incidence were measured. Isolated stomach tissues were cleaned and stored in 10% formalin for further histopathological studies.

6.3.6 Tumour Parameters:

Animals was sacrificed stomach was dissected out and tumours were observed, counted and various parameters were calculated such as

1) Tumor incidence: Number of tumor bearing rats/Number of Tested Rats*100

2) Average Tumor number: Total num of tumors/Number of tested rats

3) Average number of tumor bearing rats: Total number of tumors/number of tumor bearing rats

4) Tumor volume: (maximal length * $\{width\}^2$)/2

5) Tumor Burden: Tumor volume* Number of tumors.

6.3.7 Oxidative Stress Parameters:

6.3.7.1 Preparation of tissue homogenate:

Animals were sacrificed and stomach was dissected out rinsed with ice cold distilled water followed by washing with sucrose solution (0.25 M). The stomach tissue was rinsed again with distilled water and immediately stored at -20ºC till further biochemical analysis. 200 mg of stomach tissue was homogenized in 2 ml ice cold phosphate buffer (pH 7.2). Homogenate was centrifuged at 8000 RPM for 10 min.

6.3.8 Estimation of tissue protein levels

Principle:

Protein estimation by Lowry method is done to estimate the total protein. Determining the protein concentration depends upon the reactivity of the peptide nitrogen with the copper [II] ions under alkaline conditions which causes reduction of Folin- Ciocalteay phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalysed oxidation of aromatic acids. This was measured at 660nm wavelength for maximum absorption. Thus, change in the colour intensity varies on the presence of the above mentioned amino acids. Bovine Serum Albumin (BSA) was taken as standard protein for the estimation. The reaction is dependent and sensitive to change in pH so the working range of pH between 9 to 10.5 is required everytime. The developed colour was proportional to the total protein present in the reaction mixture (Lowry et al 1951).

Reagents:

1. Regents A: - 50 ml of 0.1N NAOH (0.4gm in 100ml) with 50ml of 2% NA_2CO_3

2. Reagent B: 10ml of 1.56% copper sulphate solution mixed with 10ml of 2.73% sodium potassium tartarate solution.

3. Reagent C: - Mix 10 ml of reagent A+ 0.2 ml of reagent B prior to use.

4. Reagent D: - 1N Folin- Ciocaltue reagent solution

5. Bovine serum albumin as standard

Procedure: -

Blank	**Test**
0.2 ml of D.W.	**0.2 ml of supernatant**
Diluted upto 1 ml with Tris HCL	**Diluted upto 1 ml with Tris HCL**
5 ml Reagent C	**5 ml Reagent C**
Allowed it for 10 minutes	
0.5 ml Reagent D	**0.5 ml Reagent D**

All reagents were mixed well and kept at room temperature for 30 min. in dark place and absorbance was read against blank at 600 nm. The protein levels were calculated using standard curve which was plotted using BSA as standard.

6.3.9 Measurement of Lipid peroxidation levels

Principle:

Lipid peroxidation by reactive oxygen species (ROS) is known to be involved in damaging the mechanism of several acute and chronic disorders. Thus, it estimates the Malondialdehyde (MDA) which is a product of the lipid peroxidation process. One molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) under mildly acidic conditions to form a pink colored chromogen. The intensity of this chromogen was measured colorimetrically at 535 nm (Babizhayev et al 1988).

Reagents:

1. Sodium lauryl Sulphate (SLS) (8%): 8 gm of SLS in 100 ml of distilled water.

2. Acetic acid (20 %): Prepared in 0.27 M hydrochloric acid (2.29 ml HCL in 100 ml water)

3. Thiobarbituric acid (TBA) (1% in Tris hydrochloride, pH 7) (Freshly prepared): 1 gm of thiobarbituric acid in 100 ml of Tris hydrochloride buffer pH 7.

Procedure: -

Blank	**Test**
0.2 ml of D.W.	0.2 ml of homogenate
0.2 ml of SLS	0.2 ml of SLS
1.5 ml acetic acid in HCl	1.5 ml acetic acid in HCl
1.5 ml TBA	1.5 ml TBA
0.6 ml DW	0.6 ml DW
Heated for 45 min in water bath at 95°C and cool	
5 ml mixture of n-butanol:pyridine (15:1)	5 ml mixture of n-butanol:pyridine (15:1)

All reagents were mixed well and pink colour developed in upper organic layer, the absorbance of which was read against blank at 532 nm. Malondialdehyde level was calculated using molar extinction coefficient of malondialdehyde.

6.3.10 Measurement of reduced glutathione (GSH) levels:

Principle:

Glutathione present in RBC consist of sulfhydryl groups. 5,5 dithiobis 2- nitro benzoic acid (DTNB), a disulphide compound, gets readily attacked by these sulfhydryl groups and forms a yellow coloured anion which can be measured colorimetrically at 412 nm.

Reagent:

1. Trichloroacetic acid (TCA) (5%) -5gm of TCA in 100 ml of distilled water.

2. 0.2 M ethylene -di -amine tetra acetic acid – disodium (EDTA Na2)– 7.44gm of EDTA Na2 in100 ml distilled water(ph. -8.3).

3. 0.2 M Tris EDTA buffer - 1.21 gm tris base in 20 ml EDTA Na2 buffer make up to 25 ml with distilled water (ph. -8.9)

4. 0.01 M DTNB(fresh) –0.099 gm DTNB in 25 ml distilled water.

Procedure: -

Blank	Test
0.2 ml distilled water	0.2 ml supernatant
1 ml TCA (10%)	1 ml TCA (10%)
Kept in ice bath for 30 min & centrifuged at 3000 rpm for 10 min at 4 ºC	Kept in ice bath for 30 min & centrifuged at 3000 rpm for 10 min at 4 ºC
0.5 ml supernatant	0.5 ml supernatant
2 ml di-sodium hydrogen phosphate	2 ml di-sodium hydrogen phosphate
0.25 ml DTNB (covered with aluminium foil) added just before measuring the absorbance at 412 nm	0.25 ml DTNB (covered with aluminium foil) added just before measuring the absorbance at 412 nm

All the reagents were mixed well and absorbance was read against blank at 412nm. The GSH levels was measured using standard curve which was plotted using glutathione. Results was expressed as µ/mole of GSH/g tissue.

6.3.11 Measurement of Superoxide dismutase (SOD) levels:

Principle:

The O_2^-, substrate for SOD is generated indirectly in the oxidation of epinephrine at alkaline pH by the action of oxygen on epinephrine. As O_2- builds in the solution, the formation of adrenochrome accelerates because O_2^- also reacts with epinephrine to form adrenochrome. Towards the end of the reaction, when the epinephrine is consumed, the adrenochrome formation slows down. If observed for long time, the adrenochrome disappears and brown insoluble products form in the solution. SOD reacts with the O_2- formed during the epinephrine oxidation and therefore slows down the rate of formation of the adrenochrome as well as the amount that is formed. Because of this slowing process, SOD is said to inhibit the oxidation of epinephrine.

Reagents: -

1. EDTA: 0.0001 M (9.3 mg/250 ml)

2. Carbonate buffer pH 9.7: (8.4 gm $NaHCO_3$ + 10.6 gm Na_2CO_3 in 500 ml)

3. Epinephrine 0.003 M: (50 mg/100 ml in 2 pH HCL and cover with aluminium foil.)

Procedure: -

Blank	Test
0.2 ml of D.W.	0.2 ml of supernatant
0.1 ml EDTA	0.1 ml EDTA
0.5 ml Carbonate buffer	0.5 ml Carbonate buffer
1 ml epinephrine	1 ml epinephrine

All reagents were mixed well and absorbance was read against blank at an interval of 30 sec for 3 min. at 480 nm. The SOD level was calculated using standard curve which was plotted using standard SOD.

6.3.12 Inflammatory markers:

a) Interleukin-6 (IL-6)

Principle: -

Interleukin-6 is an inflammatory marker which is secreted by various tumour cells which is indulged in the proliferation and differentiation of malignant cells found in the tumour tissues and serum. Thus, the levels of IL-6 can be used as prognostic value to predict the disease (Kumari, et al., 2016).

Procedure: -

The isolated stomachs of the sacrificed animals were collected. 200mg of stomach tissue was homogenized in 2 ml of ice cold phosphate buffer (pH 7.2). The homogenates were centrifuged at 8000 RPM for 10 min and the supernatants are collected. IL-6 levels were analysed using Elisa kit (Elabscience, India).

b) Tumour Necrosis Factor (TNF)-alpha:

Principle: -

Tumour necrosis factor (TNF) is a pro-inflammatory versatile cytokine which plays an important various multiple cellular events including cell proliferation, differentiation, survival and death. TNF is secreted by inflammatory cells which are involved in inflammation associated with carcinogenesis.

Procedure: -

The supernatants of tissue homogenates were used to analyse the levels of TNF-alpha levels using Elisa Kit (Elabscience, India).

c) Nuclear factor-κB (NF-κB):

Principle: -

Nuclear factor-κB (NF-κB) is involved in various pathways which mediate its activation in tumour cells. Consecutive activation of NF-κB transcription factors is been associated with several tumorigenesis, comprising of promoting cancer-cell proliferation, prevention of apoptosis, elevating tumour's angiogenic and metasis (Park and Hong, 2016).

Procedure: -

The supernatants of tissue homogenates were used to analyse the levels of NF-κB using Elisa Kit (Elabscience, India).

d) Interferon-gamma (IFN-γ):

Principle: -

Interferon-γ (IFN-γ) is a pluripotent and a prototypical antitumor cytokine. It not only controls the tumour initiation and progression but it also shapes tumour immunogenicity, this promotes the outgrowth of tumour cells which has immunoevasive properties (Street, et al., 2001).

Procedure: -

The supernatants of tissue homogenates were used to analyse the levels of IFN-γ using Elisa Kit (Elabscience, USA).

e) Interleukin-1β

Principle: -

Interleukin (IL-1β) the sub receptor of IL-1 receptor which plays a major role in inflammation. There are evidences of increase in the levels of IL-1β in several cancers. Thus estimation of the levels of IL-1β can be beneficial to identify the growth of tumour and metastasis.

Procedure: -

The supernatants of tissue homogenates were used to analyse the levels of IL-1β using Elisa Kit (Elabscience, USA).

6.3.13 Histopathological studies:

The animals were sacrificed and the stomach tissues were isolated to perform the histopathological studies. The tissues were fixed in 10% formalin for further studies.

6.3.14 Immunohistochemistry staining

Principle:

Immunohistochemistry (IHC) is a method for detecting antigens or haptens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. It is also used to identify the distribution and localization of biomarkers which are differentially expressed proteins in various parts of biological tissue.

Bcl-2 staining:

Procedure:

Solutions and reagents

1. Xylene

2. Ethanol, anhydrous denatured, histological grade (100%, 95%, 85%, 75%)

3. Washing buffer:

1XPBST, 1 L = (10X PBS 100 mL, dH2O 900 mL, dH2O 900 mL, Tween-20 1 mL)

10X PBS 100 mL = (dH2O 900 mL, Tween-20 1 mL, NaH2PO4·2H2O 2.84 g

Na2HPO4·12H2O 27.2 g, NaCl 90 g, dH2O 1000 mL)

The pH should be about 7.2. Adjust if necessary with 1 M NaOH or 1 M HCl

4. Antigen Retrieval Solution:

- 10mM Sodium Citrate Buffer, pH 6.0
- 10mM Tris Buffer with 1mM EDTA, pH 8.0 or 8.5 or 9.0

Adjust pH* to 8.0 or 8.5 or 9.0

*Note: Please refer to the antibody for individual antigen retrieval buffer and working conditions.

5. 3% Hydrogen Peroxide

6. Hematoxylin QS

7. Permanent Mounting Medium

Protocol: -

A. Deparaffinization

- Heat slides in an oven at 60 °C for 5 min.
- Wash slides 3 times for 10 min each in xylene.

- Wash slides 3 times for 3 min each in 100% ethanol.
- Wash slides in 95% ethanol, 1 min.
- Wash slides in 85% ethanol, 1 min.
- Wash slides in 75% ethanol, 1 min.
- Rinse slides for 5 min in distilled water.

B. Antigen Retrieval

- Put the slides in Antigen Retrieval Solution and keep 120℃ for 2.5 min in pressure cooker.
- Then cool down at room temperature.
- Wash slides three times with distilled water (2 min each)

*Note: Please refer to the antibody for individual antigen retrieval buffer and working conditions.

C. Staining

- Inactivate endogenous peroxidase by covering tissue with 3% hydrogen peroxide for 15 min.
- Wash slides twice with 1X PBST (2 min each).
- Dilute primary antibody in the **BCL-2** IHC Antibody Diluent per recommendation on the data sheet.
- Apply primary antibody to each section and incubate 90 min at room temperature*. Make sure the primary antibody solution covers the tissue evenly. *Note: Please refer to the antibody for individual buffer and working conditions. Wash slides three times with 1X PBST (2 min each).
- Apply to each section secondary antibody and incubate for 15 min at room temperature *. *Note: Please refer to the antibody for individual buffer and working conditions.
- Wash slides three times with 1X PBST (2 min each). Add freshly prepared DAB substrate to the sections.
- Incubate tissue sections with the substrate at room temperature until suitable staining develops (generally about 5 min). Rinse sections with water. Counterstain with Hematoxylin for 3 min.
- Rinse sections with water.

- Wash slides in 75% ethanol, 1 min.
- Wash slides in 85% ethanol, 1 min.
- Wash slides in 95% ethanol, 1 min.
- Wash slides in 2 changes of 100% ethanol rinses, 1 min each.
- Wash slides in 3 changes of xylene, 1 min each.
- Mount coverslips on slides using Permanent Mounting Medium.

Allow slides to dry overnight at room temperature and then analyze the results with microscope.

6.3.13 mRNA EXPRESSION STUDIES

Total ribonucleic acid (RNA) was extracted from intact hearts using the FastRNA® Pro Green Kit (MPBIO) according to the manufacturer's instructions. The reverse transcription (RT) reaction was performed using First Strand cDNA Synthesis kit (Imperial life sciences). Real-time polymerase chain reaction (PCR) was performed in LightCycler® 480 (Roche Applied Biosciences) using TaqMan Universal PCR Master mix for determining mRNA levels of FoxO3a and Binp3. To detect FoxO3a and Binp3, specific primers were designed with the 'Primer 3Plus' Program listed in table. All reactions were performed in duplicate. The PCR product was separated using electrophoresis on 1% agarose gel and semi-quantified as a ratio to GAPDH. The levels of TP53 were identified for identifying the process of apoptosis.

CHAPTER-7
RESULTS

7. RESULTS

7.1 IN-SILICO STUDIES

7.1.1 Molecular docking

Anti-malarial of each class was taken for the docking at caspase-3 (the effector caspase). The reported amino-acid for the inhibition of the protein which cleaves the caspase-3 are Tyr204, Arg207, Phe256, Trp206, Cys163, Gly122, Gly165, Met61, His121 and Ser120. Out of the 10 reported amino acids **His121, Tyr204** and **Cys163** are important amino-acids responsible for the inhibition of the specific protein (Ganesan, et al., 2011). Caspase-3, the effector caspase was taken for docking of standard drug (doxorubicin) and the other classes of anti-malarial agents. Chloroquine from class 4-aminoquinolines was docked to caspase-3 and amino-acids interaction which were obtained are MET61, HIS121, GLY122, CYS163, TYR204, SER205, ARG207 with gold score of 55.39, Doxorubicin an anti-cancer drug belonging to the class of anthracycline used as first-line chemotherapeutic agent in many of the cancer was taken as standard in the treatment of gastric cancer (Rivankar, 2014). The important amino-acid interactions were HIS121, CYS163, TRY204, ARG207 with gold score of 52.71. Sulfadoxine is a sulphonamide derivative which inhibits the activity of dihydopteroate synthase (DHPS) (Yaro, 2009). The important amino-acid interaction found in docking were HIS121, GLY122, CYS163, TRY204, ARG207 with a docking score of 50.87. Quinine is the oldest and most effective anti-antimalarial agent. Quinine is a bark component of cinchona (quina-quine) tree (Achan et al., 2011). The amino-acid interactions obtained were MET61, HIS121, GLY122, ARG207 with gold score of 45.26. Pyrimethamine belonging to the class of di-aminopyrimidines has shown promising anti-cancer potential in various cancers. All the three amino acids important for the caspase inhibition were found in pyrimethamine with a gold score of 42.43. The gold score of pyrimethamine is less than quinine but the amino-acid interaction was prominent in pyrimethamine than quinine. Proguanil a folic-acid antagonist (Garbis et al., 2007) was docked with the effector caspase, HIS121, SER205, TRP206 were reported amino-acid interaction with a gold score of 40.13.

TABLE. 7.1 Docking studies of anti-malarial agents and standard

Name of drug	Gold score	Amino acid interaction
Chloroquine	55.39	MET61, HIS121, GLY122, CYS163, TYR204, SER205, ARG207
Doxorubicin	52.71	**HIS121**, **CYS163, TRY204**, ARG207
Sulfadoxine	50.87	HIS121, GLY122, CYS163, TRY204, ARG207
Quinine	45.26	MET61, HIS121, GLY122, ARG207
Pyrimethamine	42.43	**HIS121, CYS163,TYR204**
Proguanil	40.13	HIS121, SER205, TRP206

7.2 IN-VITRO CYTOTOXICITY STUDY

7.2.1 MTT ASSAY

Pyrimethamine treatment in AGS cell lines caused decrease in cell proliferation and cell viability in dose dependent manner. As the dose of pyrimethamine increased, cell proliferation and viability decreased. From MTT assay, IC50 value of pyrimethamine was calculated to be 91.1 µM at 24 hours. The IC50 of Doxorubicin was found to be 4.5 µM at 24 hours.

TABLE. 7.2 Table IC50 value of pyrimethamine using MTT assay

DRUG	IC50 (24 hours)
Pyrimethamine	**91.1 µM**
Doxorubicin	4.5 µM

7.3 IN-VIVO STUDIES

7.3.1 Physical Parameters

7.3.1.1 Effect of pyrimethamine on body weight, food and water intake

The general body weight, food and water intake were measured on daily basis during 7 weeks of study for all the groups of animals. Our data reveals that there was highly significant ($p<0.001$) decrease in body weight, food intake and water intake in disease control group. Pyrimethamine treated group showed highly significant ($p<0.001$) increase in body weight, food intake and water intake as compared to disease control group.

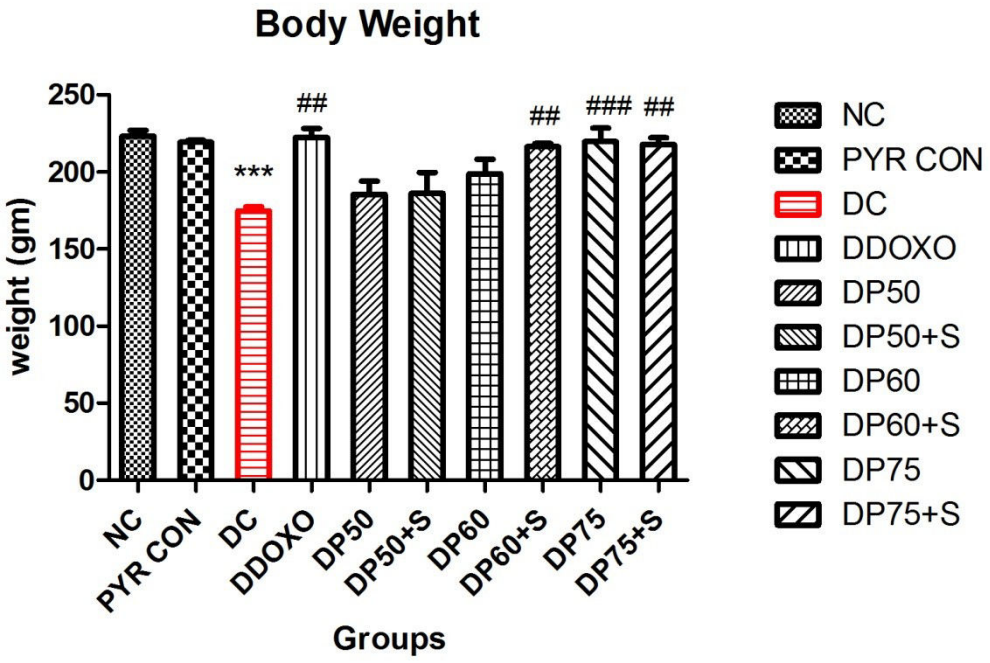


FIG. 7.3.1.1 Effect of Pyrimethamine on body weight in MNNG induced gastric cancer in rats

Each value represents mean ±SEM of n=6 animals

*** = Significant different than normal control ($p<0.001$)
= Significant different than disease control ($p<0.001$)

NC- Normal Control, PYRI CON- Pyrimethamine Control, DC-Disease Control, DDOXO- Disease treated with Doxorubicin, TP50- Disease treated with 50mg PYR,

TP50+S- Disease treated with 50mg PYR+ 1mg Doxorubicin, TP60- Disease treated with 60mg PYR, TP60+S- Disease treated with 60mg PYR+ 1mg Doxorubicin, TP75- Disease treated with 75mg PYR, TP75+S-Disease treated with 75mg PYR+ 1mg Doxorubicin.

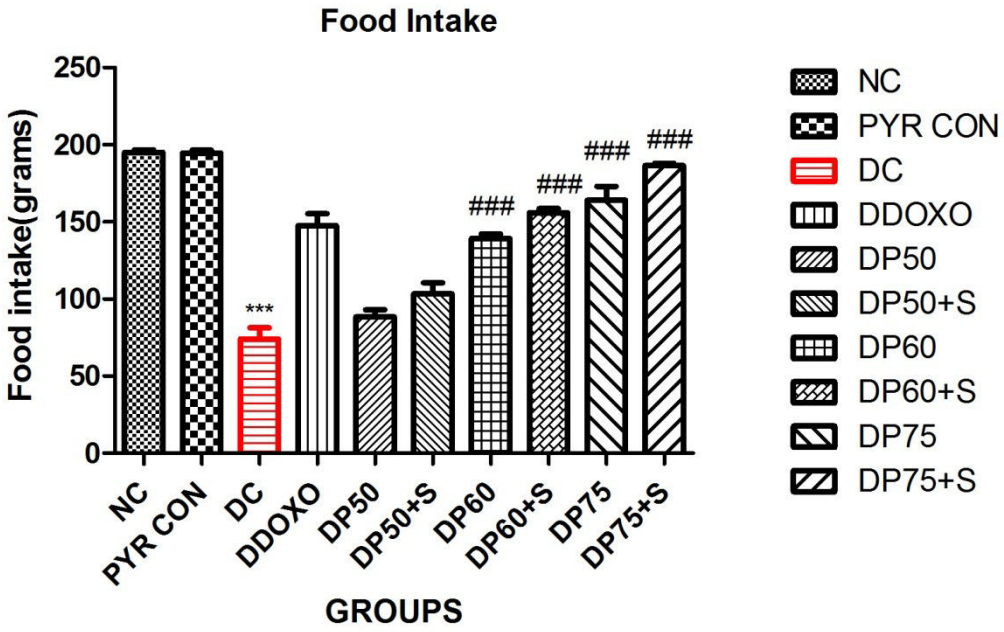
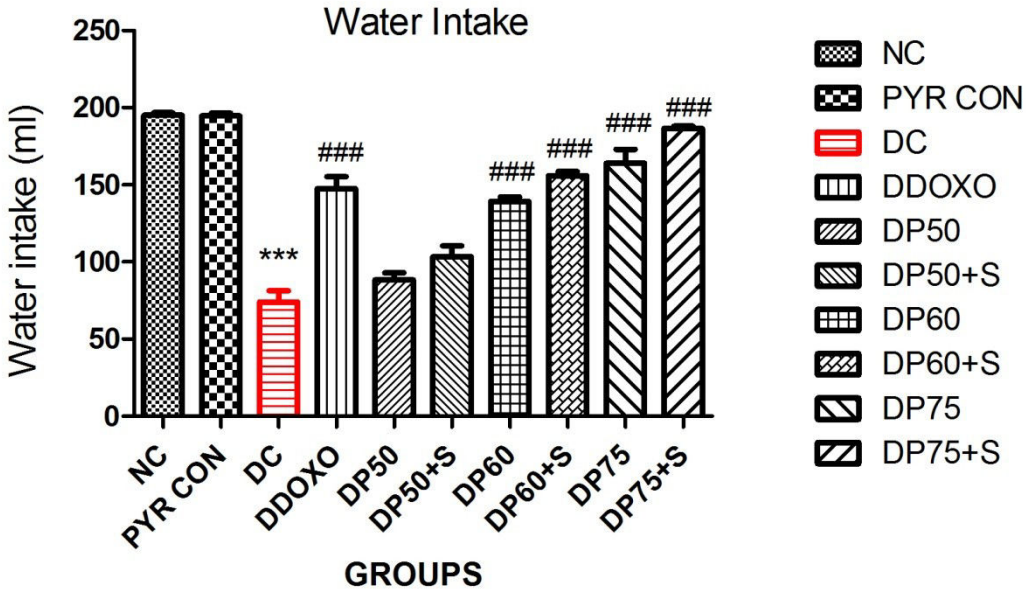


FIG. 7.3.1.2 Effect of pyrimethamine on food intake in MNNG induced gastric cancer in rats

Each value represents mean ±SEM of n=6 animals

*** = Significant different than normal control ($p<0.001$)
= Significant different than disease control ($p<0.001$)

NC- Normal Control, PYRI CON- Pyrimethamine Control, DC-Disease Control, DDOXO- Disease treated with Doxorubicin, TP50- Disease treated with 50mg PYR, TP50+S- Disease treated with 50mg PYR+ 1mg Doxorubicin, TP60- Disease treated with 60mg PYR, TP60+S- Disease treated with 60mg PYR+ 1mg Doxorubicin, TP75- Disease treated with 75mg PYR, TP75+S-Disease treated with 75mg PYR+ 1mg Doxorubicin.



7.3.1.

3 Effect of pyrimethamine on water intake in MNNG induced gastric cancer in rats

Each value represents mean ±SEM of n=6 animals

*** = Significant different than normal control ($p<0.001$)
= Significant different than disease control ($p<0.001$)

NC- Normal Control, PYRI CON- Pyrimethamine Control, DC-Disease Control, DDOXO- Disease treated with Doxorubicin, TP50- Disease treated with 50mg PYR, TP50+S- Disease treated with 50mg PYR+ 1mg Doxorubicin, TP60- Disease treated with 60mg PYR, TP60+S- Disease treated with 60mg PYR+ 1mg Doxorubicin, TP75- Disease treated with 75mg PYR, TP75+S-Disease treated with 75mg PYR+ 1mg Doxorubicin.

7.4 ANTI-TUMOUR EFFICACY STUDY

7.4.1 Tumour parameters and biomarker

7.4.1.1 Effect of pyrimethamine on tumour parameters and CEA levels:

Fig 7.4.1 depicts representative images of gross morphology of stomach at the end of 7 weeks for all the groups under investigation. Upon statistical analysis, it was found that there was highly significant ($p<0.001$) increase in average number of tumours, average % tumour volume, average tumour burden, % of tumour incidence and CEA level in disease control group. Pyrimethamine treated group showed highly significant ($p<0.0001$) decrease in all the tumour parameters such as average tumour number, average % tumour volume, average tumour burden, % of tumour incidence and CEA level in comparison with disease control animals.

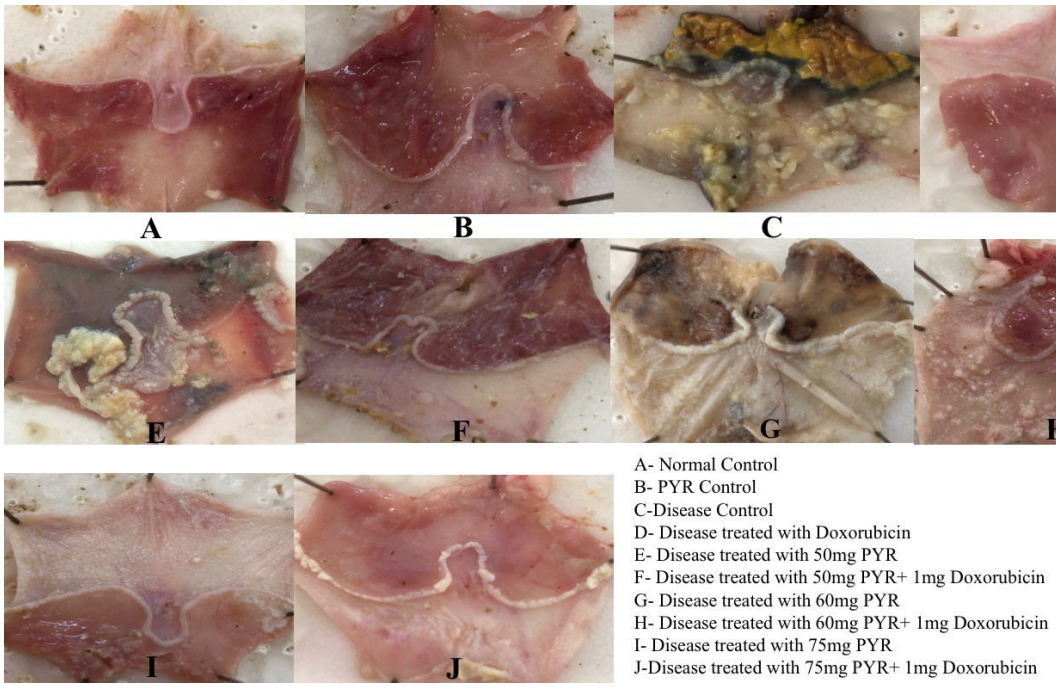


FIG. 7.4.1 Tumour incidence of different animal groups

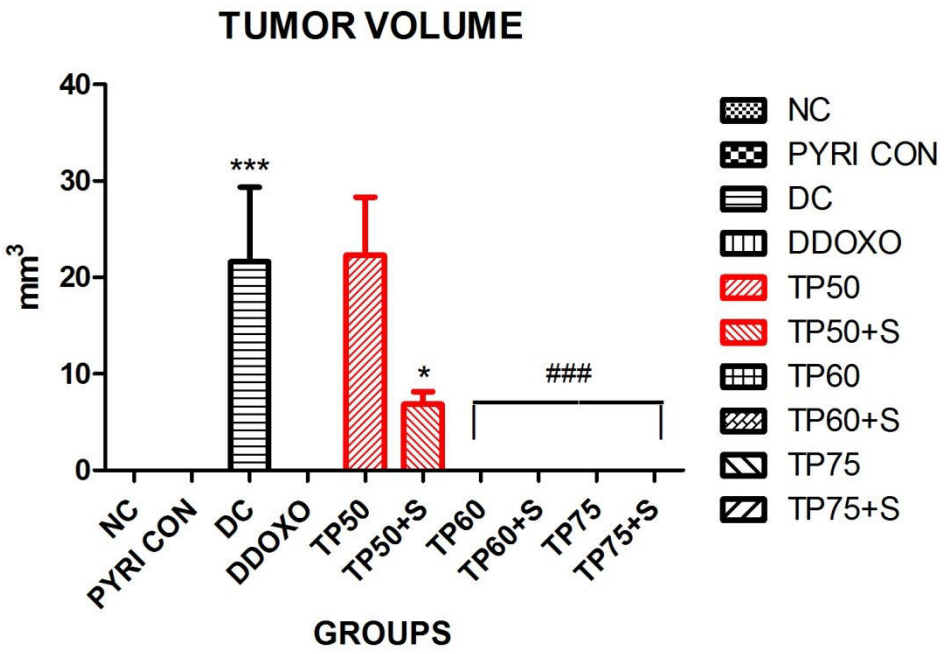
7.4.1.1 Pyrimethamine effect on tumour parameters:

GROUPS	NC	PYRI CON	DC	DDOXO	TP50	TP50+S	TP60	TP60+S	TP75
Tumour volume	0	0	21.60± 7.756***	0	22.30± 6.022#	6.862± 1.298#	0	0	0
Tumour burden	0	0	94.65± 32.13***	0	49.46± 13.36#	20.16± 4.078###	0	0	0
% Tumour Incidence	0	0	100***	0	95.56± 15.55	87.33± 13.68#	0	0	0
CEA (ng/ml)	3.418 ± 0.447	3.198 ± 0.134 5	14.37± 0.2912 ***	6.619± 0.3458	12.21± 0.4548##	10.92± 0.3687###	11.96± 0.5288##	9.020± 0.08322###	10.03± 0.1538###

Each value represents mean ±SEM of n=6 animals

*** = Significant different than normal control ($p<0.001$)
= Significant different than disease control ($p<0.001$)

NC- Normal Control, PYRI CON- Pyrimethamine Control, DC-Disease Control, DDOXO- Disease treated with Doxorubicin, TP50- Disease treated with 50mg PYR, TP50+S- Disease treated with 50mg PYR+ 1mg Doxorubicin, TP60- Disease treated with 60mg PYR, TP60+S- Disease treated with 60mg PYR+ 1mg Doxorubicin, TP75- Disease treated with 75mg PYR, TP75+S-Disease treated with 75mg PYR+ 1mg Doxorubicin.

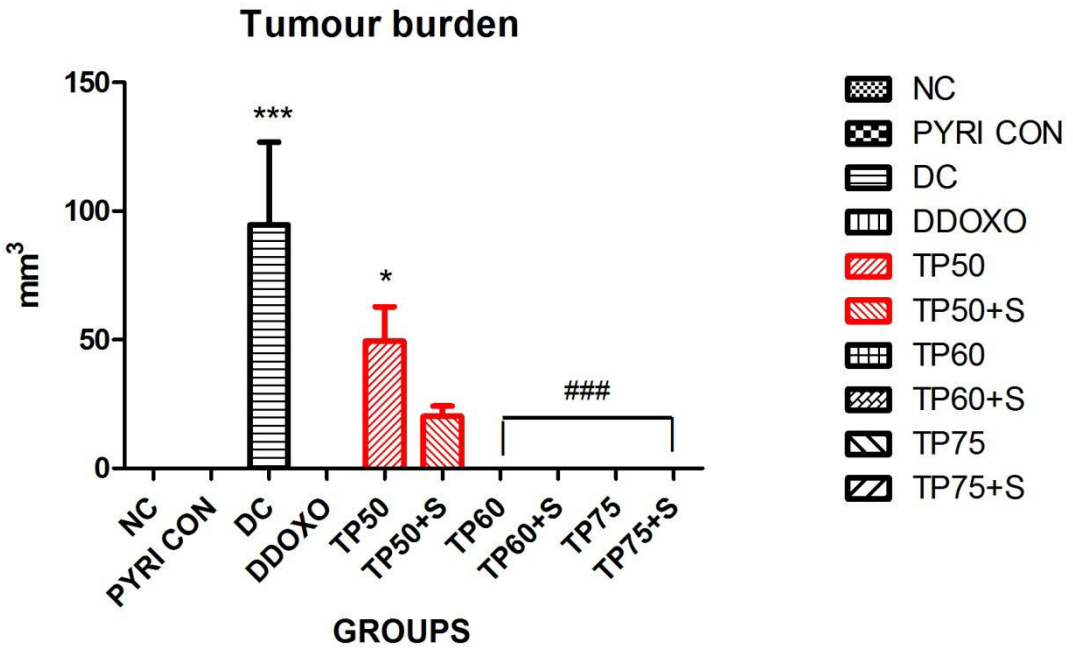


7.4.1.2 Effect of Pyrimethamine on tumour volume in MNNG induced gastric cancer in rats

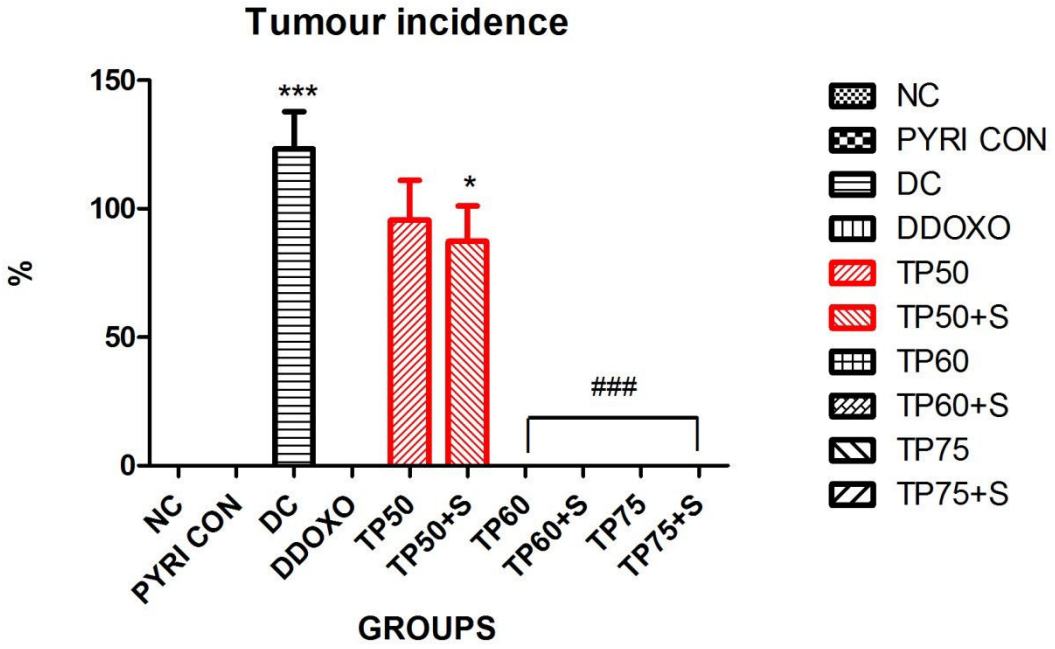
Each value represents mean ±SEM of n=6 animals

*** = Significant different than normal control ($p<0.001$)
= Significant different than disease control ($p<0.001$)

NC- Normal Control, PYRI CON- Pyrimethamine Control, DC-Disease Control, DDOXO- Disease treated with Doxorubicin, TP50- Disease treated with 50mg PYR, TP50+S- Disease treated with 50mg PYR+ 1mg Doxorubicin, TP60- Disease treated with 60mg PYR, TP60+S- Disease treated with 60mg PYR+ 1mg Doxorubicin, TP75- Disease treated with 75mg PYR, TP75+S-Disease treated with 75mg PYR+ 1mg Doxorubicin.



7.4.1.3Effect of Pyrimethamine on tumour burden in MNNG induced gastric cancer in rats

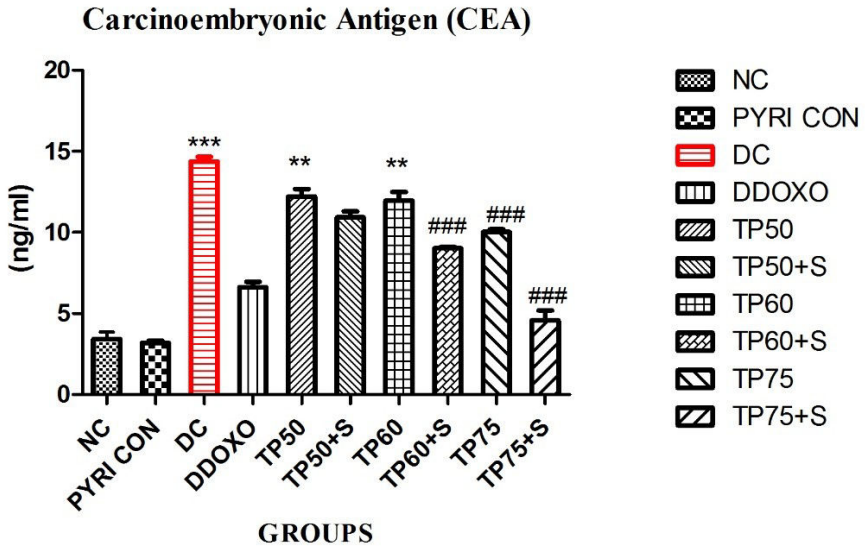


7.4.1.4 Effect of Pyrimethamine on tumour incidence

Each value represents mean ±SEM of n=6 animals

*** = Significant different than normal control (p<0.001)
= Significant different than disease control (p<0.001)

NC- Normal Control, PYRI CON- Pyrimethamine Control, DC-Disease Control, DDOXO- Disease treated with Doxorubicin, TP50- Disease treated with 50mg PYR, TP50+S- Disease treated with 50mg PYR+ 1mg Doxorubicin, TP60- Disease treated with 60mg PYR, TP60+S- Disease treated with 60mg PYR+ 1mg Doxorubicin, TP75- Disease treated with 75mg PYR, TP75+S-Disease treated with 75mg PYR+ 1mg Doxorubicin.



7.4.1.5 Effect of Pyrimethamine on CEA levels in MNNG induced gastric cancer in rats

Each value represents mean ±SEM of n=6 animals

*** = Significant different than normal control (p<0.001)
= Significant different than disease control (p<0.001)

NC- Normal Control, PYRI CON- Pyrimethamine Control, DC-Disease Control, DDOXO- Disease treated with Doxorubicin, TP50- Disease treated with 50mg PYR, TP50+S- Disease treated with 50mg PYR+ 1mg Doxorubicin, TP60- Disease treated with 60mg PYR, TP60+S- Disease treated with 60mg PYR+ 1mg Doxorubicin, TP75- Disease treated with 75mg PYR, TP75+S-Disease treated with 75mg PYR+ 1mg Doxorubicin

7.5 OXIDATIVE STRESS PARAMETERS

7.5.1 Malondialdehyde levels (MDA):-

The animals from disease control group exhibited highly significant ($p<0.001$) increased MDA levels than the animals from normal groups. Pyrimethamine treated animals have significantly ($p<0.001$) decreased MDA levels than the animals from disease control animals. However, the level of MDA of control treated animals showed no significant difference from normal control and pyrimethamine control treated animals.

7.5.2 Superoxide dismutase (SOD):-

The animals from disease control group exhibited highly significant ($p<0.001$) lowered level of SOD than the animals from normal groups. Pyrimethamine treated animals have highly significant ($p<0.001$) higher SOD level than the animals from disease control animals. However, the level of SOD of control treated animals showed no significant difference from normal control and pyrimethamine control animals.

7.5.2 Glutathione reductase (GSH):-

The animals from disease control group exhibited highly significant ($p<0.001$) lowered level of GSH than the animals from normal groups. Pyrimethamine treated animals have highly significant ($p<0.001$) higher GSH level than the animals from disease control animals. However, the level of GSH of control treated animals showed no significant difference from normal control and pyrimethamine control animals.

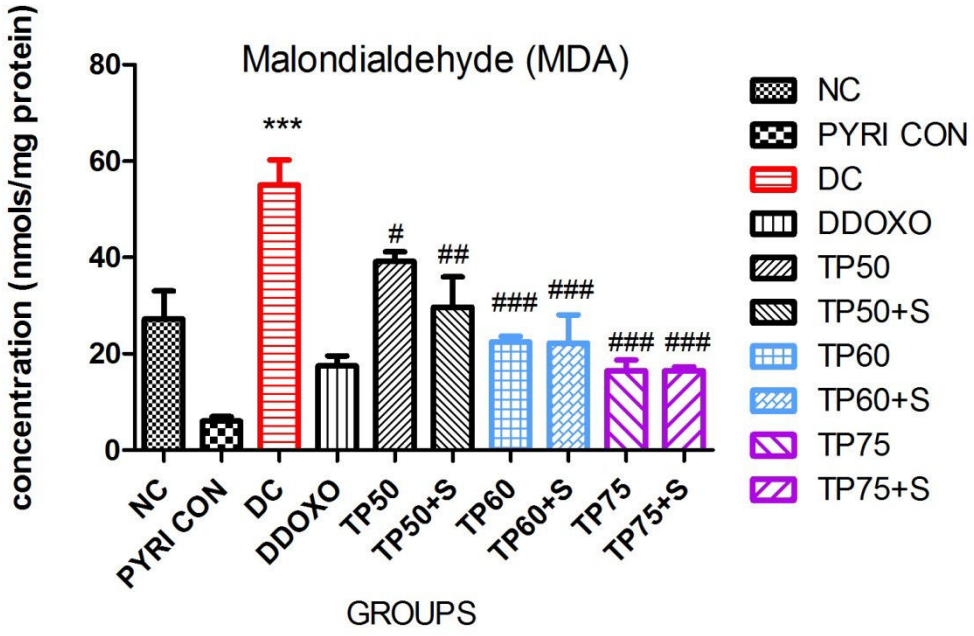
7.5.2 Effect of Pyrimethamine on Oxidative parameters

GROUPS	NC	PYRI CON	DC	DDOXO	TP50	TP50+S	TP60	TP60+S	TP75
MDA	27.26 ± 5.743	16.013± 0.9489	55.03± 9.007***	17.53± 2.016	39.17 ± 1.965	29.73± 6.267	22.44± 1.218	22.26± 5.817	16.49± 2.215
SOD	33.74 ± 0.710	32.01± 0.380	6.241± 0.492***	33.21± 1.351	8.053 ± 0.825	7.043± 1.143	13.05± 1.562##	21.47± 0.912###	25.19± 1.679###
GSH	35.58 ± 0.264	35.54± 0.890	26.57± 3.149***	39.40± 2.210#	21.08 ± 2.974	30.19± 4.322#	32.77± 1.228###	35± 0.091	40.38± 3.624##

Each value represents mean ±SEM of n=6 animals

*** = Significant different than normal control ($p<0.001$)
= Significant different than disease control ($p<0.001$)

NC- Normal Control, PYRI CON- Pyrimethamine Control, DC-Disease Control, DDOXO- Disease treated with Doxorubicin, TP50- Disease treated with 50mg PYR, TP50+S- Disease treated with 50mg PYR+ 1mg Doxorubicin, TP60- Disease treated with 60mg PYR, TP60+S- Disease treated with 60mg PYR+ 1mg Doxorubicin, TP75- Disease treated with 75mg PYR, TP75+S-Disease treated with 75mg PYR+ 1mg Doxorubicin



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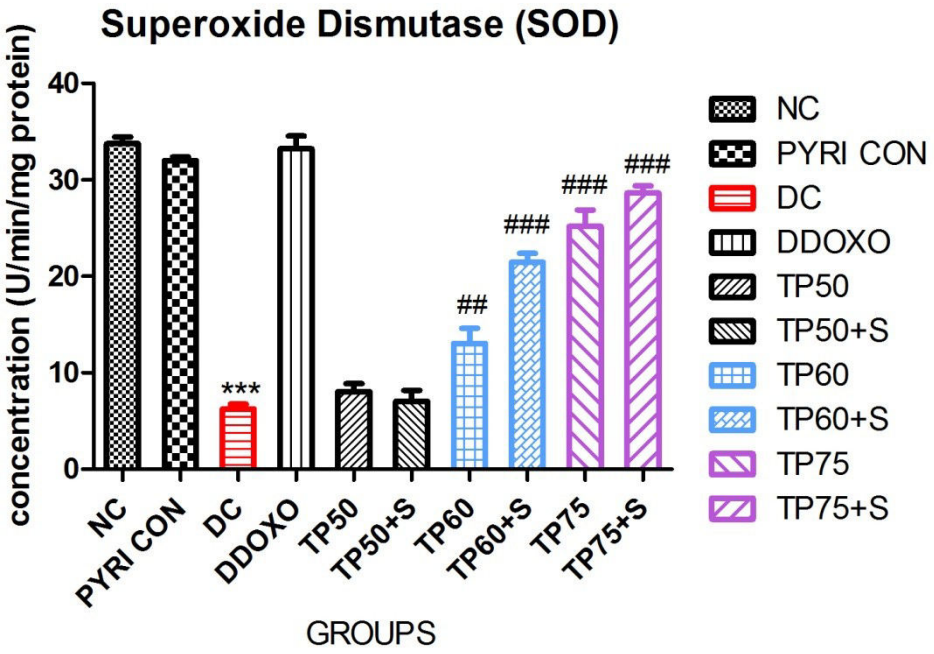
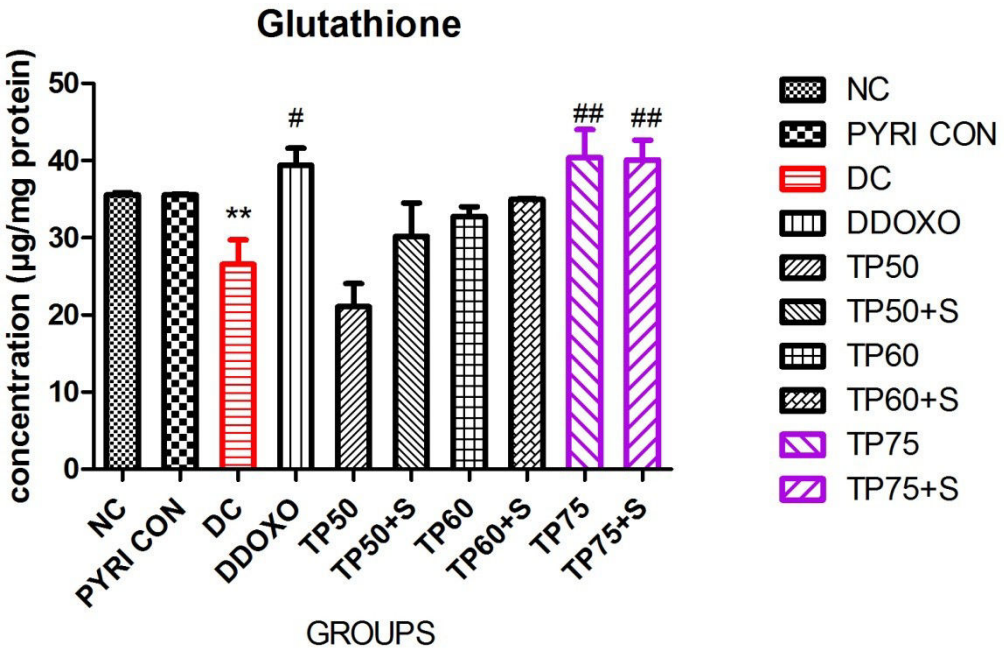
7.5.1 Effect of Pyrimethamine on Malondialdehyde (MDA) levels in MNNG induced gastric cancer in rats

*** = Significant different than normal control ($p<0.001$)
= Significant different than disease control ($p<0.001$)

NC- Normal Control, PYRI CON- Pyrimethamine Control, DC-Disease Control, DDOXO- Disease treated with Doxorubicin, TP50- Disease treated with 50mg PYR, TP50+S- Disease treated with 50mg PYR+ 1mg Doxorubicin, TP60- Disease treated with 60mg PYR, TP60+S- Disease treated with 60mg PYR+ 1mg Doxorubicin, TP75- Disease treated with 75mg PYR, TP75+S-Disease treated with 75mg PYR+ 1mg Doxorubicin.



7.5.2 Effect of Pyrimethamine on Superoxide dismutase (SOD) levels in MNNG induced gastric cancer in rats



7.5.2 Effect of Pyrimethamine on Glutathione (GSH) levels in MNNG induced gastric cancer in rats

7.6 EFFECTS OF INFLAMMATORY MARKERS:

Effect of pyrimethamine on inflammatory markers:

7.6.1 Interleulin-6 (IL-6)

The animals from disease control group exhibited highly significant ($p<0.001$) increased IL-6 level than the animals from normal groups. Pyrimethamine treated animals have significantly ($p<0.001$) decreased IL-6 level than the disease control animals. However, the level of MDA of control treated animals showed no significant difference from normal control and pyrimethamine control treated animals.

7.6.2 Tumour Necrosis Factor-Alpha (TNF- α)

The animals from disease control group exhibited highly significant ($p<0.001$) increased TNF-α level than the animals from normal groups. Pyrimethamine treated animals have significantly ($p<0.001$) decreased TNF- α level than the animals from disease control animals. However, the level of TNF- α of control treated animals showed no significant difference from normal control and pyrimethamine control treated animals.

7.6.3 Interferon Gamma (IFN- γ)

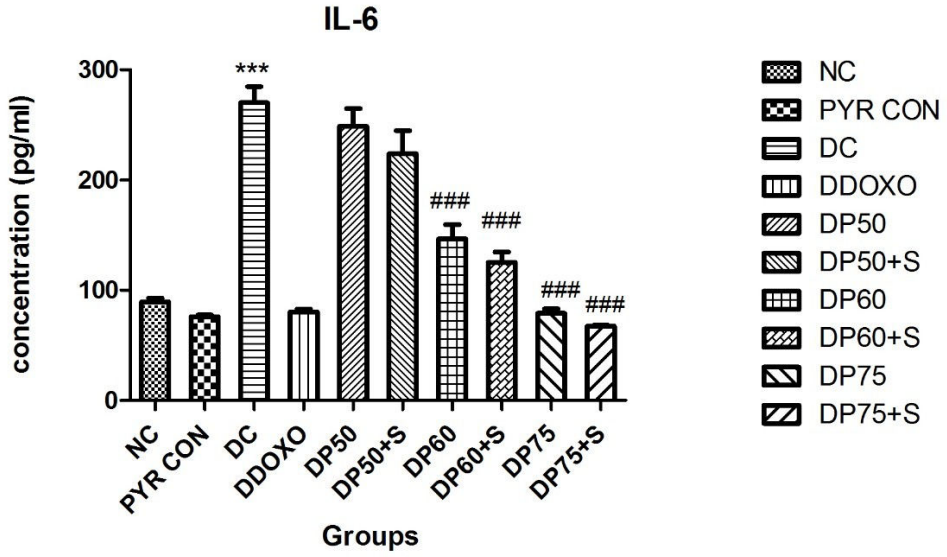
The animals from disease control group exhibited highly significant ($p<0.001$) increased IFN- γ level than the animals from normal groups. Pyrimethamine treated animals have significantly ($p<0.001$) decreased IFN- γ level than the animals from disease control animals. However, the level of IFN- γ of control treated animals showed no significant difference from normal control and pyrimethamine control treated animals.

7.6.4 Interleukin-1 beta (IL-1 β)

The animals from disease control group exhibited highly significant ($p<0.001$) increased IL-1 β level than the animals from normal groups. Pyrimethamine treated animals have significantly ($p<0.001$) decreased IL-1 β level than the animals from disease control animals. However, the level of IL-1 β of control treated animals showed no significant difference from normal control and pyrimethamine control treated animals.

7.6.5 Nuclear Factor Kappa-B (NF- ϰB)

The animals from disease control group exhibited highly significant ($p<0.001$) increased NF- ϰB level than the animals from normal groups. Pyrimethamine treated animals have significantly ($p<0.001$) decreased NF- ϰB level than the animals from disease control animals. However, the level of NF- ϰB of control treated animals showed no significant difference from normal control and pyrimethamine control treated animals.

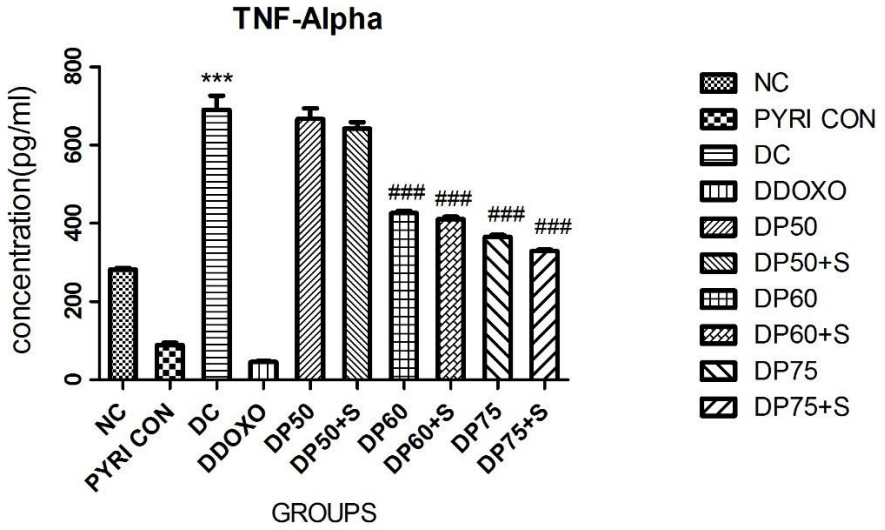


7.6.1 Effect of Pyrimethamine on interleukin-6 (IL-6) levels in MNNG induced gastric cancer in rats

Each value represents mean ±SEM of n=6 animals

*** = Significant different than normal control ($p<0.001$)
= Significant different than disease control ($p<0.001$)

NC- Normal Control, PYRI CON- Pyrimethamine Control, DC-Disease Control, DDOXO- Disease treated with Doxorubicin, TP50- Disease treated with 50mg PYR, TP50+S- Disease treated with 50mg PYR+ 1mg Doxorubicin, TP60- Disease treated with 60mg PYR, TP60+S- Disease treated with 60mg PYR+ 1mg Doxorubicin, TP75- Disease treated with 75mg PYR, TP75+S-Disease treated with 75mg PYR+ 1mg Doxorubicin.

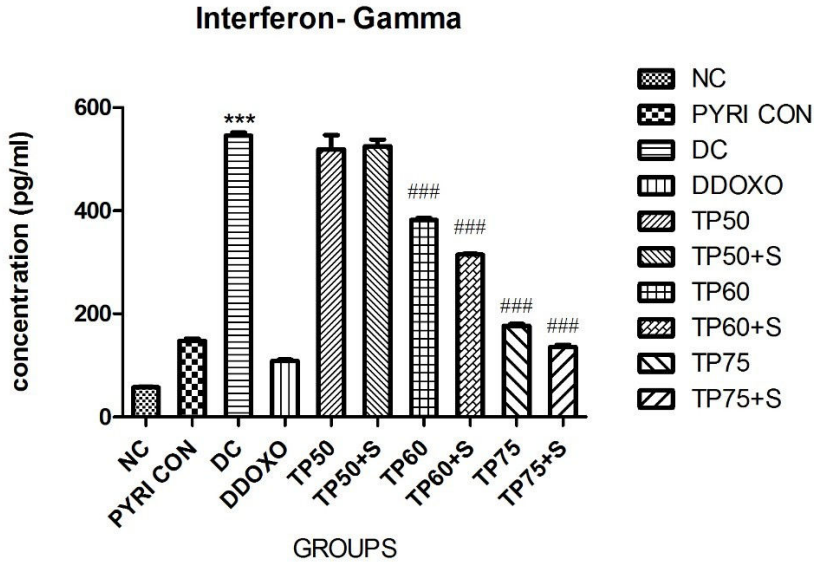


7.6.2 Effect of Pyrimethamine on tumour necrosis factor (TNF- α) levels in MNNG induced gastric cancer in rats

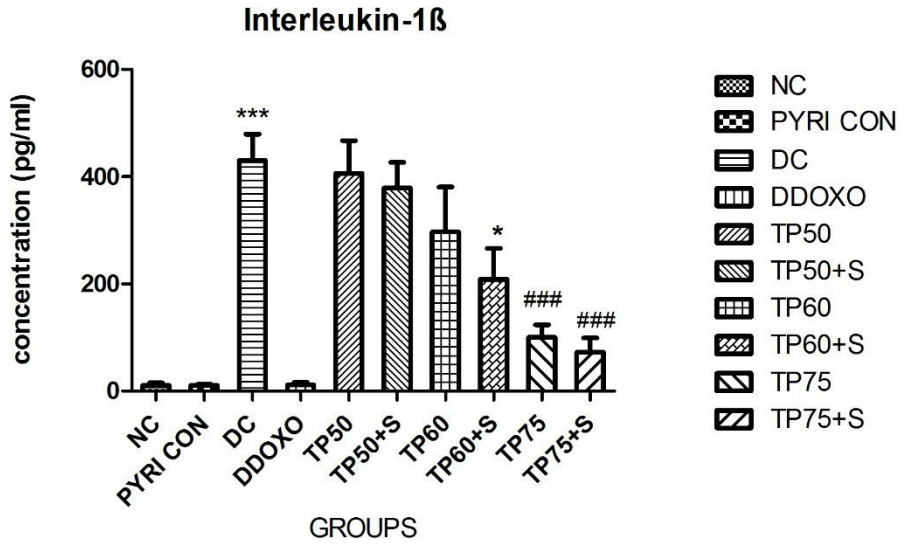
Each value represents mean ±SEM of n=6 animals

*** = Significant different than normal control ($p<0.001$)
= Significant different than disease control ($p<0.001$)

NC- Normal Control, PYRI CON- Pyrimethamine Control, DC-Disease Control, DDOXO- Disease treated with Doxorubicin, TP50- Disease treated with 50mg PYR, TP50+S- Disease treated with 50mg PYR+ 1mg Doxorubicin, TP60- Disease treated with 60mg PYR, TP60+S- Disease treated with 60mg PYR+ 1mg Doxorubicin, TP75- Disease treated with 75mg PYR, TP75+S-Disease treated with 75mg PYR+ 1mg Doxorubicin.



7.6.3 Effect of Pyrimethamine on interferon gamma (IFN- γ**) levels**



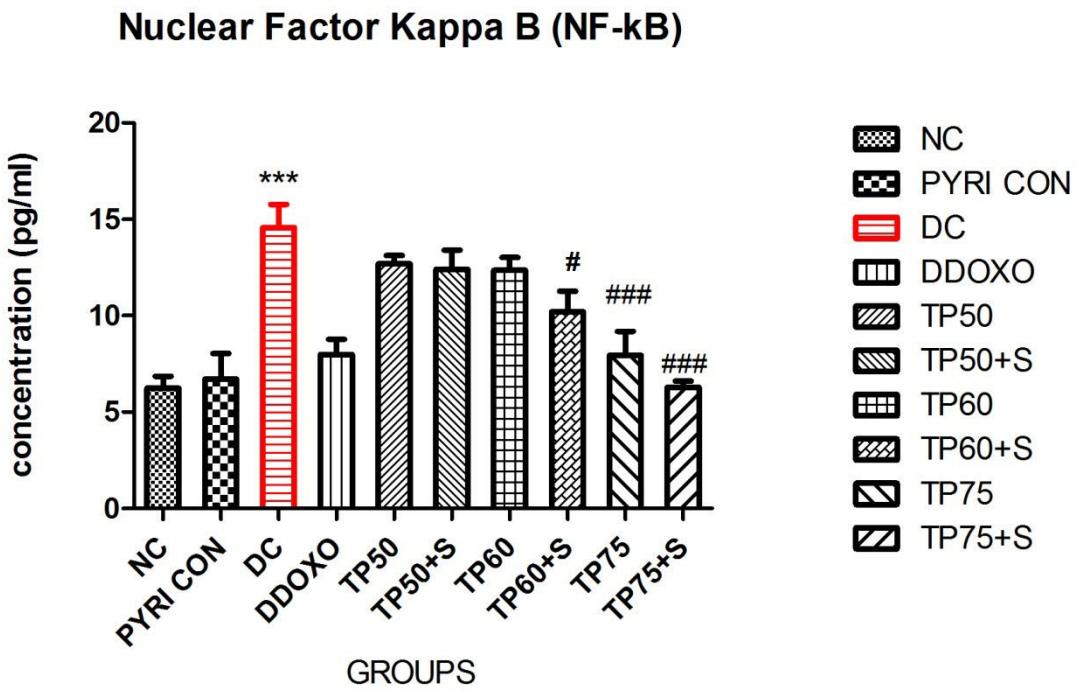
7.6.4 Effect of Pyrimethamine on interferon gamma (IL-1 β**) levels in MNNG induced gastric cancer in rats**

Each value represents mean ±SEM of n=6 animals

*** = Significant different than normal control ($p<0.001$)
= Significant different than disease control ($p<0.001$)

NC- Normal Control, PYRI CON- Pyrimethamine Control, DC-Disease Control, DDOXO- Disease treated with Doxorubicin, TP50- Disease treated with 50mg PYR,

TP50+S- Disease treated with 50mg PYR+ 1mg Doxorubicin, TP60- Disease treated with 60mg PYR, TP60+S- Disease treated with 60mg PYR+ 1mg Doxorubicin, TP75- Disease treated with 75mg PYR, TP75+S-Disease treated with 75mg PYR+ 1mg Doxorubicin.



7.6.5 Effect of pyrimethamine on nuclear factor kappa B (NF- ϰB**) levels in MNNG induced gastric cancer in rats**

Each value represents mean ±SEM of n=6 animals

*** = Significant different than normal control ($p<0.001$)
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NC- Normal Control, PYRI CON- Pyrimethamine Control, DC-Disease Control, DDOXO- Disease treated with Doxorubicin, TP50- Disease treated with 50mg PYR, TP50+S- Disease treated with 50mg PYR+ 1mg Doxorubicin, TP60- Disease treated with 60mg PYR, TP60+S- Disease treated with 60mg PYR+ 1mg Doxorubicin, TP75- Disease treated with 75mg PYR, TP75+S-Disease treated with 75mg PYR+ 1mg Doxorubicin.

7.6.6 Effect of Pyrimethamine on several inflammatory pathways

GROUPS	NC	PYRI CON	DC	DDOXO	TP50	TP50+S	TP60	TP60+S	TP75
IL-6	89.43 ± 3.481	75.78 ± 3.055	270± 14.84***	80.27± 2.581	248.8 ± 16.10	224.8± 20.97	146.72 ± 12.89	125.2± 9.36	79.24± 3.817
TNF- α	282.1 ± 4.439	89.93 ± 5.997	690.3± 35.77***	45.46± 2.875	667.2 ± 27.18	426.4± 5.072	233.05 ± 4.562##	411.0± 6.271###	365± 5.241###
IFN- γ	57.19 ± 1.534	148± 3.990	545.7± 6.165***	108.7± 2.449	519.3 ± 27.25	525.4± 13.06	382.4± 3.784##	314.9± 2.030##	176.9± 3.239###
IL-1 β	10.96 ± 4.155	10.72 ± 2.034	430± 49.25***	11.92± 4.411	406.3 ± 60.69	378.9± 48.01	295.5± 84.13###	209± 57.33###	100± 23.66###
NF- ϰB	6.238 ± 0.6267	6.705 ± 1.331	14.56± 1.220***	7.986± 0.7836	12.70 ± 0.4218	12.41± 0.9931	12.35± 0.6675	10.22± 1.063##	7.939± 1.259###

Each value represents mean ±SEM of n=6 animals

*** = Significant different than normal control ($p<0.001$)
= Significant different than disease control ($p<0.001$)

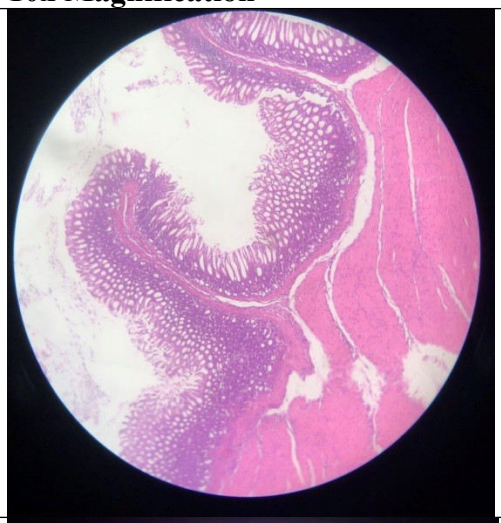
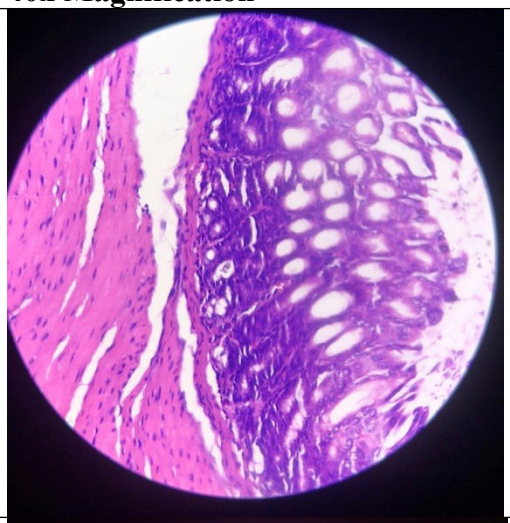
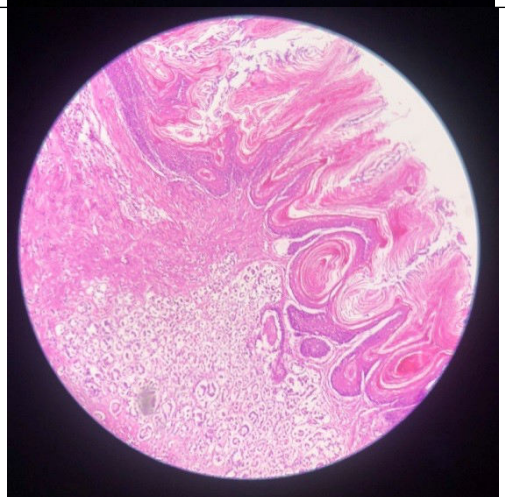
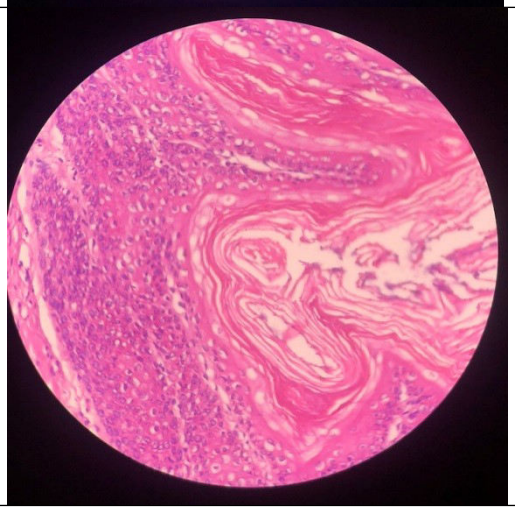
NC- Normal Control, PYRI CON- Pyrimethamine Control, DC-Disease Control, DDOXO- Disease treated with Doxorubicin, TP50- Disease treated with 50mg PYR, TP50+S- Disease treated with 50mg PYR+ 1mg Doxorubicin, TP60- Disease treated with 60mg PYR, TP60+S- Disease treated with 60mg PYR+ 1mg Doxorubicin, TP75- Disease treated with 75mg PYR, TP75+S-Disease treated with 75mg PYR+ 1mg Doxorubicin.

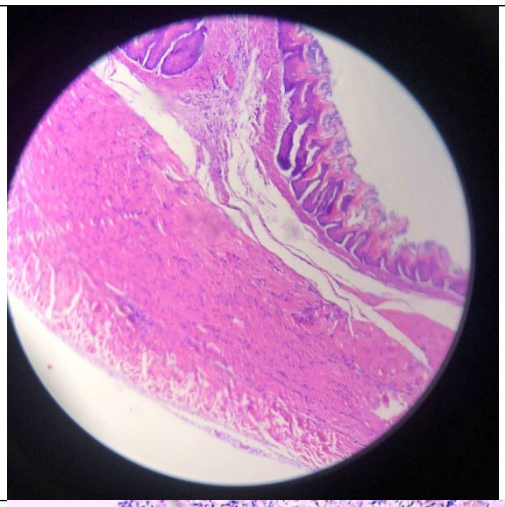
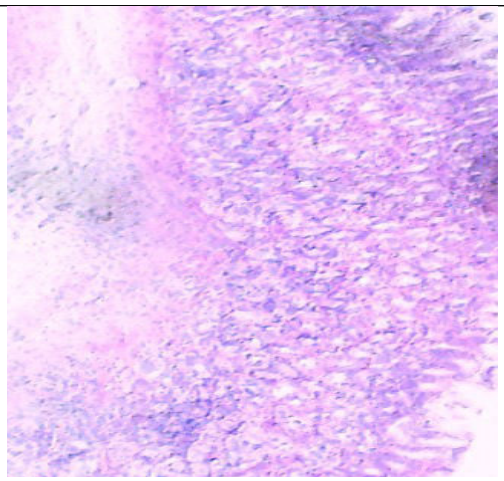
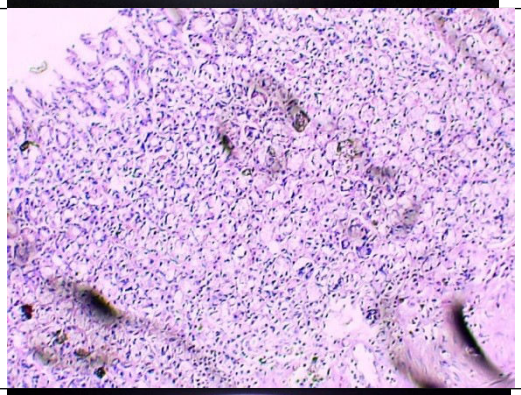
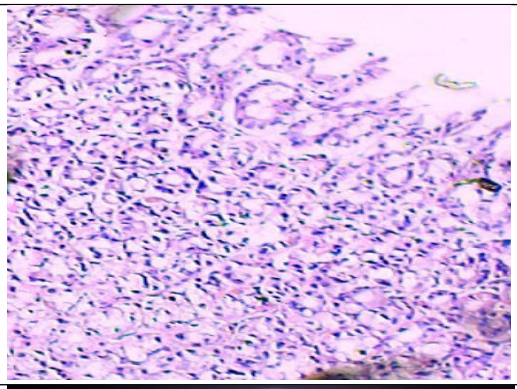
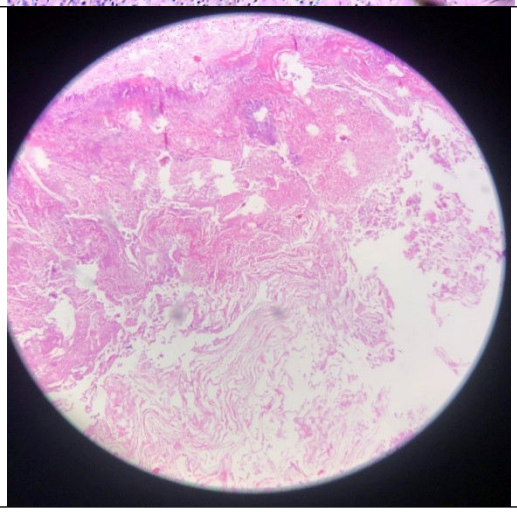
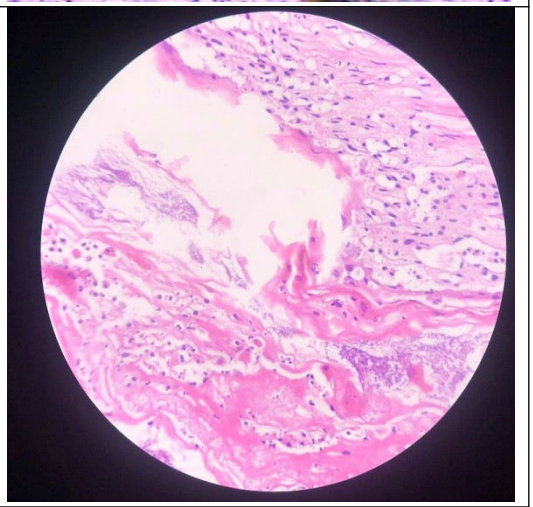
7.7 HISTOPATHOLOGICAL STUDY

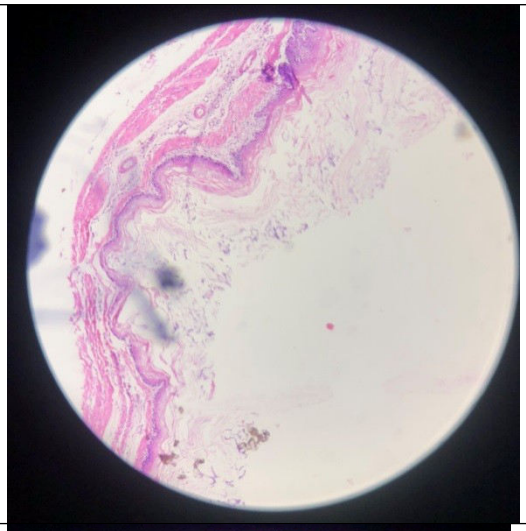
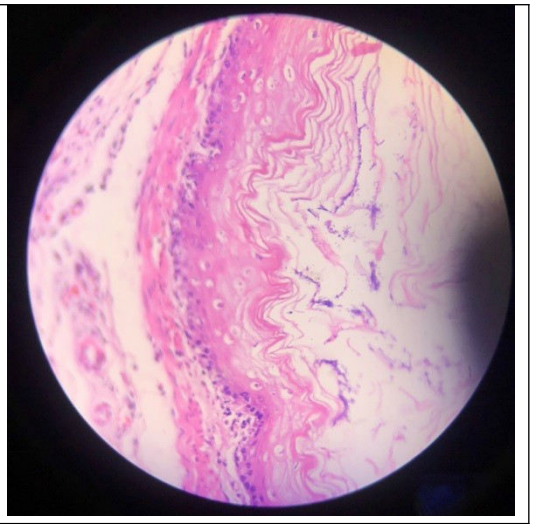
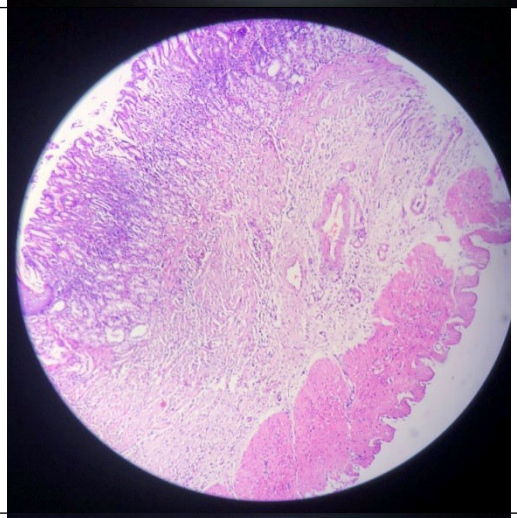
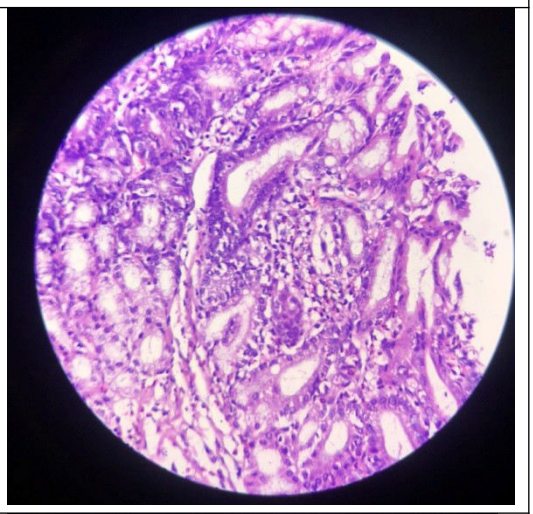
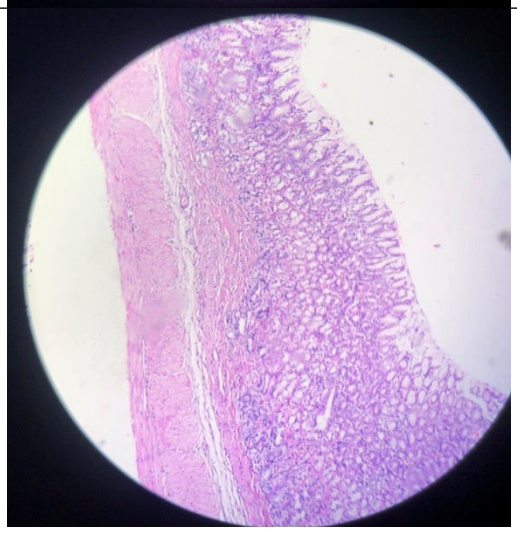
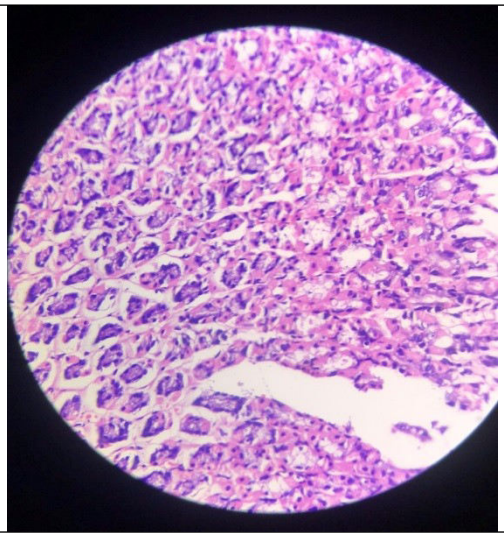
Figure depicts the Hematoxylin and eosin (HE) staining of stomach tissues from different groups of animals.

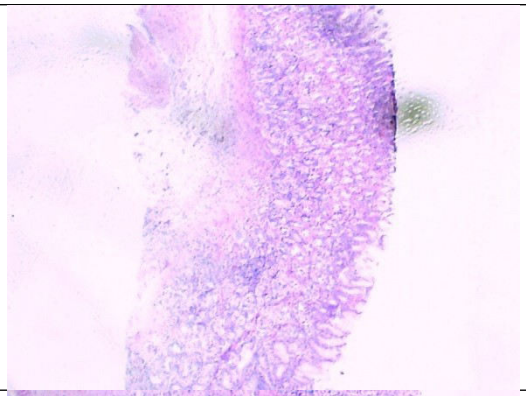
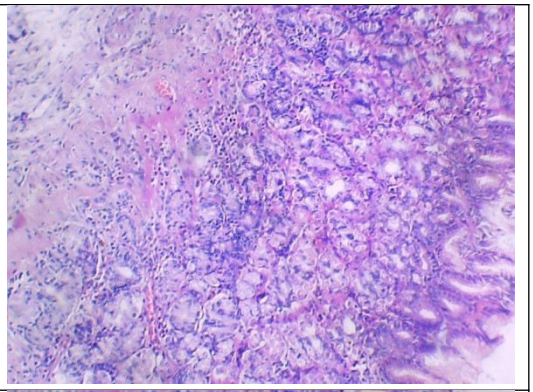
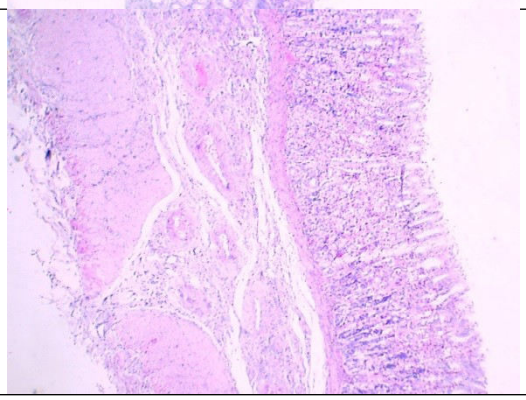
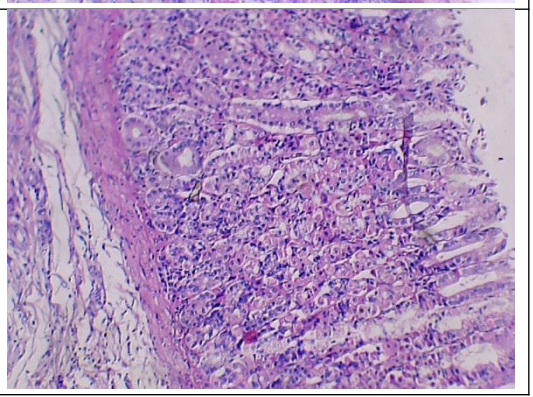
FIG A, D, G, I and J represents Intact lining of the outer-epithelium, followed by mucosal and sub-mucosal layer i.e. muscularis propria and serosa and sub-serosa layer in 10X and 40X magnification.

FIG B, E, F, G, H represents The lining of the outer epithelium is ruptured tumour invaded in the gastric epithelium with squamous cell carcinoma infiltrating lamina propria in 10X and 40X magnification.

	HISTOLOGY OF STOMACH	
	10x Magnification	**40x Magnification**
NC **FIG. A**		
DC **FIG. B**		

PYRI CON FIG. C		
DDOXO FIG. D		
TP50 FIG. E		

TP50+S **FIG. F**		
TP60 **FIG. G**		
TP60+S **FIG. H**		

TP75 **FIG. I**		
TP75+S **FIG. J**		

7.8 IMMUNOHISTOCHEMISTRY

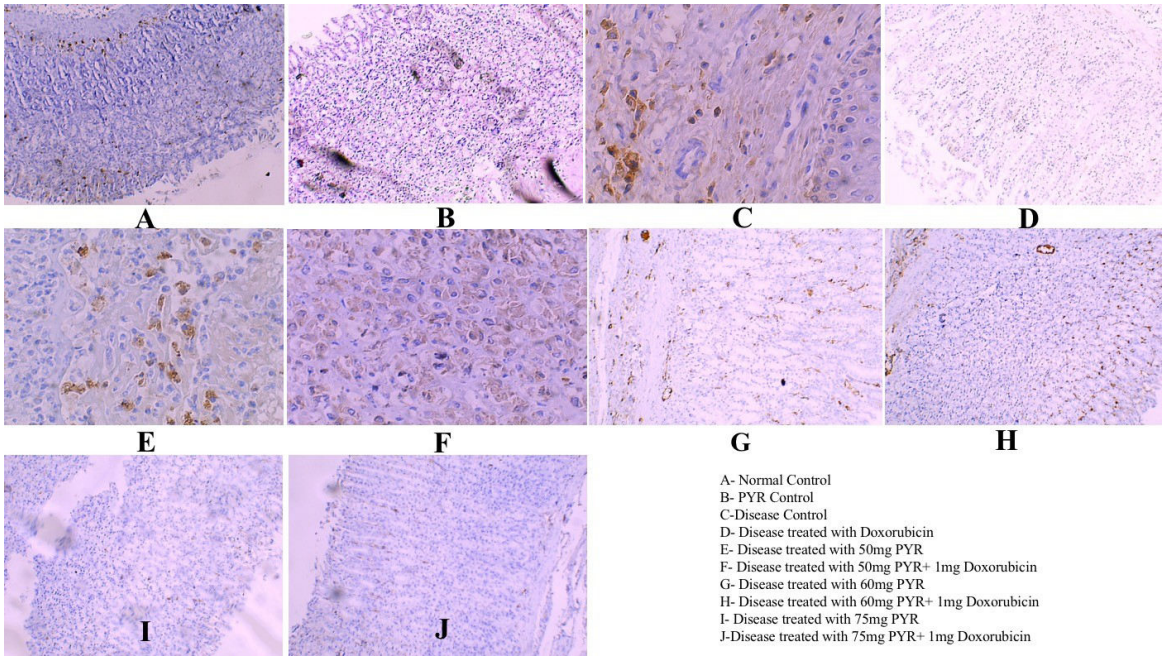
Immunohistochemistry of BCL-2 was performed; BCL-2 is an anti or pro-apoptotic gene which mediates the process of apoptosis. DAB dye stains the apoptotic protein and tissues were counterstain with Hematoxylin and eosin.

Representative images of expression of BCL-2 (brown) in stomach tissues from different animal groups in 40X magnification**.**

FIG A, D, I and J represents normal expression of BCL-2 with reduced staining of brown colour.

FIG B, E, F, represents over expression of BCL-2 with increased staining of brown colour.

However, FIG G, H showed negligible expression of BCL-2.



7.9 mRNA expression study

Figure 7.9 is representative image of mRNA expression study of TP53. mRNA expression study of TP53 showed that there was significantly ($p<0.05$) reduced expression of TP53 in disease animals compared with normal control animals. There was significantly ($p<0.001$) higher expression of TP53 in disease treated animals with doxorubicin, 75 mg pyrimethamine, 75 mg pyrimethamine in combination with doxorubicin, 60 mg pyrimethamine and 60 mg pyrimethamine in combination with doxorubicin comared with disease control animals. 50 mg dose pyrimethamine treated animals did not significantly increase expression of TP53.

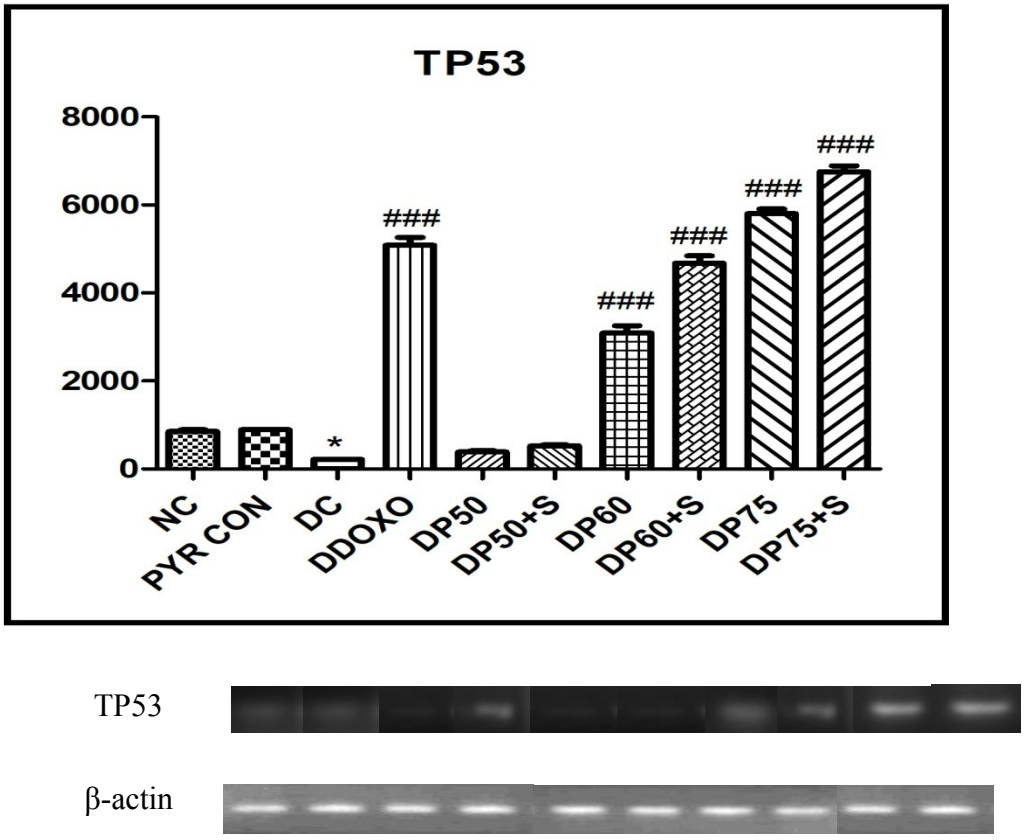


Figure 7.9 Effect of pyrimethamine on mRNA expression of TP53

CHAPTER-8

DISCUSSION

8. DISCUSSION

Gastric cancer is the fourth most common malignancy worldwide and remains the second cause of death amongst all malignancies worldwide. According to Global Cancer Statistics 2018 (GLOBOCON). Gastric carcinoma (GC) is the fourth most common malignancy worldwide (989,600 new cases per year in 2008) and remains the second cause of death (738,000 deaths annually) of all malignancies worldwide. (GLOBOCON website). Drug re-purposing (identifying new mechanism of the old drugs) has recently gained substantial attention which may be a promising strategy for the development of new anti-cancer agents (Bertolini, et al., 2015). Thus, the drugs which are originally identified as analgesic, anti-diabetic, anti-epileptic, anti-hypertensive and anti-malarial have been evaluated for their anti-cancer activity and probable mechanism of action (Gupta, et al., 2013). From the previous described class of drugs, anti-malarial agents are now reviewed as an emerging anti-cancer agents.

Here we present pyrimethamine (PYR), an FDA approved anti-microbial drug (Takakura, et al., 2011) as a promising drug for repurposing in cancer therapeutics. Our reports suggests that pyrimethamine (2,4-diamino-5-p-chlorophenyl-6-ethyl-pyrimidine) have a potent effect on reduction of tumour related parameters, efficient anti-oxidant and anti-inflammatory activity. Recently reports suggested that pyrimethamine showed a dual role as anti-tumour proliferation and metastasis via acting on dihydrofolate reductase pathway (DHFR) in lung cancer cell lines as well as in-vivo studies in C57BL/6J mice model (Liu, et al., 2019). pyrimethamine controlled tumour growth in melanoma by conducting *in-vitro* and *in-vitro* studies (Tommasino et al., 2016). Anti-tumour effects of pyrimethamine were observed *in-vitro* and *in-vitro* via acting on cathepsin B-dependent and caspase dependent apoptotic pathway in pituitary adenomas (Dai, et al., 2013). Pyrimethamine has shown potent effect in various cancers including breast cancer, small cell lung carcinoma, adrenocortical cancer, melanoma, prostate cancer and invasive pituitary adenomas. Our research suggested that pyrimethamine acts as an anticancer agent in gastric cancer through an anti-apoptotic and anti-proliferative mechanism by carrying out *in-vitro* and *in-vivo* studies.

In-silico docking is one of the major part of drug development process. Recently anti-malarials were docked to check their potential activity as anti-cancer agents. The distinct

class of anti-malarial agents were screened as well as their efficacy was checked on 91 human cancer cell lines. The results of docking and in-vitro studies depicted that pyrimethamine, a dihydrofolate reductase had a potential to become an anti-cancer agent (Van, et al., 2013). Caspases, a family of cysteine proteases which are involved in cell death often termed as apoptosis. They are present as inactive zymogens in cells and are activated at onset of apoptosis via undergoing cascade of catalytic activation. Caspase-3 an effector caspase induces apoptosis. Thus, the docking of the anti-malarial and doxorubicin was docked against caspase-3 protein. The GOLD suite software was used for the docking. Though, the GOLD score of the targeted molecule was lower than chloroquine, quinine, and sulfadoxine, but the amino acid necessary for the interaction and inhibition of the caspase-3 was present in the pyrimethamine.

Dose fixation studies was performed to fix the dose of the induction compound N-methyl-N-nitro-N-nitrosoguanidine (MNNG). A pilot study carried out with three different doses of MNNG to obtain least mortality and prominent carcinogenic changes. The reported dose of MNNG in literature review was found to be 200mg/kg (Sukumaran, et al., 2016) where 71.1% mortality was encountered with-in a day. Thus, 50mg/kg, 100mg/kg and 150mg/kg doses were checked. The 50mg/kg dose was found to be safe but there were no solid tumours found as only dysplastic changes had occurred. 100mg/kg dose of MNNG was selected as squamous cell carcinoma along with solid tumours was confirmed. There was equal mortality obtained in 150mg/kg group animals to that of 200mg/kg. As well three different doses (50, 60 and 75mg/kg) of pyrimethamine was checked alone as well as in combination with the standard chemotherapeutic drug doxorubicin (1mg/kg).

The MTT assay has been widely used to calculate the cell viability. The enzymatic reduction of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to MTT-formazan. This is catalysed by mitochondrial succinate dehydrogenase. Thus, this assay is dependent on mitochondrial respiration which indirectly quantifies the cellular energy capacity of a cell. The MTT assay is a colorimetric reaction which measures the cell monolayers that have been plated in 96-well plates (CHACON, et al., 1996). The anti-proliferative activity of pyrimethamine was confirmed by carrying out *in-vitro* study on AGS cell lines. Treatment with pyrimethamine showed a dose dependent reduction in cell viability on AGS cell lines. The IC50 of pyrimethamine was calculated at 24 hours

which was found to be 91.1 μM. As well as the IC50 of Doxorubicin was found to be 4.5 μM at 24 hours. Though, the potency of pyrimethamine was low but has lesser side effects than the standard which is used as anti-cancer drug in chemotherapy.

In the present study, gastric cancer was induced by N-methyl-N-nitro-N-nitrosoguanidine (MNNG), which mimics human gastric cancer by having various changes in the glandular as well as forestomach region including, erosive lesions, proliferation of pyloric mucosa leading to differentiated and un-differentiated stomach carcinoma with histological changes (Tsukamoto, et al., 2007). p21 protein is coded by p21 gene which repairs the DNA damage via inhibiting mismatch repair (MMP). MNNG causes alkylation, rapid degradation of p21 and reduction in recruitment of MMR protein to chromatin MNNG causes imbalance between the MMR protein which leads to imbalance in the pro and anti-apoptotic protein (Jascur, et al., 2011). Upregulation of STAT3 plays a major role in tumorigenesis in gastric cancer (Johnston, et al., 2011). Thus the inhibition of STAT3 pathway lead to the anti-tumour efficacy which subsequently causes apoptosis on tumour cells.

Current studies showed that MNNG induced gastric cancer depicted 100% tumour incidence, increased tumour volume and tumour load. Treatment with pyrimethamine 75mg alone and combination with doxorubicin (standard chemotherapeutic drug) significantly reduced ($p<0.001$) tumour volume, tumour burden and % tumour incidence rate than that of disease control animals via acting on apoptotic cell death mechanisms. However, no significant results were obtained in animals treated with the minimum dose of pyrimethamine i.e. 50mg/kg as well as pyrimethamine in combination with 1mg/kg doxorubicin. Pyrimethamine treated animals reduced the tumour growth and tumour volume in lung cancer malignancy (Liu, et al., 2019). Reportedly pyrimethamine tends to inhibit in-vivo metastatic cancer cells as well as cancer invasion in BALB/c TUMO transplant breast cancer murine mice model (Khan, et al., 2018). Anti-tumour effects of pyrimethamine was observed via reduction in tumour growth, tumour volume and weight in severely compromised immune deficient (SCID) mouse model of in metastatic melanoma (Tommasino, et al., 2016).

There are evidences indicating pyrimethamine to acting via cathepsin and caspase dependent pathway in pituitary adenoma cell which lead to suppression of tumour growth

and tumour size and significantly increases the survival rate of female NOD/ SCID mice (Dai, et al., 2013).

Carcinoembryonic Antigen (CEA) is a glycoprotein which is attached to the surface of the enterocytes and play a role in cell adhesion and programmed cell death (Căinap, et al., 2015). CEA levels are also used to detect liver metastasis and locoregional reoccurrence of cancer with the sensitivity of 100% and 60% respectively (Fletcher 1986, Pietra et al. 1998). CEA also plays a criticalrole in cancer metastasis and tumou r invasion due to its role in the regulation of the innate immune system and signal transduction mediator (Hammarström and Baranov, 2001, Li et al. 2010). CEA levels predicts cancer stage, progression and recurrence of gastric cancer (Maehara, et al., 1990). There are evidences of increased in the levels of CEA in gastric cancer, which plays an important role in the diagnosis as well as recurrence of the disease (Sun and Zhang, 2014). CEA levels can also be used as prognostic and diagnostic values in early and advanced gastric cancer (Feng, et al., 2017). There are reports which depicts increased levels of CEA in MNNG induced gastric cancer (Burkitt, et al., 2017). However, CEA is the most common marker used for the prognosis of gastric cancer (Căinap, et al., 2015). Our study shows elevated levels of CEA in disease control group in comparison with the normal control group. Treatment with two doses of pyrimethamine i.e. 75mg/kg and 60mg/kg alone and combination with doxorubicin (standard chemotherapeutic drug) significantly reduced ($p<0.001$) significant decrease in the level of CEA except the dose of 50mg/kg and combination.

MNNG induces adenocarcinoma in the glandular stomach of BALB/c and C3H mice when given orally in drinking water (Tatematsu et al., 1992). MNNG usually develops well differentiated adenocarcinoma in the glandular stomach. There are reports of high incidences of squamous cell carcinoma in the forestomach by MNNG induced gastric cancer (Tatematsu et al., 1975). Schoental et al., treated rats with MNNG using a stomach tube to induce formation of squamous cell carcinoma in the rat forestomach. Additionally, Sugimura and Fujimura generated pyloric adenocarcinomas with high frequency by administering MNNG orally to rats in drinking water (Sugimura and Fujimura, 1967). MNNG causes cancer when the animals are exposed to it on repeated and daily basis (Yu, et al., 2014). In the present study histopathological studies proved the carcinogenic

changes in the disease control group in comparison with the normal control group. Hematoxylin and eosin staining of gastric tissue depicted that MNNG induced group of animals showed well differentiated squamous cell carcinoma which has invaded to the muscularis propria. Treatment with pyrimethamine and combination significantly ($p<0.001$) reduced the dyplastic and hyperplastic changes, invasion of the tumour cells in the gastric epithelium. However, no significant effects were obtained in disease control group treated with 50mg/kg and in combination with doxorubicin dose.

The generation of oxidative stress is associated with the initiation, promotion and progression of several tumors (Valko et al. 2007). The body is constantly exposed to free radicals and other reactive oxygen species (ROS) either from external environment (pollution, sunlight and other forms of radiation) or from endogenously sources. There are evidences that oxidative stress can lead to cancer (Ames, et al., 1995). Several reports suggest that gastrointestinal tract is a major site for oxidant production as well as a source of antioxidants. Imbalance in the oxidative stress can modulate the apoptotic program (Tamimi, et al., 2002) and could lead to gastric cancer (Sihvo, et al., 2002). MNNG causes significant increase in the levels of ROS. In animal groups treated with MNNG showed significant increase in levels of MDA whereas, enzymatic (GSH and SOD) anti-oxidants were reduced (Luo and Wu, 2011). Malondialdehyde (MDA), is an oxidative stress marker also responsible for lipid peroxidation it is reactive as well as potential mutagenic (Hartman,1983). MNNG treated rats showed significant increase in the lipid peroxidase (Tandon, et al., 2004). Decreased enzymatic levels of superoxide dismutase (SOD) and Glutathione (GSH) are associated with squamous cell carcinoma (Miranda, et al., 2000). However, treatment with pyrimethamine significantly decreases in the ROS levels in hepatocellular carcinoma (HCC) (Jang, et al., 2016). In the present study, MNNG caused significant increase in the levels of MDA and significant decrease in enzymatic anti-oxidants (GSH and SOD) in disease control group to that of in normal animals. Treatment with pyrimethamine caused significant ($p<0.001$) elevation in the enzymatic anti-oxidants and reduction in MDA levels. However, treatment with 50mg/kg and in combination had no significant effect on either parameter.

Chronic inflammation is associated with several processes that contributes to the onset as well as progression of tumour (Il'yasova, et al., 2005). Interleukin-6 (IL-6) is one of the

major inflammatory cytokine in tumour microenvironment which promotes the tumorigenesis, tumour survival, angiogenesis, invasiveness and metastasis (Das and Bhatt, 2016). tumour necrosis factor-alpha (TNF-a) is a multifactorial cytokine has diverse role in cellular events including survival, proliferation, differentiation and death which may be involved in inflammation associated carcinogenesis (Wang, and Lin, 2008), Interferon-gamma (IFNγ) is antitumor cytokine which controls tumour initiation and progression by shaping tumour immunogenicity. This promotes the outgrowth of tumour cells which has immunoevasive properties (Street, et al., 2001), Interleukin-1beta (IL1B*)* is associated with tumour metasis and growth which leads to tumour progression (Apte, et al., 2006) and nuclear factor kappa B (NF-κ B). Activation of NF-κB transcription factors has been associated with tumorigeneses, comprising of promoting cancer-cell proliferation and prevention of apoptosis (Park and Hong, 2016). There are reports which states that, increase in the elevation of these inflammatory markers is associated with gastric cancer. MNNG induced gastric carcinoma significantly increased the levels of IL-6 and TNF-alpha (Luo and Wu, 2011; Ma et al., 2017). The activation and elevation of IFNγ leads to subsequent activation of NF-κB, which leads to cancer cell proliferation and metasis in MNNG induced gastric cancer (Xu, et al., 2018). *Artemisia annua* significantly reduced the levels of TNF-α, IL-1β and IL-6 by acting on toll-like receptor-4 (TLR4) in alcohol induce liver injury in mice (Zhao, et al., 2017). Pyrimethamine significantly reduced the levels of IL-6 in metastatic lung cancer cell lines in-vitro as well in C57BL/6J mice in-vivo (Liu, et al., 2019) There was significant increase in the inflammatory levels in disease control animals in comparison to that of the normal animals. Treatment with pyrimethamine and combination with standard significantly ($p<0.001$) reduced the levels of the inflammatory markers. Although no significant effects were obtained in 50mg/kg alone and in combination with standard.

Apoptosis is controlled cell suicide program which plays a major role in tissue homeostasis by eliminating the defective and unnecessary cells (Kerr et al., 1972 Raff, 1998). Dysregulation of apoptotic signaling can contribute to the various human malignancies as well as tumor pathogenesis (Plati, etal., 2008). Bcl-2 is an anti-apoptotic protein, localized in intracellular membranes including nuclear membrane, endoplasmic reticulum and outer mitochondrial membrane (Antonsson, 2001). It regulates caspase activity, ion channels, localization of cytochrome c, and has an anti-apoptotic function

(Karam, et al., 2007). Bcl-2 expression is reported in various epithelial malignant tumors including gastric cancer (Erkan, et al., 2012). Bcl-2 is over expressed in early gastric tumorigenesis (Anagnostopoulos, et al., 2005). The immunoreactivity of Bcl-2 was significantly associated with differentiation in gastric carcinoma. MNNG treated rats causes over expression of Bcl-2 protein in gastric mucosa (Dinparast-Djadid, et al., 2015). The levels of anti-apoptotic protein Bcl-2 were found significantly in disease control group in comparison to the groups treated with PYR alone as well as in combination with standards.

Tumor suppressor gene (TP53) plays a major role in induction of apoptosis. The apoptosis process may be due to other cell activities including cell stress and effects of other p53 mediators. P53 follows the extrinsic death receptor pathway of apoptosis via activating the caspase cascade as well as the intrinsic mitochondrial pathways which maintains the balance between pro-apoptotic and apoptosis inducing BCL-2 family. Thus, checking the mRNA levels (transcription levels) of TP53 gene can validate the imbalance between the apoptosis process in cancer. The level of TP53 was significantly low in disease control group leading to reduction in its gene expression. However, the expression of TP53 was significantly high in groups treated with PYR 75mg dose as well as in combination with standard.

CHAPTER-9
CONCLUSION

- The present study suggested that pyrimethamine exhibits apoptotic effects in gastric cancer. In addition to that it acts as anti-proliferative, anti-oxidant and reduces inflammation in gastric carcinogenesis. The gene study suggests that TP53 expression is important to induce apoptosis in gastric cancer. Our drug shows high expression of TP53. Thus, our study suggests that pyrimethamine may be given as adjuvant therapy with doxorubicin to have better therapeutic effects.

CHAPTER-9
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9. REFERENCES

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CHAPTER-4

IN-SILICO DOCKING STUDIES

4. IN-SILICO DOCKING STUDIES

4.1 MOLECULAR DOCKING:

Molecular docking refers to an automated computed algorithm which determines the active binding site of the protein and how the compound will bind to it. Molecular docking validates the interaction of one molecule to other molecules and predicts the scoring as well as the formation of a stable complex. This approach can be used to signify the interaction between a protein and small molecule at atomic level which allows us to characterize the protein and molecule binding and annotate basic biochemical process. The binding of the ligand to its target protein or receptor is characterized by generating different orientations and conformations selecting the most fitted in the receptor. The basic mechanism behind the molecular docking is to review the binding affinity to the active site of a receptor. The scoring of docking may be validated as binding energy, qualitative numerical value or free energy. Thus, this provides an information whether a specific compound will act as an inhibitor of that protein. Thus, using a computational tool will provide an information whether a compound or range of compounds will give biological activities. There are docking programs which are available and GOLD is the most widely used in computational drug design. GOLD is a docking program developed by collaboration between the University of Sheffield, GlaxoSmithKline, and the Cambridge Crystallographic Data Centre. It uses a fast genetic algorithm for protein–ligand docking. The algorithm can allow ligand flexibility and a measure of active site flexibility. The GoldScore, ChemScore, and ASP scoring functions come with it, and it has the ability to plug in a user defined scoring algorithm (Meng, Xuan-Yu, et al., 2011).

4.2 CASPASES

Caspases, a family of cysteine proteases which are involved in cell death often termed as apoptosis. They are present as inactive zymogens in cells and are activated at onset of apoptosis via undergoing cascade of catalytic activation. The activated caspases act as inhibitor-of-apoptosis (IAP) family of proteins. The caspases are divided into two types 1) initiator caspases e.g. caspase-9 and the effector caspases e.g. caspase-3 and 7 (Shi, 2004). An effector caspase contains 20-30 residues in its prodomain sequence whereas an initiator caspase consists an extended N-terminal prodomain (>90 amino acids which are

crucial for its functionality. The initiator caspase undergoes internal cleavage to separate the small and large subunits which causes the activation of the effector caspases. However, under apoptotic conditions the initiator caspase are auto-activated. These are the processes which are acquired and performed through many components and complex pathways (Adams and Cory, 2002; Shi, 2002) e.g. the apoptosome is responsible for the activation of caspase-9 (Rodriguez and Lazebnik, 1999).

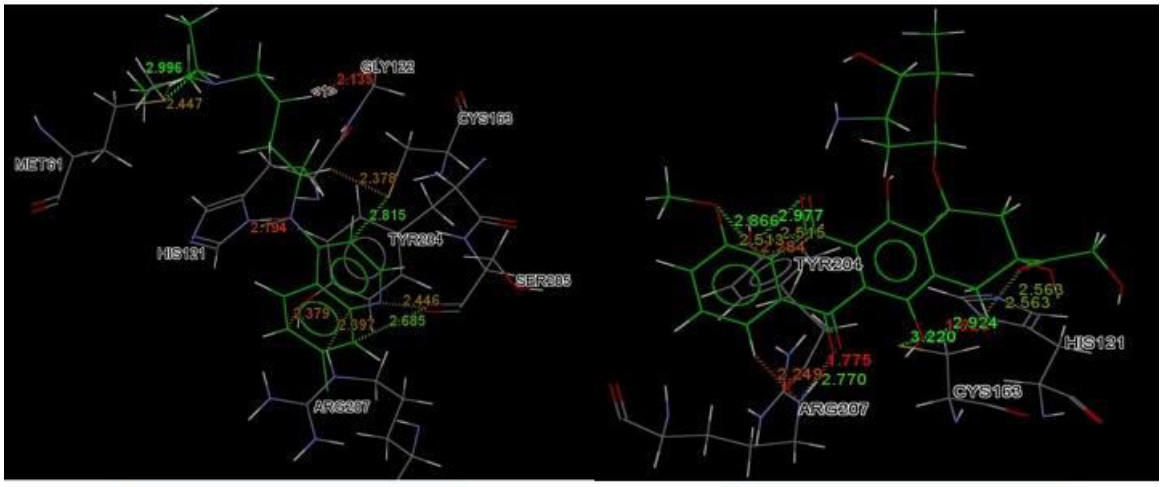
Name of drug	**Gold score**	**Amino acid interaction**
Chloroquine	55.39	MET61, HIS121, GLY122, CYS163, TYR204, SER205, ARG207
Doxorubicin	52.71	**HIS121**, **CYS163, TRY204**, ARG207
Sulfadoxine	50.87	HIS121, GLY122, CYS163, TRY204, ARG207
Quinine	45.26	MET61, HIS121, GLY122, ARG207
Pyrimethamine	42.43	**HIS121, CYS163,TYR204**
Proguanil	40.13	HIS121, SER205, TRP206

DOCKING AND GOLD SCORE:

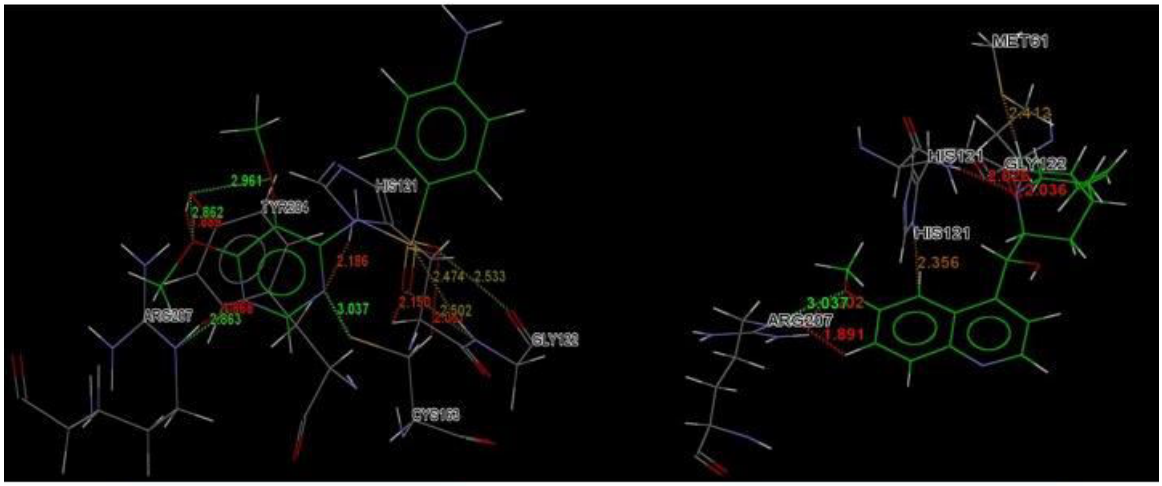
Anti-malarial of each class was taken for the docking at caspase-3 (the effector caspase). The reported amino-acid for the inhibition of the protein which cleaves the caspase-3 are Tyr204, Arg207, Phe256, Trp206, Cys163, Gly122, Gly165, Met61, His121 and Ser120. Out of the 10 reported amino acids **His121, Tyr204** and **Cys163** are important amino-acids responsible for the inhibition of the specific protein (Ganesan, et al., 2011). Caspase-3, the effector caspase was taken for docking of standard drug (doxorubicin) and the other classes of anti-malarial agents. Chloroquine from class 4-aminoquinolines was docked to caspase-3 and amino-acids interaction which were obtained are MET61, HIS121, GLY122, CYS163, TYR204, SER205, ARG207 with gold score of 55.39, Doxorubicin an anti-cancer drug belonging to the class of anthracycline used as first-line chemotherapeutic agent in many of the cancer was taken as standard in the treatment of

gastric cancer (Rivankar, 2014). The important amino-acid interactions were HIS121, CYS163, TRY204, ARG207 with gold score of 52.71. Sulfadoxine is a sulphonamide derivative which inhibits the activity of dihydopteroate synthase (DHPS) (Yaro, 2009). The important amino-acid interaction found in docking were HIS121, GLY122, CYS163, TRY204, ARG207 with a docking score of 50.87. Quinine is the oldest and most effective anti-antimalarial agent. Quinine is a bark component of cinchona (quina-quine) tree (Achan et al., 2011). The amino-acid interactions obtained were MET61, HIS121, GLY122, ARG207 with gold score of 45.26. Pyrimethamine belonging to the class of di-aminopyrimidines has shown promising anti-cancer potential in various cancers. All the three amino acids interaction important for the caspase inhibition were found in pyrimethamine with a gold score of 42.43. The gold score of pyrimethamine is less than quinine but the amino-acid interaction was prominent in pyrimethamine than quinine. Proguanil a folic-acid antagonist (Garbis et al., 2007) was docked with the effector caspase, HIS121, SER205, TRP206 were reported amino-acid interaction with a gold score of 40.13.

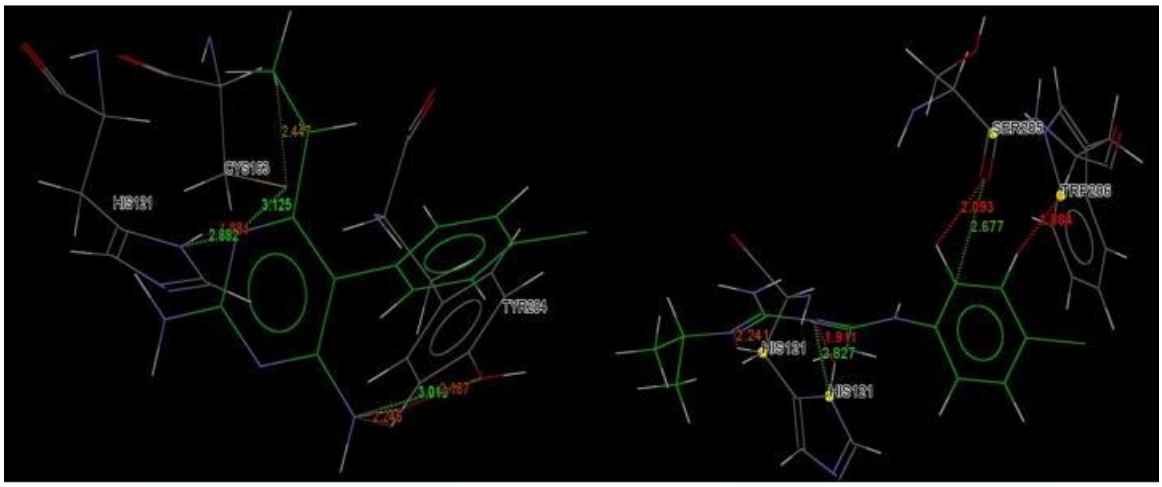
Though, the GOLD score of the targeted molecule was lower than chloroquine, quinine, and sulfadoxine, but the amino acid necessary for the interaction and inhibition of the caspase-3 was present in the pyrimethamine.



CHLOROQUINE DOXORUBICIN



SULFADOXINE QUININE



PYRIMETHAMINE PROGUANIL

URKUND

Urkund Analysis Result

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