REVERSAL OF SODIUM ARSENITE –INDUCED DELAY IN GASTRIC ULCER HEALING IN RATS BY *KOLAVIRON*, VITAMIN E AND ZINC

BY

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ABSTRACT

Arsenic acid is a major contaminant of various water sources used for human consumption and industrial activities in most developing countries. It has been reported to cause degenerative inflammation and oxidative damage in many tissues. However, there is a paucity of information on its effect on various gastrointestinal tract conditions of exposed individuals. A mechanistic study in rats on the ameliorative activities of antioxidants (kolaviron, zinc, and vitamin E) on the effects of Sodium Arsenite (SA) exposure during acetic acid - induced gastric ulcer healing was undertaken.

Wistar rats (n=125,150-200g), randomly divided into five groups were treated for two weeks as follows: control (distilled water), SA (5mg/kg, p.o), SA+ kolaviron (100 mg/kg, p.o), SA+ vitamin E (100mg/kg, p.o), SA + zinc sulphate (20mg/kg p.o). Kolaviron was obtained from *Garcinia Kola* using soxhlet extraction process. Gastric ulceration was induced by administration of acetic acid (0.06ml, 40% v/v). Indices of ulcer healing determined on days 3, 7, 14 and 21 post induction were ulcer score and area using planimetry, total gastric acidity by titration, and neutrophil/inflammatory cell infiltration using histomorphometry. Blood cells were quantified using haemocytometry and activities of Superoxide Dismutase (SOD), Catalase, protein level, Malondialdehyde and Nitric oxide (NO) concentration were determined by spectrophotometry. Stomach sections were immunostained for CD31 and Factor VIII (angiogenesis), p53 (apoptosis), Epidermal Growth Factor Receptor (EGFR) and Ki-67 (cell proliferation). Stomach tissue was also stained with H&E, and viewed under light microscope. All these variables were evaluated by days 3,7,14 and 21 post-induction. Data were analysed using ANOVA at $\alpha_{0.05}$.

In the ulcerated control animals, the ulcer areas were 0.74 ± 0.01cm², 0.57 ± 0.08cm², 0.53 ± 0.12cm² and 0.24 ± 0.04 cm² while SA exposure significantly increased ulcer areas for 81.0%, 100.0%, 65.0%, 33.0% by days 3, 7, 14 and 21, respectively. By day 21 post-ulcer induction, zinc, *Kolaviron* and vitamin E had reduced ulcer areas relative to SA-treated group by 21.0%, 0.5% and 2.0%, respectively. Sodium arsenite decreased gastric mucosal thickness and parietal cell mass but increased lipid peroxidation, malondialdehyde levels and Neutrophil-Lymphocyte ratio. These effects were reversed by vitamin E and kolaviron. Total gastric acidity reduced while the levels of SOD, catalase, total protein and NO increased as healing progressed in all groups, but at a higher rate in the SA exposed group. The expression of CD31, factor VIII, Ki67 and EGFR proteins were significantly reduced

by sodium arsenite, co-treatment with the antioxidants increased the labelling indices towards control values in the order zinc>kolaviron>vitamin E. The p53 expression was increased in SA treated animals but was reduced by the antioxidants in the same order. Gastric tissue necrosis, hyperplasia and haemorrhage observed in the SA-treated animals were reduced by the antioxidants.

Sodium arsenite delayed gastric ulcer healing in rats via oxidative stress, inflammation, alteration in proliferative and apoptotic activities and impaired angiogenesis in the stomach.

Keywords: Sodium arsenite, Gastric ulcer healing, *Kolaviron,* Gastro-toxicity

Word count: 470

CERTIFICATION

I certify that ADEBAYO GRACE IYABO carried out this work titled," REVERSAL OF SODIUM ARSENITE INDUCED DELAY IN GASTRIC ULCER HEALING IN RATS BY KOLAVIRON, VITAMIN E AND ZINC" under my supervision in the Department of Physiology, College of Medicine , University of Ibadan, Ibadan, Nigeria.

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DEDICATION

I dedicate this work to God almighty, for his grace and mercy over me. To my spouse and sons, God bless you

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I thank you God, for your benevolence, unending love, abundant grace, strength, faithfulness and wisdom upon my life and the life of my family. God, you are awesome!I want to appreciate my supervisor, Rev.(Prof.) S.B.Olaleye, for his understanding and guidance, fatherly advice in the course of my programme and especially during this work. May God continue to enlarge your coast and you will be fruitful in your endeavours in Jesus name (Amen).

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LIST OF ABBREVIATIONS

AA	Acetic acid
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CAT	catalase

CCK	Cholecystokinin
CD 31	Cluster of differentiation 31
CGRP	Calcitonin gene related peptide
COX -1	Cyclo-oxygenase 1
COX-2	Cyclooxygenase 2
Differential WBC	Differential white blood cell count
DMA	Dimethylarsenoic acid
DNA	Deoxyribonucleic acid
ECL	Enterochromaffin like cells
EGFR	Epidermal growth factor receptor
EMG	Electro-myographic technique
Factor VIII	Von Willebrand
Gi	Guanine nucleotide binding protein (activated G proteins- inhibitory)
GRP	Gastrin releasing peptide
Gs	Guanine nucleotide binding protein (activated G proteins- stimulatory)
GSH	Glutathione S-transferase
H & E	Hemotoxylin ansd eosin staining
H^+- K^+-ATPase	Hydrogen potassium proton pump
H_2	Histamine receptor
Hb	Hemoglobin
HDC	Histidine decarboxylase
HL-60	Human promyelocytic leukemic cells

IHC	Immunohistochemistry
Ki-67	Kiel 67
KV	Kolaviron
M_1-M_5	Muscarinic receptor 1-5
MAs	Methyarsenoic acid
MCL	Maximum concentration limit
MDA	Malondialdehyde
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
P53	Phosphoprotein 53
PACAP	Pituitary adenylate cyclase –activating peptide
PCV	Packed cell volume
PECAM-1	Platelet-endothelial cell adhesion molecule- 1

PPB	Parts per billion
RBC	Red blood cell count
RDA	Recommended Dietary Allowance
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction (Real time)
SA	Sodium arsenite
SAM	S-adenosylmethionine
SOD	Superoxide dismutase
TWBG	Total white blood cell count
UA	ulcer area
VE	Vitamin E
VIP	Vasoactive intestinal peptide
$VPAC_2$	Vasoactive intestinal peptide activated receptor
Zn	Zinc

CHAPTER ONE

INTRODUCTION

The Gastrointestinal tract is an interface between the body and external environment, providing the body with a continuous supply of water, electrolytes and nutrients. It is a site of biotransformation, forming a barrier against unwanted materials and its' excretion i.e. is very important in the normal body homeostasis (Barltrop and Brueton, 1990). Gastrointestinal tract has many functions such as secretions, movement and absorption of nutrient. These functions are affected by various signals from luminal contents including nutrients and non-nutrients, chemicals, mechanical factors and micro – organisms (Soybel, 2005). The gastrointestinal tract starts from the mouth, esophagus, stomach, intestines to anus and can be associated with several diseases which ranges from infectious diseases, inflammatory diseases, ulceration as well as cancer of the gastrointestinal tract.

Ulceration of gastrointestinal tract could occur in the esophagus, stomach, and duodenum. Ulceration in the stomach or duodenum is referred to as "peptic ulcer". In healthy individuals, digestive tract is coated with a mucous membrane that protects the underlying tissue against the highly corrosive digestive acid. When there is an increase in gastric acid, or pH of the acid is significantly reduced, or the mucus membrane layer becomes too thin or dry, it results in to gastric injury (Grossman, 2009, Lawande *et al.*, 2012). Peptic ulcers are open sores that develop within the digestive tract mucosal lining, specifically, the initial portion of the small intestine (duodenum), esophagus, and stomach. It is a prevalent, fairly heterogeneous diseases with worldwide distribution affecting people from all countries and different race, about 5-10% of the general population over a lifetime (Laurette *et al.*, 2015). Peptic ulcer disease represents one of the important disease causing morbidity and increased health care costs, with expenditure estimates related to work loss, hospitalization and outpatient care (Bhattacharya *et al.*, 2003). The concepts of peptic ulcer disease has undergone radical changes over 20 years especially following the recognition of *Helicobacterpylori* which was believed to be a major contributing factor: 1 case per 1000 population in Japan, 1.5 cases per 1000 population in Norway, 2.7 cases per 1000 population in Scotland (Everhart *et al.*, 1998). Its worldwide prevalence differs, as duodenal ulcer is more in the western populations while the gastric ulcer is more frequent in the Asia especially among the Japanese (Sonnenberg, 1985, Sonnenberg and Everhart, 1996).There has been at least 70-90% of patient with gastric ulcer and 80-95% with duodenal ulcer (Miniayan *et al.*, 2006). It was estimated in 2010 that about

250,000 people died of peptic ulcer globally (Lozano,2012). The epidemiology reported by Brown *et al.,* 2012, using data from in patient, the total number of cases of hospitalization of pediatric patients for peptic ulcer disease bleeding in the US in 2008 was estimated to be between 378 and 652. The overall 17.4% of insured pediatric patients with any upper gastrointestinal ulcer in 2008 were reported to have developed bleeding. The estimated incidence of peptic ulcer in the US pediatric population in 2008 ranged from 0.5 to 4.4/100.000 individuals.It has caused a high rate of morbidity particularly for the population of both industrialized and non- industrialized countries. Its true prevalence rate in Nigeria population is not certain, however, Nigeria is said to be one of the country where there is high rate of peptic ulcer disease prevalence (Ndububa and Adeyemi, 2008).

The Protection of the gastric mucosa involves factors such as mucosal barrier, mucus secretion, blood flow, cell regeneration, and the release of endogenous protective agents especially prostaglandins and epidermal growth factors (Berglindh, 1977). Increased gastric acid is one of the prevalent factors influencing gastric ulcer formation and or development (Alphin and Wards, 1967). Imbalance between gastric offensive factors (like acid or pepsin secretion, lipid peroxidation, nitric oxide,) and defensive mucosal factors (like mucin secretion mucosal cells shedding, glycoprotein proliferation and anti-oxidant enzymes like catalase, superoxide dismutase and glutathione level) results in development of peptic ulcer (Prabha *et al.,* 2009). The pathogenesis is multifactorial including *Helicobacter pylori,* use of nicotine, pepsin, gastroduodenal motility, smoking and complex interaction between so called aggressive and protective factors. There had been so many treatment/drug therapies employed such as the use of proton pump inhibitors, H_2 receptors blockers, eradication of *Helicobacter pylori,* increased mucin or mucous production just to mention a few (Kang *et al.*,2010). Despite greatly expanded knowledge of the pathogenesis and treatment of gastric ulcer, much remain unknown and there is greater incidence of gastric ulcer especially in the elderly, development of tolerance and incidence of relapse as well as side effect interfering with the clinical usefulness (Kang *et al.,* 2010). This has led to renewed search for other probable ulcerogens

Environmental factors (trace elements, pollutants), food, water or dietary intake of potential triggers of gastric injury has been found to contribute to the etiology of peptic ulcer. Certain trace elements or heavy metals such as lead, cadmium, and mercury are known to be toxicants in the body system (Vasquez *et al.,* 2015). Their toxicity in the gastrointestinal tract have been suggested to have potential(s) in promoting ulcer formation (Olaleye *et al.*,2006), Researchers

have documented the potentials of Lead (Pb) in promoting gastric ulcer formation (Olaleye *et al.*,2006, 2007) and Cadmium has been reported to cause desquamation of gastric epithelium thus increasing the severity of gastric ulcer (Trujano and Navarrette, 2011).

Arsenic is the 33rd element on the periodic table and with a common oxidation state of +5, +3, and -3. It is either in the form of a trivalent arsenite (AS^{3+}) or pentavalent arsenate (AS^{5+}) naturally occurring and ubiquitously present in the environment. Arsenical compounds are environmental toxicants with multiple effects in animal and human population (Liu *et al.,* 2001,Waalkes *et al.,* 2003, Flora *et al.,* 2008). Humans are exposed to arsenic mainly through oral or inhalation routes (Flora *et al.,* 2008). Oral exposure can occur via consumption of contaminated water, food, drugs and such exposure can be lifelong. Occupational exposure on the other hand, occurs mainly through inhalation via non-ferrous ore, smelting semiconductors, glass manufacturing, and power generation by burning contaminated coal, (Liu *et al*., 2001).The main source of environmental arsenic exposure in most population is the drinking water of which inorganic forms of arsenic predominate, (Bates *et al.,* 1992, Pott *et al.,* 2001). Assessing the risk of exposure to inorganic arsenic in water supplies is therefore a key issue in scientific community. High level of arsenic in drinking water can be found in many countries including Argentina, Mexico, Thailand, China (NRC, 1999, Pott *et al.,* 2001) and is also evident that low level of arsenic typically found in India may pose a significant health risk to human. It is frequently used in production of herbicides, insecticides rodenticides, food preservatives and by product of used fossil fuel (Flora *et al.,* 1995, Nickson *et al.,* 1998, Jana *et al.,* 2006). However, chronic dermal toxicity, nephrotoxicity and skin cancer have been found to occur with arsenic exposure. Arsenic has a multi-site carcinogenic effect in humans, causing tumors in variety of tissues such as the lungs, skin and bladder (NRC.,1999, Walkees *et al*., 2003, Jana *et al.,* 2006). Studies have also shown that acute arsenic exposure may cause gastrointestinal tract disorder (Goebi *et al*.,1990) such as vomiting, abdominal pain, bloody diarrhea (which is the most common) and other GIT effects like inflammation in the pharynx and the esophagus, however chronic exposure may exert degenerative inflammatory and neoplastic changes of respiratory, hematopoietic, cardiovascular and nervous system. The effect of both acute exposure and chronic exposure of arsenic compounds and its mechanism on gastrointestinal tract is not well established.

Arsenic acid exerts its toxicity in part by generation of reactive oxygen species (ROS) (Hei *et al*., 1998,Das *et al*., 2003,Kitchin and Ahmad, 2003, Liu *et al*., 2003, Tao *et al*., 2009,). Studies have revealed that arsenic acid induces multiple biological effects such as DNA

damage, apoptotic cell death and global DNA hypomethylation (Huang *et al*., 1999, Kirkpatrick *et al*., 2003, Chen *et al*., 2004, Jiang *et al*., 2009). When both human and animals are exposed to arsenic acid, they experienced increase formation in the level ROS/RNS, which includes the formation of peroxyl radicals (ROO), superoxide radical, singlet oxidative, hydroxyl radical via fenton reaction, hydrogen peroxide, dimethyl arsenic radical, dimethyl arsenic peroxyl radical and oxidant induced DNA damage (Jomovo *et al*., 2011).

Several studies have shown that reactive oxygen Species (ROS) play significant roles in the pathophysiology of human diseases including peptic ulcer (Repetto and Llesuy, 2002, Dotan *et al*., 2004, Paracha *et al.*,2013). These ROS are generated as by –products from the metabolism of arachidonic acid, platelets, macrophages and smooth muscle cells are potent ulcerogens in the gastric mucosa. Interestingly, several compounds which has been shown to possess the ability to scavenge free radicals and are therefore being projected to be useful in protecting the gastric mucosa from oxidative damage. These compounds, known as antioxidants apart from their free radical scavenging ability, are known to inhibit lipid peroxidation (Nakatami, 2000, Repetto and Llesuy, 2002). Other free radical –mediated processes, thus protecting the body from a number of diseases.

Kolavironis a defatted ethanol extracts from seed of *Garcinia kola*. It has mixtures of Garcinia biflavonoid GB1, GB2 and bi flavonone (Iwu *et al.,* 1990, Farombi *et al.,* 2000). It is a nutritionalsupplements that have a wide range of medicinal value and numerous biochemical importance in the human body system (Farombi *et al.,* 2002).Kolaviron especially biflavonoid complex of *Garcinia kola* have been reported to possess some many benefits, these benefits have been attributed to its anti –inflammatory (Olaleye *et al.*,2000), anti-oxidant (Farombi *et al.*,2000,) as well as antigenotoxic (Nwankwo *et al.,* 2000; Farombi *et al.,* 2005), anti-hepatoxic activities (Farombi *et al*., 2000, 2004) (Akintonwa and Essien,1990, Farombi *et al.*, 2001, 2009, 2013). It also has antimalarial, antidiabetic and anti-viral as well as anti-ulcer properties(Ibironke *et al*., 1997; Olaleye and Farombi, 2006; Olaleye and Cho, 2010)

Vitamins (C&E) have been shown to possess antioxidant activities (Dawud *et al.*, 2014). They are said to possess the ability to inhibit or delay oxidative processes (Flora *et al.*, 2008). Vitamin E,a lipid soluble antioxidant, has been used to improve blood circulation and increase the supply of oxygen so as to create good conditions for the recovery of ulcer. In addition, it can also restrain the production of helicobacter pylori to prevent the reoccurrence of ulcer. Other functions include enzymatic activities, gene expression, and neurological function(s).

The most important function of vitamin E has been suggested to be in cell signaling (Azzi, 2007). This variant can be found most abundantly in wheat germ oil, sunflower, and sunflower oils (Reboul, 2006). Vitamin C is a water soluble antioxidant and it occurs in the organism as an ascorbic anion(Flora *et al.,* 2008). It can acts as a free scavenger of free radicals such as like superoxide and hydroxyl radicals, hydrogen peroxide, singlet oxygen, and hypochlorous acid,and plays important role in the regeneration of α-tocopherol i.e. guarantees the chain breaking antioxidant action of vitamin E (Jonas *et al.*, 1993, Beyer, 1994, Young and Woodside, 2001)

Zinc is an essential trace element for humans, animals and plants (Shah and Sachdev, 2001; Das and Das, 2012). It is a micronutrient which is very important in numerous aspects of cellular metabolism and is required for catalytic activity of several enzymes (Anonymous, 2001). It can limit oxidant –induced damages in several ways (Disilvestro,2000), protect against vitamin E depletion (Noh and Koo, 1998),and stabilize membrane structure (Bray and Bettger, 1990) and restriction of endogenous free radical production (Disilvestro, 2000). It also plays a major role in immune function, protein synthesis, wound healing and DNA synthesis, in cell division and growth (Prasad, 1995, Prasad *et al*., 1997 and Lansdown *et al.*, 2007).

1.1. STATEMENT OF PROBLEM

The deleterious effects of exposure to inorganic arsenic acid such as sodium arsenite in water supplies have attracted the attention of the researchers in recent years. Studies have shown that sodium arsenite toxicity could be via oxidative stress, induction of apoptosis, cytotoxicity in so many tissues of the body thereby causing injuries to these tissues (Jomovo *et al.*, 2011, Calatayud *et al.*, 2014, 2015). From previous experimental studies, it has been shown that sodium arsenite aggravated ulcer formation in indomethacin induced ulcer thereby reducing the cyto-protection in the stomach mucosa (Unpublished data). Its modification and transformation also start from the gastrointestinal tract and reports on its ability to induce oxidative stress, apoptosis and DNA damage has been established. It has been evident that oxidative stress is a major culprit in the etiology of ulcer formation. Therefore, an interest in studying the effect of sodium arsenite on ulcer formation and healing was developed .The treatment of sodium arsenite toxicity over the years has been the use of chelation therapy and the use of some antioxidants such as vitamin E, β-carotene,α-Lipoic acid, etc., and the use of herbal medicines has been suggested to be a better option for treatment of arsenic poisoning (Flora *et al.*, 1998). Kolaviron; [defatted ethanol extract] from the seeds of *Garcinia kola* (Iwu *et al.*, 1990), which has been documented to possess antioxidant, scavenging, anti-inflammatory and hepato-protective properties against several carcinogens in various experimental models (Akintonwa and Essien, 1990; Olaleye *et al.*,2000; Farombi *et al.*, 2000; Farombi *et al.*, 2001,Ayeola *et al.*,2014,Abarikwu, 2015). The protective effect of vitamin E supplementation against sodium arsenite induced oxidation has been reported in humans and rats (Kumar *et al.*, 2002, Goldfarb, 2006) Zinc homeostasis has also been shown to be important for the integrity of gastric mucosal cells and is a key factor for the preservation of the intestinal barrier.(Lambert *et al*., 2004). Therefore, treatment with kolaviron, vitamin E andzincwas suggested to be use in intervening the gastro toxicity of sodium arsenite exposure in the rat's stomach.

1.2. AIM

To investigate the effect of sodium arsenite toxicity on gastric ulcer healing process in experimentally induced chronic gastric ulcer.

1.3. OBJECTIVES

1. Investigate the toxicity of sodium arsenite on formation of experimental gastric ulcer.
2. Study the effects of sodium arsenite on healing of experimental gastric ulceration in rats.
3. Study the role oxidative stress play in the observed effects of sodium arsenite on experimental ulceration.
4. To inquire if known antioxidants –kolaviron, vitamin E and zinc sulphate will affect the observed effects of sodium arsenite on experimental ulceration
5. To study the mechanisms involved in the observed actions in 1 to 4 above.

CHAPTER TWO
LITERATURE REVIEW

2.0. THE STOMACH

The stomach is an organ of the digestive system and is an expanded section of the digestive tube between the esophagus and small intestine. It is shaped like the letter 'J'. The right side of the stomach is called the greater curvature and the left the lesser curvature. The most distal and narrow section of the stomach is termed the pylorus - as food is liquefied in the stomach it passes through the pyloric canal into the small intestine. The wall of the stomach is structurally similar to other parts of the digestive tube, with the exception that the stomach has an extra oblique layer of smooth muscle inside the circular layer, which aids in performance of complex grinding motions. In the empty state, the stomach is usually contracted, has its mucosa and submucosa thrown up into distinct folds called rugae; when distended with food, the rugae are straightened and flat (Soybel, 2005).The lining of the stomach is covered with numerous small holes. These are the openings of gastric pits which extend into the mucosa as straight and branched tubules, forming gastric glands. The stomach is so special an organ, that it can regenerate the layer of mucus lining in 2 weeks. In adult humans, the stomach has a relaxed, near empty volume of about 45 ml. It is a distensible organ; it normally expands to hold about one litre of food, but can hold as much as two to three litres. The stomach of a newborn human baby will only be able to retain about 30 ml. (Soybel, 2005, Sherwood *et al.*, 1997).

2.1.0. TYPES OF SECRETORY EPITHELIAL CELL

There are four major types of secretory epithelial cells cover the surface of the stomach and extend down into gastric pits and glands:

- **Mucous cells:** secrete alkaline mucus that protects the epithelium against shear stress and acid.
- **Parietal cells:** secrete hydrochloric acid.

- **Chief cells:** secrete pepsin, a proteolytic enzyme.
- **G cells:** secrete the hormone gastrin.

There are differences in the distribution of these cell types among regions of the stomach - for example, parietal cells are abundant in the glands of the body, but virtually absent in pyloric glands.

2.1.1. THE SECTIONS OF STOMACH

The stomach is divided into four sections, each of which has different cells and functions. The sections are:

Cardia	Where the contents of the esophagus empty into the stomach.
Fundus	Formed by the upper curvature of the organ.
Body/ Corpus	The main, central region.
Pylorus	The lower section of the organ that facilitates emptying the contents into the small intestine

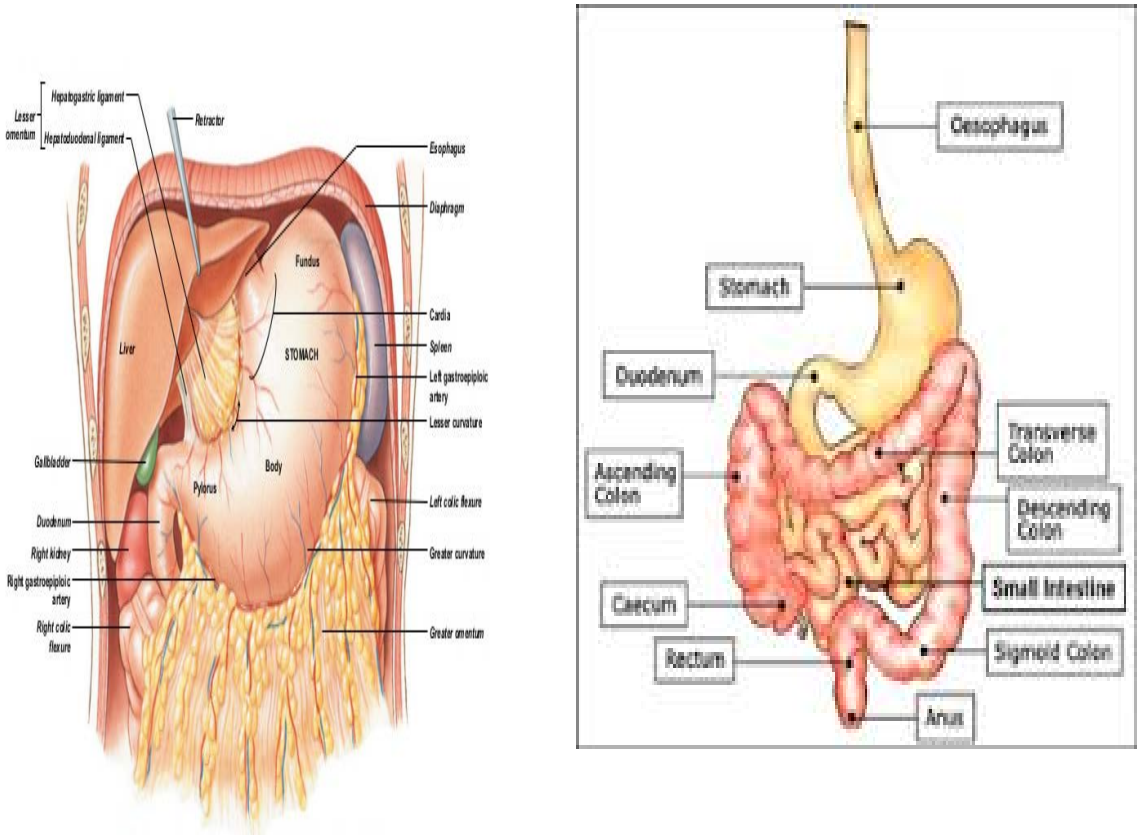


Figure 2.1. The surface structure of stomach (pearson education,2012) and the gastrointestinal tract (pepticulcersociety,2010).

When the bolus (masticated food) enters the stomach through the esophagus via the esophageal sphincter. The stomach releases proteases (protein-digesting enzymes such as pepsin) and hydrochloric acid, which kills or inhibits bacteria and provides the

acidic environment (pH = 2) for the proteases to work. Food is churned by muscular contractions of the stomach wall called peristalsis. This process reduces the volume of the fundus, before looping around it and the body of stomach, thus converting the boluses into chyme (partially digested food). Chyme slowly passes through the pyloric sphincter and into the duodenum, where the extraction of nutrients begins. Depending on the quantity and contents of the meal, the stomach will digest the food into chyme anywhere between 40 minutes and a few hours (Sherwood *et al.,* 1997).

2.1.2. GASTRIC SECRETION

The stomach plays many functions including partial storage of food, accommodation of food, mixing of food with digestive juices. About 1.5 – 2.0 liters of gastric juice is produced daily. The secretions from the stomach includes digestive enzymes, hormones and mucus and these play many roles. The gastric juice is made up of water, hydrochloric acid, digestive enzymes e.g. pepsin, hormones (e.g. Gastrin), intrinsic factor, mucous.

2.1.3. PHASES OF GASTRIC SECRETION

There are three major phases of gastric secretion which includes:

a. **Cephalic phase:** this is initiated by the thought, sight, taste and smell of food and it is dependent on the parasympathetic innervations of the stomach. In this phase, there is secretion of HCL from the parietal cells and gastrin from the G-cells, secretion of pepsinogen from peptic chief cells and these are stimulated by the vagal efferent fibres. Almost half of the gastric secretion released during a meal occurs in this cephalic phase via vagal stimulation.
b. **Gastric phase:** is initiated by the entry of food into the stomach. Food entering the stomach buffers acid and raises the pH and allows other stimuli to release acid e.g. vagal efferent nerves. Distention of the corpus acting through vagal reflexes increases secretion of HCL.
c. Distention of the antrum initiates vagal reflexes which results in the release of gastrin from the antral G-cells. Gastrin release is inhibited at a low pH of less than 3; this low pH activates local reflexes which enhances pepsinogen secretion. Although, the volume of gastric secretion during this phase is less than during the cephalic phase, it continues for a longer period, thus the two phases in the long run contribute the same amount of secretion.

d. **Intestinal phase:** This begins as chyme empties from the stomach to the duodenum. Overall, little gastric secretion occur during the intestinal phase.

2.1.4. GASTRIC SECRETORY CELLS

In the mucosa of the stomach, there are glands buried within it, these glands contain various cells.

Oxyntic gland:they are located within the fundus and corpus of the stomach and contains three secretory cells

a. Parietal cell that secretes HCl for antibacterial function and stimulates the conversion of pepsinogen to pepsin for protein digestion. They also secrete intrinsic factor which is responsible for absorption of vitamin B12 from the stomach.
b. Chief cells/ peptic cells: secretes pepsinogen a precursor of the proteolytic enzyme pepsin.
c. Mucous cells: they secrete mucus for protecting gastric mucosa from corrosive effects of HCl.

Pyloric Gland**:** located in the antrum and pyloric region of the stomach. They contain G-cells and some mucus secreting cells. The G-cells are responsible for the release of the hormone gastrin which increases the rate of gastric functions.

2.1.5. MECHANISM OF HYDROCHLORIC ACID SECRETION

HCL indirectly plays a role in the digestion of protein because it provides optimal pH for the action of pepsin. HCL also hinders the growth of pathogenic bacteria. There is an active transport of K^+ and Cl^- into the cannaliculi of parietal cells through a channel. The flow of Cl^- creates a negative potential in the cannaliculi. This activates the H^+ and K^+ exchanger and hydrogen ion moves into the cannaliculi. It binds with chloride ion. Water moves into the cannaliculi and causes the formation of HCL. In another mechanism, hydrogen ion entering the cannaliculi is supplied by the dissociation of carbonic acid within the parietal cell.

2.1.6. CONTROL OF GASTRIC SECRETION:

a. THE ROLE OF HISTAMINE IN REGULATION OF GASTRIC ACID SECRETION

Gastric acid secretion is stimulated or inhibited by endocrine, paracrine, and neurocrine signals via at least three messenger pathways; gastrin-histamine, CCK-somatostatin, and acetylcholine.Histamine is produced by decarboxylation of L-histidine. Most histamine in the body is stored in mast cells and basophil leukocytes, although some is found also in eosinophil and platelets. In the gastric mucosa, histamine occurs mainly in enterochrommaffin like cells and mast cells **(**Håkanson *et al,* 2006**).**

Acetylcholine and histamine directly stimulate the parietal cell to secrete acid. Histamine is released from enterochromaffin-like (ECL) cells by gastrin in mammalian and amphibian stomach. Acetylcholine coming from enteric nerve terminals in the vicinity of the gland also releases histamine in the amphibian stomach and perhaps in mammals. However, acetylcholine and histamine activate acid secretion differently. Acetylcholine binds to M_3-muscarinic receptors leading to increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Kato *et al,* 2005). Histamine binds to H_2 receptors resulting in the elevation of both $[Ca^{2+}]_i$ and cyclic AMP (cAMP). Therefore, the composition of primary gastric juice in the gland may vary according to the stimulus.

Pepsin secretion is stimulated by agonists acting on β-adrenergic, muscarinic (M_1 and M_3), and cholecystokinin (CCK_1) receptors (Dimaline and Varro, 2007,). Chief cells are not stimulated by histamine since they do not bear H_2 receptors. Mucous neck cells are stimulated by muscarinic and β-adrenergic agonist.

B. THE GASTRIN-HISTAMINE PATHWAY

Circulating gastrin acts on the CCK_2 receptors of the enterochrommafin like cells, resulting in increased histidine carboxylase mRNA expression and accelerated release and synthesis of histamine, which, in turn, stimulates gastric acid secretion by activating the histamine H_2 receptors of the parietal cells (Chen *et al,* 2002, 2004). In an experiment carried out, the greater impairment of acid secretion in CCK_2 receptor knockout mice was suggested to be due to the loss of typical ECL cells and their replacement by histamine-free endocrine-like cells, displaying an ultra-structure distinct from that of the ECL(Enterochrommafin like) cells (ECL cell replacements) (Chen *et al,* 2002). Histidine decarboxylase knockout mice had little or no de novo histamine synthesis in the gastric mucosa, resulting in severely impaired acid secretion and a failure to respond to gastrin (Furutani, 2003). H_2-receptor knockout mice showed a complete lack of acid response to both histamine and gastrin (Kobayashi *et al.*,

2000). Thus targeted gene disruption of gastrin, CCK_2 receptor, histidine decarboylase, and H_2 receptor showed that histamine has a key role in the gastrin-triggered pathway that controls acid secretion.

C. GASTRIN-HISTAMINE AND THE CCK-SOMATOSTATIN PATHWAYS

CCK_1 receptors recognize CCK preferentially; in fact, sulfated CCK-8 binds to the receptor with 500 times greater affinity than gastrin-17. CCK mobilizes somatostatin from D cells by acting on CCK_1 receptors in both the antral and oxyntic mucosa, thereby inhibiting the gastrin-histamine pathway (G cells and ECL cells) and the activity of the parietal cells by an effect of somatostatin on sst_2 receptors (Allen *et al,* 2002). However, acid secretion in CCK_1-receptor knockout mice was not much different from that in wild-type mice as found in previous studies (Allen *et al.,* 2002).

Further studies also found that the CCK-somatostatin pathway has been uncovered by generating gastrin and CCK double-knockout mice, which were found to have normally functioning parietal cells despite inactive enterochrommafin like cells (Chen *et al,* 2004). In gastrin and CCK double-knockout mice, little or no histamine was mobilized from the ECL cells to stimulate the H_2 receptors. At the same time, there was no circulating CCK to stimulate gastric mucosal D cells to release somatostatin. Despite the lack of gastrin (and CCK), the parietal cells were still capable of producing gastric acid in response to vagal stimulation (pylorus ligation) and a single injection of histamine (but not gastrin). The low acid secretion in the gastrin knockout mice and the relatively high acid output in the gastrin and CCK double-knockout mice can be explained by assuming that CCK somehow counteracts the acid-stimulating effect of gastrin. Indeed, administration of sulfated CCK-8 to gastrin and CCK double-knockout mice effectively inhibited acid secretion (a single injection) and increased fundic somatostatin mRNA expression more than twofold (2-day infusion) (Chen *et al,* 2004).

Thus, the net acid output may be determined by the balance between stimulating signals from the gastrin-histamine pathway on one hand and inhibiting signals from the CCK-somatostatin pathway on the other. In fact, the studies of somatostatin receptor knockout mice indicated that endogenous somatostatin acts on the sst_2 receptor to suppress gastric acid secretion by an inhibitory action directly on the parietal cell and by inhibition (probably together with galanin) of the action of gastrin on the ECL cells (Martinez, 1998).

D. NEURAL PATHWAY (ACETYLCHOLINE AND NEUROPEPTIDES)

The central mechanisms control the sympathetic and parasympathetic inputs to myenteric and submucosal ganglia in the stomach wall; also command neurons in these ganglia control nerve signaling to the parietal cells. Thus parietal cell function is regulated not only by circulating hormones (e.g., gastrin and CCK) and paracrine messengers (e.g., histamine and somatostatin) but also by neurotransmitters from enteric neurons (e.g., acetylcholine, catecholamines, and neuropeptides such as pituitary adenylatecyclase-activating peptide (PACAP), VIP, and galanin (Hakanson *et al,* 2001). Acetylcholine acts on muscarinic receptors, which are of five subtypes ($M_{1–5}$). All $M_{1–5}$ receptors seem to be expressed in the stomach wall, and $M_{1–4}$ is expressed in the oxyntic mucosa as revealed by RT-PCR analysis (Kato *et al,* 2005). Identification of the precise cellular localization of these different receptor subtypes has turned out to be difficult.

However, it has been suggested that M_1 receptors are expressed by chief cells and surface mucous cells, M_2 and M_4 receptors occur on D cells, M_3 on parietal cells and G cells, and M_5 on postganglionic enteric nerve fibers (Kato *et al,* 2005). Acetylcholine is known to stimulate acid secretion. In fact, M_3-receptor knockout mice had an impaired parietal cell function as evidenced by elevated intragastric pH, reduced acid output in response to pylorus ligation, reduced proportion of secreting parietal cells, and elevated serum gastrin concentration in the fasted state (Furutani*et al,* 2003).It may be noted that pylorus ligation induces acid secretion through vago-vagal reflexes independent of gastrin and ECL cells (enterochromaffin like cells) (Zhao *et al,* 1996). Although the ECL cells seem to lack muscarinic receptors, they mobilize histamine in response to adrenaline/noradrenaline (acting on M_2-receptors) and to certain neuropeptides that occur in enteric neurons, such as (Pituitary adenylatecyclase-activating peptide) PACAP (acting on PAC_1 receptor) and VIP (acting on $VPAC_2$ receptor). Also, Galanin inhibited gastrin-induced mobilization of ECL cell histamine in vitro as well as in vivo by acting on Gal_1 receptors. Carbachol (a stable acetylcholine analog) stimulated acid secretion in wild-type mice but reduced acid secretion (pylorus ligation model) in gastrin and CCK double-knockout mice (Chen *et al,* 2004). Conceivably, carbachol mobilizes somatostatin from the D cells (by acting on M_2 and/or M_4 receptors), which leads to inhibition of the parietal cells (by acting on (somatostatin $_2$ receptors- sst_2 receptors), resulting in low acid secretion. In fact, the somatostatin concentration in the oxyntic mucosa was

almost doubled, and secretory granules were notably numerous in the D cells of the oxyntic mucosa of gastrin and CCK double-knockout mice (Chen *et al*, 2007).

E. THE H_2, CCK_2, M_3, AND SST_2 RECEPTORS OF THE PARIETAL CELL

Parietal cells harbor at least three types of acid-stimulating receptors (H_2, M_3, and CCK_2) and one type that inhibit acid secretion (sst_2). The targeted gene disruption of any of the three stimulating receptors will result in impaired acid secretion. Activation of the H_2 receptor (by histamine) appears to play a crucial role in acid secretion. Thus, when histamine is missing from the ECL cells, as is the case in Histidine decarboxylase knockout mice in study by Furutani *et al*, 2003 and CCK_2-receptor knockout mice (Chen *et al*, 2002), gastrin, carbachol, or pylorus ligation induced little or no acid secretion. When the H_2 receptor was missing (as in H_2-receptor knockout mice), there was no acid response to gastrin, and the acid response to carbachol was impaired at 3–4 months of age (Fukushima *et al*.,2003) and lost at 6 and 14 months of age (Okabe, 2002).

In HDC knockout mice, the use of histamine and carbachol, was able to induce stimulation of acid in the mice and the effect that could be prevented by H_2-receptor blockade (famotidine) (Furutani, 2003). On the other hand, in the M_3-receptor knockout mice , they were unable to respond to stimulation of acid secretion by 2-deoxy-D-glucose (a vagal stimulant) and they also responded poorly to both gastrin and histamine, suggesting that the M_3 receptor on the parietal cell is needed to ensure full secretory capacity (Aihara, 2005). In somatostatin-receptor knockout mice, the acid response to gastrin was enhanced greatly but not to histamine. This became evident that cross-link exists between these receptors on the parietal cells, probably via overlapping intracellular second messenger systems. Studies of isolated parietal cells or isolated oxyntic glands from pig, rat, guinea pig, or rabbit have shown that the CCK_2 and M_3 receptors are coupled to $G_q$trimeric protein, which upon stimulation activates phospholipase C to induce a rise in inositol trisphosphate, causing a release of intracellular calcium. The H_2 receptor is coupled to both G_q and G_s transduction pathways. The G_s pathway activates adenylatecyclase and increases intracellular cAMP. Moreover, the sst_2 receptor has been suggested to be coupled to the $G_i$trimeric protein that inhibits the PAC_1 receptor-coupled G_s pathway in the ECL cells and perhaps also the CCK_2 receptor-coupled G_q pathway in ECL cells and parietal cells. It has been suggested that an increase in both cAMP and intracellular calcium is needed to stimulate acid secretion.

2.2.0. DEFENSE SYSTEM OF THE STOMACH

The stomach is lined by a complex epithelium that forms a selective barrier between the external environment and the body. The epithelium is folded into several branching, tubular gastric glands that reach deep into the muscularis mucosa. There are diverse range of functions performed by gastric epithelial cells and it is maintained within the hostile luminal environment that can contain up to 150 ml HCl and aggressive proteases, which can digests the tissue, as well as a variety of noxious pathogens (Dimaline and Varro,2007). Despite continuous exposure to these injurious factors, large number of defense mechanisms prevent the local damage and helps maintain structural and functional mucosal integrity (Tulassay and Herszényi, 2010).

In general, gastric defense mechanism consist of the gastric mucosal "barrier" which is a multilayer system, which include a pre-epithelial mucus-bicarbonate barrier, epithelial barrier (surface epithelial cells connected by tight junctions), and a sub epithelial component including blood flow and nerves (Henriksnäs *et al.*, 2006, Dimaline and Varro, 2007, Nayeb-Hashemi and Kaunitz, 2009).

2.2.1. THE MUCUS – BICARBONATE BARRIER

Exposure of the stomach to endogenously produced acid and degrading enzymes requires the presence of an efficient gastric mucosal barrier. From studies done in three decades ago, Allen and Garner in 1980 established that mucous bicarbonate barrier as a key component of the gastro duodenal mucosal protective mechanisms against noxious agents (Allen and Flemström, 2005). This barrier constitutes the first line of mucosal defense and is formed by mucus gel, bicarbonate (HCO_3^-), and surfactant phospholipids, which covered the mucosal surface (Lichtenberger, 1999, Allen and Flemström, 2005).

The gastric mucus however consists of a viscous, elastic, adherent and transparent gel secreted by apical expulsion from surface epithelial cells. It is formed by approximately 95% water and approximately 5% mucin, glycoproteins that covers the entire gastrointestinal mucosa, and its luminal surface is coated with a film of surfactant phospholipids with strong hydrophobic properties. The bicarbonate is secreted by surface epithelial cells and its role is to neutralize acid diffusing into a stable, adherent mucus gel layer and to be quantitatively sufficient to maintain a near neutral pH (approximately=7.0) at the mucus-mucosal surface interface (Hills *et al*., 1983, Lichtenberger 1999, Repetto and Llesuy, 2002, Tulassay and Herszényi, 2010).

Also, pepsin is another endogenous aggressor in gastric juice which has received a little attention. Pepsin damages the stomach and is characterized by focal areas of discontinuity in the adherent mucus layer, localized hemorrhagic punctuate ulcers with bleeding into the lumen, and shows no evidence of re-epithelialization or mucoid cap formation (Allen and Flemström, 2005). Thus, the unstirred mucus gel layer is also a physical barrier that prevent luminal pepsin to access the underlying mucosa. It retains HCO_3^- secreted by surface epithelial cells, preventing penetration of pepsin and therefore preventing proteolytic digestion of the surface epithelium (Tulassay and Herszényi, 2010). Therefore, a dissipation of the mucus gel and phospholipid layer by ulcerogenic substances (such as aspirin and bile salts) leads to both acid back-diffusion and mucosal injury (Darling *et al*., 2004, Allen and Flemström, 2005).

Intracellular mucus can scavange oxygen radicals generated in the surface epithelium acting as endogenous antioxidant and reduce mucosal damage mediated by oxygen free radicals. (Penissi and Piezzi, 1999, Repetto and Llesuy, 2002).Furthermore, cells containing mucus can release intracellular mucus in the gastric tissue when the stomach is encountered with the extracellular oxygen species thereby preventing damages and scavenging them as well. (Seno *et al*., 1995)

The efficacy of protective properties of the mucus barrier also depends on the amount or thickness of the layer covering the mucosal surface (Penissi and Piezzi, 1999, Repetto and Llesuy, 2002). The thickness of this layer is the result of a dynamic balance between its secretion and its erosion mechanically by shear forces of digestive process and by proteolytic degradation, particularly from luminal pepsin in stomach. The adherent mucus gel form is physically unique compared with other secretion in the stomach. Studies have shown that adherent mucus gels from stomach, duodenum, and colon are all well-defined viscoelastic gels that do not dissolve on dilution. They can be for a relatively long time (30–120min), re-annealing when sectioned. Thus, mucus gels are said to be stable substances, and exposure of isolated gastric mucus gel to pH 1–8, hypertonic salt, or bile does not disperse or affect its rheological properties. In functional terms, these recognized properties contribute to the adherent mucus gel layer forming a continuous and effective protection over the mucosa (Allen and Flemström, 2005).

2.2.2. THE EPITHELIAL CELLS BARRIER

The continuous layer of surface epithelial cells is another line of defense, which can secrete mucus, bicarbonate and also generate prostaglandins (PGs), heat shock proteins, trefoil factor family peptides (TFFs), and cathelicidins. The epithelial barrier serves to separate the digestive lumen from the internal compartments of the organism. Its main role is to maintain a selective exchange of different substances (secretions, nutrients, etc) between these two compartments, and to ensure the protection of the organism against the penetration of micro-organisms and other exogenous antigens, essentially contained in food. However, two important components of the digestive epithelial barrier carry out these functions; the epithelial cells and the intercellular junctions (tight junctions). Both structures provide two pathways for transepithelial transport: transcellular and paracellular routes, respectively (Matysiak-Budnik *et al.,* 2003,Laine *et al.,* 2008, Tulassay and Herszényi, 2010,). The presence of phospholipids on epithelial cells surfaces makes the cells to be hydrophobic and can repel acid- and water soluble damaging agents (Lichtenberger *et al.,* 1983).

The paracellular pathway seems to be the major route of trans-epithelial macromolecular permeation. This route is a complex array of structures that are mainly controlled by tight junctions between epithelial cells. It appears to be the key regulator of gastrointestinal permeability to macromolecules such as endotoxin and other bacterial products. Also, interconnected by tight junctions, the surface epithelial cells form a "barrier" which can prevent back diffusion of acid and pepsin (Werther, 2000, Farhadi *et al.,* 2003, Laine *et al*., 2008). However, this dynamic gateway is able to change its size under various physiological and pathological conditions. For instance, an earlier study (Madara, 1983) showed that increases in guinea pig intestinal transepithelial resistance induced by osmotic loads were accompanied by alterations in absorptive-cell tight junction structure. This alteration in intestinal permeability after meal ingestion enhances the ability of the small intestine to harvest the maximal amount of nutrients, as well as also increase the risk of exposure to luminal pro-inflammatory compounds. Tight junctions are also composed of other structural proteins including actin anchoring protein (ZO-1) and occludins, which could be the target of oxidative or other toxin injury and result in disruption of gastrointestinal barrier integrity (Nusrat *et al.,* 2001, Farhadi *et al.,* 2003).The gastric epithelial cells can also generate heat shocks that are essential for the maintenance cellular homeostasis during normal cell growth and for survival during various cellular stresses, such as increased temperature, oxidative stress, and cytotoxic agents, preventing protein denaturation and protecting cells against injury.

Activation of heat shock protein response is one of the mucosal protective mechanisms of the antacid hydrotalcite. Also, cathelicidin and ß defensins are cationic peptides that play roles in the innate defensive system at mucosal surfaces preventing bacterial colonization. These elements have been demonstrated in gastric epithelial cells, and they can accelerate ulcer healing (Tarnawski *et al.,* 1999,Tarnawski *et al.,* 2005, Oyaka *et al.,* 2006, Tanaka *et al.,* 2007, Tulassay and Herszényi, 2010).The trefoil factor family (TFFs) also plays important role in the mucosal intergrity (Taupin and Podokyl, 2003). It comprises a group of small peptides (6.5–12 kDa) secreted abundantly by surface epithelium, they help to regulate re-epithelialization by stimulating cell migration and exert mucosal protective action from a broad range of toxic chemicals and drugs (Laine *et al.,* 2008), as well as inhibiting apoptosis and inflammation, and augmenting the barrier function of mucus (Taupin and Podolsky, 2003, Hernández *et al.*,2009, Tulassay and Herszényi, 2010).

Prostaglandins (PGs) are also important in preventing mucosal damage. They are synthesized by gastric mucosal epithelial cells from arachidonate metabolism through the action of cyclooxygenases (COX). Exogenous PGs has been able to attenuate or even completely prevent mucosal damage caused by corrosive substances such as absolute ethanol, concentrated bile or hyperosmolar solutions (Farhadi *et al.,* 2003). However, there are two forms of prostaglandin and they includes prostaglandin E2 and prostacyclin. They are said to have "cytoprotective" effects on the gastrointestinal epithelium and therefore are crucial for the maintenance of the gastric integrity. It is well established that inhibition of their synthesis results in the reduction of gastric mucosal blood flow and gastric mucosal damage (Abdel Salam *et al.,* 1997).

The cytoprotective action of prostaglandins is as a result of its complex ability to stimulate mucosal mucus and bicarbonate secretion, to increase mucosal blood flow and sulfhydryl compounds and limit back diffusion of acid into the epithelium in the stomach (, Tarnawski *et al*., 1985, Farhadi *et al*., 2003; Kato *et al*., 2005). Certain growth factors, such as epithelial growth factor (EGF) have been confirmed to inhibit non-steroidal anti-inflammatory drugs (NSAID) induced gastric ulcerations in animals in absence of prostaglandins. Also, growth factors can stimulate prostaglandin production in rat endometrial cells through a mechanism that involves increase in COX activity.

Mediators such as nitric oxide (NO), calcitonin gene related peptide(CGRP) as well as some hormones including gastrin and cholecystokinin (CCK), ghrelin, leptin and gastrin-releasing

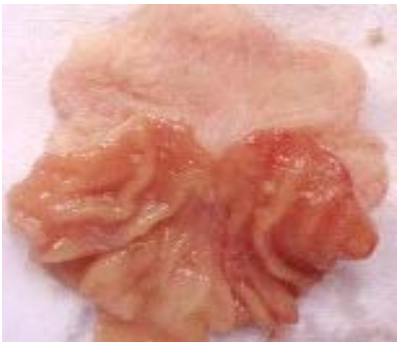
peptide (GRP) have also been found to protect gastric mucosa against the damage induced by corrosive substances. This protective action has also been attributed in part to the release of PGs because it could be abolished by the pretreatment with indomethacin (a non-selective inhibitor of COX 1 and 2) and restored by the addition of exogenous PGE2.

2.2.3. MICROCIRCULATION AND SENSORY INNERVATIONS

Gastric mucosal microcirculation also plays an essential role in the maintenance of gastric integrity, especially for delivering oxygen and nutrients and removing toxic substances. In the muscularis mucosae, most gastric arteries branch into capillaries, which enter the lamina propria and travel upward in proximity to gastric glandular epithelial cells. At the base of surface epithelial cells, the capillaries then converge into collecting venules (Laine *et al.*, 2008, Tulassay and Herszényi, 2010).In restitution process, (a process whereby denuded areas of the mucosa are covered by rapidly migrating cells from adjacent mucosa), depends extensively on adequate blood flow, thus adequate blood flow is essential (Lacy and Ito, 1984,Guttu *et al.*, 1994; Abdel-Salam *et al.*, 2001).It has been studied that exposure of the gastric mucosa to an irritant or acid back diffusion occurrence leads to a marked increase in mucosal blood flow. This increase allows removal and/or dilution of the back-diffusing acid and/or noxious agents and seems to be essential for mucosal defense because its abolition through mechanical restriction of blood flow leads to hemorrhagic necrosis (Holzer, 2006, Laine *et al.*, 2008).

The endothelial cells are also able to generate potent vasodilators agents such as nitric oxide (NO) and prostacyclin (PGI_2). NO is produced from L-arginine in a reaction catalyzed by the enzyme nitric oxide synthase (NOS) (Bredt and Snyder, 1990). It is an important biological signaling molecule that influences circulation by regulating vascular smooth muscle tone and modulating systemic blood pressure. Therefore, it has been shown to exert positive effects on mucosal defense in the gastrointestinal system (Berg *et al*., 2004). Both NO and PGI_2 oppose the mucosal damaging action of vasoconstrictors such as thromboxane A2, leukotriene C4, and endothelin. Consequently, these agents maintain viability of endothelial cells and prevent platelet and leukocyte adherence to the microvascular endothelial cells preventing compromise of the microcirculation and thus protecting the gastric mucosa against injury (Laine *et al.*, 2008). In addition to maintaining gastric blood flow, NO helps to protect the gastrointestinal tract by inhibiting gastric acid secretion from parietal cells, stimulating mucus and bicarbonate secretion and by promotion angiogenesis *invivo* and *invitro* (Brown *et al.*, 1993; Ma and Wallace, 2000).

Gastric mucosa and submucosal vessels are also innervated by primary afferent sensory neurons gand nerves forming a dense plexus at the mucosal base. Afferent neurons constitute an emergency system that is activated when the gastric mucosa is endangered by noxious agents. Thus, activation of these nerves in presence of gastric acid promotes releasing of neurotransmitters such as substance P and CGRP, which relax the smooth muscle surrounding the arterioles, increasing mucosal blood flow, thereby increasing mucus gel and surface cell intracellular pH in stomach. This mucosal protective action occur through vasodilatation of submucosal vessels mediated by NO generation. Therefore, interference with any aspect of the sensory innervations impairs the hyperemic response and therefore diminishes resistance of the gastric mucosa to injury (Tulassay and Herszényi, 2010, Laine *et al.*, 2008;; Holzer, 2007; Tanaka *et al.*, 1997).



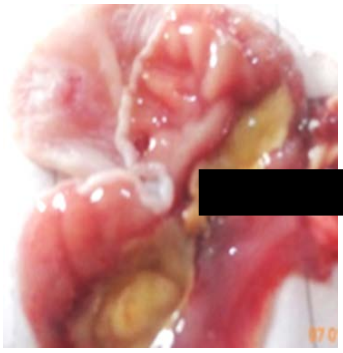


Figure 2.2.Normal mucosa of the stomach **Ulcer at the pyloric region of the stomach**

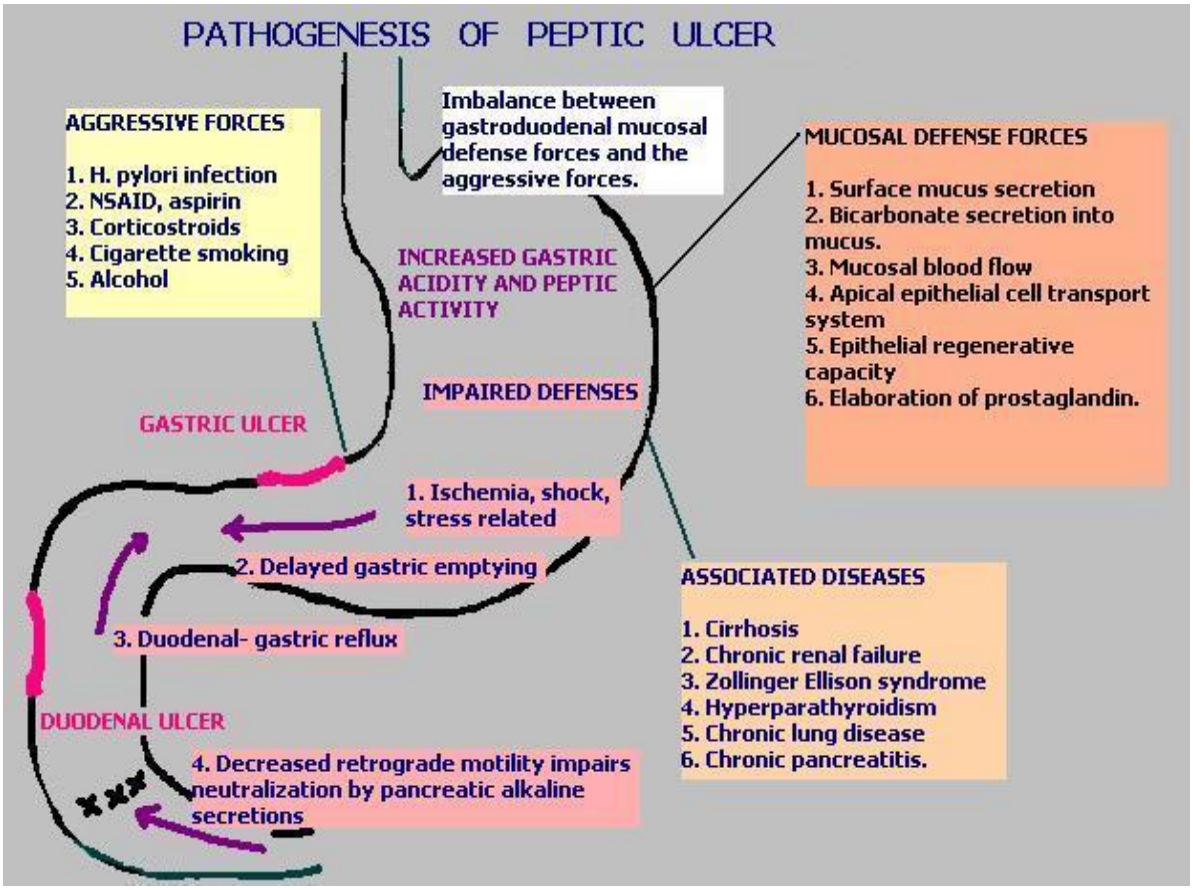


Figure 2.3. Pathogenesis of ulcer (histopathology, 2010)

2.3.0.ETIOLOGY OF ULCER

Many factors are directly related to impairment in mucosal defense and can alter the epithelial barrier increasing the formation of mucosal injury. Gastric acid secretion, bacteria and their products, non-steroidal anti-inflammatory drugs, alcohol, reactive oxygen species, as well as different chemical compounds are important in etiology of ulcer and their effects on the gastric barrier represent important mechanisms of the pathogenesis of gastric ulcers, chronic gastritis and other gastric diseases, which are frequently generated through an imbalance between mucosal aggressive and defensive factors (Tulassay and Herszényi, 2010,Peskar and Marici, 1998, Wallace, 1992).

A. GASTRIC ACID SECRETION

Gastric acid facilitates the digestion of proteins and absorption of calcium, iron, and vitamin B-12, as well as it is the first line of mucosal defense to avoid microorganisms colonization thus preventing the bacterial overgrowth and consequent enteric infection(such as by *Helicobacter pylori*). However, levels of acid (and pepsin) can overwhelms mucosal defense mechanisms, common and potentially serious acid-related clinical conditions occur, including gastroesophageal reflux disease, Barrett's esophagus, where the usual squamous mucosal lining becomes replaced by columnar epithelial cells of putative specific aspect, peptic ulcer disease, and stress-related erosion/ulcer disease (Schubert and Peura, 2008; Schubert, 2008).

When histamine-2 blockers (like cimetidine) was produced, it effectively eliminated acid and thus many patients found that their ulcer disease was healed, these observations validated the dictum “no acid, no ulcer” (Gustfson and Welling, 2010). With time, the prevalence as well as the management of these disorders has changed. The advent of newer pharmacological therapy (potent antisecretory medications such as proton pump inhibitors) and the understanding of the role of *Helicobacter pylori* in the pathogenesis of peptic ulcer disease, more ulcers were successfully treated medically and the number of surgical cases drastically decreased (Lorentzon *et al*., 1987,Lindberg *et al.,* 1990,Meyer-Rosberg *et a*l., 1996, Fock *et al.,* 2008,Schubert and Peura, 2008). As a result, the quantitative measurement of gastric acid secretion, for the most part, has become obsolete. Nevertheless, there are multiple processes involved in the development of gastric lesions, hyper secretion of acid continues to be a necessary condition for ulcer production and for a variety of common gastrointestinal disorders, since medical therapy for these illnesses involves both removing the injurious agent (e.g. NSAIDs or *H. pylori*) and inhibiting acid secretion (Richardson *et al.,* 1998,Schubert &

Peura, 2008).Parietal cells secrete hydrochloric acid at a concentration of approximately 160 mmol/L or pH (0.8).

Acid is thought to gain access to the lumen by means of channels in the mucus layer created by the relatively high intraglandular hydrostatic pressures generated during secretion(approximately 17 mm Hg) (Johnson *et al*., 2001). Thus, luminal acid interferes with the process of restitution, resulting in the conversion of superficial injury to deeper mucosal lesion and inactivates the acid-labile growth factors important for maintenance of mucosal integrity and repair of superficial injury. A large amount of studies show that the rate of acid secretion by the human stomach changes little with aging unless there is coexisting disease of the oxyntic mucosa such as atrophic gastritis, infection with *H. pylori* or both (Trey *et al*., 1997; Schubert & Peura, 2008).To prevent acid-induced mucosal damage, gastric acid must be precisely regulated through a highly coordinated interaction of neural, hormonal, and paracrine pathways (Schubert and Peura, 2008). The principal stimulants of acid secretion include gastrin, histamine, gastrin-releasing peptide (GRP), orexin, ghrelin, and glucocorticoids, while the main inhibitor is somatostatin, released from oxyntic and pyloric D cells (paracrine).

B. NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs)

Another important factor directly related to gastric injury initiated by impairment in mucosal defense is the prominent non-steroidal anti-inflammatory drugs (NSAIDs) use. As the prevalence of *H. pylori* infection has declined, because of continued efforts to eradicate the organism, the prevalence of NSAID-induced ulcers has risen and is taking on a greater clinical importance. Studies have shown that NSAIDs are among the most commonly used drugs in the world. In United States, approximately 70 million prescriptions are written each year, while in Europe these medications represent more than 7.7% of all prescriptions (Graumlich, 2001; Jones, 2001). NSAIDs use is more frequent among women and it increases with age, as well as the incidence of rheumatic diseases. In fact, more than 90% of prescriptions for NSAIDs are made to patients aged >65 years. The major problem with the use of these drugs is that they induce predictable gastric mucosal injury, including complications in both upper and lower gastrointestinal tract (Laine *et al*., 2008; Sostres *et al*., 2010).The major mechanism via which NSAIDs cause ulcers and gastrointestinal complications is said to be by inhibition of cyclooxygenase (COX), a key enzyme in the biosynthesis of prostaglandins (PGs). There are two well identified isoforms of COX(S), COX-1 and COX-2(Laine *et al*., 2008; Sostres *et*

al., 2010). COX-1 isoform is expressed in most tissues, producing prostaglandins that play an essential protective role in the stomach by stimulating the synthesis and secretion of mucus and bicarbonate, increasing mucosal blood flow and promoting epithelial proliferation. So, the COX-1-mediated PG synthesis is mainly responsible for maintaining gastric mucosal integrity at baseline. On the other hand, COX-2has little or no expression in most tissues but is rapidly induced in response to inflammatory stimuli. Therefore, this isoform is the primary target for anti-inflammatory drugs. The traditional NSAIDs nonselective inhibitors of both COX-1 and COX-2 are the indomethacin, ibuprofen, they cause damage in the stomach with a marked decrease in the gastric mucosal PGE2 content. This effect occurs via COX 1 isoform inhibition, creating a gastric environment that is more susceptible to topical attack by endogenous and exogenous factors (Vane & Botting, 1995). Moreover, the inhibition of the COX 1 blocks platelet production of thromboxane, which increases bleeding when an active gastrointestinal bleeding site is present (Lanas and Scheiman, 2007; Sostres *et al.*, 2010).

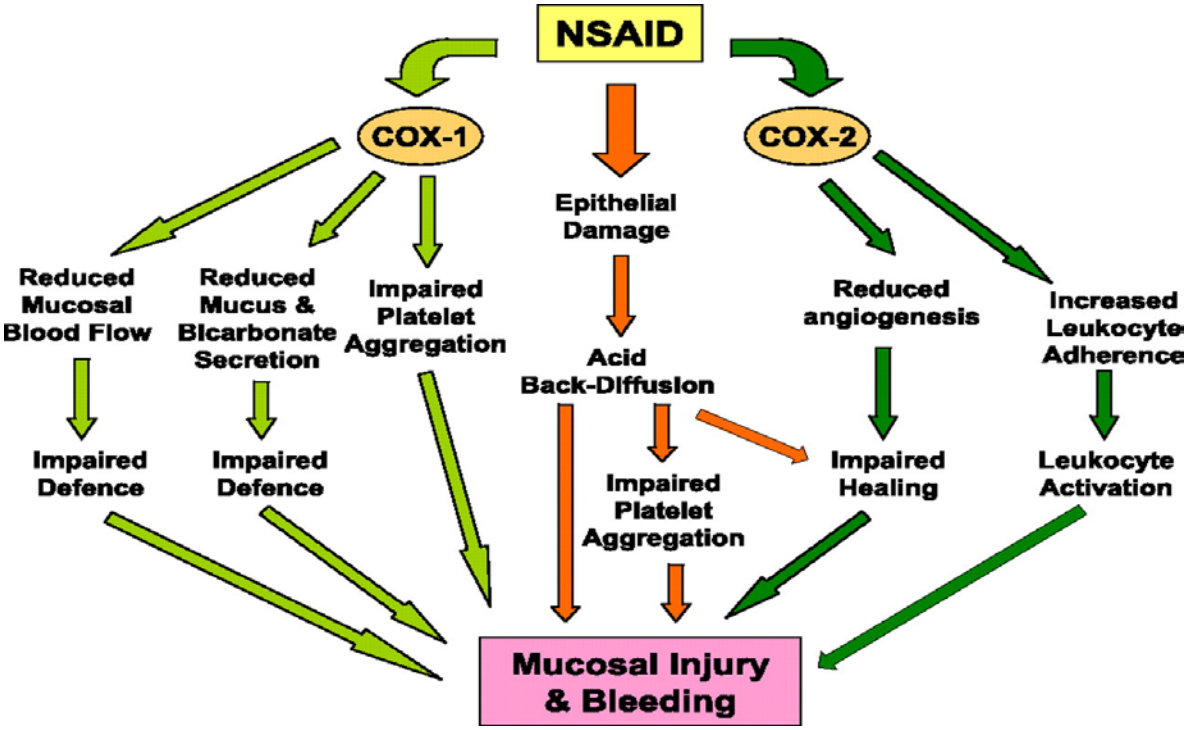


Figure 2.4. Pathogenesis of NSAID-induced gastric injury and bleeding (adapted from Wallace, 2008)

C. ALCOHOL

Throughout the world, alcohol has been used for centuries in social, medical, cultural, and religious settings. Currently, it is considered to be one of the most commonly abused drugs, related to a wide range of physical, mental, and social harms, and responsible for 3.8% of deaths and 4.6% of disability-adjusted life years lost worldwide. The World Health Organization (WHO) has estimated that there are about 2 billion people worldwide who consume alcoholic beverages and 76.3 million with diagnosable alcohol use disorders(Stermer, 2002; WHO, 2004, 2008; Rehm *et al.,* 2009).Among the various organ systems that mediate alcohol's effects on the human body and its health, the gastrointestinal tract plays a particularly important role. The alcohol absorption into the bloodstream occurs throughout the gastrointestinal tract and its direct contact with the mucosa can induce numerous metabolic and functional changes. These alterations may lead to marked mucosal damage, which can result in a broad spectrum of acute and chronic diseases, such as gastrointestinal bleeding and ulcers (Bode and Bode, 1997). Pathogenesis of ethanol-induced gastric lesions is complex. Alcohol may interact directly with the gastric mucosa or it may act through a more general mechanism affecting the release of hormones and the regulation of nerve functions involved in acid secretion (Chari *et al*., 1993, Bode and Bode, 1997). Intra-gastric application of absolute ethanol has long been used as a reproducible method to induce gastric mucosa lesions in experimental animals (Olaleye *et al.,* 2007, Santos *et al.,* 2012). The effects of acute administration of absolute ethanol to rats and mice on the gastric mucosa are dose-dependent and the damage appears as early as 30 minutes after ingestion and reaches a peak at about 60 minutes. The ethanol-induced gastric mucosal lesions and erosions are similar to those occurring in gastric ulcer (Repetto &Llesuy, 2002, Stermer, 2002).

D.OXIDATIVE STRESS

From previous reports, reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, are said to be involved in the etiology(pathophysiology) of several human diseases including neurodegenerative disorders, viral infections, inflammation, autoimmune pathologies, as well as in digestive disturbances such as gastrointestinal inflammation and gastric ulcer (Repetto & Llesuy, 2002).During gastric oxidative stress, the imbalance of aggressive and defensive factors in the stomach plays a pivotal role in gastric hemorrhage and ulcer formation (Hung, 2005).

Overproduction of ROS has been concerned as one of the major pathogenic factors that directly results in oxidative damage, including lipid peroxidation, protein oxidation, and DNA damage, which can lead to cell death. Additionally, these agents are known to act as second messengers to activate diverse redox-sensitive signaling transduction cascades, including mitogen-activated protein kinases (MAPKs) and downstream transcription factors such as NF-kB and AP-1, which regulate the expression of several pro-inflammatory genes and, thereby, lead to the elaboration of chemical and humoral mediators of tissue inflammation and injury (Sun and Oberley, 1996; Ali and Harty, 2009). This is frequently evidenced by pro-ulcerative factors in the *stomach* and gut such as *H pylori*, use of NSAIDs, ethanol, smoking, psychological stress, corticosteroid use, and loss of sleep, while defensive factors involve glutathione (GSH), an important endogenous sulfhydryl compound, and mucus biosynthesis (Olaleye *et al.*, 2007).

In the illness state, oxidative stress of the stomach may occur and result in an elevation of mucosal lipid peroxide that are generated from the reaction of oxy-radicals and cellular polyunsaturated fatty acid, while GSH may act to prevent this aggressive action that can damage gastric mucosal cells. Malondialdehyde (MDA) is an end product resulting from peroxidation of polyunsaturated fatty acids and related esters within cell membranes, and the measurement of this substance represents a suitable index of oxidative tissue damage.

On the other hand, sulfhydryl compounds such as GSH are involved in the maintenance of gastric integrity, particularly when reactive oxygen species are implicated in the patho-physiology of tissue injury (Blandizzi *et al*., 2005). Thus, the appearance of lipid free radicals and MDA in the blood and gastric juice could result from ROS-initiated chain reactions or initiated by indirect mechanisms that suppress the antioxidant capacity in both blood and gastric wall to scavenge ROS (Dotan *et al.*, 2004; Tuorkey and Abdul-Aziz, 2011). Infact, numerous studies have demonstrated a decrease in GSH level in inflammatory and ulcerated gastric mucosa, as well as the protective effect of GSH on gastric damage induced by ethanol, non-steroidal anti-inflammatory drugs, or lipopolysaccharide has been well documented (Hung, 2000; Hung, 2005; Silva *et al*., 2009; Al-Hashem, 2010).

E. HELICOBACTER PYLORI

Helicobacter pylori is a common human pathogen and public health problem associated with the pathogenesis of gastritis and peptic ulcers. With a prevalence of up to 90% in developing populations, this microorganism is the second most common pathogen for human beings. It is

a non-sporulating, gram-negative microaerophilic *bacilli,* spiral-shaped, having one to six polar-sheathed flagellae emerging from one of its rounded ends and a smooth surface (Dye *et al.;* 1989). This pathogen multiplies with great efficiency in the hostile environment within the stomach but survives poorly in the gastric lumen. It is mainly found under the mucous layer and in close proximity, or even attached, to gastric superficial epithelial cells, without substantial invasion of host tissue (Dubois, 1995).

H. pylori induces chronic gastritis of varying severity in infected subjects, which in around 10-15% progresses to peptic ulcer, while in 1-2% of subjects ultimately results in MALT lymphoma or gastric adenocarcinoma. The initial response to infection is an interaction of the host epithelial cells with the bacteria, however, the pathogenetic mechanisms of chronic infection with *H. pylori* and gastric ulcer are yet to be full determined (Parsonnet *et al*., 1991; Ernst and Gold, 2000; Calvino-Fernández and Parra-Cid, 2010).

A characteristics feature of this pathogen is the synthesis of urease, which was its first virulence factor studied. This enzyme may explain the extraordinary ability of bacteria to colonize the gastric mucosa and survive in an acid environment (Smoot, 1991). It catalyzes urea hydrolysis with the formation of ammonium (NH_3), carbon dioxide and hydroxyl ions. By this mechanism, *H. pylori* neutralizes the surrounding gastric acid and protects itself from the strong acidicity of the stomach (Smoot, 1991). Although, the neutralization of gastric acid benefits the bacteria, metabolites from urease activity are toxic to gastric epithelial cells. The formed ammonium reacts with hypochlorite(OCl^-) produced by activated neutrophils to form highly toxic monochloramine (NH_2Cl) in the stomach, a hallmark of *H. pylori* infection.In fact, inhibition of *H. pylori* urease has been showed signifycantly decrease this toxicity, suggesting that ammonia is at least partially responsible for the cytotoxicity found in association with this bacterium. Moreover, hydroxide ions are also considered toxic to gastric epithelial cells (Smoot, 1991; Handa *et al.*, 2010).

Besides urease activity, further important virulence factors from *H. pylori* are their spiral shape and the motility of their flagellae, which render them resistant to peristaltic flushing of the gastric contents and enable them to persist in the mucous layer.

F. ACETIC ACID INDUCED ULCER

In science, the most applicable ulcer research is the techniques that involve establishing an experimentally induced ulcer that resembles human ulcers. The use of acetic acid in ulcer

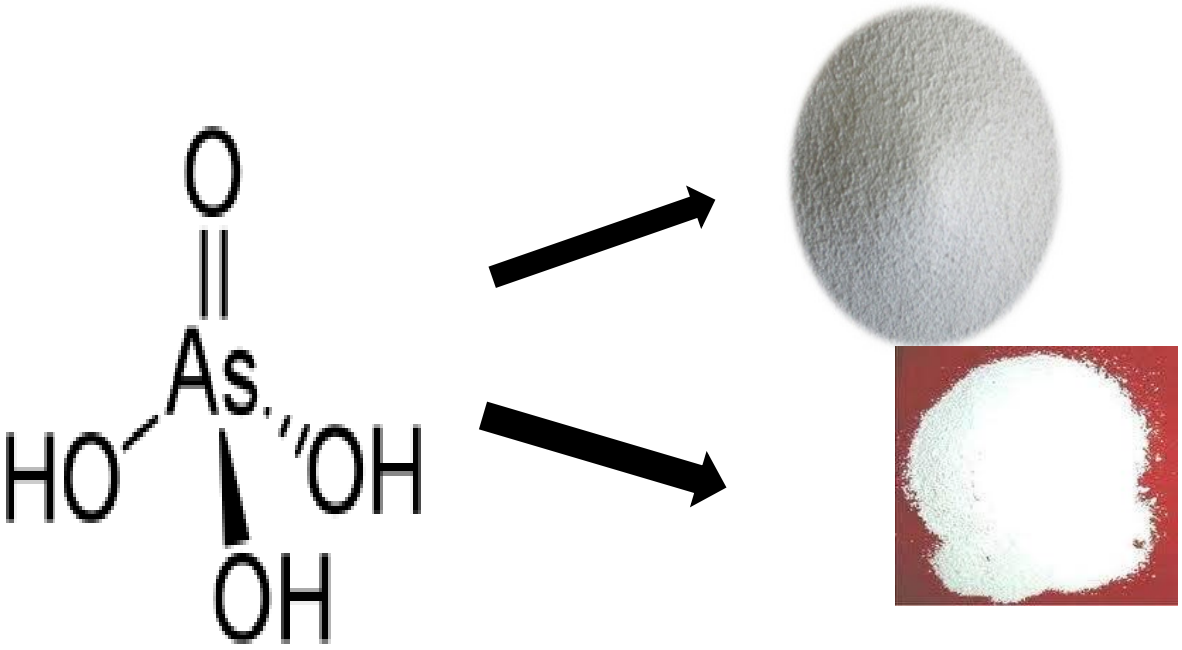
model starts in 1969, and now it is well established(Okabe and Amagase, 2005). The technique or the procedure is quite simple, readily result in to ulcers with consistent size and severity at an incidence at 100%. The model highl resembles human ulcer interms of its pathological features and healing mechanisms. Spontenous relapse of ulcer is also observed in this model as found in the peptic ulcer patient. This form of ulcer model respond to various antiulcer drugs including acid pump inhibitors, sucraflate, growth factors etc.

It is used as standard for screening potential antiulcer drug and study the process of ulcer healing. Acetic acid has been extensively used in research to induce ulcer and its mechanism is via increase in the acidity of the stomach contents (Shirisha and Subash, 2012), stimulating gastric acid hypersecretion which could be due to the release the capillary permeability and back diffusion of HCl.

2.4.0. ARSENIC ACID

Arsenic is a semi-metallic poison known since ancient times. Geber, an Arab alchemist of eighth century produced arsenious oxide from naturally occurring ore found in lead and iron mining and thus one of the most cruel, deadly and widely administered poisons during the medieval history was made available to the humanity(Tripathi and Kumar, 2010). Arsenic is a common environmental pollutant that is threatening life of many people especially in developing countries of the world (Chatterjee *et al.*,1995). It is widely distributed in nature in many forms and its compounds are used extensively as components of herbicides, insecticides, rodenticides, food preservatives, and drugs (Mustafa *et al.,* 2010, Baxley *et al.,* 1985). Arsenic is a ubiquitous element that can come in many oxidative states: -3, 0, +3 and +5. It ranks 12th in the water body, 14th in seawater and 20th in the earth's crust and can be found in an inorganic or organic form (Mandal and Suzuki, 2002). The trivalent inorganic are one of the most common forms that humans are exposed. This exposure can be from natural sources like soil, water, or food sources and from human sources like industrial pollution, mining (copper smelting) and medication.

2.4.1 BIOCHEMICAL STRUCTURE.



Arsenic acid is a tetrahedral species of idealized symmetry C_{3v} with As–O bonds lengths ranging from 1.66 to 1.71 Å .Being a triprotic acid, its acidity is described by three equilibria:

$$H_3AsO_4 + H_2O \rightleftharpoons H_2AsO^{-}_4 + H_3O^+ \ (K_1 = 10^{-2.19})$$
$$H_2AsO_4^{-} + H_2O \rightleftharpoons HAsO_4^{2-} + H_3O^+ \ (K_2 = 10^{-6.94})$$
$$HAsO_4^{2-} + H_2O \rightleftharpoons AsO_4^{3-} + H_3O^+ \ (K_3 = 10^{-11.5})$$

These K_a values are close to those for phosphoric acid. The highly basic arsenate ion (AsO_4^{3-}) is the product of the third ionization. Unlike phosphoric acid, arsenic acid is an oxidizer, illustrated by its ability to convert iodine to iodide (Lee and Harrison, 2007).

Arsenic acid is prepared by treating arsenic trioxide with concentrated nitric acid and dinitrogen trioxide is produced as a by-product.

$$As_2O_3 + 2\ HNO_3 + 2\ H_2O \rightarrow 2\ H_3AsO_4 + N_2O_3$$

The resulting solution is cooled to give colourless crystals of the hemihydrate $H_3AsO_4 \cdot ½H_2O$, although the dihydrate $H_3AsO_4 \cdot 2H_2O$ is produced when crystallisation occurs at lower temperatures (Brauer, 1963).

Also, Arsenic acid is slowly formed when arsenic pentoxide is dissolved in water, and when *meta*- or pyroarsenic acid is treated with cold water. Arsenic acid can also be prepared directly from elemental arsenic by moistening it and treating with ozone.

$$2As + 3\ H_2O + 5\ O_3 \rightarrow 2\ H_3AsO_4 + 5\ O_2$$

2.4.2. DISTRIBUTION

An inorganic arsenical compound consists of arsenite, considered to be the most toxic form, and arsenate the less toxic form, and organic forms the least toxic ones. Arsenic is a metalloid widely present in the environment. It is found in well water, soil, and air, and is also released from mining residues and industrial debris, among other anthropogenic sources. Exposure to arsenic occurs either occupationally in several industries, including, mining, pesticide, pharmaceutical, glass and microelectronics, or environmentally from both industrial and natural sources (Tchounwou *et al.,* 2002, Balakumar *et al.,* 2010).

The most common pathway for an elevated environmental exposure to inorganic arsenic worldwide is through drinking water. Chronic exposure to arsenic causes a wide range of toxic effects and thus this metalloid is classified as Group I carcinogen in humans. Drinking water contamination by arsenic remains a major public health problem.

Chronic arsenicism has been observed in workers and in groups of the general population living in some areas of the United States(Welch *et al.,* 2000; Shaw *et al*., 2005), United Kingdom (Goyer *et al.,* 1995), China, Taiwan(Chen *et al.,* 2005; Tseng *et al.,* 2006), Mexico (Cebrian *et al.,* 1993), Chile (Smith *et al.,* 1998),Argentina (Rich *et al.,* 1998), India (Mazumdar, 1988; Rahman *et al.,* 2005)and Bangladesh (Gamble *et al.,* 2005).

Today, a number of countries are affected by arsenic and its detrimental effects but Bangladesh and West Bengal have the largest affected areas. In these countries, the surface water is contaminated with pathogens, so tube wells were dug to reach the groundwater to use for drinking (Karn and Harada, 2001). Unfortunately, the well water contains high amounts of inorganic arsenic (Chowdhury *et al.,* 2001). When arsenic is found in high concentrations in the bedrock it is usually paired with sulfide minerals. The most common mineral forms are arsenian pyrite ($Fe(S,As)_2$) and arseno pyrite (FeAsS),and it can also come in the forms of realgar (AsS), orpiment (As2S3), or scorodite($FeAsO_4$.2H2O). Large concentrations of arsenic in drinking water usually come from aquifers that are located in alluvial and delta planes. They create the best circumstances for the arsenic to separate from its mineral form through redox reactions that normally occur between a pH of 6.5-8.5. Hydrologically closed basin regions and a high pH of saline groundwater will also affect the adsorption of the arsenic (Welch, 2000). The poor adsorption of arsenic into evaporate minerals will only occur at a high salinity (> 9molar) and as long as the redox stability of As(V) are at moderately

reducing conditions and high pH values occur, it will decrease As affinity to the mineral surfaces . In south eastern Bangladesh alone, 90% of the boreholes have an arsenic concentration greater than 50μg/L, which is due to the area being part of a large delta plane. The World Health Organization (WHO) has changed its limits of arsenic in the groundwater throughout the decades with the increasing concern for its adverse effects on human health.

In 1958, the regulation was 200 ppb, this amount was lowered to 50 ppb in 1963, and finally in 1993 was lowered to 10 ppb (WHO, 2003). The U.S. itself made the standard of 10 ppb effective on February 22, 2002, with water systems needing to be in compliance with the new standard by January 23, 2006. In the U.S., 31,350 public and private water supplies were tested for arsenic concentration from 1973 to 2001. In certain areas like California (highest concentration was 2600ppb), and Nevada (highest concentration was 2200 ppb) (Ryker, 2001), the high concentrations are largely due to evaporation and concentration in shallow ground water.

Exposure to trivalent and pentavalent forms of arsenic, which occurs worldwide primarily through occupational and environmental exposure, causes characteristic skin alterations (ulceration), including hyperkeratosis and skin cancer (Yoshida *et al.*,2004).Epidemiological studies conducted in Taiwan(Chou *et al.*,1995), Chile(Smith *et al.*,1998) and Japan indicated a connection between arsenic exposures from contaminated drinking water among the inhabitants. It is also known that arsenic interact with other substances, metals inclusive there by potentiating its effects and/or vice versa (Odunola *et al*., 2007). There is growing evidences that sodium arsenite intoxication can compromise the integrity of the liver in mouse, rat, fish, and goat (Sharma *et al*., 2009, Roy *et al*.,2009, Vutukuru *et al.*,2007). Recently, some studies suggest the use of antioxidants and antioxidant rich foods and herbal medicinal plant for the management of arsenicosis (Das *et al.*,2010) Induction of cancer is frequently associated with DNA damage, changes in ploidy of cells, and non-random chromosome aberrations which can result from exposure to arsenic(Sinha et al.,2005).

In Nigeria, according to the reports by Garba *et al*., 2012, one hundred and twenty samples of drinking water fom well and boreholes were collected from eight local government in Northern Kaduna senatorial district and were randomly subjected to arsenic elucidation. The data from the result of the study stated that the concentration of arsenic compound from the well water concentration was amount to 0.28mg/L while from the borehole it was 0.09mg/l which exceeded the maximum concentration limit of 0.01 mg/L set by WHO.

The arsenic concentration in wells and boreholes water in the southern parts of Kaduna has been found to be 0.34mg/L which is higher than the boreholes samples (0.14mg/L) and exceeded the maximum concentration limit of 0.01 mg/l set by the World Health Organization (adopted by the Standard Organization of Nigeria).

Musa *et al.* 2008, reported that the concentration of arsenic distribution in Zaria is from 0.02 mg/L – 0.51 mg/L, their finding also reported a high level of arsenic concentration of 0.81mg/L in Kutama and 0.77 mg/L in Getso, Gwarzo Local Government area, Kano State, above the Maximum Concentration Limit (MCL) of 0.01mg/L. These high concentrations were likely be due to disposal of arsenic containing materials, burning of solid wastes, natural processes and human activities(Garba *et al.,* 2008, Musa *et al.,* 2008). High concentration of arsenic was recorded in wells from the sampled areas because most of them are open and the areas where they are located have high human, agricultural activities and natural processes.

It was therefore expected that the people of Kaduna North Senatorial District of Kaduna State, Nigeria may likely suffer because the arsenic concentrations in both the well and the borehole waters are high enough to cause the arsenicolysis as they are taking the water directly without proper treatment.

In parts of Biu volcanic pronvince, in the North-Eastern Nigeria, the values of arsenic concentration observed in surface water in Biu volcanic Province was higher than the permissible level of WHO set in 2008, there was obvious symptons of its poisonous effect on theinhabitant of the area (Usman and Lar, 2013), in some underground and waters from some major towns in Ogun state (Kayode *et al.*,2011),high concentration above MCL was also observed.In the south west of Ibadan,such was also reported by Egbinola and Amanambu in 2014. In Odede waters in Ogun state, there was high level of arsenic acid higher than the WHO permissible limit (Amori *et al,* 2011).

In the reports by Atobatele and Olutona in 2015, the arsenic concentration in Aiba reservoir as assessed to determine its level and distribution in sedimaent and tisues of fish, it was observed that the first and second Canonical Variate showed 49.82% and 34.75% between-species variation respectively. This report suggests that fish at the lower level of the food web have higher levels of As compared to those at a higher trophic status. The current low levels of arsenic in the abiotic component of the reservoir suggest that the contamination is mainly from anthropogenic rather than from natural sources and the report suggested that there is a

seasonal, municipal and agricultural sources of arsenic contamination of Aiba Reservoir (Atobatele and Olutona, 2015).

Assessment carried out by Maduabuchi *et al.,* 2007 showed that there was 33.3% of the canned beverages had arsenic levels that exceeded the maximum contaminantlevel (MCL) of 0.01 mg/L set by U.S. EPA while 55.2% of non-canned beverages had their arsenic levels exceedingthe MCL. The arsenic concentrations ranged from 0.003 to 0.161 mg/L for the canned and 0.002 to 0.261 mg/l for thenon-canned beverages.

2.4.3. SOURCES OF ARSENIC ACID

Exposure to arsenic may come from natural source, from industrial source, or from administered i.e. accidental source. Self-administration of arsenic, unintentionally i.e., accidental consumption by children or deliberate i.e., homicidal or suicidal in attempts by adults, represents the rare causes of acute poisoning. The source of such self-administration is typically an arsenic-containing insecticide, herbicide, or rodenticide. From a clinical perspective, massive exposures are now not usually seen in suicidal or homicidal setting accidental exposures, usually not serious yet largely preventable, are usually seen in children, and chronic or intermittent exposures often are the most diagnostically challenging. Exposure to arsenic via drinking water, air, food, and beverage has been reported occurring at many places in the world. Exposure through drinking water is increasing due to contamination from industrial operation and over withdrawal of groundwater for irrigation.Occupational and environmental health problems can result from the frequent commercial presence of arsenicals.

Exposure to arsine gas is also an environmental health hazard of concern in numerous occupational circumstances. Arsine is a colourless, odourless, tasteless, nonirritating gas that causes a rapid and unique destruction of red blood cells and may result in kidney failure, which is uniformly fatal without proper therapy. Most cases of arsine poisoning have occurred with the use of acids and crude metals of which one or both contained arsenic as an impurity.

The two usual routes of absorption of arsenic are by ingestion and/or inhalation. There may be some degree of skin absorption of trivalent arsenic oxide since it is more lipid-soluble than the pentavalent form (Winship,1984, Hua *et al.,* 2004). If the contact is by ingestion, then

symptoms caused by acute gastrointestinal irritation will dominate the reaction. Ingested arsenic has a shorter half-life than inhaled ‘arsenic due to more rapid biotransformation in the liver(Vater,1988).If the inhalation is the route of initial contact, then respiratory irritation will be a major determinant of early symptoms. However, once the arsenic is absorbed, the vascular circulation will have contact with a wide variety of potential symptoms reflecting the diversity of possible organ damages.

Arsenic enters the human body through ingestion, inhalation, or skin absorption. Most ingested and inhaled arsenic is well absorbed through the gastrointestinal tract and lung into the blood stream. 95% of the ingested trivalent arsenic is absorbed from the gastrointestinal tract. It is distributed in a large number of organs including the lungs, liver, kidney, and skin (Hunter,1942). After absorption through lungs and the gastro-intestinal tract, 95 to 99 % of the arsenic is located in erythrocytes, bound to the globin of hemoglobin and is then transported to the other parts of the body. About 70% of the arsenic is excreted mainly through urine. Most arsenic absorbed into the body is converted by the liver to less toxic methylated form that is efficiently excreted in the urine. The rate of decrease of arsenic in the skin appears to be especially low compared with the rate for other organs.

2.4.4. CLINICAL USES OF ARSENIC

Arsenic trioxide has been used as treatment for different types of leukemia, like multidrug-resistant human myeloid leukemia, acute promyelocytic leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, non-Hodgkin’s lymphoma,Hodgkin’s disease, chronic lymphocytic leukemia, myelodysplastic syndrome, and multiple myeloma (Chen *et al.*, 2002, Li and Broome, 1999).

The arsenic has been used to induce cell cycle arrest and apoptosis in the leukemia cells, but the same outcomes may also happen in noncancerous cells (Qian *et al.*, 2007).In a study done by Perkins in 2000, human promyelocytic leukemic cells (HL-60) and myelogenous leukemia cells (K562) that were incubated in arsenic trioxide saw a reduction in growth with a LC50 between 0.8 and 1.5 μmol/L. At 2 μmol/L of arsenic trioxide, after 7 days of incubation, 30 to 50% of cells, in all cell lines, showed morphologic features of apoptosis, like blebbing and an expression of the cell-surface phosolipid phosphatidylserine, that is only released from the cell’s cytosol to the cells surface during apoptosis (Perkins *et al.*, 2000).

Another study showed that arsenic trioxide disrupted tubulin polymerization in K562 cells at concentrations ranging from 0.5–5 μmol/L (Li and Broome, 1999). Its use dates back to 1681 as pesticide, herbicides and as poison. Parenterally arsenic was used in the past for the treatment of syphillis, topical eosinophilia, trapanosomiasis, Lichen planus, verruca planum and psoriasis. Domestic, agricultural and industrial uses of arsenic in the form of insecticides, weedicides, rodenticides and arsine are becoming rarer because of advent of low toxic pesticides. It has also been used in Fowler solution in the treatment of arthritis (Klaasen, 2000).

2.4.5. OTHER USES OF ARSENIC ACIDS

1. Wood preservatives:

Inorganic arsenic is an active component of chromated copper arsenate, an antifungal wood preservative used to make “pressure-treated” wood for outdoor applications. Chromated copper arsenate is no longer used in residential applications, following a voluntary ban on its use in Canada and the United States of America at the end of 2003 (ATSDR, 2007).

2. Agricultural uses:

In the agricultural industry, arsenic has historically been used in a range of applications, including pesticides, herbicides, insecticides, cotton desiccants, defoliants, and soil sterilants. Inorganic arsenic pesticides have not been used for agricultural purposes in the USA since 1993. Organic forms of arsenic were constituents of some agricultural pesticides in the USA. However, in 2009, the US Environmental Protection Agency issued a cancellation order to eradicate the use of organic arsenical pesticides by 2013 (EPA, 2009). The one exception to the order is monosodium methanearsonate (MSMA), a broadleaf weed herbicide, which will continue to be approved for use on cotton. Small amounts of disodium methane arsonate (DSMA, or cacodylic acid) were historically applied to cotton fields as herbicides, but its use is now prohibited under the aforementioned US EPA 2009 organic arsenical product cancellation. Other organic arsenicals (e.g. roxarsone, arsanilic acid and its derivatives) are used as feed additives for poultry and swine to increase the rate of weight gain, to improve feed efficiencies, pigmentation, and disease treatment and prevention (EPA, 2006, 2008; FDA, 2008).

3. Industrial purpose:

Arsenic and arsenic compounds are used for a variety of other industrial purposes. Elemental arsenic is used in the manufacture of alloys, particularly with lead (e.g. in lead acid batteries) and copper. Gallium arsenide and arsine are widely used in the semiconductor and electronics industries. Because of its high electron mobility, as well as light-emitting, electromagnetic and photovoltaic properties, gallium arsenide is used in high-speed semiconductor devices, high power microwave and millimeter-wave devices, and opto-electronic devices, including fibre optic sources and detectors (IARC, 2008). Arsine is used as a doping agent to manufacture crystals for computer chips and fibre optics. Arsenic and arsenic compounds are used in the manufacture of pigments, sheep-dips, leather preservatives, and poisonous baits. They are also used in catalysts, pyrotechnics, and antifouling agents in paints, pharmaceutical substances, dyes and soaps, ceramics, alloys (automotive solder and radiators), and electrophotography.During 1990–2002, approximately 4% of arsenic produced was used in the manufacture of glass, and 1–4% was used in the production of non-ferrous alloys (NTP, 2005)

2.5.0.SODIUM ARSENITE

Sodium arsenite is a compound with formula $NaAsO_2$. It is the sodium salt of arsenous acid. It is a crystalline solid consisting of sodium cations, Na^+, and *catena*-arsenite anions, $[AsO_2]n-n$, which are infinite -O-As(=O)- chains, similar in structure to selenium dioxide, SeO_2 . It is produced from arsenic trioxide and sodium nitrate.It is also a waste product from manufacturing industries (Oyadeji *et al*, 2007). Sodium arsenite is used for the production of insecticides and pesticides for agricultural purposes (Nickson *et al.*, 1993, Morakinyo *et al*, 2010). Its use dates back to 1681. The poisonous properties of arsenic oxide led to the use of sodium arsenite as poison.When applied on plants, it is washed by rain into water bodies e.g. Rivers and leeches into underground water. It is ingested orally by drinking from these water bodies or eating foods that it has been applied on. It is un-degradable and may accumulate gradually. Also prolonged exposure has adverse effects on the skin. It can be inhaled and on prolonged exposure, it causes serious effects.

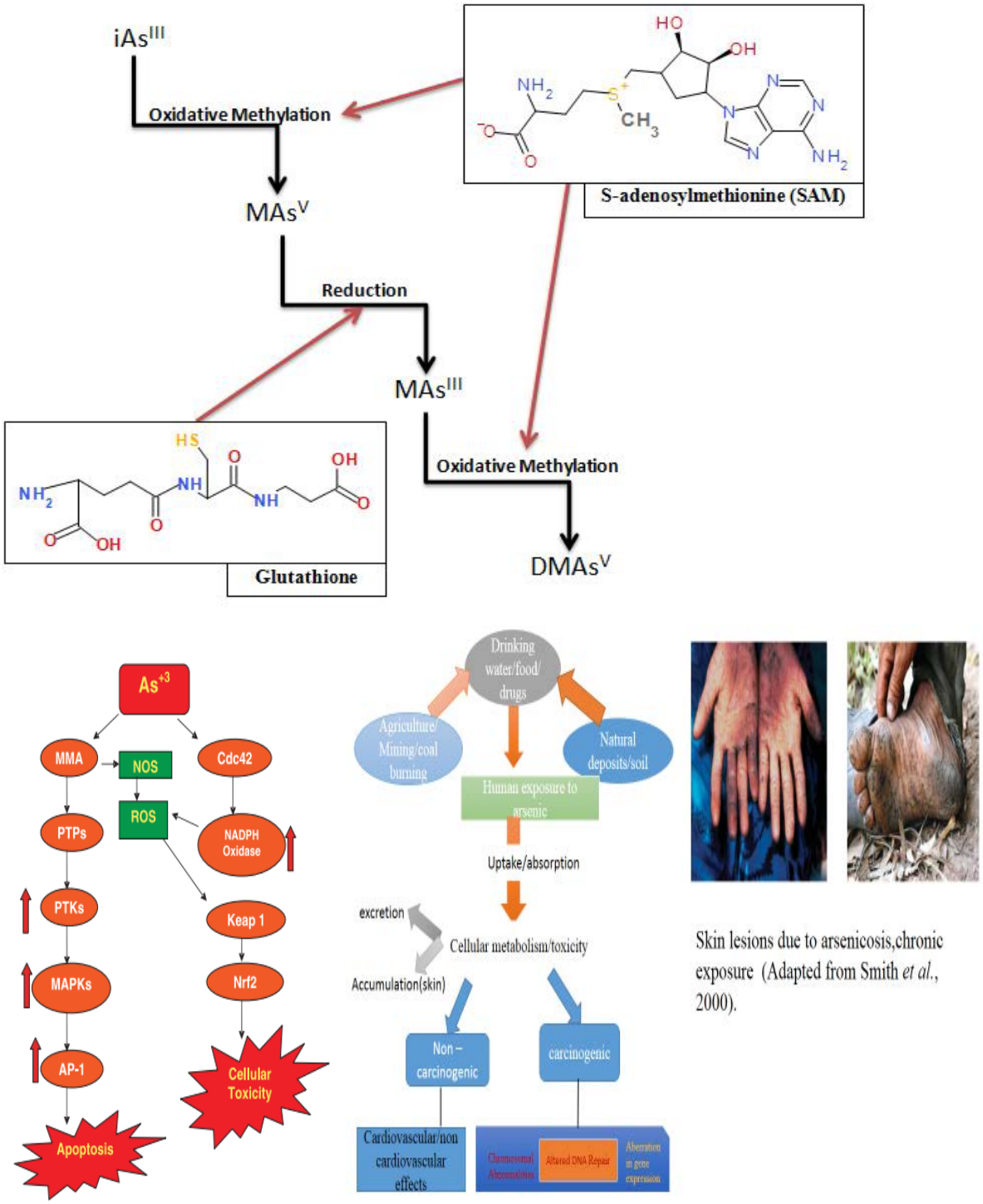
2.5.1.FEATURES OF SODIUM ARSENITE

It has appearance of gray-white powder, odourless and very soluble in water with the specific gravity of 1.87g/$cm^3$and very stable under ordinary condition of use and storage.it can emits fumes of arsenic when heated to decomposition.

2.5.2. METABOLISM

When inorganic arsenic enters the body, the proposed metabolic pathway starts with the liver where it is metabolized through an oxidative process in which the trivalent inorganic arsenic will gain a methyl group that is donated from S-adenosylmethionine (SAM). This process changes the oxidative state to a pentavalent form, creating methylarsenoic acid (MAsV). From there, two electrons are removed from arsenic through a thiol oxidation process to reduce As(V) to As(III).

Another oxidative methylation can take place creating dimethylarsinic acid (DMAsV) (Thomas *et al.*, 2004). Normally the methylation and reduction process would be considered a detoxification step, with the arsenic metabolites exiting through the urine, but some studies have shown that the methylated species might also contribute to the effects seen with arsenic exposure (Styblo, 2000)



Skin lesions due to arsenicosis,chronic exposure (Adapted from Smith *et al.*, 2000).

Figure 2.6a: Methylation process with the methyl don or SAM and the reducing cofactor glutathione,adapted from Steffens, 2009. F**igure**.2.6b**.** Arsenic induced ROS generation and its impact on cellular pathways **(**from adapted Flora *et al.*, 2008) **.Figure 2.6c:**Skin lesions due to arsenicosis(Adapted from Smith *et al.*, 2000)**.**

An *in vivo* study examined the effects that methylarsonic acid, $CH_3AsO(OH)_2$(MAA), dimethylarsininc acid, $(CH_3)_2AsO(OH)$ (DMAA), and trimethylarsine oxide when orally administered to mice. DMAA had the highest toxicity of the methylated species with a LD_{50} of 1.2 g/kg, while MAA and TMAO had LD_{50} of 1.8 and 10.6 g/kg respectively. These concentrations are below arsenic trioxide's acute toxicity in which two mouse studies had an oral LD_{50} of 26-48 mg/kg (Harrison *et al.*,1958). Even though methylated species are not lethal it is important to consider their biological effects. The toxicity of inorganic arsenic, trivalent and pentavalent methylated arsenicals, was investigated in cultured human hepatocytes, bronchial cells, keratinocytes and urothelium cells, along with rat hepatocytes (Styblo *etal.*, 2000). Trivalent monomethylated arsenic was found to be less cytotoxic when compared to inorganic arsenic (III) in all cell types (Styblo *et al.*, 2000). The cell line with the greatest methylation capacity were the rat hepatocytes followed by the human hepatocytes, keratinocytes and bronchial cells. Another study also investigated the effects of exposed human epidermal keratinocytes to the different oxidative states of arsenic (Vega *et al.*, 2001). They observed that the lower the oxidative state, the more cytotoxic the arsenic was and within the pentavalent oxidative states the methylated versions had the higher cytotoxicity: iAsIII (arsenite) > MAsIIIO (methyl arsine oxide) > DMAsIIIGS (dimethylarsinous acid with glutathione) > DMAsV (dimethylarsinic acid) >MAsV (methylarsonic acid) >iAsV(arsenate) (Vega *et al.*, 2001). This shows that the methylated species of arsenic may contribute to the adverse effects connected with exposure to arsenic.

2.5.3. MOLECULAR EVENTS

Multiple aliments has been found to be connected to chronic exposure but there is no specific order in which they appear, so diagnosis of arsenic exposure can be difficult. External characteristics can consist of dermal changes like lesions, pigmentation changes, increase of keratoses, or skin cancers (Tondel, 1999; Mandal, 2002). The internal ailments associated with exposure range from cardiovascular diseases, different forms of cancer, respiratory or gastrointestinal problems to developmental problems (Shi *et al.*, 2004, Soffritti *et al.*, 2006). A clear mechanism or pathway for these ailments is still unknown but there have been various hypotheses like alteration of the DNA methylation pattern, impairment of DNA repair mechanisms, genetic damage or programmed cell death (Hughes, 2002).

For example, it has been shown that arsenic can modify DNA methylation patterns(Richard *et al.*, 2007). Gene transcription can be regulated through methylation and an alteration to DNA

methylation patterns can cause genes to be over- or under-expressed itself (Newell-Price *et al.*, 2000). A study showed that when the A549 human adenocarcinoma cell line was exposed to various concentrations of sodium arsenite, sodium arsenate, and DMAsV, both sodium arsenite (0.08-2 μM) and sodium arsenate (30-300 μM) hypermethylated the promoter region of the tumor suppressor gene P53 (Mass and Wang, 1997), which indicates that increasing arsenic concentrations likely suppressed p53expression more efficiently, allowing for deregulation in the cell cycle process. Arsenic exposure can both increase genetic damage as well as alter DNA repair mechanisms (Andrew *et al.*, 2006). There are three levels of genetic damage that can occur and as follows.

- Mutation and frame shift mutation;
- Chromosome or chromatid breaks and aneuploidy, otherwise known as a gain or loss of a whole chromosome.
- Genetic damage is spindle disruption at metaphase and anaphase during the cell cycle and dicentric chromosome formation, where a chromosome contains two centromeres instead of one (Gonzalgo and Jones, 1997).

Its exposure finally results in programmed cell death which follows a highly regulated pathways and can lead to either apoptotic or autophagic cell death (Burchell *et al.*, 1991). In apoptosis, the activation of various caspases lead to the formation of blebs, bulges that separate from the cell, taking a portion of cytoplasm and containing the organelles of the cell so that other neighboring cells will not be affected.

Autophagic cell death differs in that it forms vacuoles in which organelles of the cell are destroyed while maintaining membrane formation (Cuervo, 2004). A variety of studies have shown that arsenic is able to induce programmed cell death (Li and Broome, 1999,Soignet *et al.*, 1998; Kong *et al.*, 2005; Bashir *et al.*, 2006; Binet *et al.*, 2006; Cheung *et al.*, 2006; Qian *et al.*, 2007; Banerjee *etal.*, 2008; Leu and Mohassel, 2009). Other reports also found that exposure to 4μM arsenic trioxide result not only in apoptosis in two leukemia cell lines (Molt-4 and Mutz-1) but an increase in non-apoptotic cell death known as autophagy (Qian *et al.*, 2007). Additionally, male Wistar rats exposed to 2.5 and 5 mg/L sodium arsenite in their drinking water had an increase in apoptotic cells in their livers (Bashir *et al.*, 2006).

2.5.4. TRANSFORMATION OF ARSENIC IN THE BODY

Once arsenic is ingested, soluble forms of arsenic arereadily absorbed from the gastrointestinal tract. Absorption rate estimates range from 40 to 100% in humans. Arsenate, As(V) whether inorganic or organic, is better absorbed than As(III) arsenite becausearsenate is less reactive with membranes of the gastrointestinal tract(NAS,1977). Arsenic in drinking water is mostly inthe arsenate form, and complete absorption of arsenic from water may occur.

Once absorbed, arsenic is transported by the blood to different organs in the body, mainly in the form of MMA. Typical levels in the blood of people who are not exposed to a significant source of arsenic pollution range from 1 to 5 mg/l As, levels in soft tissue range from 0.01 to 0.1 mg As/gm (The highest levels may be found in nails and hair (0.1 to 1mg As/g) where arsenic accumulates over time(EPA, 1993).

Metabolism of arsenic in humans involves two processes. After entering a cell, arsenate is reduced to arsenite. Arsenite is then methylated to form MMA and DMA; this process occurs primarily in the liver (Tseng,1968, McKinney,1992) .Trimethylarsine oxide, which is third species has not yet been identified in human, is expected to be produce during arsenic metabolism and its significance in organic metabolism is still not known.

Inorganic As(V) and As(III) have different mechanisms of action. Arsenate As(V) behaves very much like phosphate consequently, it can substitute for phosphate in normal cell reactions, interesting with normal cell functions(NAS,1977).In contrast, arsenite [As(III)] has a high affinity for thiol (-SH) groups in proteins, causing inactivation of a variety of enzymes (Abernathy,1993, NAS,1977, Tseng,1968). Because arsenate is reduced in the body to arsenite, arsenate in drinking water may have a biological effect identical to arsenite. In contrast to inorganic arsenic, neither MMA nor DMA binds strongly to molecules in humans.Hence, their relative acute toxicity is less than that of inorganic arsenic form(Abnerthy,1993). In general, inorganic As(V) is one tenth as toxic as inorganic As(III), and MMA and DMA are less toxic than inorganic As(V).After ingestion, inorganic arsenic that is not immediately excreted or absorbed by tissues is progressively detoxified through the methylation process. However, the chronic effects of a MMA and DMA are not known, only a few studies have evaluated DMA (EPA, 1993).

The inorganic arsenic is excreted primarily via urine as the parent form of the ingested arsenic. After methylation, it is also excreted as MMA and DMA. Humans rapidly excrete

most blood arsenic, with 50 to90% cleared in two to four days. The remainder is cleared 10-100 times more slowly (NAS,1977, EPA,1993).The pharmacokinetics of arsenic in the human body are not well understood. Although, several pharmacokinetic models have been developed, thinly apply to short-term exposure (two to four rats) and have several limitations that cause them to inaccurate projection.However, further development and refinement of pharmacokinetic and pharmacodynamic models are important, especially its effects on health when there is exposure to low level of arsenite.

2.5.5. ARSENIC EFFECT ON BODY ENZYMATIC SYSTEM

Arsenite compound mainly absorbed to the human gastrointestinal tract and it deposited hugely to the various cells in the body. As a result, it affect the enzyme activity in the cell and finally the affected cells becomes dead slowly. It involves two steps

1st step.

Pyruvic acid (which is obtained from the glucose of inside the cell mitochondria) breaks with the help of a special type of enzyme. The pyruvate oxidase complex is necessary for oxidative decarboxylation of pyruvate produce to acetyl coenzyme A and carbon dioxide before it enters the tricarboxylic acid cycle. In this process energy is store for workable of cells.

The enzyme system comprises several enzymes and cofactors one protein molecule of enzyme having one lipoic acid. In each lipoic acid there are two sulfhydryl (-SH) or thiol group, which is essential for its workability.In the presence of trivalent arsenic (Arsenite) it replace the two Hydrogen from the thiol group and attached with sulfur molecule and formed a dihydrolipoyl-arsenite chelate complex, which prevents the re-oxidation of the dihydrolipoyl group necessary for continued enzymatic activity, and this pivotal enzyme step is block. As a result amount of pyruvate in blood increases, energy production is reduced and finally the cell damage slowly.In the same manner arsenic destroy workability of another enzyme and reduced production of succinyl coenzyme A and finally production of ATP reduced. If arsenic is deposited in long time then itbreaks the ATP block the energy supply to the cells.

2nd Step

The arsenate form of inorganic arsenic is available in nature. This also blocks the enzymatic activity in mitochondria but in different way. The next steps of ADP from the continuing

enzymatic activity combine with inorganic phosphate and produce ATP. This reaction is called oxidative phosphorylation. Since arsenic can replace phosphorus, so it combines with ADP to replacing phosphate and subsequent formation of an unstable arsenate ester bond that is rapidly hydrolysate. As a result of this, production of ATP through phosphorylation is hampered and source of energy in cell reducing continually not only this but also it disturb the electron transfer of inorganic phosphorus with ATP. Thus, the so-called high-energy bonds of adenosine triphosphate are not conserved in the presence of arsenate. This process is termed "arsenolysis", Arsenic may therefore be highly toxic to cellular respiration by inhibiting energy-linked functions of the mitochondria in two very different ways.

1. Trivalent arsenic inhibits the reduction of nicotinamide adenine dinucleotide by deactivating critical enzymes in the tricarboxylic acid cycle.

2. Pentavalent arsenic uncouples oxidative phosphorylation by arsenolysis.

Another important enzymatic reaction is the production of ATP with succinic acid or succinate through flevo protein reduction, Arsenate compound disturbed in this reaction also, as a result energy supply in cells reduced.

2.5.6. ACUTE ARSENITE POISONING

The symptoms and signs of acute arsenic poisoning usually appear within thirty minutes to one hour after ingestion but may be delayed several hours. Garlic odour of the breath and faeces is present and helps to identify toxicant in severely poisoned patient. It is often metallic taste in the mouth. Adverse GI effects include inflammation, vesicle formation and eventual sloughing of the mucosa in the mouth, pharynx and esophagus. Other effect includes vomiting, abdominal pain and rice- water or bloody diarrhea being the most common. These effects result from the action of an arsenical metabolite on blood vessels generally and the splanchnic vasculature in particular, causing dilation and increased capillary permeability.

The central nervous system is also commonly affected during acute exposure. Symptoms may begin with headache, dizziness, drowsiness and confusion. Symptoms may progress to include muscle weakness and spasms, hypothermia, lethargy, delirium, coma and convulsions (Malachowski,2000).Renal injury is manifest as proteinuria, hematuria, glycosuria, oliguria, casts in the urine and in severe poisoning, acute tubular necrosis. Cardiovascular manifestations include shock, cyanosis and cardiac arrhythmia (Goldsmith, 2008) which are

due to direct toxic action and electrolyte disturbances. Liver damages may be manifested by elevated liver enzymes and jaundice. Injury to blood forming tissues may cause anemia, leukopenia and thrombocytopenia.

Death usually occurs one to three days following onset of symptoms and is often the result of circulatory failure, although renal failure may contribute (Malachowski, 2000). If patient survives, painful paresthesias, tingling and numbness in the hand and feet maybe experienced as a delay sequela of acute exposure. This sensorimotor peripheral neuropathy which may include muscle weakness and spasms typically begins 1-3 weeks after exposure. (Heyman, 1956). The muscle weakness may be confused as Guillain- Barre syndrome.

2.5.7. CHRONIC ARSENITE POISONING

From repeated absorption of toxic amounts generally has an insidious onset of clinical effects and may be difficult to diagnose. Neurological, dermal and non-specific manifestations are usually more prominent than gastrointestinal effects that characterize acute poisoning. Muscle weakness and fatigue can occur, as can anorexia and weight loss. Hyperpigmentation is a common sign and tends to be accentuated in areas that are already more pigmented such as the groin and areola. Hyperkeratosis is another very common sign especially on the palms and soles (Maloney, 2006, Navarro, 2006) subcutaneous edema of the face, eyelids and ankles, stomatitis, white striations across the nails (Mees lines), and sometimes loss of nails or hair are other signs of chronic continuous exposure (Malachowski, 2000, Navarro, 2006). On occasion, these hyperkeratotic papules may undergo malignant transformation (Maloney, 1998)

Neurological symptoms are also common with chronic exposure. Peripheral neuropathy, manifested by paresthesia, pain, anesthesia, paresis, and ataxia may be a prominent feature. It may often begin with sensory symptoms in the lower extremities and progress to muscular weakness and eventually paralysis and muscle wasting. Although less common, encephalopathy can develop with speech and mental disturbances very much like those seen in thiamine deficiency (Wernicke’s syndrome).

Other organ systems are affected with arsenic toxicity. Liver injury reflected in hepatomegaly and jaundice may progress to cirrhosis, portal hypertension and ascites. Arsenic has direct glomerular and tubular toxicity resulting in oliguria, proteinuria and hematuria. Electrocardiographic abnormalities (prolongation of the Q-T interval) and peripheral vascular

disease have been reported. The latter includes acrocyanosis, Raynaud's phenomenon and frank gangrene (Malachowski, 2000, Lin *et al*, 1998). Hematologic abnormalities include anemia, leukopenia and thrombocytopenia (Malachowski, 2000). Late sequela of protracted high intakes of arsenic includes skin cancer and an increased risk of lung cancer. (Malachowski, 2000, Ellen horn, 2007)

2.5.8. HARMFUL EFFECTS

Despite the fact that it can be useful, it causes serious medical problems which have been studied over time. Arsenic exposure results in arsenic dermatosis along with hyperkeratosis, gangrene and skin cancer. Other studies indicate that the kidney, liver, Uterus and prostate may also be target sites of arsenic carcinogenesis in humans (Waalkes *et al,* 2000). Also arsenic intoxication in experimental animals has been associated with hepatic tumors (Waalkes *et al*, 2003), inhibition of testicular steroidogenic and ovarian functions, incapacitation of leydig cell function, negative effects on spermatogenesis and also severe metabolic disorders e.g. diabetes in humans (Tseng *et al*, 2002). In mice, it causes structural changes in thymus of pregnant and newborn mice.

Arsenic exposure has been associated with elevation of adrenocortical steroidogenesis and plasma corticosterone level (Ghosh *et al*, 1991).Acute exposure to sodium arsenite causes gastrointestinal disorders such as diarrhea and hyper motility, Garlic ordour breath and rice like watery stool (Goebi *et al.,* 1990).Chronic exposure may cause degenerative, inflammatory and neoplastic changes in the respiratory, cardiovascular and nervous systems (Neiger, 2001). It is easily absorbed from the tract because of its characteristic solubility. There is however a lack of literature data on the possible effects of arsenite on nociceptive processing, a possibility that is very strong in the light of arsenite induced inflammatory perturbations on one hand (Neiger *et al*, 2001) and its peripheral neuropathic effect on the other (Liu *et al*, 2001).The delirious effect of arsenite has been studied and has been attributed to oxidative damage, thus an antioxidant should protect against or reverse its toxicity (Hei *et al*, 1998). Studies has shown that sodium arsenite is a hormone disrupter which causes reproductive damage (Sarkar *et al,* 2003) by damaging the hypothalamo-pituitary testicular and ovarian activities. The poisonous effect of this chemical has been established as it affects many body systems thus it should be handled with care to avoid intoxication. In a study carried out to determine the effect of arsenite on gastric mucosa of frogs, it was discovered that arsenite

sloughs the gastric mucosa which inhibits oxygen uptake by the mucosal cells and this leads to a decrease in gastric secretion. (Sarkar *et al*, 2003).

I. TREATMENT OF ARSENITE POISONING

a. Skin decontamination: wash arsenical pesticide with copious amounts of water and soap from skin and hair. Flush contaminant from eyes with clean water. If irritation persists, specialized medical treatment should be obtained.

b. Gastrointestinal contamination: administer intravenous fluids to restore adequate hydration, support urine flow and electrolyte imbalances. Monitor intake/output continuously to guard against fluid overload. If acute renal failure occurs, monitor blood electrolytes regularly. Blood transfusions and oxygen by mask may be needed to combat shock.

II. PRIMARY SYMPTOMS AND DIAGNOSIS OF ARSENIC DISEASES

a. Clinical Symptoms:

The clinical symptoms occurs in the early stage of human arsenic poisoning and are unspecific. The clinical manifestations of arsenic poisoning are myriad, and the correct diagnosis depends largely on awareness of the problem. Among the people who were taking high-arsenic water, early symptoms included, following non-specific symptoms, which can be present in many other diseases.

- Palpitations
- Fatigue
- Headache, dizziness, insomnia, weakness
- Nightmare
- Numbness in the extremities, anaemia

III. STAGES OF CLINICAL FEATURES OF ARSENIC TOXICITY

Arsenical toxicity or arsenicosis develops insidiously after six months to two years or more depending on the amount of intake of arsenic found in the ground water and arsenic concentration in the water. The higher the concentration above the maximum permissible level (0.05 mg/L) or higher the amount of daily water intake, the earlier the onset of symptoms.

The features of arsenical toxicity was classified by Saha in 1998 which are now known as Saha's classification of stages. These are the Preclinical, Clinical, Internal complication and malignancy.

A. Pre-Clinical (asymptomatic) Stage: This may be subdivided into

a. i. Stable, sub-clinical oroccult phase or **tissue phase (persistent)**. Body tissue showing high arsenic concentrations with no apparent clinical symptoms.

b. ii. Bloodphase *(*Labile*):* After the intake of arsenic contaminated water, bloodand urine examination reveals arsenic products but on withdrawal of it, urine becomes free of arsenic. The nature of arsenic revealed in urine is dimethyl arsonic acid (DMAA) and trimethyl arsinic acid (TMAA).

Tissuephase *(*stable*):* In this phase, examination of nails, hair and skin scales or other body tissues reveals high arsenic concentration, though the features of arsenic toxicity are absent.

B. Clinical Stage(symptomatic or overt phase): The presence of clinical symptoms is confirmed by detection of higher arsenic concentration in nail, hair and skin scales. Idea of skin scales for arsenic was also first observed by Saha in 1995.

Onset: The features of arsenical toxicity appear gradually and slowly with time. Six month to ten years(average 2 years) may be required for the development of clinical features. If the arsenic concentration in water consumed is not very high or the daily water intake is low or if the patient spends most of the day in other unaffected areas for business or service or if the nutritional status of the patient is good, the clinical features may not developed for years and if it develops at all, the sign are often mild. On the other hand if these conditions are not satisfied, the symptoms may develop between 6 months to 2 years.

Major Dermatological Signs:

(i) **Melano-keratosis*:*** Melanosis i.e., dark pigmentation-diffuse and/or spotted keratosis i.e., dry, rough spotted nodules in palms and/or soles are the chief symptoms of arsenical dermatosis (ASD). It should be noted that there are various causes of melanosis and keratosis, spotted and diffuse, genetic and acquired.

(ii) The combination of the two features-melanosis and keratosis-in the same patient in adults points to the diagnosis of arsenical dermatosis. Genetic disorders are often present since childhood and acquired diseases like arsenicosis appear in later life.

The (Diffuse darkening of skin) starts in the palm and gradually spreads to the whole body. Mild melanosis can be revealed by comparing with normal palm.

(iii) The ***Spotted or rain drop pigmentation (spotted melanosis)*** is usually seen on chest, back or limbs. This is a fairly common symptom. 50% of the patients show spotted melanosis in chest, back and sometimes in the limbs, i.e., hands and legs.

(iv) **The *Spotted and Diffuse Keratosis*** of palms and soles are signs of moderate to severe toxicity. Rough, dry, spotted nodules (spotted keratosis) appear after 5-10 years in the palms and feet. Still later (>10 years), the skin becomes dry and thickened. This stage is called diffuse keratosis. Gradually, thickening of soles can give rise to cracks and fissures (hyperkeratosis).G1

(v) **Leucomelanosis** : About one third of the patients develop pigmented and depigmented spots in legs or trunk, found in advanced stage of the disease.Probably stimulation of melanocytes produces the pigmentation and damage in later stage is responsible for the de-pigmentation spots. Leucomelanosis is common (white and black in colour) in persons with advanced arsenicosis or who have stopped drinking arsenic-contaminated water but had spotted melanosis earlier.

(vi) **Dorsal keratosis**i.e., rough dry skin often with palpable nodules (spotted keratosis) on dorsum of hands, feet and legs are the signs seen in severe case. If the arsenic intake is high or the disease is of long duration- more than 10-15 years, keratosis also develops in the dorsal skin of hands, feet, legs or even other parts of the skin (whole body keratosis).

IV. EFFECTS OF ARSENIC ACID ON DIFFERENT SYSTEMS OF THE BODY

There is good evidence that arsenic is carcinogenic in humans if exposed orally or by inhalation.

A. Respiratory effects:

Effects of arsenic on the human respiratory system have been reported both from occupational exposure as well as from tube-well water. Humans exposed to arsenic dust or fume inhalation are more opt to be encountered in mining and milling of ores, in industrial processing, such as smelting industry which often produces irritation of the mucous membrane, resulting in laryngitis, bronchitis, rhinitis and tracheo-bronchitis, causing stuffy nose, sore throat, hoarseness and chronic cough etc. A very high exposure of unprotected workers may manifest perforated nasal septum after 1-3 weeks of exposure, but such effects are minor or absent at exposure levels of 0.01-1mg/m(Armstrong,1984,Ide& Bullough,1988). A fatal case of arsenic trioxide inhalation manifested widespread tracheobronchial mucosal and sub mucosal hemorrhages with mucosal sloughing, alveolar haemorrhage, and pulmonary edema (FAO,1983).Chronic asthmatic bronchitis and asthma is a common complication of ground water arsenic toxicity(Saha,1995).

B. Cardiovascular effects:

It has been suggested by several epidemiological studies that chronic inhalation of arsenic trioxide can increase the risk of death in humans from cardiovascular disease. Long term inhalation of inorganic arsenic could injure the blood vessels or the heart. Zaldivar in 1974 reported several cases of myocardial infarction and arterial thickening in children who consumed water containing about 0.6 mg/l arsenic.

Arsenic ingestion through food or water may have serious effects on the human cardiovascular system. Both acute and chronic arsenic exposure cause altered myocardial depolarization and cardiac arrhythmias that may lead to heart failure. Low level arsenic exposure by humans may also cause vascular system damage, a classical example of which is

Blackfoot disease, which is endemic in an area of Taiwan where most drinking water contains 0.17 to 0.8 ppm arsenic, corresponding to doses of about 0.01 to 0.5 mg As/kg/day.

Effects of arsenic on the vascular system have also been reported in a number of other populations. In Chile, ingestion of 0.6 to 0.8 mg/l arsenic in drinking water (equivalent to 0.02 - 0.06 mg As/kg/day) increased the incidence of Raynaud's disease and of cyanosis of fingers and toes. Thickening of blood vessels and their occlusion were noticed due to arsenic in beer poisoning (Saha,1995). In acute voluntary massive arsenic intoxication, the muscles showed hyper-contracted fibres, myo-fibrillar disruption, mitochondrial abnormalities and cytoplasmic vacuoles.

C. Gastrointestinal effect:

Gastrointestinal symptoms are common in acute poisoning but not in chronic like ground water arsenicosis. Workers exposed to high levels of arsenic dusts or fumes suffer from nausea, vomiting and diarrhoea. Clinical signs of gastrointestinal irritation from acute arsenic poisoning include burning lips, painful swallowing, thirst, nausea and several abdominal colic. These symptoms are usually not detectable at exposure levels below 0.01mgAs/kg/day and they decline within a short time after exposure ceases. The efficiency of absorption or inorganic arsenicals from the gastrointestinal tract is related to their water-solubility. Chakraborty and Saha,1987 reported three deaths in India due to chronic arsenic poisoning by drinking water from tube wells having mean arsenic content of 0.64mg/l. The most likely mechanism of gastrointestinal toxicity is damage to the epithelial cells, with resulting irritation.

D. Hematological effects

The hematopoietic system is also affected by both short-and long-term arsenic exposures. Anemia and leukopenia are common effects of poisoning and have been reported as resulting from acute, intermediate(Franzblau,1989), and chronic oral exposures. These effects may be due to a direct hemolytic or cytotoxic effect on the blood cells and a suppression of erythropoiesis. No such effects were noticed in humans exposed chronically to 0.07 mg As/kg/day or less. Relatively high doses of arsenic have been reported to cause bone marrow depression in humans. Mizuta *et al.*, 1956 reported anemia and leukopenia in adults ingesting 3 mg As/day in soy sauce. The malnutrition is a major causes of anemia is underdeveloped country like India and Bangladesh. Hence the anemia in patients with arsenicosis should be

properly examined for the amount of the arsenic in the system and malnutrition. High concentration of arsine (10 ppm) cause death within hours due to red blood cell hemolysis. Low levels of arsenic (0.5-5.0 ppm) bring about these effects in a few weeks. Renal damage is secondary and occurs due to clogging of nephrons with hemolytic debris. Mono-, di-, and trimethylarsines are strong irritants but are less hemolytic than arsine. Arsine exposure by humans is usually fatal without proper therapy (Sittig, 1985). Arsine breaks down in the body to inorganic arsenic and methylated derivatives (less toxic than arsine).The mechanism of hemolysis involved depletion of intracellular GSH, resulting in oxidation of sulfhydryl groups in the hemoglobin from ferrous to ferric in mice and rats. Haemocyanin combines with arsenic, which reduced oxygen uptake by cells.

E. Hepatic effect

Arsenic was the one of first chemical agents which can cause damage to the liver cells in humans. The liver tends to accumulate arsenic with repeated exposures, hepatic involvement has been reported most commonly as a complication of chronic exposures over periods of months or years). The symptoms seen in Patients are bleeding esophageal varices, ascites, jaundice, or simply an enlarged tender liver. Hepatic lesion that formed after prolonged ingestion of arsenic-containing medicines (Fowler's Solution) have been described. Clinical examination often reveals that the liver is swollen and tender. The analysis of blood sometimes shown elevated levels of hepatic enzymes. These effects are most often observed after chronic exposures to as little as 0.02 to 0.1 mg As/kg/day(Silver and Weinman,1952,Mazumdar *et al*,1988). Arsenic has been observed to produce mitochondrial damage and impaired mitochondrial functions, and accordingly might be expected to affect porphyrin metabolism. Franklin *et al.*, 1950, found that in people using fowler's solution shows evidence of hepatic fatty infiltration and cirrhosis of the liver(Oladosu *et al*.,2012)

F. Renal effects:

The kidneys will accumulate arsenic in the presence of repeated exposures like the liver cells. The kidneys are the major route of arsenic excretion, as well as major site of conversion of pentavalent arsenic into the more toxic and less soluble trivalent arsenic. Sites of arsenic damage in the kidney include capillaries, tubules, and glomeruli. Damaged proximal tubular cells lead to proteinuria and casts in the urine. Mitochondrial damage is also prominent in tubular cells. Oliguria is a common manifestation, but if acute arsenic poisoning is sufficiently severe to produce shock and dehydration, there is real risk of renal failure, although dialysis

has been effective in overcoming this complication. Arsine-induced hemolysis may likely cause tubular necrosis with partial or complete renal failure, requiring hemodialysis for removal of the hemoglobin bound arsenic (Valentine, 1985, Saha, 1995)

G. Dermal effects

From previous epidemiological studies, it was shown that people exposed to drinking water that contained 0.01 to 0.1mg of arsenic acid showed characteristic effects of arsenic ingestion which includes generalized hyperkeratosis, warts or corns on the palms and soles, and areas of hyperpigmentation interspersed with small areas of hypopigmentation on the face, neck, and back(Borgono and Greiber, 1972, Cebrian *et al.,* 1983, Brickley *et al.,*1989).

H. Neurological effects:

Several studies have indicated that ingestion of inorganic arsenic can result in neural injury. It has been shown that both the peripheral and central components of the nervous system can be damaged by arsenic (Pershagen *et al.,*1981). In acute high exposures (1 mg As/kg/day or more) often cause encephalopathy with such symptoms as headache, lethargy, mental confusion hallucination, seizures, and coma. Individuals with repeated arsenic exposures frequently contract sensorimotor polyneuropathy, which usually, but not always displays symmetrical involvement and which may resemble Landry-Guillain-Barre Syndrome in its presentation. Also, Neuropathy usually appears in 1 to 5 weeks after an acute exposure and is produced mainly by axonal degeneration. Symptoms of chronic encephalopathy include persistent headache, diminished recent memory, distractibility, abnormal irritability, restless sleep, loss of libido, increased urinary urgency, and increased effects of small amount of ethanol. Secondary depression, anxiety, panic attacks and somatizations are also common, in addition to the organic cognitive impairment documented by neuropsychological testing.

Electro-myographic technique (EMG) has been used to detect neuropathy and it showed decreased nerve condition amplitude with little change in nerve condition velocity. From previous studies, it was reported that there was asymmetric bilateral phrenic nerve involvement in a patient who was poisoned by arsenic. Inhalation of inorganic arsenic can cause neurological injury in humans. These may include peripheral neuropathy of both sensory and motor neurons causing numbness loss of reflexes, and muscle weakness (NIOSH,1979)

2.6.0. OXIDATIVE STRESS

Oxidative stress reflects or can be described as an imbalance between the systemic manifestation of reactive oxygen species and the ability of biological system to readily detoxify the reactive intermediates or to repair the resulting damage (Gwen *et al*, 2005). It has been shown that disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Furthermore, some reactive oxidative species act as cellular messengers in redox signaling. Thus, oxidative stress is thought to be involved in the development of cancer, Parkinson's disease, Alzheimer's disease, atherosclerosis, heart failure, myocardial infarction, fragile X syndrome, Sickle Cell Disease, lichen planus, vitiligo, autism, and chronic fatigue syndrome(Gwen *et al*, 2005). However, reactive oxygen species can be beneficial, as they are used by the immune system as a way to attack and kill pathogens (Segal, 2005).

Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses, such as glutathione. The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stress may cause necrosis (Lennon *et al*,1999).

2.6.1. CAUSES OF OXIDATIVE STRESS.

Free radicals can be produced through several chemical processes, both within and outside the organism. Depending on the origin of its production, the peroxynitrite can be in equilibrium with its conjugate acid (ONOOH). In neutral solution it is a powerful oxidizing agent able to

form nitrate tyrosine residues, nitrating and oxidizing guanosine, degrade carbohydrates, initiate lipid peroxidation, and fragment DNA (Beckman and Koppenol, 1996, 2001).

The production of O_2^- and NO in vivo is however different. The peroxynitrite production always occurs when there is an excess of one or the other (Grisham *et al.*, 1999). Some researchers established that both reactions of oxidation and nitration are mediated by the peroxynitrite are influenced largely by the relative flow of production of O_2^- and NO (Jourd'Heuil *et al*., 2001).

It was also established that the highest rates of oxidation occur with an excess of NO, producing oxidation through the .OH and from the peroxynitrite NO_2 formed. However, the reaction of peroxynitrite with CO_2 is the most important way that the peroxynitrite decomposes in vivo (Lymar and Hurst, 1995), forming the end product N_2O_3, which is a potent nitrating agent. In addition to the reactions of oxidation, the peroxynitrite has the ability to nitrate phenolic compounds under physiological conditions, such as the rings of tyrosine (Goldstein *et al.,* 2000). Tyrosine residues are oxidized by the radical derivatives of the peroxynitrite forming the radical tyrosyl, which in turn reacts with NO to form 3-nitrotyrosine. The nitration mediated by peroxynitrite in vivo might be inhibited by a relative overproduction of O_2^-, because of competition between them by the radical tyrosyl, by which the formation of 3-nitrotyrosine would be inhibited when the rate of formation of O_2^- exceeded that of NO (Goldstein *et al*., 2000) in the exogenous and endogenous sources (Freeman & Crapo, 1982).

2.6.2. EXOGENOUS ROS PRODUCTION

Many antineoplastic agents such as the Adriamycin, bleomycin, daunorubicin, and other antibiotics (Doroshow and Hochstein, 1982) depend on quinoide groups or joining metals for their activity. Some of the effects of these drugs have been attributed to their ability to reduce oxygen to superoxide, the hydroxyl radical, and hydrogen peroxide, also the irradiation of organisms by electromagnetic radiation (x-rays and gamma rays) or by particle radiation (electrons, protons, deuterons, and neutrons) also cause free radicals (Bielsky and Gebieki, 1989).

Environmental factors, such as photochemical air pollutants as ozone, hyperoxia, pesticides, tobacco smoke, solvents, anaesthetics, and aromatic hydrocarbons are a source of reactive

species. These agents have free radicals, such as in tobacco smoke, or become reactive species with cellular metabolism and detoxification processes (Mason, 2000). Environmental pollution is also another important source of oxidation (Searing and Rabinovitch, 2011; Bhalla, 1999). Studies have shown that ozone pollution causes serious damage to human health and is a determining factor in the progression of neurodegenerative diseases (Zawia *et al*., 2009; Schwela, 2000). This gas acts to produce ROS in the body, causing an increase in oxidants, increasing the state of oxidative stress in the organism and thus contributing to increase the neurodegenerative process in human (Cretu *et al*., 2010).

2.6.3. ENDOGENOUS ROS PRODUCTION

There are a variety of soluble components able to produce phosphorylation in the cell, such as thiols, hydroquinone, catecholamines, flavins, and tetrahydropterins. In all these, the superoxide radical is the radical primarily formed by the dioxygen reduction by these molecules (Baccarini, 2000). Hydrogen peroxide is also produced as a byproduct from the disproportionation of the superoxide radical, either spontaneously or enzymatically catalyzed by superoxide dismutase (SOD).

Others Causes:

Nutritional deficiencies: adequate nutritional intake is very important as Antioxidant systems require a variety of cofactors (glutathione, sulfate, vitamin A, vitamin E, or minerals like selenium and copper) that should be present in our food. Proper fatty acid supplementation is also a determinant; it could compensate for the damage caused by oxidative stress (polyunsaturated fatty-acids are very sensitive to oxidative damage).

Exposure to toxic chemicals: excessive exposure to toxic chemicals present in our environment can cause severe oxidative damage. For instance, dioxin increases the production of reactive oxygen species by the mitochondria, leading to oxidative damage in the endothelium, liver and brain. Exposure to certain chlorinated compounds is associated with an increase of 8-OhdG, a marker of DNA oxidative damage. Organophosphate pesticides generate free radicals and alter the antioxidant defense system in erythrocytes.

Heavy metal exposure also causes strong oxidative damage: arsenic, lead, cadmium, and mercury promote the formation of hydrogen peroxide and at the same time inhibit anti-oxidant

enzymes (GSH synthetase, GSH reductase). Most chemicals can to some extent generate oxidative stress, exposure to low doses of different chemicals may finally, when they combine, lead to a significant oxidative burden.

Infections: infections and inflammatory processes are associated with production of pro-oxidative molecules. Phagocytic cells indeed kill bacteria by producing hypochlorous acid (HOCl) from hydrogen peroxide (a reaction catalyzed by myeloperoxidase). Hypochlorous acid is itself a strong oxidant. Chronic infections are therefore associated with increased oxidative burden; for instance, Helicobacter pylori infections cause severe oxidative damage to the gastric mucosa.

2.6.4. IMPORTANCE OF OXIDATIVE STRESS

The immune system uses the lethal effects of oxidants by making production of oxidizing species a central part of its mechanism for killing pathogens. Activated phagocytes produces both ROS and reactive nitrogen species. These include superoxide(O_2^-),nitric oxide (NO) and their particularly reactive product, peroxynitrite (ONOO-) (Nathan and Shiloh, 2000). The use of these highly reactive compounds in the cytotoxic response of phagocytes also caused damage to host tissues, the non-specificity of these oxidants is an advantage as they will damage almost every part of their target cell. This prevents a pathogen from escaping this part of immune response by mutation of a single molecular target (Rice-Evans and Gopinathan, 1995).

2.6.5. MARKERS OF OXIDATIVE STRESS

The following are the basic markers of Oxidative stress:

- Oxidative DNA damage markers (Formation of 8-OHdG by oxidative radicals),
- Protein oxidation markers (Dityrosine),
- Lipid Peroxidation markers (Hexanoyl-lysine) ,
- Antioxidants detection ,
- Specific oxidative stress markers antibodies.

The anti- oxidative network acts as a defense mechanism against stress. The human body has anti-oxidative enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH),

and catalase, which scavenge free radicals. These enzymes make up a preventive type of anti-oxidative network (Yoshikawa *et al.*, 2000).

General types of anti-oxidative substances are able to respond directly to and eliminate free radicals and are therefore called radical-scavenging antioxidants. These are divided into water-soluble substances, such as vitamin C, and fat-soluble substances, such as vitamins A and E as well as coenzyme Q10(Aoi *et al*, 2004).

The water- and fat-soluble antioxidants mutually react with each other and individually form sophisticated networks that protect the body against oxidative damage and systems that repair and regenerate lipids, proteins, and DNAs by free radicals (repair/regeneration type of antioxidative activities) also exist. In these systems, phospholipase, protease, transferase, and DNA repair enzymes are the primary workhorses (Naito *et al*, 2005).

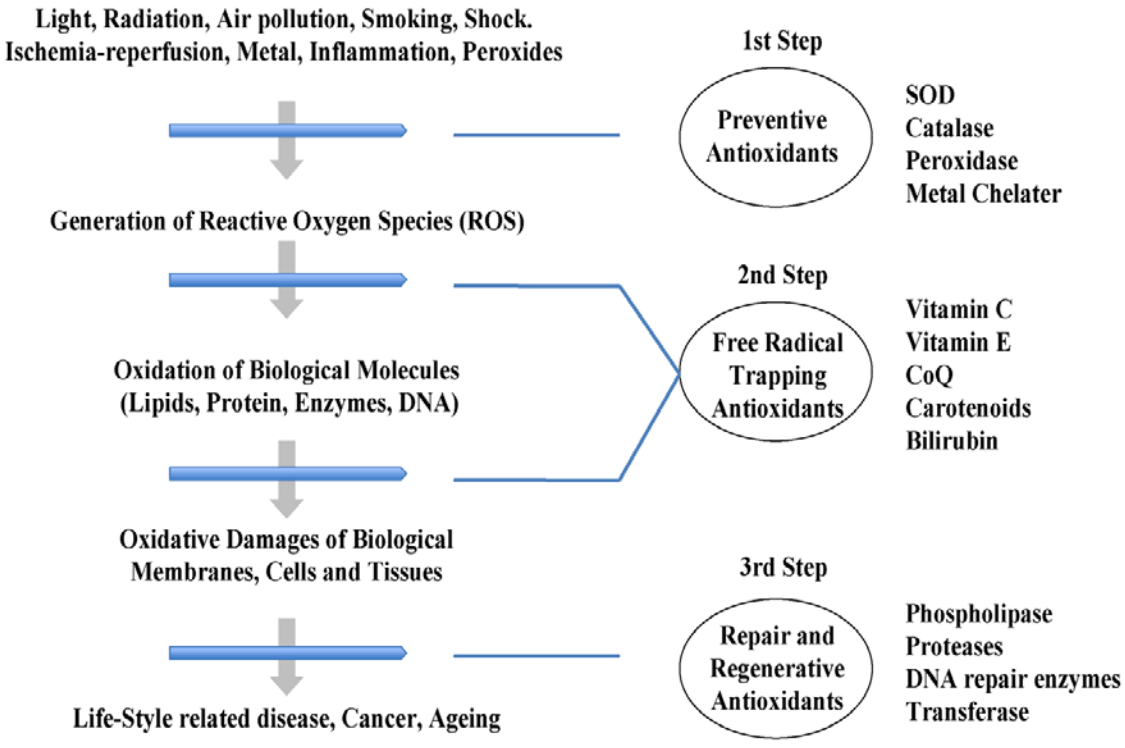


Figure.2.7. Anti - oxidative network in biological system()

2.7.0 ANTIOXIDANTS

Antioxidants are important molecules that helps to prevent oxidation of other molecules and oxidation is a chemical reaction that help transfers electrons or hydrogen from a substance to an oxidizing agent, it is a reaction can produce free radicals which starts a chain reactions. These chain reaction cause damages in the cells or death to cell. Antioxidants terminates these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions .i.e. they are important reducing agents and they includes thiols , ascorbic acid ,polyphenols(Sies, 1997).

2.7.1. IMPORTANCE.

The antioxidants are widely used as dietary supplement and have been found to prevent several diseases such as cancer, corornary heart diseases, ulcer healing etc. several studies suggested that antioxidant supplements might promote health, and excessive supplementation with certain putative antioxidants might be harmful (Jha *et al*, 1995; Bjelakovic *et al*, 2007; Baillie *et al*, 2009). They are also possesses many industrial uses as it is used as food preservatives, cosmetics, upgrade/to prevent the degradation of rubber and gasoline (Dabelstein *et al*, 2007).

It has been used as medication in the treatment of various brain injury as the brain is uniquely vulnerable to oxidative injury. Itshigh metabolic rate and elevated levels of polyunsaturated lipids, makes it a target for lipid peroxidation. Examples are superoxide dismutase mimetics, sodium thiopental and propofol used to treat reperfusion injury and traumatic brain injury

while some experimental drugs such as disufenton sodium, ebselen are used in the treatment of stroke, they are suggested to prevent oxidative stress in neurons, prevent apoptosis and neurological damage (Warner *et al*, 2004). The antioxidant has been suggested to possibly used in treatment of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis(Di Matteo and Esposito, 2003), and used to prevent noise-induced hearing loss.

Targeted antioxidants may lead to better medicinal effects for instance mitochondria-targeted ubiquinone, for example, may prevent damage to the liver caused by excessive alcohol (Kopke *et al*, 2007).

2.7.2. TYPES OF ANTIOXIDANT

Antioxidants are classified into two broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (lipophilic). In general, water-soluble antioxidants react with oxidants in the cell cytosol and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation (Sies, 1997). These compounds may be synthesized in the body or obtained from the diet (Vertuani *et al*, 2004). The different antioxidants are present at a wide range of concentrations in body fluids and tissues, with some such as glutathione or ubiquinone mostly present within cells, while others such as uric acid are more evenly distributed. Some antioxidants are only found in a few organisms and these compounds can be important in pathogens and can be virulence factors (Miller and Britigan, 1997).

The relative importance and interactions between these different antioxidants is a very complex question, with the various metabolites and enzyme systems having synergistic and interdependent effects on one another. The action of one antioxidant may therefore depend on the proper function of other members of the antioxidant system. The amount of protection provided by any one antioxidant will also depend on its concentration, its reactivity towards the particular reactive oxygen species being considered, and the status of the antioxidants with which it interacts (Vertuani *et al*, 2004).

Some compounds contribute to antioxidant defense by chelating transition metals and preventing them from catalyzing the production of free radicals in the cell. Particularly important is the ability to sequester iron, which is the function of iron-binding proteins such as transferrin and ferritin. Selenium and zinc are commonly referred to as antioxidant nutrients, but these chemical elements have no antioxidant action themselves and are instead required for the activity of some antioxidant enzymes (Imlay, 2003).

2.7.3. VITAMIN E

Vitamin E, is a natural antioxidant, believed to prevent diseases associated with oxidative stress (Kappus, and Diplock, 1992). Vitamin E is fat soluble and a potent antioxidant that reduces ROS formed during fat oxidation; ROS can cause a chronic inflammatory response (Herrera and Barbas, 2009). Vitamin E is the term for a group of tocopherols and tocotrienols, of which α-tocopherol has the highest biological activity. Due to the potent antioxidant properties of tocopherols, the impact of α-tocopherol in the prevention of chronic diseases believed to be associated with oxidative stress has often been studied, and beneficial effects have been demonstrated. Recent observations that the α-tocopherol transfer protein in the liver specifically sorts out *RRR*-α-tocopherol from all incoming tocopherols for incorporation into plasma lipoproteins, and that α-tocopherol has signaling functions in vascular smooth muscle cells that cannot be exerted by other forms of tocopherol with similar anti-oxidative properties, have raised interest in the roles of vitamin E beyond its anti-oxidative function. Also, γ-tocopherol might have functions apart from being an antioxidant. It is a nucleophile able to trap electrophilic mutagens in lipophilic compartments and generates a metabolite that facilitates natriuresis. Vitamin E is considered safe in pregnancy, although experiments that evaluate the safety of high-doses of Vitamin E in pregnancy have not been reported (Cohen-Kerem and Koren, 2003).

2.7.4. SOURCES OF VITAMIN E

In general, food sources with the highest concentrations of vitamin E are vegetable oils, followed by nuts and seeds including whole grains. Adjusting for typical portion sizes, however, for many people in the United States the most important sources of vitamin E include commercial breakfast cereal and tomato sauce. Although, originally extracted from wheat germ oil, most natural vitamin E supplements are now derived from vegetable oils, usually soybean oil (Bauernfeind, 1990, USDA Agricultural Research Service, 2009).

Vitamin E deficiency causes neurological problems due to poor nerve conduction. These include neuromuscular problems such as spino-cerebellar, ataxia and myopathies. Deficiency can also cause anemia, due to oxidative damage to red blood cells. Commercial vitamin E supplements can be classified into several distinct categories: Fully synthetic vitamin E, "dl-alpha-tocopherol", the most in expensive, most commonly sold supplement form usually as the acetate ester; Semi-synthetic "natural source" vitamin E esters, the "natural source" forms used in tablets and multiple vitamins. These are highly fractionated d-alpha tocopherol or its esters, often made by synthetic methylation of gamma and beta d, d, d-tocopherol vitamers extracted from plant oils. Less fractionated "natural mixed tocopherols" and high d-gamma-tocopherol fraction supplements.

2.7.5. BRIEF HISTORICAL REVIEW OF VITAMIN E

In 1922, Evans and Bishop discovered vitamin E—a micronutrient essential for reproduction in rats. During feeding experiments with rats Herbert McLean Evans concluded in 1922 that besides vitamins B and C, an unknown vitamin existed (Evans and Bishop, 1992).Although every other nutrition was present, the rats were not fertile. This condition could be changed by additional feeding with wheat germ. It took several years until 1936 when the substance was isolated from wheat germ and the formula C29H50O2 was determined. Evans also found that the compound reacted like an alcohol and concluded that one of the oxygen atoms was part of an OH (hydroxyl) group. As noted in the introduction, the vitamin was given its name by Evans from Greek words meaning "to bear young" with the addition of the -ol as an alcohol (Evans *et al,* 1996). The structure was determined shortly thereafter in 1938 (Fernholz, 1938). Vitamin E was rediscovered in the 1950s as factor 2 by Klaus Schwarz (Schwarz, 2005) and placed in the context of cellular antioxidant systems, together with sulfur amino acids (factor 1) and selenium (factor 3). Vitamin E subsequently proved to be effective in preventing lipid

peroxidation and other radical-driven oxidative events (Tappel, 2002, Burton and Ingold, 2006, Esterbauer *et al*, 2009).

The antioxidant activity of vitamin E has persuaded many groups to study its ability to prevent chronic diseases, especially those believed to have an oxidative stress component such as cardiovascular diseases, atherosclerosis, and cancer. Epidemiological studies have reported that high vitamin E intakes are correlated with a reduced risk of cardiovascular diseases, whereas intakes of other dietary antioxidants (such as vitamin C and β-carotene) are not, suggesting that vitamin E plays specific roles beyond that of its antioxidant function.

The possibility that vitamin E has an ameliorative effect in chronic disease has spurred interest in determining its specific molecular functions and whether these are related to its antioxidant function (Stampfer. *et al*, 2003, Rimm *et al*, 2006).

2.7.6. PREVIOUS STUDIES ON VITAMIN E AS ANTIOXIDANTS.

Antioxidant defence system comprises of enzymes such as catalase, superoxide dismutase, glutathione peroxidase and non-enzymatic antioxidants including vitamin A, vitamin C, vitamin E, ubiquinone and flavonoids. Antioxidants are molecules which interact with ROS and scavenge the free radicals before cellular vital molecules are damaged preventing cellular damage and disease (Rokitzki *et al*, 2004).

Vitamin E, a potent naturally occurring lipid-soluble antioxidant possesses the ability to directly quench free radicals and function as a membrane stabilizer. It protects critical cellular structures against the damage from oxygen free radicals and reactive products of lipid peroxidation. The protective effect of vitamin E supplementation against sodium arsenite induced oxidation has been reported in humans and rats (Kumar *et al*, 2002, Goldfarb *et al*, 2006)

2.8.0. ZINC

Zinc is a metallic chemical element which has the symbol **Zn** with atomic number 30. It is the first element of group 12 of the periodic table and is chemically similar to magnesium, its ion is of similar size and has a common oxidation state of +2. Zinc is the 24th most abundant element in the Earth's crust and has five stable isotopes. The most common zinc ore is sphalerite (zinc blende), a zinc sulfide mineral. The largest mineable amounts are found

in Australia, Asia, and the United States. Zinc production includes froth flotation of the ore, roasting, and final extraction using electricity.

The element was probably named by the alchemist Paracelsus after the German word *Zinke*, a German chemist Andreas Sigismund Marggraf was given credit in the western world for discovering pure metallic zinc in 1746. Work done by Luigi Galvani and Alessandro Volta uncovered the electrochemical properties of zinc by 1800. A variety of zinc compounds are commonly used, such as zinc carbonate and zinc gluconate (as dietary supplements), zinc chloride (in deodorants), zinc pyrithione (anti-dandruff shampoos), zinc sulfide (in luminescent paints), and zinc methyl or zinc diethyl(in the Soil) contains 5–770 ppm of zinc with an average of 64 ppm. Seawater has only 30 ppb zinc and the atmosphere contains 0.1–4 μg/m^3. (Emsley, 2001).

The element is normally found in association with other base metals such as copper and lead in ores. Zinc is a chalcophile, meaning the element has a low affinity for oxides and prefers to bond with sulfides. Chalcophiles formed as the crust solidified under the reducing conditions of the early Earth's atmosphere. Sphalerite, which is a form of zinc sulfide, is the most heavily mined zinc-containing ore because its concentrate contains 60–62% zinc. (Emsley, 2001).

Other minerals from which zinc is extracted include smithsonite (zinc carbonate), hemimorphite (zinc silicate), wurtzite (another zinc sulfide), and sometimes hydrozincite (basic zinc carbonate). With the exception of wurtzite, all these other minerals were formed as a result of weathering processes on the primordial zinc sulfides. Identified world zinc resources total about 1.9 billion tonnes. (Tolcin, 2011).

Zinc is a very important micronutrient as well as essential trace element for human, animals and plants (Shah and Sachdev, 2001; Das and Das, 2012). It is involved in numerous aspects of cellularmetabolism and necessary for catalytic activity of several enzymes (Anon., 2001).it is not an antioxidant but can limit oxidant-induced damages in several ways (Disilvestro,2000). For instance, protection against vitamin E depletion (Noh and Koo, 1998),stabilization of membrane structure (Bray and Bettger, 1990) and restriction of endogenousfree radical production (Disilvestro, 2000). It also plays a major role in immune function (Prasad *et al.*, 1997), protein synthesis, wound healing and DNA synthesis (Lansdown *etal.*2007). Zinc also plays a role in cell division and growth (Prasad, 1995). It is "typically the second most abundant transition metal in organisms" after iron and it is the only

metal which appears in all enzyme classes,the primary source of zinc is food. Some of the major sources include red meat, poultry, seafood, whole cereals, cereals and dairy products.

Zinc is however required for growth, optimum performance and modulation of immune system, partly because its role as a co-factor of more than two hundred enzymes (Zago and Oteiza, 2001). The mechanism of its antioxidant activity is not well defined but is been suggested to increase the synthesis of metallothionen (a cysteine rich protein) which acts as a free-radical scavenger (Webb and Cain, 1982; Oteiza *et al.*, 1996). In 1998, it was proposed by Kim *et al.,* (1998) that the mode of action of zinc as antioxidant is by studying its interaction with vitamin E. For instance, vitamin E is impaired in animals that has deficiency of zinc. It has the capability of occupying the iron and copper binding sites on the lipids, proteins and DNA, thus can exert direct antioxidant (Tate *et al*., 1999). Its supplementation decreases malondialdehyde (MDA) concentrations (Anderson *et al*., 2001, Sahin and Kucuk, 2003). Zinc is a recognized micronutrient with an outstanding and diverse biological, clinical and global public health importance (Hambidge *et al*., 2010). Zinc is therefore an important metallo-enzyme and constituent of cell membrane and subcellular structures and maintains stability (WHO, 1996). It has been reported to improve healing of leg ulcer (Stromberg 2009). About 2-4 grams of zinc is distributed throughout the human body. Most zinc is in the brain, muscle, bones, kidney, and liver, with the highest concentrations in the prostate and parts of the eye. Semen is particularly rich in zinc, which is a key factor in prostate gland function and reproductive organ growth. (Berdanier *et al.*, 2007).

In humans, it plays ubiquitous biological roles. It can interacts with a wide range of organic ligands and has roles in the metabolism of RNA and DNA, signal transduction, and gene expression. It also regulates apoptosis. (Hambidge and Krebs, 2007).

In the brain, zinc is stored in specific synaptic vesicles by glutamatergic neurons and can "modulate brain excitability". It plays a key role in synaptic plasticity and so in learning.However it has been called "the brain's dark horse" since it also can be a neurotoxin, suggesting zinc homeostasis plays a critical role in normal functioning of the brain and central nervous system. (Bitanihirwe and Cunningham, 2009).

Zinc is an efficient Lewis acid, making it a useful catalytic agent in hydroxylation and other enzymatic reactions. The metal also has a flexible coordination geometry, which allows proteins using it to rapidly shift conformations to perform biological reactions. Two examples of zinc-containing enzymes are carbonic anhydrase and carboxypeptidase, which are vital to

the processes of carbon dioxide (CO_2) regulation and digestion of proteins, respectively(Stipanuk, 2006).

In blood plasma, zinc is bound to and transported by albumin (60%, low-affinity) and transferrin (10%). Since transferrin also transports iron, excessive iron reduces zinc absorption, and vice-versa. A similar reaction occurs with copper. The concentration of zinc in blood plasma stays relatively constant regardless of zinc intake. Cells in the salivary gland, prostate, immune system and intestine use zinc signaling as one way to communicate with other cells. (Rink and Gabriel, 2000).

2.8.1. DIETARY SUPPLEMENT

Zinc deficiency has been associated with major depressive disorder (MDD), and zinc supplements may be an effective treatment (Swardfager *et al.*, 2013), it serves as a simple, inexpensive, and critical tool for treating diarrheal episodes among children in the developing world. Zinc becomes depleted in the body during diarrhea, but recent studies suggest that replenishing zinc with a 10- to 14-day course of treatment can reduce the duration and severity of diarrheal episodes and may also prevent future episodes for up to three months. (Bhutta *et al.*, 2000). It has been determined that zinc can be part of an effective treatment for age-related macular degeneration.and is an effective treatment for acrodermatitis enteropathica, a genetic disorder affecting zinc absorption that was previously fatal to babies born with it. (Emsley, 2001).

Gastroenteritis is strongly attenuated by ingestion of zinc, and this effect could be due to direct antimicrobial action of the zinc ions in the gastrointestinal tract, or to the absorption of the zinc and re-release from immune cells (all granulocytes secrete zinc), or both(Valko *et al.*, 2005).

2.8.2. DIETARY INTAKE

In the U.S., the Recommended Dietary Allowance (RDA) is 8 mg/day for women and 11 mg/day for men. Median intake in the U.S. around 2000 was 9 mg/day for women and 14 mg/day in men. (Connie and Christine, 2009). Oysters, lobster and red meats, especially beef, lamb and liver have some of the highest concentrations of zinc in food (Connie and Christine, 2009).Zinc supplements should only be ingested when there is zinc deficiency or increased zinc necessity (e.g. after surgeries, traumata or burns). Persistent intake of high doses of zinc

can cause copper deficiency. (Colin, 2010). The concentration of zinc in plants varies based on levels of the element in soil.

When there is adequate zinc in the soil, the food plants that contain the most zinc are wheat (germ and bran) and various seeds (sesame, poppy, alfalfa, and celery, mustard). Zinc is also found in beans, nuts, almonds, whole grains, pumpkin seeds, sunflower seeds and blackcurrant. (Colin, 2010). Other sources include fortified food and dietary supplements, which come in various forms. In 1998, it was reviewed that zinc oxide and zinc carbonate are nearly insoluble and poorly absorbed in the body (Allen, 1998). It is also noted that excessive supplementation of zinc could become harmful. Harmful, therefore it is suggested that intake should not exceed 20mg/day. Although, a tolerable upper intake was set at 40mg/day by U.S. National Research council (Maret and Sandstead, 2006). The dietary supplements could be in different forms, which areacceptable by the regulatory authorities and they includes zinc gluconate, zinc sulphatemonohydrate; zinc sulphate heptahydrate, zinc carbonate, chelated zinc and amino acids,zinc acetate and dehydrate. Others are zinc chloride, monohydrate; zinc oxide, zinc lactate,trihydrate; and zinc propionate by which the percentage of elemental zinc varies by form ofsupplement (Disilvestro,2004; Strnadova *et al.*,2011), however, the absorption, bioavailability or tolerability of the different forms are yet to be determined(Maret and Sandstead, 2006)

2.8.3. ZINC DEFICIENCY AND RISK FACTORS

It plays an important role in biological functioning of the body and its deficiency cause adverse / detrimental effects on the human health (Das and Das, 2012).

Deficiency of zinc can lead to retardation of growth and development, delayed wound healing, alopecia, poor pregnancyoutcomes with teratogenic effects and decreased immune function and increasedsusceptibility to disease (Maret and Sandstead, 2006). It has been reviewed over the years that more than 400,000children die every year due to zinc deficiency in developing countries (Shah and Sachdev,2001). The WHO advocates zincsupplementation for severe malnutrition and diarrhoea (WHO, 2004).

Severe zincdeficiency depresses immune function (Shankar and Prasad, 1998) and mild to moderate degrees of zinc deficiency may impair macrophage and neutrophil functions and naturalkiller cell activity (Rink and Gabriel, 2000).

2.9.0. *GARCINIA KOLA* (BITTER KOLA)

Garcinia kola Heckel, also called bitter kola is an important and highly valued ingredient in the African Medicine.it is cultivated mainly for its edible fruit and seed in the tropical rain forest, which over the years have used in folk medicine as a rejuvenating and a general antidote (Nwaneri *et al.,* 2010). It belongs to the *family Guttiferea* and is generally eaten in the West African and contains high bio-flavonoids. The seeds have been used for treatment of different ailment and diseases such as catarrh, pain, liver disorder etc (Cotterhill, 1978). It is also called orogbo in Yoruba land, Miji-goro by Hausas and Akiilu by Igbos.it has ellipsoid shape.

The plant has a height of about 14m and produces a reddish, yellowish or orange colour fruits containing 2-4 seeds. It is endemic in the humid rainforest in the coastal areas and low land plains above the sea level. The extract from the bark of the plant are used in the traditional medicine for the treatment of liver cirrhosis and hepatitis (Galam *et al.,* 2013).

The seed are taken as refreshment in time past in Nigeria, it is however reported to contain a complex mixture of prephenylated benxophenones, xanthoses and bioflavonoids. Various isolation form *Garcinia kola* Includes kolaviron, xanthoses, guacinoic acid, garenial and tocotrienol (Orie, 1993)

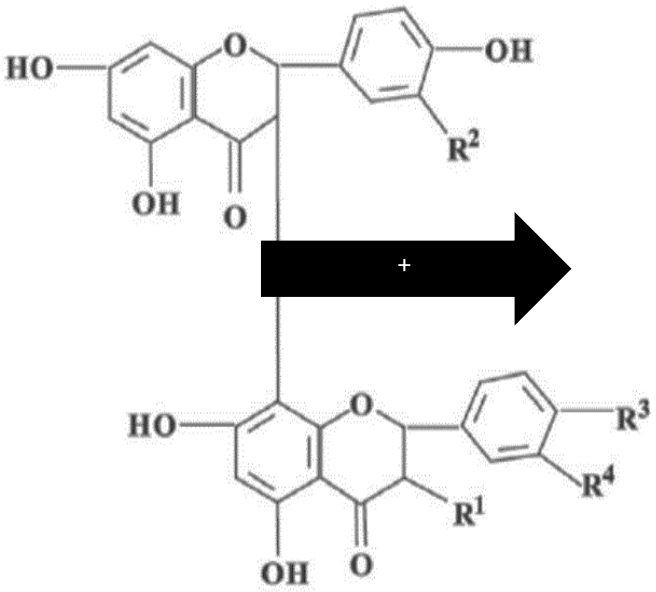
2.9.1.IMPORTANCE.

1. The extract of garcinia has been used to enhance testosterone production (spermatogenic effect) in Sprague dawley rats (Galam *et al.,* 2013).
2. It has been reported to be used as anti-hepatoxic agent in liver toxicity (Iwu, 1987)
3. It has also been used as antioxidant in oxidative damage of renal system in mice (Adaramoye, 2009).
4. It also possess neurotoxic and bronchodilatory effects.
5. It also possesses antimicrobial, anti-inflammatory, anti-diabetic and anti-viral properties, analgesic and antiulcer properties (Ibironke *et al.,* 1997, Olaleye *et al.,* 2000).

2.9.2. KOLAVIRON

Kolaviron was extracted from *Garcinia kola,* is a defatted ethanol extract from the seeds of *Garcinia kola*. It is a complex of three compounds namely; the Garcinia bioflavonoids GB1.GB2 and Flavonone. Itis in the ratio of 2:2:1(Kubanga, 1987, Iwu *et al.,* 1990).

Kolaviron has been reported to possess antioxidant properties antioxidant (Farombi *et al.*,2002, 2007; Terashima *et al.*, 2002; Farombi and Nwaokeafor, 2005), anti-inflammatory (Olaleye *et al.*,2000), anti-hepatotoxic (Farombi *et al.*, 2000, 2004), analgesic (Olaleye *et al.*, 2000), anti-genotoxic agent(Nwankwo *et al.*, 2000; Farombi *et al.*, 2005) and anti-carcinogenic (Farombi *et al.*, 2009),inhibition of apoptosis(Olaleye and Cho,2010).



	R^1	R^2	R^3	R^4
GB 1	OH	H	OH	H
GB 2	OH	H	OH	OH
Kolaflavanone	OH	H	OMe	OH

Figure 2 8: *Garcinia kola*(Amazing health benefits of bitter kola, 2013)and structure of kolaviron

2.10. GASTRIC H^+-K^+ ATPase

Gastric H^+- K^+-ATPase/pump is an intrinsic membrane protein localized on plasma membranes of the parietal cells. It is the key enzyme involved in the final step of acid secretion by catalyzing electroneutral exchange of luminal K^+ for cytoplasmic H^+ and externally coupled with ATP hydrolysis (Shin *et al.,* 2007). The enzyme comprises of two subunits of designated α and β subunits which are encoded by separate genes. The α-subunit is a catalytic subunit responsible for ion exchange while the β-subunit is heavily glycosylated and is necessary for delivery of the α-subunit to plasma membranes. It can be stimulated by several intracellular signals involving H_2 receptors, M_3 receptors and gastrin receptors to secrete acid and decrease in its activities leads to decrease in gastric acid secretion (Helander and Keeling, 1995, Sachs *et al.*, 2005, Thong-Ngam *et al.*, 2005).

The parietal cells are highly specialized cells located in the inner lining of the gastric mucosa, it possesses an extensive secretory membrane system, H^+-K^+ ATPase is the major protein constituent of the membranes. The H^+- K^+ ATPase can also be found in the renal medulla (Shin *et al.,* 2009).

It is a heterodimer protein with two genes, gene ATP4 A which encodes α- subunit. The α -subunit is approximately 1000 amino acid protein and contains catalytic sites of the enzymes. It can form pore through the cell membrane and allows for transport of ions. H+ ions binds to the α-subunit has phosphorylation site (Berg, 2012, Chourosa *et al.*,2005).

The β- subunit is encoded by ATP 4B , is approximately 300 amino acid protein with the 36 amino acid N-terminal cytoplasmic domain, heavily glycosylated extracellular domain with single transmembrane . The β subunit supports and stabilizes α subunit and is necessary for its functioning, prevents the pumps from operating or moving in the reverse. β- subunit contains signals that direct heterodimer to membrane destinations within the cell. These signals subordinate α- subunit.

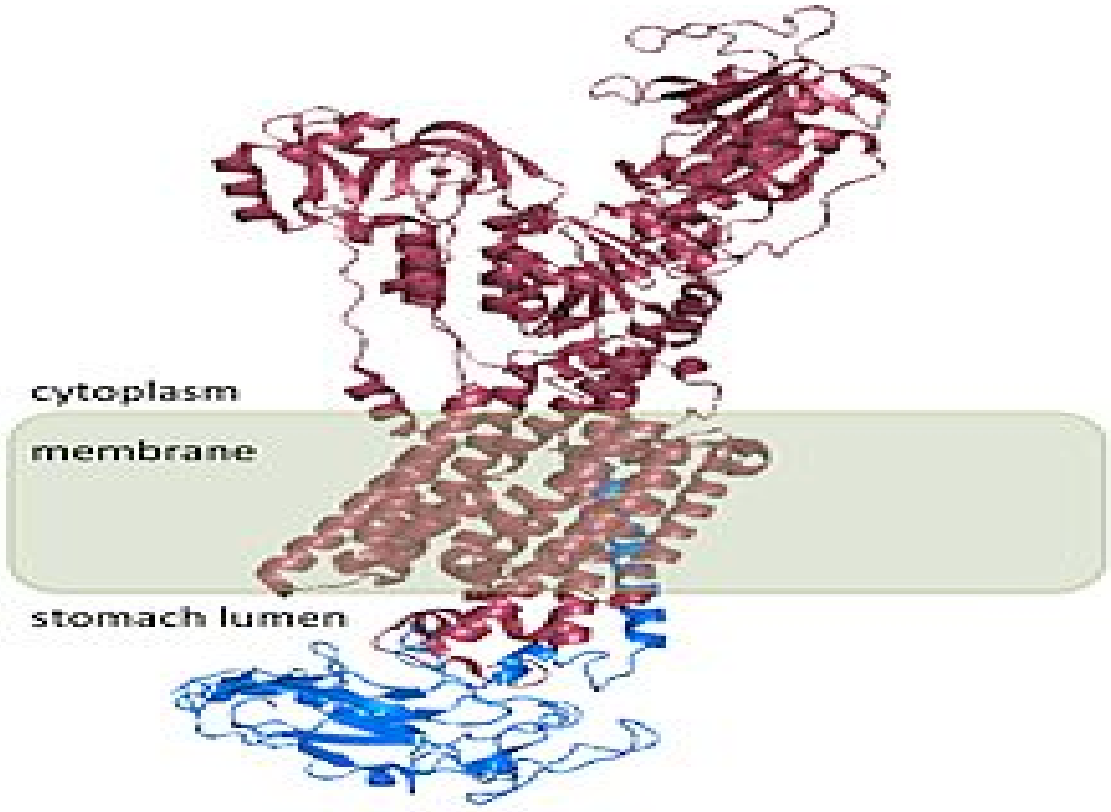


Figure2.9: Structure of the hydrogen potassium ATPase. The α subunit is shown in pink; the β subunit is shown in blue(encyclopedia.m.http//en.wikipedia./org/wiki/, 2016)

2.10.1. MECHANISMS OF ACTION

The H^+- K^+- ATPase is a member of Eukaryotic class like the Ca^{2+} and the Na^+/K^+ ATPases. It functions as an α, β promoter and electro-neutral transporting one proton in to the stomach lumen per potassium retrieved from the gastric lumen. It is able to transport against the concentration gradient using energy derived from the hydrolysis of ATPases. A phosphate group is transferred from adenosine triphosphate (ATP) to the H^+- K^+- ATPase during the transport cycle. The phosphate transfer cause a conformational change in the enzyme that helps drive ion transport and the hydrogen potassium ATPase activated indirectly by gastrin thereby causes ECL cells to release histamine (Prinz *et al*., 1992). The histamine binds to H2 receptors on the parietal cell and activates the cAMP-dependent pathway which makes the enzyme to move from the cytoplasmic tubular membranes to deeply folded canaliculi of the stimulated parietal cell. Once localized, the enzyme alternates between the two conformations namely the E1 and E2 to transport ions across the membranes.

The E1 conformation binds with a phosphate from the ATP and hydronium (H+) ion on the cytoplasmic side, then the enzymes changes to the E2 conformation which allows the H^+ to be released in to the lumen. The E2 inturns bind with the potassium ions and reverts the E2 confromation to release the phosphate and K+ in the cytoplasm so that another ATP can be hydrolyzed to continue the cycle. The β- subunit makes the proton pumping unidirectional by preventing the E2-P conformation from reverting in to the E1-P conformation (Abe *et al.,* 2009, Shin *et al.,* 2009). The numbers of ions transported depends the pH of the stomach.

2.11.0. THE IMMUNOHISTOCHEMISTRY TECHNIQUE

The immunohistochemistry/immunocytochemistry technique is a technique used for localizing specific antigens or cells based on antigen- antibody recognition and seek to exploit the specificity provided by binding of an antibody with its anitigen observed or at the level of light microscope. It is therefore used in for identification of cell or tissue antigens ranging

from amino acids and proteins to infectious agents and specific cellular populations (Coons *et al.*,1941, Brandtzaeg, 1998). It combines histological, immunological and biochemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label.

IHC makes it possible to visualize the distribution and localization of specific cellular components within a cell or tissue. The method comprises of two phases: (1) slide preparation (specimen fixation and tissue processing) and stages evolved for the reaction (in order: antigen retrieval, non-specific site block, endogenous peroxidase block, primary antibody incubation, and the employment of systems of detection, revealing and counterstaining and also slide mounting and storage); (2) interpretation and quantification of the obtained expression (Brandtzaeg, 1998). Brandtzaeg, (1998) stated that immuno-staining for cell markers represents a way of communication with the cells, because it allows not only the histological origin of the cell to be identified but also indicates its function *in vivo*, when duly investigated with the correct antibodies.It was concluded in his study that *in vitro* and *in situ* trials are in fact "pictures" of the situations that occur *in vivo* and therefore constitute one of the pillars of biomedical research. This includes immunohistochemistry, the importance of which is growing (Brandtzaeg, 1998).

2.11.1. HISTORY

The history of IHC (immunohistochemistry) began more than half of a century when Marrack produced reagents against typhus and cholera microorganisms, using a red stain conjugated to benzidin tetrahedro (Marrack,1934).Professor Albert H. Coons from Harvard School of Medicine, Boston, U.S.A. believed that the antigen detection provided by red colour in tissue slices had very low sensitivity under optical microscopy and, in the early 1940's , he demonstrated that localizing antigens, especially microorganisms, are possible in tissue slices using antibodies against *Streptococcus pneumoniae* stained with fluorescein, visualized by ultra-violet light (fluorescence microscopy) (Coons *et al.*, 1941). Subsequently, the introduction of enzymes as marked antibodies, developed by Nakane (1968) imported a new and important era for immunohistochemistry, as it became possible to see these reactions through optical microscopy. These results had great impact in the 1960(s) (Nakane, 1968; Avrameas and uriel, 1966). In the 1990s, the technique became a general application in the surgical pathology (Taylor, 1994a, 1994b, Taylor and Cotes, 1994). This innovation took immunohistochemistry beyond the exclusive sphere of laboratories equipped with

fluorescence microscopes, and the technique spread to a broad group of researchers and pathologists (Haines and West, 2005).There are discoveries of the unlabeled antibody peroxidase-antiperoxidase (PAP) method by Sternberger *et al.,* (1970), and the alkaline phosphatase-antialkaline phosphatase (APAAP) method by Mason and Sammon (1978). Significantly expanded the application of immunohistochemistry technique (Sternberger *et al.,* 1970; Cordell *et al.,* 1984).

The diaminobenzidine molecule (DAB) was conjugated to antibodies during the same period, (Singer,1959), currently representing the most used chromogen for peroxidase, and as it produces an electro-dense precipitate which is also used in electronic microscopy, substituting ferritin (Sternberger,1967).

Subsequently, gold colloidal particles were used as immuno-histochemical colorations (Faulk and Taylor, 1971) and this finding rapidly led to an important method of sub cellular immune staining (Roth *et al.,* 1982). The discovery of antigen retrieval methods (exposure of antigen epitopes in tissue study favors the antigen-antibody reactions for the next stages of the technique) by Huang *et al.,* (1976). The use of systems of secondary antibody detection (for example the avidin-biotin-peroxidase complex) (ABC) and the labelled streptavidin-biotin complex(LSAB) by Hsu *et al.*,(1981a,b,c) also allowed immunohistochemistry to be used in fresh specimens as well as in fixed tissues, which further increased the applicability of the technique in pathology diagnostic routines.

The immunohistochemistry became incorporated in to diagnostic routine of pathological anatomy after tissue antigen was demonstrated by immunoperoxidase technique in tissues fixit is a greated in formalin and embedded in paraffin (Leong and Wright, 1987; Werner *et al.,* 2005). It is an important tool for scientific research and also a complementary technique in the elucidation of differential diagnosis which are not determinable by conventional analysis with haematoxylin and eosin (Nadji, 1986; Leon and Wright, 1987; Rickert and Maliniak, 1989; Schmit, 1991; Rosai, 1996; Torres, 1998; Raab, 2000; Taylor, 2000; Werner *et al.,* 2000; Hsi, 2001; Bodey, 2002). However, it became a great improvement in the contribution and application of immunohistochemistry in pathological anatomy(brown revolution" of the histopathology laboratory) (Leong and Wright, 1987).

2.11.2. APPLICATIONS AND IMPORTANCE

The IHC reactions can be used in different situation, and as follows;

1. Histo-genetic diagnosis of morphologically non-differentiated neoplasia which is the most important aspect.
2. Sub-typing of neoplasias (such as lymphomas, for example);
3. characterization of primary site of malignant neoplasias;
4. Research for prognostic factors and therapeutic indications of some diseases;

Discrimination of benign *versus* the malign nature of certain cell proliferations; identification of structures, organisms and materials secreted by cells (Leong and Wright, 1987, Werner*et al.,* 2000, Bodey, 2002, Jaffer and Bleiweiss,2004).

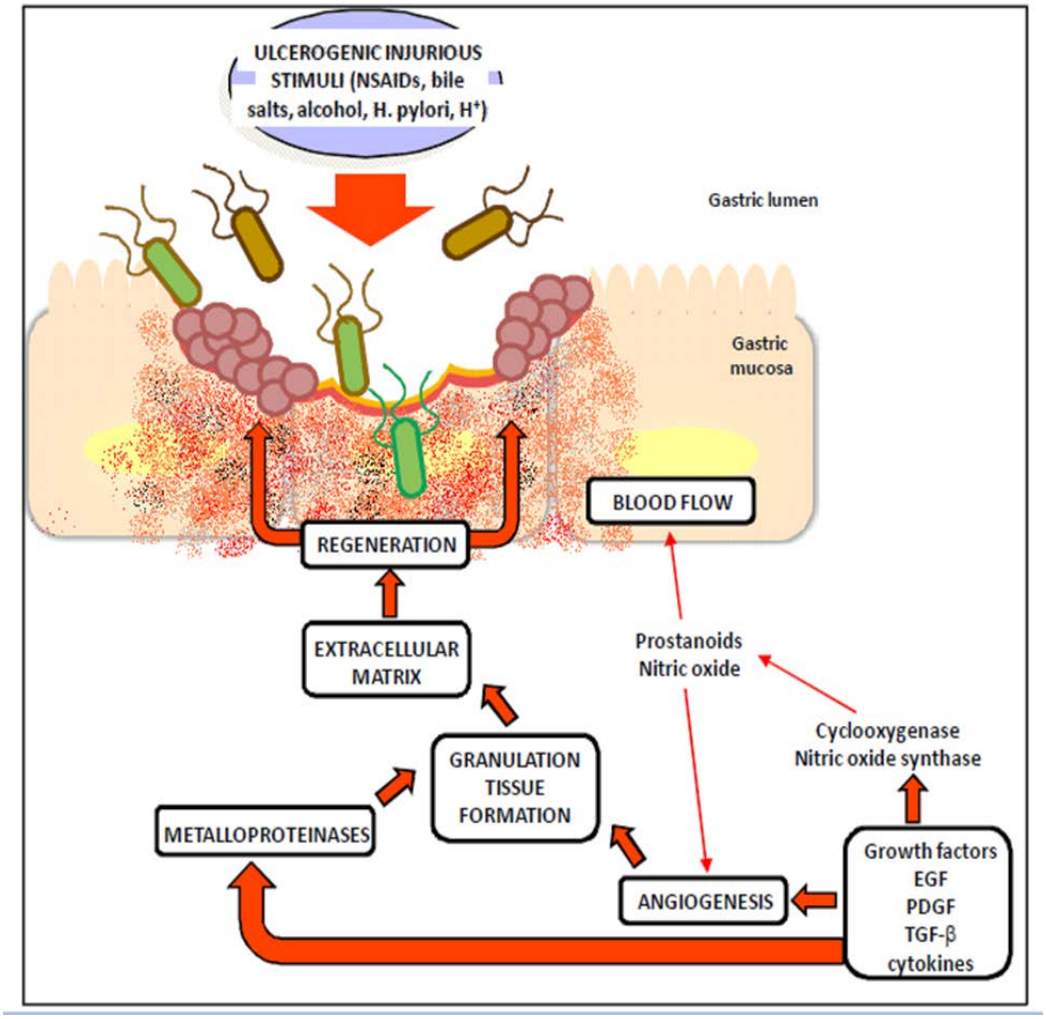


Figure 2. 10: Ulcer healing process, Fornai *et al*, 2011

2.12. ULCER HEALING

Ulcer healing is a genetically programmed repair process which includes inflammation, cell proliferation, re-epithelialization, formation of granulation tissue, angiogenesis, interactions between various cells and the matrix and tissue remodeling, all resulting in scar formation. These process are controlled by various mediators, such as prostaglandins (PGs), cytokines, and growth factors(Folkman *et al.,* 1991,Takahashi *et al.,* 1997,Tarnawski, 2005, Wallace *et al.,* 2006).

a. CD31 (PLATELET-ENDOTHELIAL CELL ADHESION MOLECULE)

CD31 or platelet-endothelial cell adhesion molecule- 1 (PECAM- 1) is endothelial marker which can be expressed by platelets and megakaryocytes, and its role is associated with participating in cell adhesion and angiogenesis. It is hematopoeitic progenitor cell antigen that is expressed on vessels or tumor cell especially in mesenchymal tumors (classification of certain diagnosis) (Pusztaszeri *et al.,* 2006, Marelli-Berg *et al.,* 2013)

CD31 is a member of the immunoglobulin super family,is about 130-kDa transmembrane glycoprotein which is designated as PECAM-1 (platelet endothelial cell adhesion molecule 1). It is present on the surface of platelets, monocytes, macrophages, and neutrophils and is a constituent
of the endothelial intercellular junction. It is very important in the adhesion cascade between EC and the inflammatory cells during inflammation in facilitating leucocyte migration and between EC during angiogenesis. It has been recently been recognized for its angiogenic role (DeLisser *et al.,* 1997; Matsumura *et al.,* 1997, Zhou *et al.,* 1999). It is one of the best markers for benign and malignant vascular tumors (De Young *et al.,* 1993) but also stains plasma cells, monocytes, and megakaryocytes.

b. Factor VIII(von Willebrand factor (vWF)

Factor VIII is also known as the von Willebrand factor (vWF), is a glycoprotein synthesized by endothelial cells and megakaryocytes. It can function in the body in two-fold; it combines with other proteins to form anti-hemophilic factor and can acts as an activator of factor X in the clotting system also plays a major roles in platelet aggregation (Ogawa *et al.,*1946) . From study carried out by Kang *et al.,* (2010) has been used to described the density of micro - vascular vessels and reacting lymphatic endothelium during ulcer healing process.

c. EGFR (EPIDERMAL GROWTH FACTOR RECEPTOR)

Epidermal growth factor is an important peptides that promotes gastro-intestinal mucosal repair (Basson, 2003). It exerts its action by binding to its receptor on epithelial cell surface, a process that triggers a number of intracellular events that culminate in to cell migration and proliferation (Boonstra *et al.,* 1995). Members of the EGF family of peptide growth factors are mitogenic in many tissues, they mediates their effects after binding to specific receptors. The induction of EGFR tyrosine kinase plays an important role in the initiation of mucosal repair after acute gastric injury in rats (Relan *et al.,* 1995). EGFR is usually expressed on all mucosal cells of the gastrointestinal tract, but the expression has been shown to be increased in gastric cancer, ulcer of the stomach (Kajikawa *et al.,* 1991; Tokunga *et al.,* 1995). Although EGFR mediated signal transduction mechanisms have been studied extensively in several cell systems, these pathways have not been thoroughly investigated in gastric epithelial cells. In the healing zone on the ulcer margin, there is dedifferentiation and expression of epidermal growth factor receptor(Tranawski, 2000, Tarnawski,1993). This is usually initiated within 3days after ulcer formation, as it is essential for ulcer healing. The stimulus for increased epithelial cell proliferation in the mucosa of the ulcer margin is most likely initiated by EGF and/or TGF-α (Pou).

The EGF mediates its biological effects on target enterocytes via binding to a specific 170-kDa membrane-bound glycoprotein receptor, the EGF receptor. The EGF receptor has been found localized in the foetal and adult gastrointestinal tract, liver, and pancreas (Forgue-Lafitte *et al.,* 1984). The binding of the EGF receptor activates the intrinsic tyrosine kinase, which then leads to a complex cascade of cellular events that ultimately result in DNA synthesis and cellular growth (Barnard *et al.,* 1995). Chronic administration of EGF produces a significant increase in mucosal DNA, RNA, and protein content (Johnson and Guthrie, 1980). This proliferative action of EGF- R is believed to contribute to the normal maintenance of mucosal integrity and functions within the gastrointestinal tract (Playford and Wright, 1996).It has been shown that EGF-R is beneficial in pathophysiological processes in the gastrointestinal tract by either reducing injury (Konturek *et al.,* 1988; Konturek *et al.,* 1995) or accelerating repair (Skov-Olsen *et al.,* 1986), capable of providing protection against a variety of gastric insults, both acid dependent (Konturek *et al.,* 1988; Konturek *et al.,* 1992) and acid independent (Konturek *et al.,* 1992; Konturek *et al.,* 1995). Its importance of EGF in ulcer healing is thereby demonstrated by a marked increase in its receptors and EGF-

producing cells in experimental gastric ulcers in rats induced by acetic acid (Skov-Olsen *et al.,* 1986; Tarnawski *et al.,* 1992) or cryoprobe (Alison *et al.,* 1995).

d. P53 (PHOSPHOPROTEIN OR ONCOGENETIC REPRESSOR)

P53 is an oncogenetic repressor or tumor suppressor or cellular tumor antigen/ phosphoprotein P53 seen in many cancers and some chronic inflammation, its anti- cancer function has been realized by triggering apoptosis (Xu *et al.,* 2001). P53 is the most striking example of a gene that control normal cell proliferation, cellular function such as DNA repair, differentiation, genomic plasticity, and programmed cell death (Harris, 1993, Greenblat *et al., 1995*). It is very important or crucial in the multicellular organism as it regulates the cell cycle and thus can function as tumor suppressor. It encoded by the TP53 gene (Kern *et al.,* 1991). P53 is described as guardian of the genome due to its role in conserving and maintaining the stability of the gene by preventing mutation of the gene (Cordon-Cordo *et al.,* 1994, Molina and Segui, 1998). Some investigators have shown that P53 mutations may occur in precancerous gastric lesions, such as atrophic gastritis and intestinal metaplasia, as well as adenomatous polyps (Shiao *et al.,* 1994; Correa, 1994). Abnormalities in P53 expression represent the most common molecular change not only in cancer, but also in precancerous gastric lesions, including gastric dysplasia (Sasano *et al.,* 1993). An increased wild-type P53 expression may also represent a cellular response to DNA damage (Shiao *et al.,* 1994). ; P5*3* is part of a pathway invoked upon DNA damage in mammalian cells through the induction of G and mitotic arrest (Blattener *et al.,* 1994,Cross *et al.,*1995).

e. Ki-67 (KIEL 67)

Ki-67 is a well-recognized nuclear antigen present in the proliferating cells, is usually expressed in all stages of cell cycle except the G0 and early part of G_1(Lopez *et al.,* 1991, Weidner *etal.,* 1994,Young –Eun Joo *et al.,* 2006). A dynamic balance between epithelia cell proliferation and apoptosis is essential for maintaining the normal mucosal integrity (Moss *et al.,* 1996). Alterations in the balance of epithelial cell proliferation and apoptosis (Correa and Miller, 1998) contribute to gastric ulcerogenesis or even carcinogenesis (Que and Gores, 1996; Moss, 1998). It was discovered in 1980s, its role in the proliferation of cancerous cell especially in lymphomas, breast, endocrine and brain cancers. It is commonly used as a complement to grading systems that include mitotic counting as a sign of proliferation (Oakman *et al.,* 2009).

2.13.0. NITRIC OXIDE

Nitric oxide (NO) is very essential and plays important roles in mammalian life (Moncada *et al.*, 1991, Schimdt and Walter, 1994). It is a very simple molecules and its synthesis involved one of the most complicated enzymes in nature, the nitric oxide synthase. The synthesis of NO synthases required several cofactors and are highly regulated (Nathan and Xia, 1994). It has multiple physiological and pathophysiological functions, which is achievable by the diverse classes of nitric oxide (neuronal NOS (nNOS), endothelial NOS (eNOS), inducible NOS (iNOS) and mitochondria NOS (mNOS).

It has been shown that unregulated production of nitric oxide can cause nitrosative stress, leading to damages of proteins/DNA and to cell injury and death (Hausladen and Stamler, 1999, Murphy, 1999). NO has shown to be involved in a number of important human diseases and its production is not limited to mammalian life but are also produced in the plants and bacteria (Watmough *et al.*, 1999, Lum *et al.,* 2002). The measurement of NO in the biological system requires a careful consideration, it is easily / rapidly oxidized in to nitrite or nitrate by oxygen. The Half-life of NO in the biological matrix is very short, ranges from 1 secs in the presence of hemoglobin to ~30 seconds (Knowles and Moncad, 1992, Fieldman *et al.*, 1993, Eich *et al.,* 1996).NO can be measured *in situ* by using fast-response amperometric electrode sensors and by *using in-vivo* trapping techniques with fluorescent or EPR-active reporter molecules (Kojima *et al.,* 1998, Kleschyov *et al.,* 2002). It can alternatively be estimated from determining the concentrations of nitrite and nitrate end products. The measurement of nitrate/nitrite concentration or of total nitrate and nitrite concentration (NOx) is routinely used as an index of NO production (Moshage *et al.,* 1995). Griess reaction assay technique have been used to determined nitrite concentration in biological matrixes, and related techniques of quantitative reduction of nitrate to nitrite.

2.14.0. NITRIC OXIDE SYNTHASES

The nitric oxide synthases are family of enzymes that catalyses the production of nitiric oxide from the L-arginine. It plays important roles in cellular signaling molecule, helps to modulates vascular tones, insulin secretion, air way tone, peristalsis, function in angiogenesis and neural development. It may also function in retrograde neurotransmitter. It is mediated by calcium-calmodulin controlled isoenzymes eNOS(endothelial NOS) and nNOS(Neuronal NOS), the inducible isoform, iNOS, is also involved in immune response, binds calmodulin at physiologically relevant concentrations, and produces NO as an immune defense mechanism, as NO is a free radical with an unpaired electron(Knowles, 1994). The endothelial isoform is the primary signal generator in the control of vascular tone, insulin secretion, and airway tone, is involved in regulation of cardiac function and angiogenesis (growth of new blood vessels). NO produced by eNOS has been shown to be a vasodilator identical to the endothelium-derived relaxing factor produced in response to shear from increased blood flow in arteries. This dilates blood vessels by relaxing smooth muscle in their linings. eNOS is the primary controller of smooth muscle tone.

Table 2.1: The different forms of NO synthase and their classification

Name	**Gene(s)**	**Location**	**Function**
Neuronal NOS (nNOS or NOS1)	NOS1 (Chromosome 12)	nervous tissue, skeletal muscle type II	cell communication
Inducible NOS (iNOS or NOS2) Calcium insensitive	NOS2 (Chromosome 17)	immune system, cardiovascular system	immune defense against pathogens
Endothelial NOS (eNOS or NOS3 or cNOS)	NOS3 (Chromosome 7)	endothelium	vasodilation
Bacterial NOS (bNOS)	Multiple	various Gram-positive bacteria	defense against oxidative stress, antibiotics, immune attack

Adapted from Stuehr,1999

2.14.1. NITRIC OXIDE IN ULCER HEALING

Nitric oxide is very important and beneficial gastric ulcer healing. Konturek *et al.,* 1993 demonstrated that inhibition of NO synthase (NOS) by *N* Gnitro-L-arginine (L-NNA) or *N*G-monomethyl-L-arginine(L-NMMA) delayed ulcer healing, reduced gastric blood flow around the ulcer, as well as impaired angiogenesis in the granulation tissue. On the other hand, administration of an NO donor (glyceryl trinitrate) or L-arginine, the substrate of NOS enhanced the ulcer healing (Elliott *et al.,*1995, Konturek *et al.,* 1993) while reversed NOS inhibitor induced delayed healing **(**Brzozowski *et al,* .1995). It can also stimulates mucus and bicarbonate secretion (Bilski *et al., 1994*, Brown *et al, 1993*, Ma and Wallace,2000) and maintaining gastric blood flow.

NO also promotes angiogenesis in vivo and in vitro. Angiogenesis, a process of generation of new blood vessel from preexisting vessels, requires a concerted interaction of a variety of cellular systems. It starts with the dissociation of basement membrane beneath endothelial cells, followed by endothelial migration, adhesion, proliferation, and tube differentiation. Ziche *et al*.,1994, 1997 have demonstrated that NO acts downstream of vascular endothelial growth factor(VEGF) and substance P in mediating angiogenesis in an in vivo corneal implant model in rabbits. NO has also been shown to stimulate angiogenesis in response to tissue ischemia in mice. NO donors promote endothelial cell proliferation and migration. It has been suggested that the presence of eNOS protein in the vascular endothelium of mucosa, sub mucosa, and especially granulation tissue suggests that eNOS might be involved in angiogenesis during ulcer healing(Ma and Wallace, 2000). This was demonstrated by Lee *et al.,* 1999 that angiogenesis and skin wound was impaired in eNOS deficient mice (Lee *et al.,* 1999).

Similarly, Murohara and colleagues (1998) reported that angiogenesis in response to tissue ischemia was significantly suppressed in eNOS-deficient mice. Thus, several studies have shown and are consistent with the hypothesis that eNOS-derived NO is a downstream signal

for growth factor-induced angiogenesis. The possible mechanism through which NO modulates angiogenesis is the suppression of protein kinase C-d (PKC-d). NO donors like sodium nitroprusside, reduced PKC-d activity and thereby promoted endothelial migration and proliferation. NO can also modulate angiogenesis is by modifying adhesion molecule expression on endothelial cells. NO has been shown to maintain the functional expression of avb3-integrin, a mediator for endothelial migration, survival, and angiogenesis. INOS can produced large amounts of NO relative to eNOS (Brown *et al,* 1994, Prinz *et al.*,1996) and can be detected in ulcerated stomach, primarily localized in the inflammatory cell at the ulcer base. The iNOS mRNA and protein expression are usually greatest at the early phase of ulcer healing.

CHAPTER THREE

MATERIALS & METHODS

3.1. EQUIPMENT

Microscopes, syringes and needles, conical flask, dissecting sets, cotton wool, dissecting board, weighing balance, hand gloves, blade, slides, animal cages, EDTA bottles, rubber catheter, tefflon homogenizer, cold centrifuge, spectrumlab 32A spectrophotometer, UV/VIS Spectrophotometer 1700, epoch microplate reader, improved Neubauer chamber.

3.1.1 DRUGS, SOLVENTS AND REAGENTS

Sodium arsenite (Sigma Chemical, Co),vitamin E(Sigma-Aldrich,Germany), zinc sulphate(Chemiet PVT,India),acetic acid, copper sulfate ($CuSO_4$. $5H_2O$), sodium tartarate, potassium iodide (KI), thiobarbituric acid (TBA), tris base,potassium chloride (KCl)(BDH, England), stock bovine serum albumin (standard) (Sigma Chemical Co., USA), trichloroacetic acid (TCA) (Oxford laboratory reagent, India), ketamine (900-B-2370, Arendonk, Belgium) and xylazine(Rotex. Med. ICA., Trittau, Germany).

50% ethanol, 0.0025N NaOH,phenopthalein,HCl,naphthylethylenediamine dihydrochlorate(NED) and sulfanilic acid,phosphoric acid, sodium phosphate dibasic anhydrous ($NaH2PO_4$), primary antibody (P53 Ab-6, EGFR Ab-I, Factor 8 Ab-2, KI67 Ab-1, CD31 Endothelial marker NCL-END, (Novacastro laboratories, UK),distilled water. All other reagents of analytical grade were obtained from department of physiology, University of Ibadan**.**

3.1.2. *GARCINIA KOLA*

Seeds of *Garcinia kola* Heckel (Family, Guttiferae) were collected from cultivated plants in Offa market, Kwara state, Nigeria. It was identified in the hebarium by Esimekhuai, D.P.O. of the department of Botany, University of Ibadan, Nigeria.

3.1.3.EXTRACTION OF KOLAVIRON:

Peeled seeds were sliced, pulverized with an electric blender and air-dried. Kolaviron (KV) was extracted from the powdered seeds of *Garcinia kola* according to the method of Iwu *et al.*, 1990. Briefly, the powdered form of *Garcinia kola* seeds (4 kg) was extracted with N-hexane (b. pt 40–60 °C) in a soxhlet for 24 h. The defatted, dried marc was extracted with acetone. The extract was concentrated and diluted with twice its volume of water and extracted with ethyl acetate (6 × 300 ml). The concentrated ethyl acetate fraction yielded kolaviron (165 g), which has been shown to consist of *Garcinia biflavanones* GB-1, GB-2 and kolaflavanone (Iwu, 1985).

1000mg was dissolved in DMSO as stock solution and 100mg/kg of kolaviron was administered orally to the animals.

3.1.4.ZINC SULPHATE

Zinc sulphate (Zn) was obtained from Loba Chemiet PVT, India, 100mg was dissolved in 1000mL of distilled water to make a stock solution, 20mg/kg was administered orally to each rat,and solution was made fresh on the day of experiment.

3.1.5. VITAMIN E

100mg/kg of vitamin E(VE)(Sigma-Aldrich, Germany) was purchased from Kunle Ara, Ibadan. The vitamin E was squeezed out from the capsule with syringe and was administered according to the weights of the animals.

3.2. EXPERIMENTAL ANIMALS

Male wistar were used (n=125,150g-200g), and was randomly divided in to five phases. Animals were purchased from the Central Animal House, Faculty of Basic Medical Science, College of Medicine, University of Ibadan,Ibadan, Nigeria. They were housed in clean standard metabolic cages and fed with standard rat pellet(vital feeds containing 14% protein,7% fats,10% crude fibers, 1% calcium, 0.35% of phosphorus, manufactured by UAC, Jos but purchased in Ibadan) with free access to tap water under standard housing conditions

(temperature: 25°C±2 with a photoperiod of 12h natural light/dark cycle and humidity: (50-55%).

3.2.1. EXPERIMENTAL DESIGN

This study was carried out in five phases and is as follows;

1. Phase I: Evaluation of the effect of sodium arsenite on the formation of experimental ulcer
2. Phase II: Study on the effects of sodium arsenite on healing of experimental gastric ulcer.
3. Phase III: Intervention studies on the effect of antioxidants; kolaviron,vitamin E and zinc sulphate on normal states of the gastrointestinal tract in sodium arsenite exposed rats.
4. Phase IV: Mechanisitic study of the effect of kolaviron and vitamin E on the healing of acetic acid induced ulcer in sodium arsenite exposed rats.
5. Phase IV: Mechnaisitic study of the effects of zinc sulphate on healing of acetic acid induced ulcer in sodium arsenite exposed rats.

Phase 1: Evaluation of the effect of sodium arsenite on the formation of experimental gastric ulcer.Forty- eight animals (n=3 animals per day) were used in this phase and grouped as follows;

1. Control(water,p.o)
2. Control(acetic acid induced ulcer (AA)
3. 5mg/kg SA exposed (p.o)+ acetic acid induced ulcer(AA)

4. 10mg /kg SA exposed (p.o) +acetic acid induced ulceration (AA)

The animals were exposed to sodium arsenite for two weeks and experimental gastric ulcer was induced using acetic acid. Parameters such as total gastric acidity, ulcer area was observed on day 3, 7,14 and 21 as shown in the table 3.1.

Table 3.1. The groups, treatments, experimental procedure and parameter studied in phase I

Group	N=number of animals	Treatment	Experimental procedure	Parameter studied
I	3	Normal saline	None	Total gastric acidity(TGA), ulcer area (UA)
	3		None	
	3		None	
	3		None	
II	3	Normal saline	AA	Total gastric acidity(TGA), ulcer area (UA)
	3			
	3			
	3			
III	3	5 mg/kg SA	AA	Total gastric acidity(TGA), ulcer area (UA)
	3			
	3			

	3			
IV	3	10 mg/kg SA	AA	Total gastric acidity(TGA), ulcer area (UA)
	3			
	3			
	3			

Total gastric acidity and the ulcer area was observed on day 3, 7,14, 21 post ulcer induction.

Phase II: Study of the effect of sodium arsenite on healing of experimental gastric ulceration.

Thirty-Six animals (150-200g), (n = 3 animals per day) were used in phase II and grouped as follows;

I. Control (water, p.o)
II. AA
III. 5 mg/kg SA (p.o.) + AA.

The animals were exposed to 5mg/kg of SAfor two weeks and experimental gastric ulcer was induced after the exposure. Parameters studied as stated in table 3.2 were observed on day 3, 7,14, 21.

Table 3.2. The groups, treatments, experimental procedure and parameter studied in phase II

Group	N=number of animals	Treatment	Experimental procedure	Parameter studied
I	3	Normal saline	None	Total gastric acidity (TGA), ulcer area (UA), relative weight of stomach,ulcer depth, parietal cell density, mucosal thickness
	3		None	
	3		None	
	3		None	
II	3	Normal saline	AA	Total gastric acidity (TGA), ulcer area (UA), relative weight of stomach,ulcer depth, parietal cell density, mucosal thickness
	3			
	3			
	3			
III	3			

	3	SA	AA	Total gastric acidity (TGA), ulcer area (UA), relative weight of stomach,ulcer depth, parietal cell density, mucosal thickness
	3			
	3			

Phase III: Intervention studies on the effects of *Kolaviron* (KV), Zinc and vitamin E on the normal states of the gastrointestinal tract of the sodium arsenite exposed rats.

Fifteen animals (150-200g, n=3)were used in this phase and are grouped as follows;

1. Control (water, p.o.)
2. 5 mg/kg SA (p.o.)
3. 5 mg/kg SA + KV (p.o.)
4. 5mg/kg SA + Zn (p.o.)
5. 5 mg/kg SA + VE (p.o.)

Animals were exposed to sodium arsenite (SA),Kolaviron (KV), Zinc Sulphate (Zn), vitamin E (VE) for two weeks. The animals were sacrificed under anesthesia and the stomach was excised and homogenized. Parameters were studied and as shown in table 3.3.

Table 3.3. The groups, treatments, experimental procedure and parameter studied in phase III

Group	N=number of animals	Treatment	Experimental procedure	Parameter studied
I	3	Normal saline	None	NO, H^+-K^+ATPase, SOD, CAT, protein level, MDA, PECAM-1, Ki – 67, P53, H& E
II	3	5 mg/ kg SA(p.o.)	None	NO, H^+-K^+ATPase, SOD, CAT, MDA, protein level, PECAM-1, Ki -67, P53, H& E
III	3	5 mg/kg SA + 100 mg/kg KV (p.o)	None	NO, H^+-K^+ATPase, SOD, CAT, MDA, protein level, PECAM-1, Ki- 67, P53, H& E

IV	3	5 mg/kg SA + 100 mg/kg VE (p.o)	None	NO, H^+-K^+ATPase, SOD, CAT,protein level, MDA, PECAM-1, Ki- 67, P53, H& E
V	3	5 mg/kg SA + 20 mg/kg Zn (p.o)	None	NO, H^+-K^+ATPase, SOD, CAT, protein level, MDA, PECAM-1, Ki-67, P53, H& E

Phase IV: Mechanistic study of the effects of Kolaviron(KV) and vitamin E on the healing of acetic acid induced ulcer in sodium arsenite exposed rats.

Eighty- four animals (150- 200g, n= 3 animals per day) were used and grouped as follows

1. Control(water, diets)
2. AA
3. SA (p.o.) + AA
4. SA + VE (p.o.) +AA
5. VE (p.o.)+ AA
6. SA + KV (p.o.) +AA
7. KV (p.o) +AA

Animals were exposed to sodium arsenite (SA) for two weeks and treated with 100 mg/kg KV and VE. After two weeks of exposure, experimental gastric ulcer was induced. Hematological parameters (TWBG, RBC, PCV, Hb, Differential WBC), proton pump (H^+-K^+ATPase), nitric oxide (NO), antioxidant (SOD, CAT), and other markers was studied on day 3,7,14, and 21 as shown in table 3.4.

Table 3.4: The groups, treatments, experimental procedure and parameter studied in phase IV

Group	N=number of animals	Treatment	Experimental procedure	Parameter studied
I	3	Normal saline	None	UA, PCV, RBC, Hb, TWBC, SOD, CAT, MDA, protein level, NO, H^+-K^+ATPase, PECAM-1, Ki-67, P53, H& E
II	3	Normal saline (p.o.)	AA	UA, PCV, RBC, Hb, TWBC, SOD , CAT, MDA, protein level, NO, H^+-K^+ATPase, PECAM-1, Ki-67, P53, H& E
	3			
	3			
	3			
III	3	5mg/kg SA(p.o.)	AA	UA, PCV, RBC, Hb, TWBC, SOD , CAT, MDA, protein level, NO, H^+-K^+ATPasPECAM-1,Ki-
	3			

	3 3			67, P53, H& E
IV	3 3 3 3	5 mg /kg SA + 100 mg/kg VE (p.o)	AA	UA, PCV, RBC, Hb, TWBC, SOD , CAT, MDA, protein level, NO,H^+-K^+ATPase,PECAM-1, Ki-67, P53, H& E
V	3 3 3 3	VE	AA	UA, PCV, RBC, Hb, TWBC, SOD , CAT, MDA, protein level, NO,H^+-K^+ATPasPECAM-1, Ki-67, P53, H& E
VI	3 3 3 3	SA+ KV	AA	UA, PCV, RBC, Hb, TWBC, SOD , CAT, MDA, protein level, NO,H^+-K^+ATPasPECAM-1, Ki-67, P53, H& E
VII	3 3 3 3	KV	AA	UA, PCV, RBC, Hb, TWBC, SOD , CAT, MDA, protein level, NO,H^+-K^+ATPasPECAM-1, Ki-67, P53, H& E

Phase V: Mechanistic study of the effects of Zinc sulphate on the healing of acetic acid induced ulcer in sodium arsenite exposed rats

Forty – eight animals (150-200g, n = 3 animals per day of observation) were used and grouped as follows

1. Control(water, p.o)
2. AA
3. SA (p.o.) + AA
4. SA+ Zn(p.o.)

The animals were administered with sodium arsenite (5mg/kg) and treated with Zinc sulphate for two weeks. After two weeks of exposure, experimental gastric ulcer was induced and on day 3, 7, 14, 21 post ulcer, parameters were studied as shown in the table 3.5.

Table 3.5. The groups, treatments, experimental procedure and parameter studied in phase V

Group	N=number of animals	Treatment	Experimental procedure	Parameter studied
I	3	Normal saline (p.o.)	None	TGA, UA, PECAM-1, factor VIII, K-i67, EGFR, and P53, H & E
	3		None	
	3		None	
	3		None	
II	3	Normal saline (p.o.)	AA	TGA, UA, PECAM-1, factor VIII, K-i67, EGFR, and P53, H & E
	3			
	3			
	3			
III	3	SA	AA	TGA, UA, PECAM-1, factor VIII, K-i67,
	3			

	3			EGFR, and P53, H & E
	3			
IV	3	SA+ zn	AA	TGA, UA, PECAM-1, factor VIII, K-i67, EGFR, and P53, H & E
	3			
	3			
	3			

3.3. EXPERIMENTAL PROCEDURE

3.3.1. ACETIC ACID INDUCED ULCERATION

Gastric ulcer was induced by acetic acid. According to Jainu *et al.*, 2006 and Okabe *et al.*, 1972 and 2010.

Principle

Acetic acid has been extensively studied to enhance ulceration in the stomach mucosa by increasing the acidity of stomach contents(Shirisha and Subash, 2012).i.e. stimulating gastric acid hypersecretion which could be due to release of histamine which inturn increases the capillary permeability and back diffusion of HCl (Jainu *et al.*, 2006, Umamaheswari *et al.*, 2007,Okabe *et al.*, 1972,2010)

Procedure

At the end of 14 weeks of sodium arsenite, Food was withheld for 24-36hrs before ulcer induction, with access to water. The animals were anesthesized with ketamine (60mg/kg) and xylazine(5mg/kg) and laparotomy was performed to expose the stomach. Gastric ulcer were induced using acetic acid with a little modification of the method described by Tsukimi and

Okabe (1994). 0.06 mL of 40% acetic acid was instilled into the submucosal layer of the gastric wall for 60 sec. The acid was removed by aspiration and the area washed with sterile saline and dabbed with cotton wool. The abdomen was sutured and the animals were returned to their diets and water. The animals were sacrificed at intervals and examined for ulcer formation (i.e. on days 3, 7, 14 and 21 post-ulcer inductions).

3.3.2. ULCER AREA MEASUREMENT

The area of ulceration was evaluated by macroscopic examination with 2X magnification hand lens. The stomach were opened along the greater curvature, and was bathed in normal saline, then spread out with pins on a cork board, and then measured. The ulcerated area in (cm^2) was calculated according to the collection of guiding principles of Drug administration of Ministry of Health Beijing in 1993. It was calculated thus

$x = \pi (d1/2) \times (d2/2)$,

Where X represents the ulcerated area (cm^2), d1represent the longest longitudinal diameter of the ulcer, d2represents the longest transverse diameter of the ulcer.

3.3.3.MEASUREMENT OF GASTRIC ACID SECRETION

Gastric acidity was performed as earlier described by Blandizzi *et al.*, 2005 with modification. At 3rd, 7th, 14th and 21st day induction of gastric ulcer, the animal were sacrificed with overdose of anesthesia; the abdomen were opened to remove the stomach. The stomach was opened along the greater curvature and the gastric content was drained into a centrifuge tube. Samples with more than 0.5ml was discarded and the resultant solution was centrifuged at 3,000rpm for 10 minutes. Gastric acid output was determined in the supernatant by titration with 0.01N NaOH.0.5ml of gastric juice pipetted into a 25 mL conical flask, 2 drops of phenolphthalein solution was added and titrated with 0.01 NaOH until a purple colour appears. The volume of NaOH added was also noted. Acidity was calculated by using the formula below;

$$\text{Acidity} = \frac{\text{Volume of NaOH x Normalty of NaOH}}{0.1} \text{ x mEq/L/100g}$$

3.4. HAEMATOXYLIN & EOSIN STAINING

Principle:

In order to microscopically view a tissue specimen that has been processed, sectioned (thinly sliced), and mounted on a slide, the specimen must be stained in order to make the cells visible. The basic nature of histology slide staining is to stain the slide with two or more contrasting dyes that will highlight specific areas or entities with one color, and leave a counterstaining background colour. The standard or **'**routine stain' is the hematoxylin and eosin stain, better known as the **'**H&E'stain. The H & E stain uses two separate dyes, one staining the nucleus and the other staining the cytoplasm and connective tissue. Hematoxylin is a dark purplish dye that will stain the chromatin (nuclear material) within the nucleus, leaving it a deep purplish-blue color. Eosin is an orangish-pink to red dye that stains the cytoplasmic material including connective tissue and collagen, and leaves an orange-pink counterstain. This counterstain acts as a sharp contrast to the purplish-blue nuclear stain of the nucleus, and helps identify other entities in the tissues such as -cell membrane (border), red blood cells, and fluid.

Procedure

1. The tissue was dewax in xylene for 10mins and rinse in absolute alcohol (six slow dips), it was taken through low grades of alcohol, i.e 80%, 70%,50% (six slow dips in each) and rinse in water
2. It was then stained in Harris haematoxylin for 5 minutes and rinse in water.
3. The tissue was later deffrentiate in 1% acid alcohol briefly and was rinse and blue under tap water for 10 minutes, it was then counterstain in 1% aqueous eosin for 3 minutes and rinse in water.
4. It is then dehydrate through ascending grades of alcohol (70%,80%,90% and absolute) and clear in xylene, then mount with dpx mountant. The nuclei appeared blue- purple and the cytoplasm appear pink

3.5. HISTOMORPHOMETRY

a. Measurement of depth of ulcer and mucosal thickness

Histomorphometric analysis was done according to the method described by Ofusori *et al*., 2008. Haematoxylin and eosin stained stomach tissue sections were subjected to morphometric analysis recommended by World Health Organization(WHO,1991) which

includes: dividing the eye piece oculometer into two 100 small divisions, the stage micrometer scale was made up to 1mm divided into 0.1mm divisions and each 0.1mm was divided into 0.01mm, the eye piece scale (oculometer) was inserted into the eye piece of the microscope by removing the superior lens, thus placing the scale on the field stop, the stage micrometer was also placed on the stage of the microscope, the stage scale was focused by the low power objective lens (x4), the stage and the eye piece scales were adjusted until there was a parallel point between the two scales, the number of the eye piece divisions and its corresponding stage measurements was noted; (if 70 oculometer divisions equal to 14µm, all the objective lens were thus calibrated). Calibration was needed for each microscope use. The oculometer fixed into the Olympus Microscope was then focused through stained sections of the tissue to allow for the measurement of the depth of ulcer and thickness of *muscularis externa*.

b. Measurement of parietal cell density

The diameters and the density the parietal cells were measured from H&E slides. H&E stained slides were prepared and studied under light microscope at 10x, 20x, 40x and 100x magnifications. All the cells were enumerated and corrections weremade for cells bisected by the edges or borders of the grid.The cell population was estimated at number of cells/mm^2 of tissue. The counting was done at a magnification of 400x. Sala *et al.*, (1981) mathematical correction was used to correct for the actual number of cells counted.

3.6. HEMATOLOGICAL PARAMETERS

Procedure for collecting blood sample

- Blood was collected via retro-orbital plexus with care and adequate safety precautions to ensure tests results are reliable, contamination of sample was avoided and infection from blood transmissible pathogen was prevented. Protective gloves were worn when collecting and handling blood samples, and all materials needed were sterilized and dried.
- Haemoglobin, PCV, WBC count, differential white blood cell count, and reporting blood cell morphology, but it is not suitable for coagulation test.

3.6.1. PACKED CELL VOLUME (PCV)

Value of the test:

Packed cell volume (PCV) also known as hematocrit is used to calculate blood indices. It is use for the diagnosis and understanding the treatment of anemia, polycythemia, determination of extent of dehydration and recovery from dehydration after treatment).it was carried out acoording to method of Bush, 1975

Principle of test*:*

The packed cell is the proportion of blood occupied by RBCs expressed in percentage. The normal value of PCV ranges from 40-45% in males and 38-42% in femlaes.

Test method:

- Three quarters of heparinized capillary tube was filled with well mixed EDTA anti-coagulant blood (tested within 3hrs of collection). The filled part was sealed with a sealant (plastacine).
- The filled capillary tube was carefully located in one of the numbered microhaemacrit rotor with the sealed end against the rim gasket (to prevent breakage); when the inner lid was used, it was carefully positioned to avoid dislodging the tubes. It was centrifuged for at 3000rpm for 5 minutes.
- After centrifuging, immediately the PCV was read in a hand-held microhaematocrit reader, it was done by aligning the base of the red cell column (above the sealant) on the zero line and the top of the plasma column on the 100 line.
- The RBC is the packed cell volume and the plasma remains above this. The reading point which is at the top of the red cell column, just below the buffy coat layer (consisting of WBCs and platelets) is the site where the PCV is read from.
- The test was performed repeatedly respectively, and red cells were ensured that they were packed correctly by re-centrifuging for 2-3mins of several PCV samples.

3.6.2 MEASUREMENT OF HEMOGLOBIN

Principle: Measurement of amount of haemoglobin in the blood is called hemoglobinometry. Hemoglobin is measured to detect anemia and its severity and to monitor an anemic patient's response to treatment.The blood was treated with a dilute solution of potassium ferricyanide and potassium cyanide at a slightly alkaline pH (Drab kin solution).

Test method

- 20 µl (0.02 ml) of EDTA blood was carefully measured and dispensed into a 4ml drabkins neutral diluting fluid.
- The diluted blood was mixed and left at room temperature, protected from sunlight for 4-5 minutes.
- The wave length of the colorimeter was set at 540nm.
- The colorimeter was zeroed with drabkins fluid or distilled water (blank) and the absorbance was read.
- The result was read from the table prepared from the calibration graph or calculated from the formulae below(ICSH,1967 and Benjamin,1985)

$$\frac{\text{Optical density of test} \times \text{concentration of standard}}{\text{Optical density of standard}}$$

3.6.3. THE RED BLOOD CELLS COUNT

Principle: Estimation of the red cells is carried out in general practice more often to confirm gross changes in the number of cells. It is useful for the calculation of M.C.V and M.C.H because estimation of PCV and hemoglobin gives sufficient information about blood picture.

Test method(according to Schalm *et al*., 1975)

- 0.02ml (20ul) of blood was put into the EDTA anticoagulant sampling bottle.
- 0.38ml of diluting fluid was measured and dispensed into the anticoagulated venous blood with the help of a micro pipette.
- The counting chamber and cover glass was cleaned properly and placed on a flat horizontal surface. Using a firm pressure, the cover slip was slide into position on a counting chamber obtaining a rainbow effect on both sides (Newton's ring). Prior moistening of the chamber surface on each side of the grid areas will help the cover glass to adhere to the chamber.
- The diluted sample was remixed. Using a capillary held at an angle of 45 degree, the chamber was filled with the sample, with precaution taken to prevent over fill of the area. It is important that the fluid is not allowed to over flow into the channels. Too much fluid in the chambers may raise the cover glass, causing a variation in the depth, resulting in gross errors.

- The chamber was placed on the microscope stage, allowed several minutes for the cells to settle. Using a 4mm objective and x 10 eye-piece, the objective was focus onto the central square millimeter of the counting chamber and the cells contained within 80 of the 400 small squares (5 groups of 16 small squares) were counted. Cells touching the center line boarding the top and hand right side of each of 16 square were included with the count. Those which touch the center line on the left and lower boarder were disregarded.
- The number of cells per cubic millimeters was reported using the following calculation as shown below

  Let N equal the number of cells counted in 80 small squares.

  The area of each small square is 1/400sq.mm; the depth of the chamber is 1/10mm.

  The volume of the fluid over a small square is therefore 1/400 × 1/10= 1/4000c./mm.

  If N cells of diluted blood are counted in 80/4000c.mm, therefore 1cmm of undiluted blood, contains N × 4000/80 × 200 cells = N × 10,000 cells. Expressed as 10^6 μl (millions/cumm) and converted to 10^{12}/L

3.6.4. WHITE BLOOD CELL COUNT

Principle

A white blood cell is used to investigate infections and unexplainable fever and to monitor treatments which can cause leucopenia. In most cases when a WBC count is requested it is also required to perform a differential WBC count

Test method

- 0.02 mL (20ul) of blood was put into the EDTA anticoagulant sampling bottle.
- 0.38mL of diluting fluid was measured and dispensed into the anticoagulated venous blood with the help of a micro pipette.
- The counting chamber and cover glass was cleaned properly and placed on a flat horizontal surface. Using a firm pressure, the cover slip was slide into position on a counting chamber obtaining a rainbow effect on both sides (Newton's ring). Prior

moistening of the chamber surface on each side of the grid areas will help the cover glass to adhere to the chamber.

- The diluted sample was remixed. Using a capillary held at an angle of 45 degree, the chamber was filled with the sample, with precaution taken to prevent over fill of the area. It is important that the fluid is not allowed to over flow into the channels. Too much fluid in the chambers may raise the cover glass, causing a variation in the depth, resulting in gross errors.
- The chamber was placed on the microscope stage, allowed several minutes for the cells to settle. Using a 4mm objective and x 10 eye-piece, with the condenser iris closed to give good contrast, the ruling of the chamber and white blood cells were focused. The cells were focused until they appear as small black dots.
- Using the improved Neubauer chamber, the cells were counted in the four large corner square millimeters of the chamber including the cells touching the center line boarding the top and the right hand side of each group of 16 squares.

  Calculation;

  Let N = No of cells counted in 4sq.mm. Since the depth of the chamber is 1/10mm. N cells are counted in 0.4 = $\frac{4cmm}{10}$ of diluted blood.

  1cmm of diluted blood contains N × 10/4 cells since blood was diluted 1 in 20

  1cmm of blood (undiluted) contains

  N × 10/4 × 20= 50N cells

In practice, with a dilution of 1 in 200 and when 4 millimeters squares are counted, the number of WBC may be multiplied by 50, e.g. number of WBC counted = 200. Multiply this by 50 = 10,000 per cmm.Total leukocytes were calculated (Coles, 1986) and expressed in 10^3 µl (thousands/cumm) and converted to 10^6 L

3.6.5. DIFFERENTIAL COUNTING OF WHITE BLOOD CELL COUNT

Reference range- Adult absolute value in percentage

Neutrophil 40-45%, Lymphocyte 21-40%, Monocyte 2-10%, Eosinophil 1-6%, Basophil 0-1%**.**

Test method

i. A drop of fresh blood was gently dropped to one end of a clean free slide, allowing the drop to spread.
ii. Holding the slide and 'spreader' at a suitable angle, the spreader was pushed along the slide, drawing the blood behind it until whole of the blood has been smeared. Do not have toenlarge the drop, or incline the spreader at too great an angle, as the film will be too thick for satisfactory microscopic examination. The smeared slide is allowed to dry off.
iii. The slide was placed in a staining rack for staining.
iv. Drops of stain were applied on the film until the whole film is covered with stain. The drops added were counted. This undiluted stain was allowed to act for one and half minutes.
v. After the one and half minutes, twice the number of drops of distilled water was applied to dilute the stain. It was mixed by rocking and by blowing through an ordinary pipette. The film was allowed to stain for 10 minutes; the excess stain was then washed with distilled water. The excess water was drained, and the back side of the slide was wiped with a clean and dry filter paper and kept in a vertical position to drain and dry.
vi. After drying the well stained film, a drop of immersed oil on the lower third of the blood film was placed and covered with a clean cover glass.
vii. The film was observe microscopically; the cells were focused using 10× objective with the condenser iris closed sufficiently to see the cells clearly, the staining and the distribution of the cells were also checked.

The blood film was examined and the absolute number of each different white blood cell type were counted seen in each field and calculated.

3.7. DETERMINATION OF PROTEIN CONCENTRATION

The protein concentrations of the various samples were determined by means of the Biuret method as described by Gornal *et al*., (1949) a slight modification: potassium iodide was added to the reagent to prevent precipitation of Cu^{2+} ions as cuprous oxide.

Principle

Proteins form a colored complexwith cupric ions in an alkaline solution as exemplified by the Biuret reagent containing $CuSO_4$, KI and sodium potassium tartarate. The protein and Biuret reagent form complex with maximum absorbance at 540 nm. The procedure is usually

calibrated with a standard BSA curve. The sample homogenate were diluted 10 times with distilled water inorder to reduce the level of protein in the samples to the sensitivity range of Biuret method. 1ml of the diluted sample was taken and added to 3ml of Biuret reagent in triplicate. The mixture was incubated at room temperature for 30 minutes after which the absorbance was read at 540nm using distilled water as blank. The protein content of the samples were usually extrapolated from the standard curve and multiplied by 100 to get the actual amount in the fraction.

3.7.1. DETERMINATION OF OXIDATIVE STRESS

Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation. This was carried out by the method of Varshney and Kale (1990).

Principle

This method is based on the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde: an end product of lipid peroxide during peroxidation. On heating in acidic pH, the product is a pink complex which absorbs maximally at 532nm and which is extractable into organic solvents such as butanol.

Procedure: 0.4ml of reaction mixture (that is, sample already quenched with 0.5ml of 30% TCA) was added to 1.6M of Tris HCL. Addition of 0.5ml TBA(0.75%) and incubated for 45 minutes at 80°C. This was then cooled in ice and centrifuged at3000g for 15minutes. The absorbance of the clear pink supernatant was then read at 532nm and the absorbance was measured against the blank of distilled water at 532nm. The MDA level was calculated according to the method of Adam-Vizi and Seregi (1982). Lipid peroxidation in units/mg protein or gram tissue was computed with a molar extinction coefficient of 1.56×10^5 $M^{-1}Cm^{-1}$.

Calculation:

$$\text{MDA (Units/mg protein)} = \frac{\text{Absorbance} \times \text{Volume of mixture}}{E_{532} \times \text{Volume of sample} \times \text{mg protein}}$$

Where E532 is molar absorptivity at 532nm

3.7.2. DETERMINATION OF CATALASE ACTIVITY

Catalase activity was determined according to the method of Claiborne (1985).

Principle

The method is based on the loss of absorbance observed at 240 nm as catalase splits hydrogen peroxide. Despite the fact that hydrogen peroxide has no absorbance maximum at this wavelength, its absorbance correlates well enough with concentration to allow its use for a quantitative assay. An extinction coefficient of 0.0436 $mM^{-1}cm^{-1}$ (Noble and Gibson, 1970) was used.

Hydrogen peroxide (2.95 ml of 19 mM solution) was pipetted into a 1 cm quartz cuvette and 50 µl of sample added. The mixture was rapidly inverted to mix and placed in a spectrophotometer. Change in absorbance was read at 240 nm every minute for 5 min.

Calculation

$$\text{Catalase activity} = \frac{\Delta A_{240}/\text{min} \times \text{reaction volume} \times \text{dilution factor}}{0.0436 \times \text{sample volume} \times \text{mg protein/ml}}$$

$$= \mu\text{mole } H_2O_2/\text{min/mg protein}$$

3.7.3. DETERMINATION OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY

The level of SOD activity was determined by the method of Misra and Fridovich (1972).

Principle

The ability of superoxide dismutase to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for this dismutase. Superoxide ($O_2^{\bullet -}$) radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per $O_2^{\bullet -}$ introduced increased with increasing pH (Valerino and McCormack, 1971) and also increased with increasing concentration of epinephrine. These results led to the proposal that autoxidation of

epinephrine proceeds by at least two distinct pathways, only one of which is a free radical chain reaction involving superoxide ($O_2^{\bullet -}$) radical and hence inhabitable by superoxide dismutase.;

Procedure

1ml of sample was diluted in 9ml of distilled water to make a 1 in 10 dilution. An aliquot of 0.2ml of the diluted sample was added to 2.5ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3ml of freshly prepared 0.3mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5ml buffer, 0.3ml of substrate (adrenaline) and 0.2ml of water. The increase in absorbance at 480nm was monitored every 30 seconds for 150 seconds.

3.8. DETERMINATION OF TOTAL NITRITE

Nitrite determination was done using the method described by Ignarro *et al.*, 1987. The assay relies on a diazotization reaction that was originally described by Griess in 1879. The procedure is based on the chemical reaction which uses sulfanilamide and naphthylethylenediamine dihydrochlorate (NED) under acidic condition. Sulfanilamide and NED compete for nitrite in the Griess reaction.

Procedure: Griess reagent was prepared by mixing equal volume of 0.1% NED and 1% sulfanilic acid in 5% phosphoric acid. 50µl of sample was added to a 96 plate well in duplicate and 100µl of griess reagent was added. The mixture was incubated at room temperature protected from light for 10minutes to allow for colour development. The absorbance was measured within 30 minutes in a microplate reader at 550nm.

1. 0.1g naphthylethylenediamine dihydrochloride (NED) was dissolved in distilled water and made up to 100ml to make 1% of NED. It was protected from light in order to prevent the influence of light and its oxidation when exposed to light.

2. 5% of Phosphoric acid (H_3PO_4) was prepared by adding 5ml of the H_3PO_4 to 95ml of distilled water. Then 1g of sulfanilic acid was dissolved in the 5% phosphoric acid to concentrate it. Equal volume of step 1 and step 2 was mixed together to form griess reagent. It was prepared on the day of experiment.

A standard was prepared to equilibrate the test sample (0.1M Sodium Nitrite (NaN03) was dissolve in 10ml distilled water as the standard).

3.9. IMMUNOHISTOCHEMISTRY

Immunohistochemistry study was done by the use of *Avidin-biotin peroxidase complex (ABC) in immunoperoxidase techniques by Hsu et al.,* 1981.

Principle:

Monoclonal antibodies or affinity purified polyclonal antibodies can be used for localization of specific antigen in the tissue sections. Antigen masked during fixing and embedding procedures are unmasked by heat treatment with metallic Salt solutions, followed by which the primary antibodies bind to specific protein to form a complex. This binding is then visualized by using second step biotinylated antibody, followed by amplification with streptavidin horseradish peroxidase enzyme which converts the substrate diamino benzidine into a brown coloured precipitate at the site of reaction, and can be observed as positive reaction.

Reagents:

1. Xylene

Graded ethanol: (70%, 90%, 95%, 100%)

Phosphate Buffered Saline: (8g sodium chloride,1.44g disodium hydrogen phosphate, 0.24g potassium dihydrogen phosphate, 0.2g potassium chloride was in 1 liter distilled water. The pH was adjusted to 7.4 using HCI)

100 mM citric acid: (stock solution)

Hydrogen peroxide: (0.3% in methanol)

Blocking reagent: Egg Avidin

Primary antibody: (1:50 - 1:I00 dilution)

Secondary antibody: (biotinylated anti-immunoglobulin antibody, Streptavidin horseradish peroxidase conjugate 1:200 dilution).

Substrate reagent: In 100 ml of PBS dissolve 5mg of Diaminobenzidine and add 0.1 ml of 0.3% hydrogen peroxide.

Haematoxylin.

DPX mountant.

Procedure:

1. The cut paraffin was embedded at 3 microns thick and allowed to heat on hot plate for 1 hour at 70^0c.

2. The sections was de-paraffinized in xylene and then rehydrated through descending grades of ethanol to distilled water (i.e. 100%, 95%, 90%, 70% H_20).

3. Antigen retrieval was carried out for the sections. They were pre-warmed with (Target Unmasking Fluid) citric acid solution with pH 6.0 (1:10 dilution) in a microwave at power 100 for 15 minutes.

4. The sections were equilibrated gradually with cool water to displace the hot citric acid for at least 5min for the section to cool.

5. The sections was blocked with peroxidase block ($0.3\%H_20_2$) for 15 minutes.

6. It was then washed with excess water and stabilized with PBS mixed with tween 20 for 2 minutes.

7. The non-specific binding was blocked with egg Avidin protein block in humidified chamber for 15 minutes, was removed gently, then washed for 2 minutes in PBS.

8. The sections was incubated with primary antibody in humid chamber for 45 minutes.

9. It was then washed extensively with PBS for 3 minutes.

10.The section was incubated with secondary biotinylated antibody for 45 minutes at room temperature followed by washing in PBS thrice.

11. Polymer was added to initiate polymerization, incubated with Streptavidin horseradish peroxidase system (Dako AS, Denmark) for 15 minutes.

12. It was washed again with PBS twice. Peroxidase substrate DAB was added for 15 minutes and brown precipitate developed indicating positive reaction.

13. It was then washed with water and counterstained for 2 minutes with haematoxylin.

14. Dehydrated in graded ethanol and leared with xylene.

15. Then mounted with DPX and examined under light microscope at magnification x40.

Evaluation of immunohistochemistry results

The expression of P53, factor8, KI67, CD31 and EGFR proteins, the intensity of staining and quantity of cells stained were evaluated in proportion to the ulcerated areas. Brown staining of the cytoplasm and nucleus of cells were viewed under x40 magnification. Area of expression was quantified with immunohistochemistry image analysis tool box (Image J)(Tuominen*et al.*,2010).

3.10. ASSAY OF H^+- K^+- ATPASE (PROTON PUMP) ACTIVITY

Proton pump activity was done with little modification (Maeda *et al.*,1988, Beil *et al.*,2001). Assay mixture consist of an aliquot of enzyme in 20mM Tris HCl (pH7.4), 2mM $MgCl_2$, and 2mM KCl. This was incubated for 5 minutes at 37^0C. The reaction started with the addition of 2mM ATP and incubated at same temperature for 30 minutes. The reaction was terminated by the addition of 10% TCA followed by centrifugation at 2000rpm for 5 minutes at 4^0C. Reagent mixture (H2SO4-Ammonium molybdate-Ascobate) was added and allowed to stand at room temperature for 30 minutes for colour development. Absorbance was read at 820 nM and values extrapolated from standard curve.This assay was carried out by the method of Ronner *et al.,* 1977 as modified by Bewaji *et al.,* 1985.

Standard curve preparation

1mM of $Na_2HPO_4.2H_2O$ was used in the standard curve for the determination of the inorganic phosphate liberated. The procedure is based on a coloured reaction developed by the addition

of a reagent mixture (H2SO4-Ammonium molybdate-Ascobate mix) and then allowed to stand for 30 minutes at room temperature. This was read against a reagent blank containing no $Na_2HPO_4.2H_2O$ at 820nm. The absorbance obtained were used to plot a standard phosphate curve of absorbance against concentration.

Calculation

$$\text{Specific activity (µmole } P_i\text{/mg Prot./hr)} = \frac{[P_i] \times 2 \times D.F}{1000 \times \text{Protein Conc (mg/ml)}}$$

Where;

$[P_i]$ = Concentration of inorganic phosphate in nmoles (obtain from standard Curve).

2 = Factor introduced to obtain the amount of P_i released per hour

DF = Dilution Factor

1000 = factor introduced to convert the P_i release to µmoles.

Concentration of inorganic phosphate of samples were determined from the standard curve.

3.11. STATISTICAL ANALYSIS

The data was analyzed using graphpad prism. All the values were presented as mean and standard error of mean (SEM) for rats in each groups. The differences between the means were statistically analyzed with analysis of variance (ANOVA), $\alpha_{0.05}$ (95%, confidence interval)

CHAPTER FOUR

RESULTS

4.0. PHASE I

4.1.0. DOSE AND DURATION DEPENDENT EFFECTS OF SODIUM ARSENITE ON TOTAL GASTRIC ACDITY IN ACETIC ACID INDUCED ULCER

The total gastric acidity showed a significant increase in the AA group as compared with the control on the day 3 and this reduced signififcantly from day 7 to day 21, $p < 0.05$. In the SA exposed group (5 mg/kg, 10mg/ kg), the total gastric acidity reduced significantly as compared with the AA and the control. The reduction observed was dose and duration dependent as shown in figure 4.1.

4.1.1. DOSE AND DURATION DEPENDENT EFFECTS OF SODIUM ARSENITE ON ULCER AREA IN ACETIC ACID INDUCED ULCER

Induction with acetic acid cause ulceration in the gastric mucosa, which significantly reduced from day 3-day 21. Exposure to 5 mg/kg SA significantly increase the ulcer area from day 3-21 as compared with the AA. There was significant increase in the ulcer area in the 10 mg/kg as compared with the 5 mg/kg SA on day 3 – day 21. There was a great incidence of ulceration in the gastric mucosa of group exposed to SA (5 mg/kg, 10 mg/kg) as compared with the AA as shown in figure 4.2.

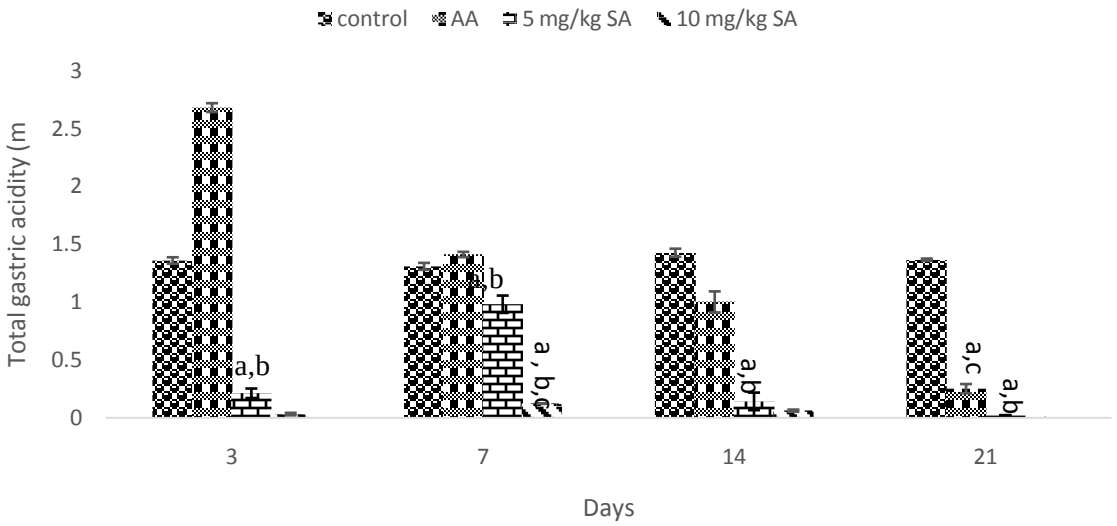


Figure 4.1. Effect of different doses of sodium arsenite on total gastric acidity in acetic acid induced ulcer by day 3,7,14 and 21 $p< 0.05$

a- significant as compared with control

b- significant as compared with the AA

c- significant as compared with 5 mg/kg SA

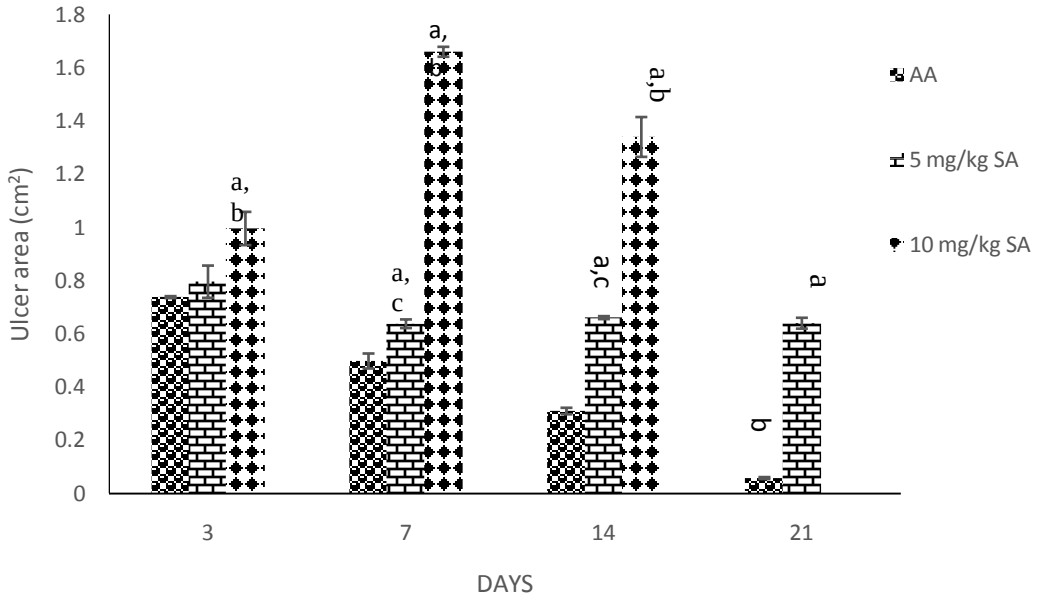


Figure 4.2. Effect of sodium arsenite on the ulcer area by days 3, 7, 14, and 21. P<0.05 significant as compared with the acetic acid induced ulcer group and control.

a – significant as compared with AA

b – significant as compared with 5 mg/kg SA

c – significant as compared with 10 mg/kg

PHASE II

4.2.0 EFFECT OF SODIUM ARSENITE ON TOTAL GASTRIC ACIDITY IN ACETIC ACID INDUCED ULCER

In the AA group, the total gastric acidity was significantly increased on day 3 and reduced as healing process proceed from day 7-21 as compared with the control group. SA exposure significantly reduced the total gastric acidity on day 3 as compared with the control and the AA group. The reduction in the total gastric acidity was continuous from day 7- day 21 as compared with other groups, $p<0.05$ as shown in figure 4.3.

4.2.1. EFFECT OF SODIUM ARSENITE ON ULCER AREA IN ACETIC ACID INDUCED ULCER

Exposure to SA increase the ulcer area significantly as compared with the AA on day 3. The ucer area reduced significantly on day 7 and increased on day 14 and there was no significant difference on day 21 as compared with other days in SA exposed group. From day 7 -21, the reduction was significantly high as compared with the AA group.There was significant reduction in the ulcer area in the AA group, $p<0.05$ as shown in plate 4.1.

4.2.2. EFFECT OF SODIUM ARSENITE ON THE RELATIVE WEIGHT OF STOMACH IN ACETIC ACID INDUCED ULCERATION

The relative weight of the stomach in SA exposed was significanlty increased as compared with the control and AA. The relative weight of the stomach was significantly reduced on day 7, 14 and 21 and significantly increased as compared with the control and AA as shown in figure 4.4. The relative weight of stomach in the AA group was significantly increased on day as compared with the control, it significantly reduced from day 3-day 21 as shown in figure 4.4.

4.2.3. EFFECT OF SODIUM ARSENITE ON DENSITY OF PARIETAL CELL IN ACETIC ACID INDUCED ULCERATION

During the healing of gastric ulcer induced by acetic acid, the parietal cell mass decreased on day 3 which progressively increase in day 7, day 14 and day 21. In the figure 4.5., in SA exposed, there was significant reduction in the parietal cell density which slightly increase on day 7, day 21 while on day 14, there was no appearance of parietal cells density as compared with the control and AA group, $p < 0.05$.

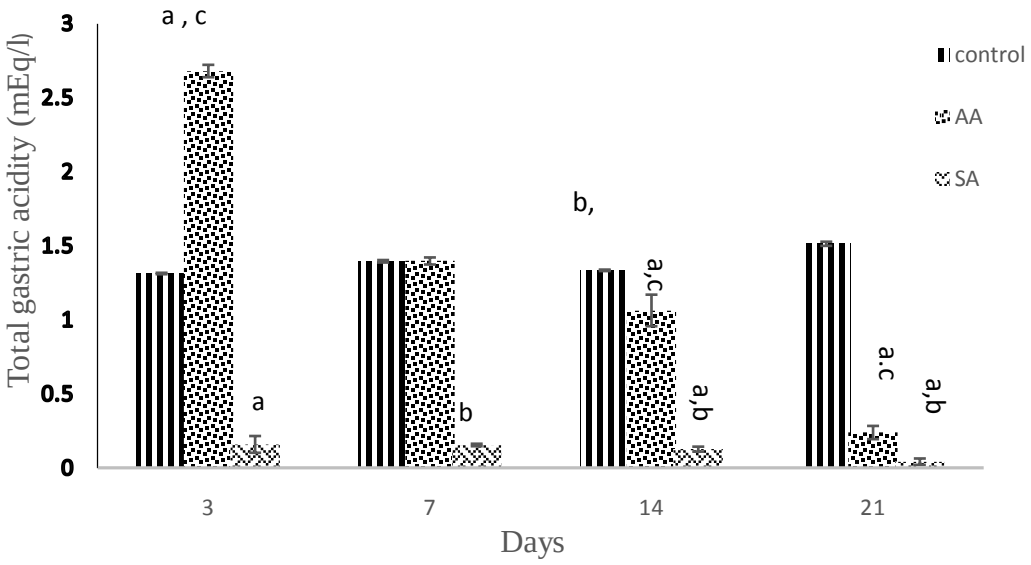


Figure 4.3. Effect of sodium arsenite on total gastric acidity on day 3, day 7, day 14, and day 21. P<0.05

- **a- significant as compared with the control**
- **b- significant as compared with the AA**
- **c- signififcant as compared with the SA**

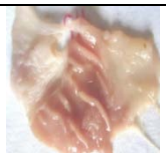
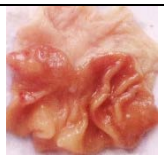
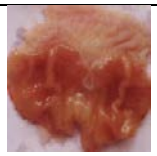
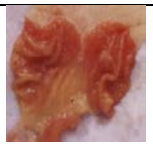
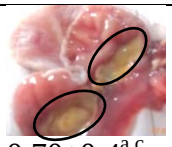
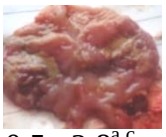
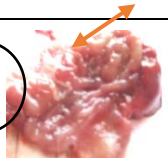
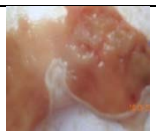
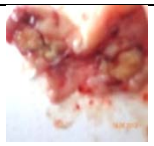
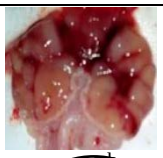
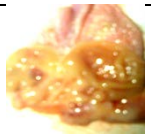
Groups	3days	7days	14days	21days
Control	 0.0 ± 0.0	 0.0 ± 0.0	 0.0 ± 0.0	 0.0 ± 0.0
Acetic acid induced ulcer	 0.70±0.4[a,c]	 0.5± 2.8[a,c]	 0.3 ±1.2[a,c]	 0.1±0. 4[a,c]
SA+ACet	 0.8 ± 6.1[a,b]	 0.6 ±1.6[a,b]	 0.7 ±0.3[a,b]	 0.6 ± 1.6[a.b]

Plate 4.1. Effect ofsodium arsenite on ulcer area experimental gastric ulcer. The circle indicates the Gastric kissing ulcerated area, P<0.05.

a- significant difference as compared with the control
b- significant difference as compared with the AA
c- signififcant difference as compared with the SA

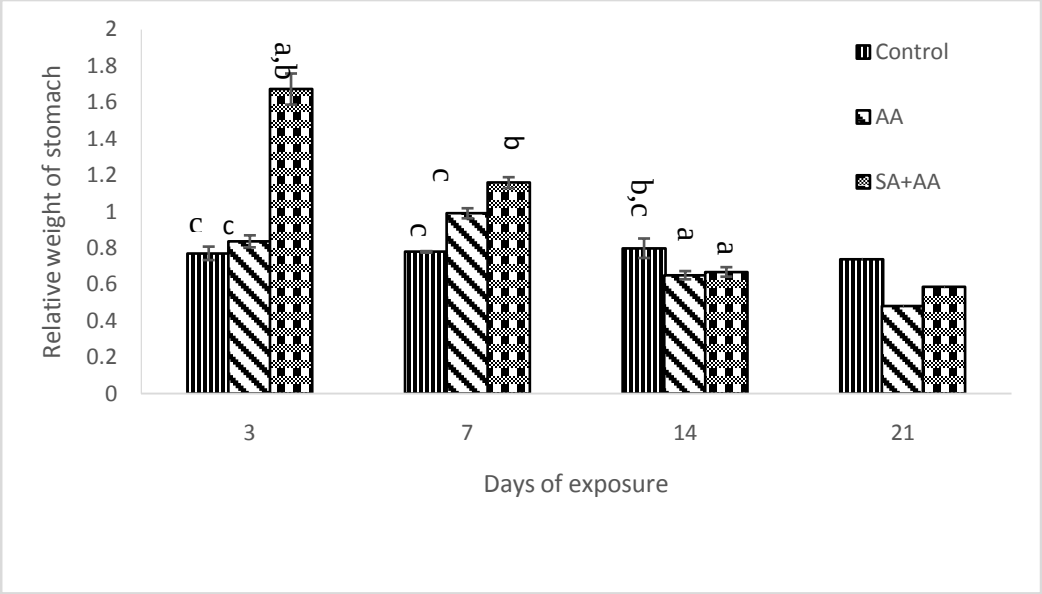


Figure 4.4. Effect of sodium arsenite on the relative weight of the stomach on day3,7,14, 21 post ulcer induction. All values are expressed as mean±SEM, P <0.05.

a- significant difference as compared with control
b- significant difference as compared with AA
c- significant as compared with SA

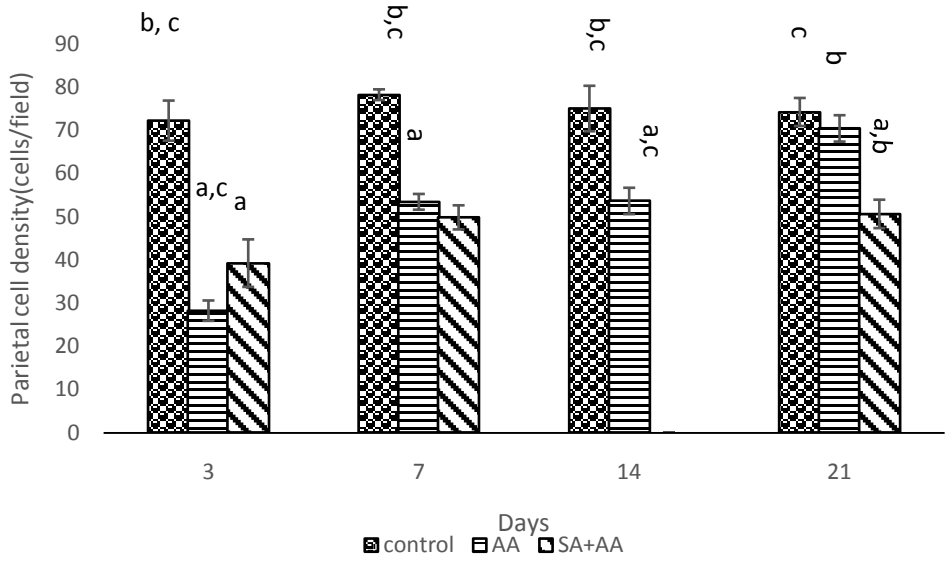


Figure 4.5. effect of sodium arsenite on the parietal cell density on day 3 , day 7, day 14 and day 21 post ulcer induction ,$P<0.05$.

a – significant as compared with the control

b- significant as compared with the AA

c - significant as compared with SA

4.2.4. EFFECT OF SODIUM ARSENITE ON ULCER DEPTH IN ACETIC ACID INDUCED GASTRIC ULCER

Figure 4.6 shows the effect of SA on the ulcer depth in the acetic acid induced ulcer. The ulcer depth was significantly increased in the AA and significantly reduced from day 7- day 21. In SA exposed, the ulcer depth was significantly increased as compared with the AA. On day 7, there was significant increase in ulcer depth as compared with day 14 and day 21, $p<0.05$ as shown in figure 4.6.

4.2.5. EFFECT OF SODIUM ARSENITE ON MUCOSAL THICKNESS IN ACETIC ACID INDUCED GASTRIC ULCER

The mucosal thickness in the control was significantly increased as compared with the AA and SA on day 3. In the AA group, the mucosal thickness increased significantly from day 7 - 21 as compared with the SA exposed. SA exposure significantly reduced the mucosal thickness as compared with the AA on day 7- day 21 as shown in figure 4.7.

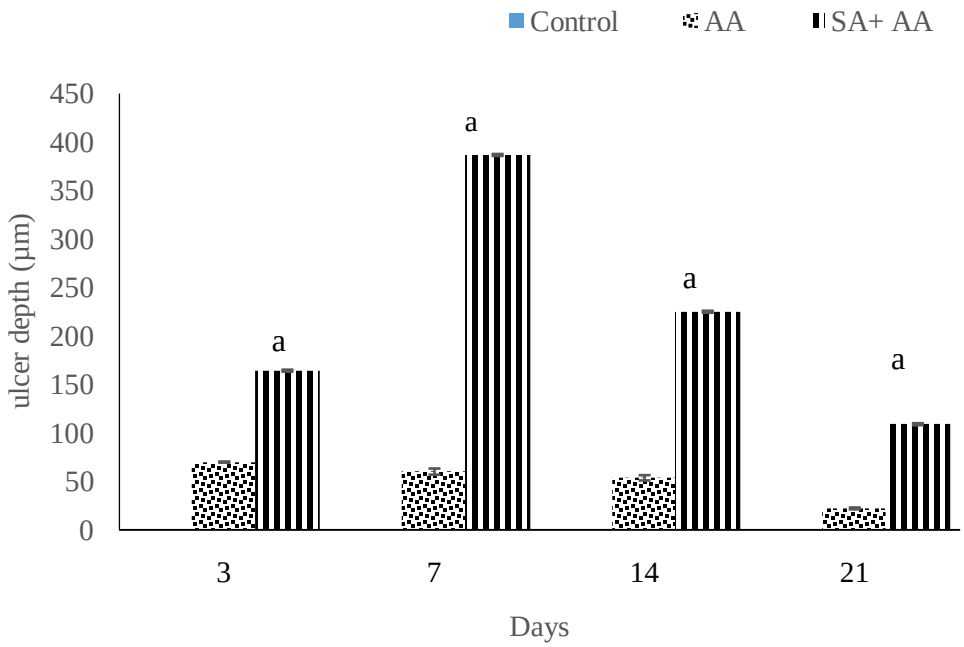


Figure 4.6. Effect of sodium arsenite on Ulcer depth in day 3,7,14,21 post ulcer, all values are expressed as Mean ± SEM, $p < 0.05$

a - significant as compared with acetic induced ulcer.

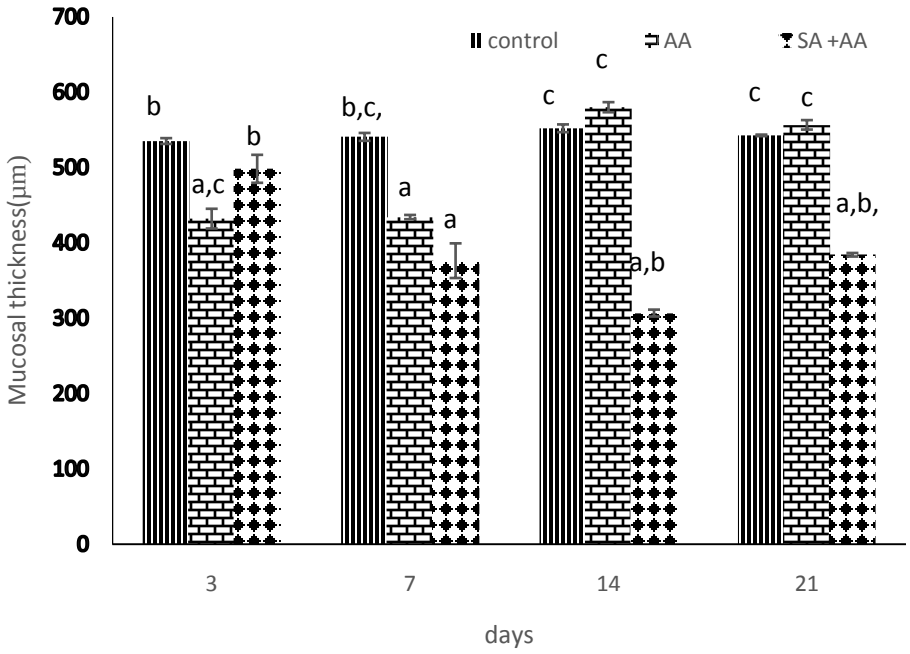


Figure 4.7. Effect of sodium arsenite on mucosal thickness on day3, 7,14,21, all values are expressed as Mean ± SEM, $p \leq 0.05$, a significant as compared with the control, b significant as compared with AA

PHASE THREE

4.3.0. EFFECT OF ZINC, VITAMIN E AND KOLAVIRON ON NITRITE CONCENTRATION (NO)IN SODIUM ARSENITE EXPOSED RATS

Zn significantly increased the nitrite concentration as compared with the control (23.44±0.91 µg/tissue in SA+Zn vs 15.01±0.59 µg/tissue in control). It was significantly increased as compared with the group treated with KV (15.51±0.54 µg/tissue) and VE (7.05 ±0.98 µg/tissue). The VE has the lowest nitrite concentration as compared with other groups, $p<0.05$ as shown in figure 4.8.

4.3.1. EFFECT OF ZINC,VITAMIN E AND KOLAVIRON ON H+- K+ ATPASE ACTIVITIES IN SODIUM ARSENITE EXPOSED RATS

Administration of KV to SA exposed animals significantly reduced the pump level as compared with the VE treated group and Zn treated as well as the control. In the VE treated group, there was reduction in the pump level but significantly increased as compared with the other groups, $p<0.05$ as shown figure 4.9.

4.3.2. EFFECT OF ZINC, VITAMIN E AND KOLAVIRON ON SOD IN SODIUM ARSENITE EXPOSED RATS

From the figure 4.10, administration of vitamin E, there was significant increase in the SOD level as compared with the other groups (SA+KV, SA+Zn and control, $P<0.05$). In the SA+ VE, the SOD was significantly increased as compared with the SA + KV, SA + Zn, SA and the control.

4.3.3.EFFECT OF KOLAVIRON, VITAMIN E AND ZINC ON CAT IN SODIUM ARSENITE EXPOSED RAT

The activities of CAT was significantly reduced in all the treated groups as compared with the control group, $p<0.05$. In the SA+KV (342±15.33), the catalase was significantly increased as compared with the SA+VE (144.5±15.11), and SA+ZN (60.80±7.76), $P<0.05$ as shown in figure 4.11.

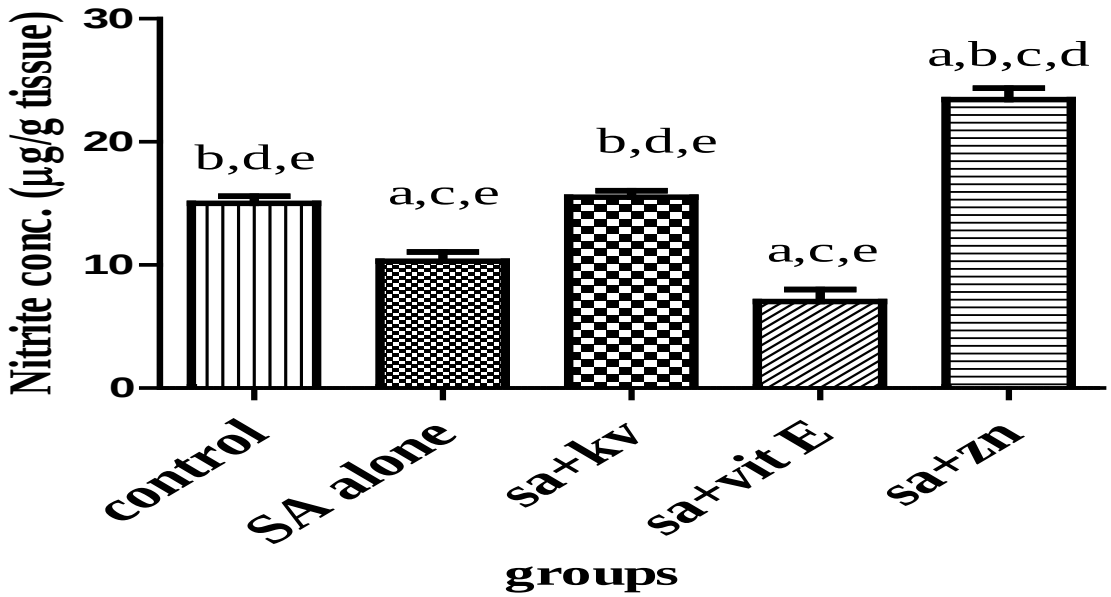


Figure 4.8. effect zinc ,kolaviron and vitamin E on nitrite concentration in sodium arsenite exposed animals a- significant as compared with control, b – significant as compared with SA, c- SA+KV, d- significant as compared with SA + VE, e –significant as compared with SA + Zn, $p < 0.05$

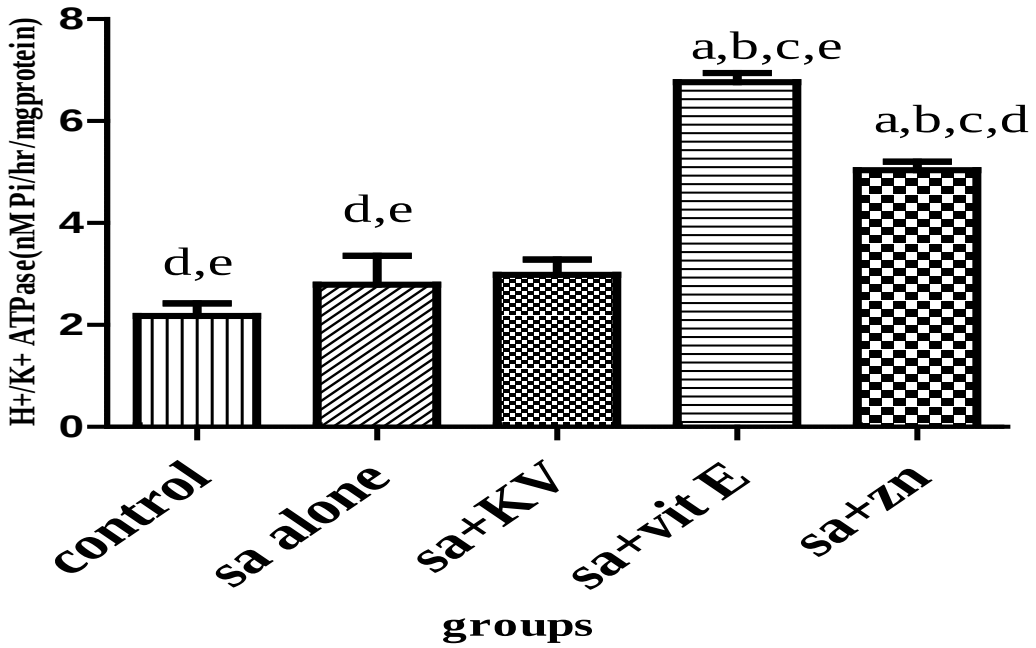
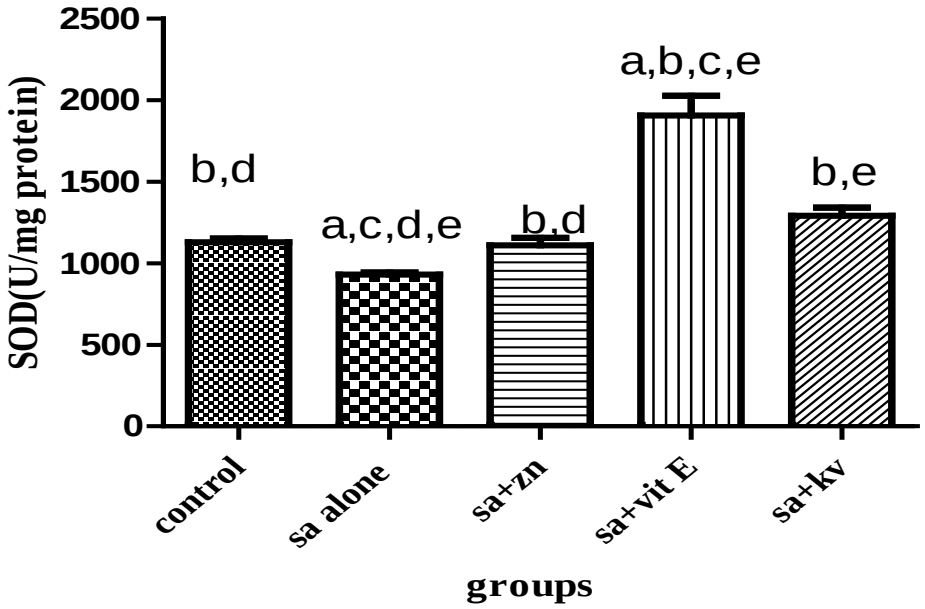
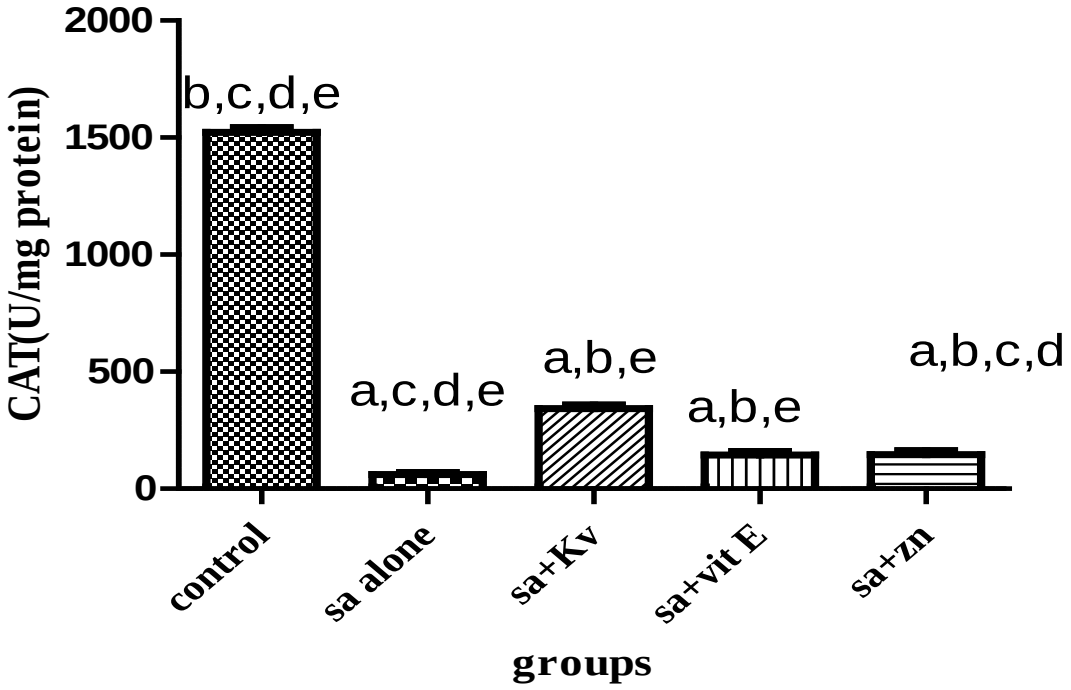


Figure. 4.9: Effect of zinc, kolaviron, and vitamin E on H^{+}-K^{+} -ATPase in sodium exposed rats. a- significant as compared with SA+ KV, b – significant as compared with SA+ZN, c- SA+VIT.E, d- significant as compared with control, $p<0.05$.



VIT E >KV>ZN>CONTROL>SA

Figure 4.10. Effect of Zn, KV and VE on SOD in SA exposed animals. a- significant as compared with control b – significant as compared with SA, c –significant as compared SA+ Zn, d- significant as compared with VE, e – significant as compared with SA +KV, $p<0.05$,$P<0.05$



CONTROL>KV>VIT E=ZN>SA

Figure 4.11. Effect of Zn, KV, and VE on CAT in sodium arsenite exposed. a- significant as compared with control, b – significant as compared with SA, c- significant as compared with the SA+KV, d- significant as compared with SA VE, e – significant as compared with SA + Zn, $p<0.05$.

4.3.4. EFFECT OF KOLAVIRON, VITAMIN E AND ZINC ON MDA IN SODIUM ARSENITE EXPOSED ANIMALS

In SA exposed, the MDA was significantly increased as compared with the treated groups and the control, $p<0.05$. There was significant reduction in all the treated groups as compared with KV, ZN and VE as shownin figure 4.12.

4.3.5. EFFECT OF KOLAVIRON, VITAMIN E AND ZINC ON PROTEIN LEVEL IN SODIUM ARSENITE EXPOSED ANIMALS

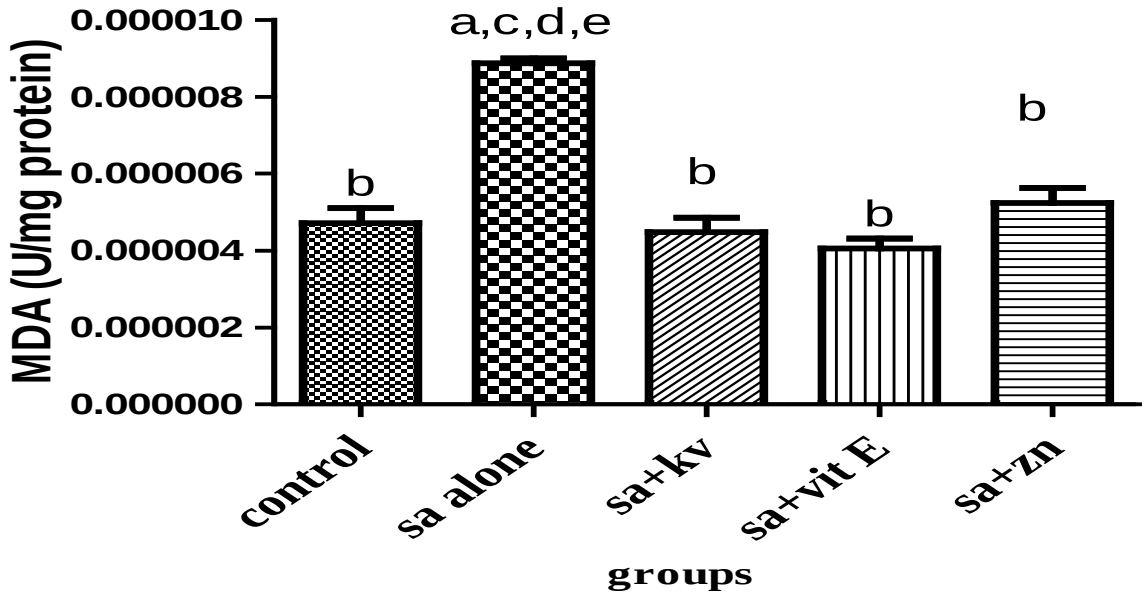
Protein level was significantly increased as in the figure 4.13 in the treatment groups as compared with the SA exposed. It was significantly increased in the SA+VE (0.4309±0.0065mg/ml) as compared with the control (0.3766±0.0069mg/ml), $p<0.05$. In the SA + KV (0.3577±0.0164), the protein level was significantly reduced as compared with the SA + VE, $p<0.05$.

4.3.6. EFFECT OF KOLAVIRON, VITAMIN E AND ZINC ON HISTOLOGY OF GATRIC MUCOSA IN SODIUM ARSENITE EXPOSED RATS

Histological photomicrographs showed severe ulceration and necrosis alongside infiltration of inflammatory aggregates into the submucosa in the SA- exposed as compared to the control with intact epithelium and mucosa layers. Treatment with KV significanlt decreased the degree of ulceration in to mild with few inflammatory cells as compared with VE and Zn treated that showed a moderate degree of ulceration with infilteration of inflammatory cells. The degree of ulceration was significantly reduced in the treatments group as compared with the SA exposed. The result is presented in plate 4.2.

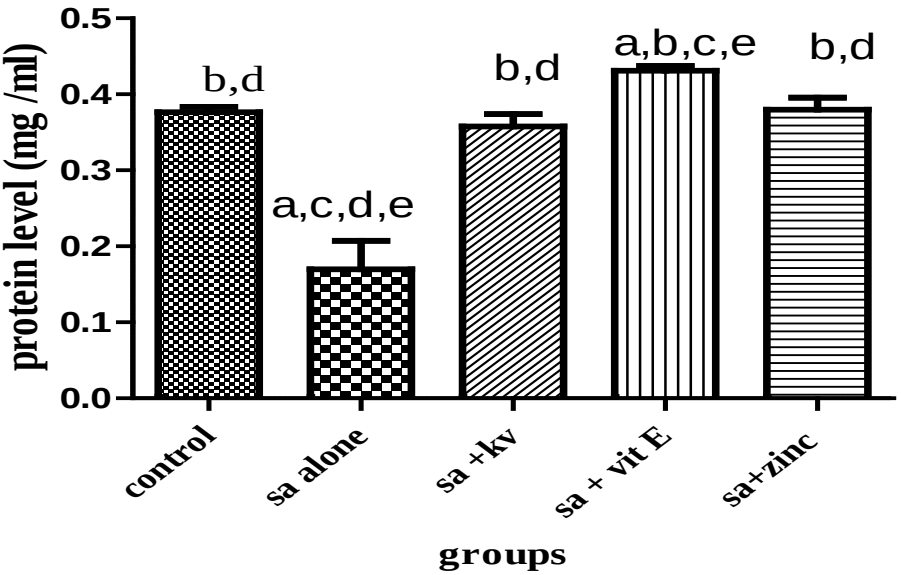
4.3.7. EFFECT OF KOLAVIRON, VITAMIN E AND ZINC ON EXPRESSION OF PECAM-1(CD31) IN SODIUM ARSENITE EXPOSED TISSUE

From plate 3, the CD 31 ezpression was significantly increased in SA+Zinc (90.3%) as compared with control (53.9%), SA (55.6%).and other treated groups . However, in the SA +VE treated (76.0%), the expression was significantly increased as compared with the SA+ KV treated (65.6%)), SA exposed, $p < 0.05$. The expression in the SA +KV was signififcantly increased as compared with the control and SA as shown in plate 4.3 and figure 4.14.



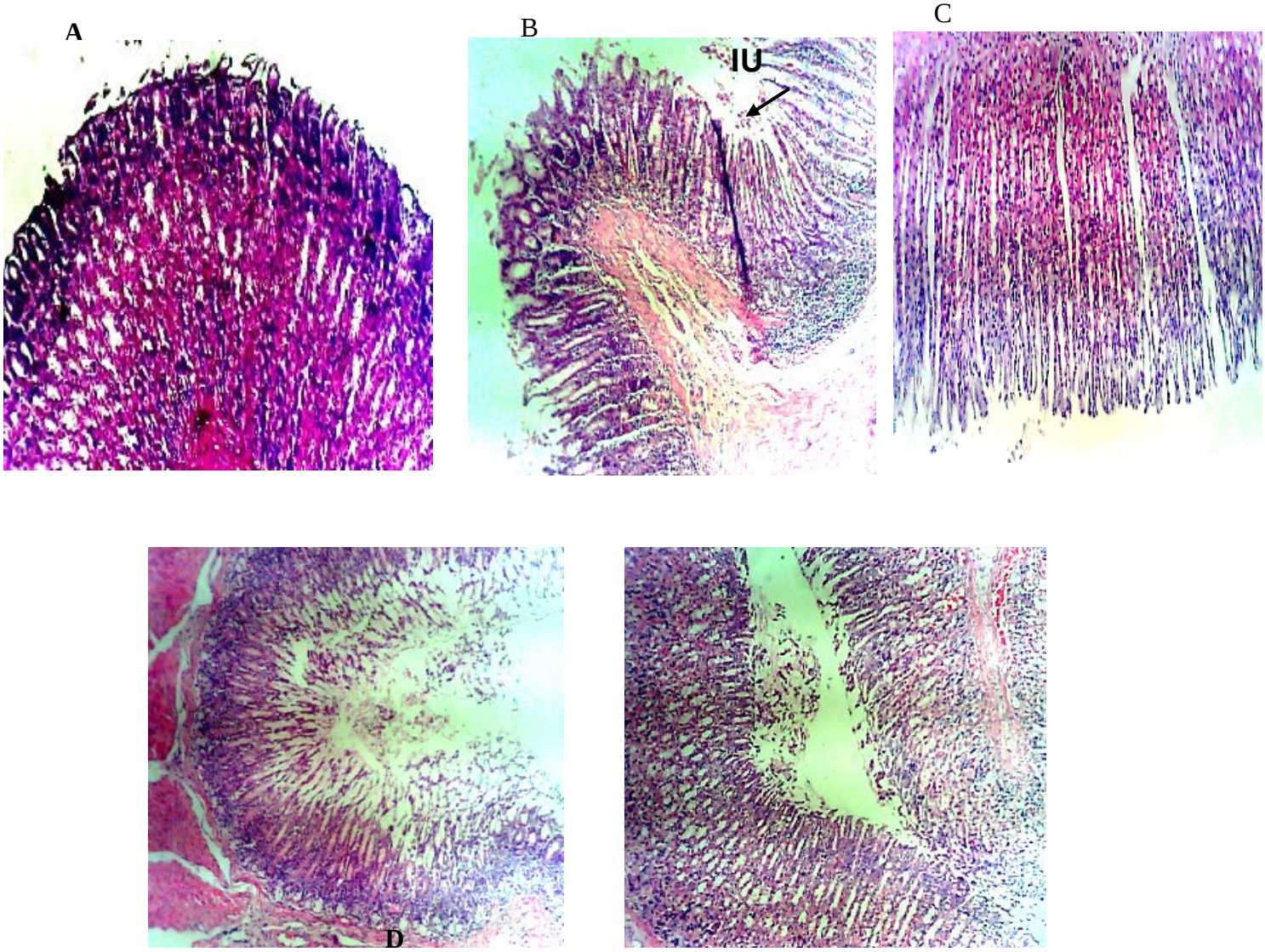
SA>ZN>KV>VIT E

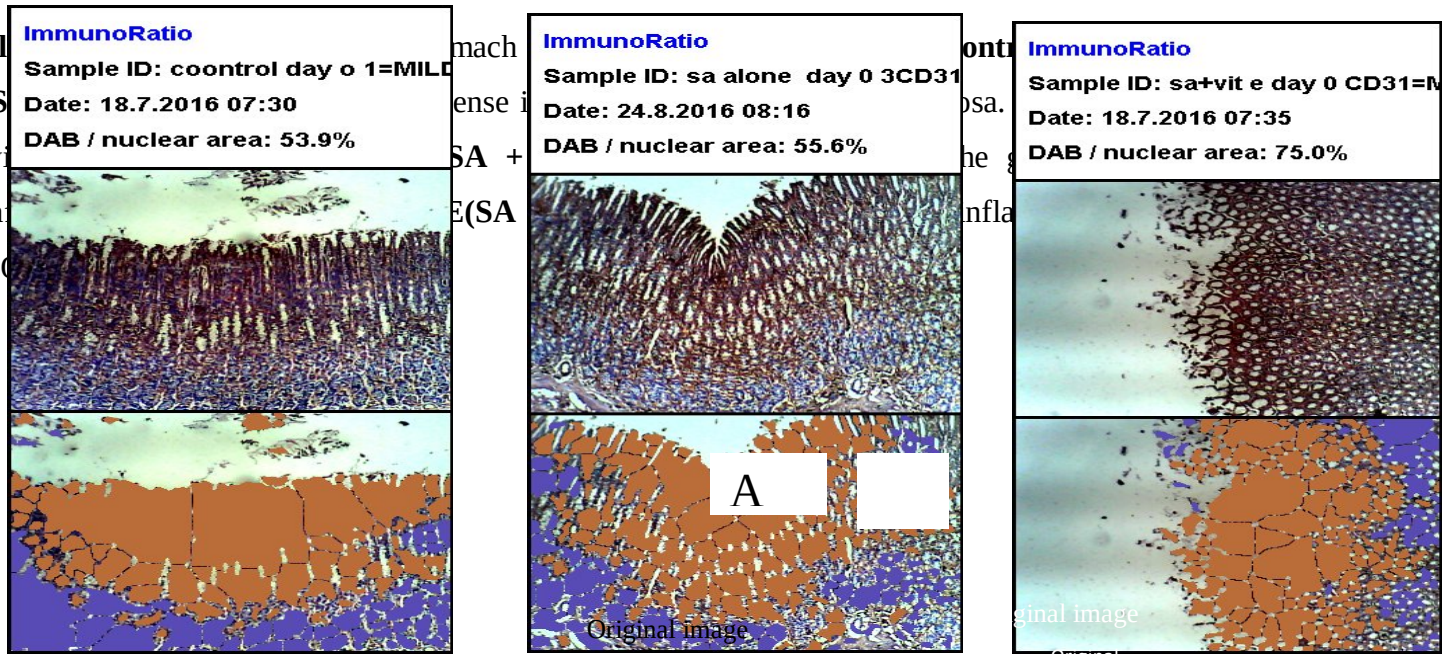
Figure 4.12. Effect of Zn, KV, and VE on MDA in sodium arsenite exposed animals. a-significant as compared with control, b – significant as compared with SA, $p<0.05$.



Vit E >ZN=KV=control>SA

Fig. 4.13: Effect of KV, VE, and Zn on protein level in sodium arsenite exposed. a- significant as compared with control, b – significant as compared with SA, c- significant as compared with SA+ KV, d- significant as compared with SA + VE, e- significant as compared with the SA +Zn, $p<0.05$


A
B
IU
C
D

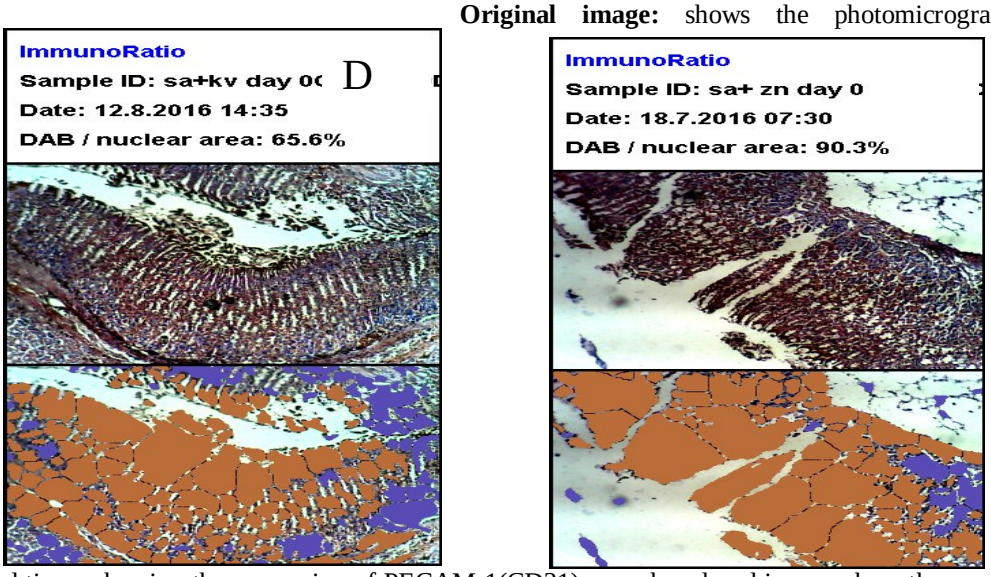

ImmunoRatio
Sample ID: coontrol day o 1=MILD
Date: 18.7.2016 07:30
DAB / nuclear area: 53.9%
ImmunoRatio
Sample ID: sa alone day 0 3CD31
Date: 24.8.2016 08:16
DAB / nuclear area: 55.6%
A
Original image
ImmunoRatio
Sample ID: sa+vit e day 0 CD31=M
Date: 18.7.2016 07:35
DAB / nuclear area: 75.0%

Original image

Pseudo-colored image showing the staining component

Pseudo-colored image showing the staining component

Plate 4.3: Effect KV, VE and Zn on CD31 expression in the gastric mucosa of the animal exposed to sodium arsenite**.** **Original image:** shows the photomicrograph of



immunostained tissue showing the expression of PECAM-1(CD31), pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate(area of expression), blue colour- H& E counterstain.**A**: **control** (mild expression) B: **SA exposed**(Mild expression) C: **SA+ VE** (moderate expression) **D: SA+ KV**(moderate expression) **E: SA+ Zn** (strong expression)

30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong express

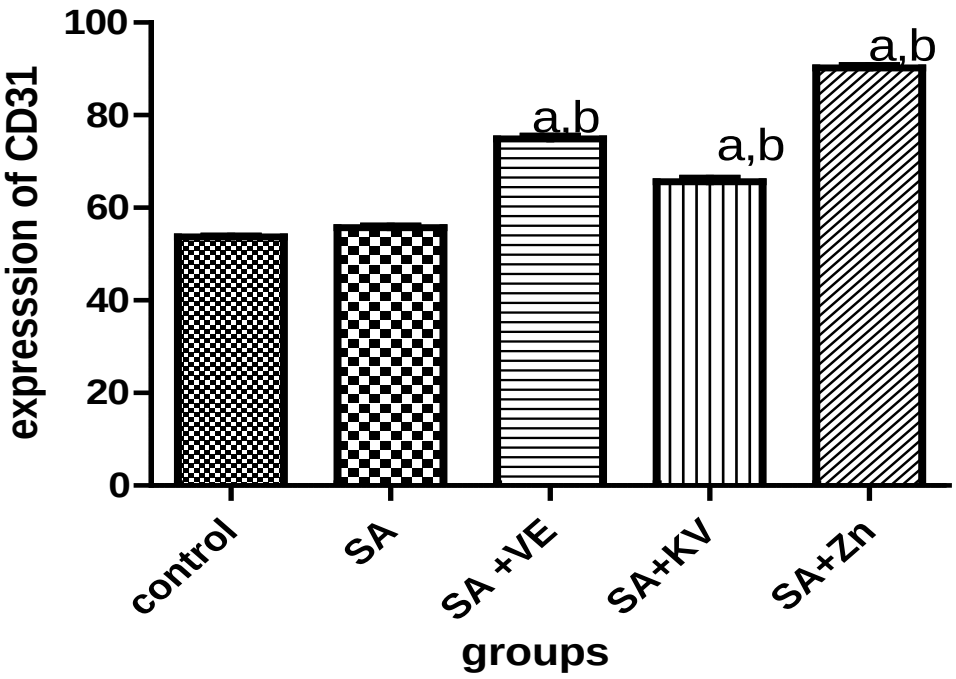


Figure 4.14: Effect KV, VE and Zn on CD31 expression in the gastric mucosa of the animal exposed to sodium arsenite**.** a – significant as compared with the control, b – signififcant as compared with the SA exposed, $p < 0.05$.

4.3.8. EFFECT OF KOLAVIRON, VITAMIN E AND ZINC ON EXPRESSION OF KI-67 IN SODIUM ARSENITE EXPOSED ANIMALS

From plate 4, ki- 67 was significantly increased in the control, SA+ KV and SA + ZN as compared with the SA exposed. There was a moderate expression in the SA + KV as compared with the SA + VE and SA. There was strong expression in the SA+ Zn as compared with the other treated groups and SA as shown in plaet 4.4 and figure 4.15.

4.3.9. EFFECT OF KOLAVIRON, VITAMIN E AND ZINC ON EXPRESSION OF P53 IN SODIUM ARSENITE EXPOSED ANIMALS

From plate 4.5, the percentage of expression of P53 was significantly decreased in the SA+ZN (53.6%) as compared with SA (97.1%). There was mild expression on treatment with zinc. In the SA + VE (76.0%) and SA + KV(80.1%) showed a significant decreased as compared with SA, however, the expression was moderate as compared with the SA + Zn and the control (43.6%).,$p< 0.05$. The percentage of expression is presented in plate 4.5 and figure 4.16

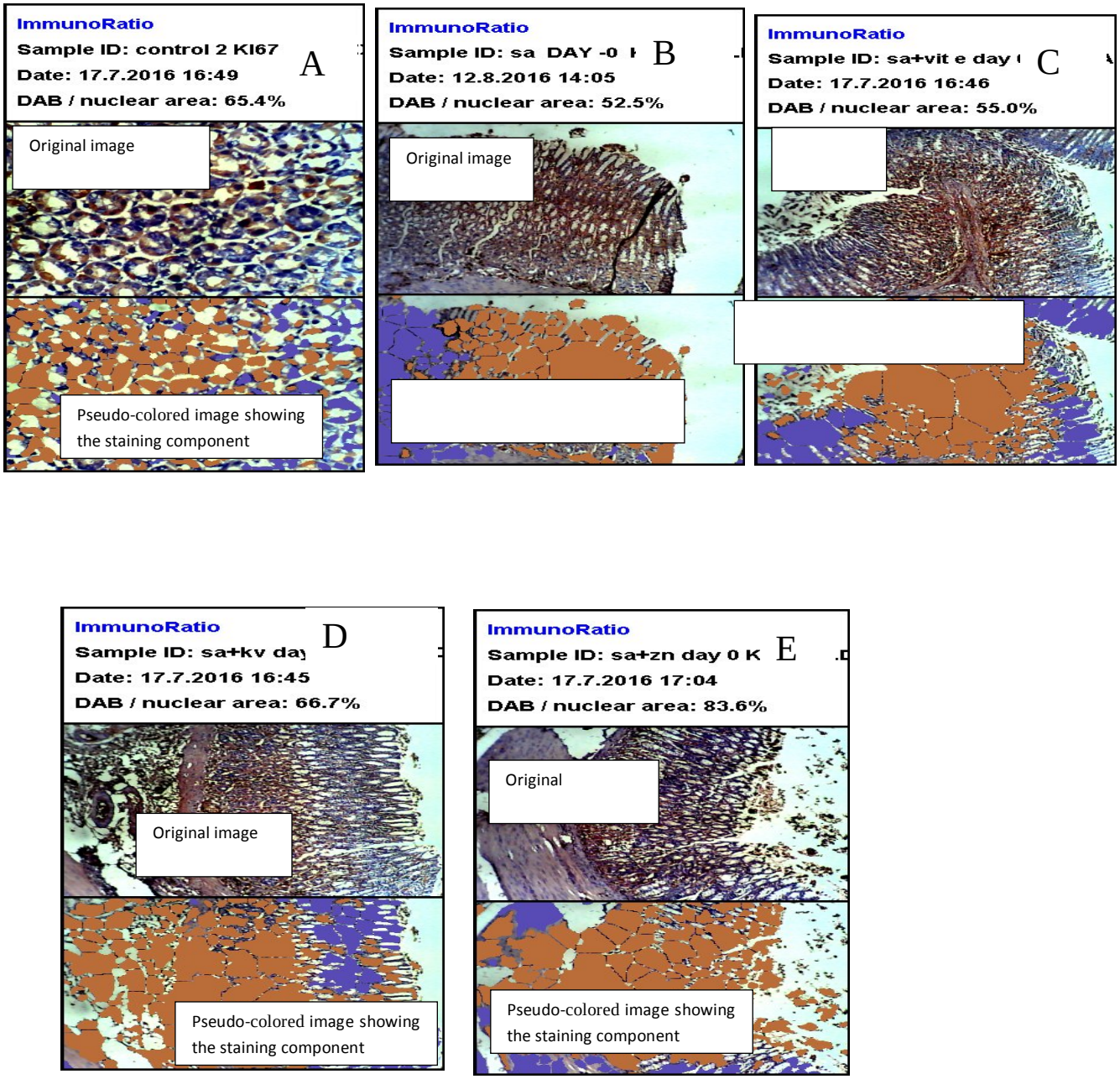


Plate 4.4: Effect KV, VE and Zn on Ki-67 expression in the gastric mucosa of the animal exposed to sodium arsenite**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of Ki-67, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain.**A**: **control** (modrate expression) B: **SA exposed** (Mild expression) C: **SA+ VE** (mild expression) **D: SA+ KV** (moderate expression) **E: SA+ Zn** (strong expression)

30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong express

.

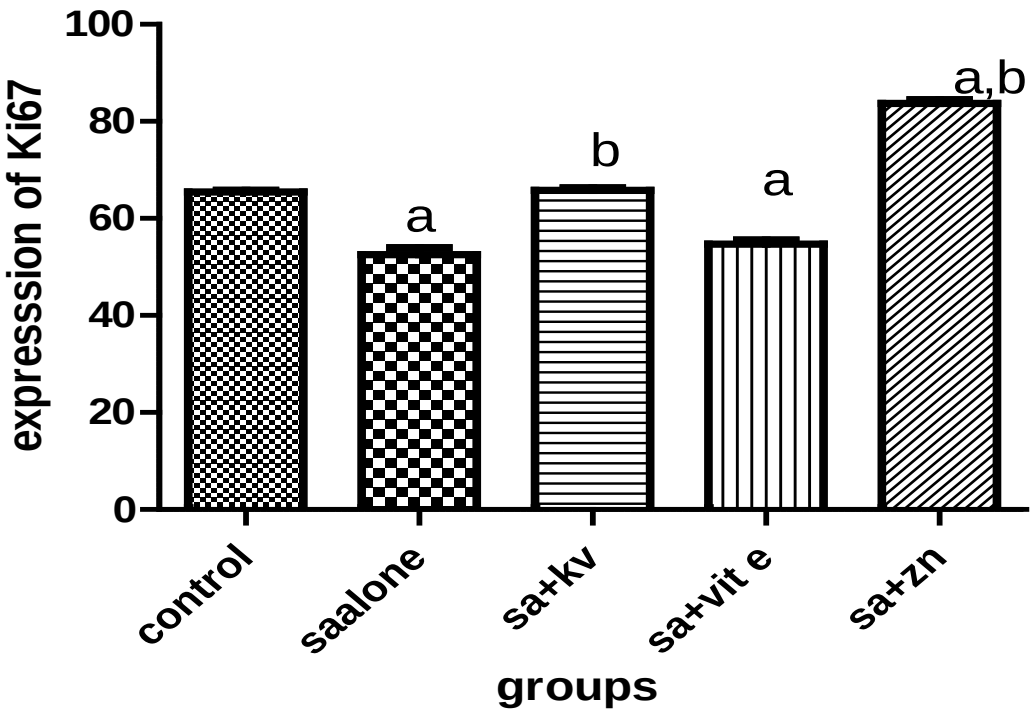
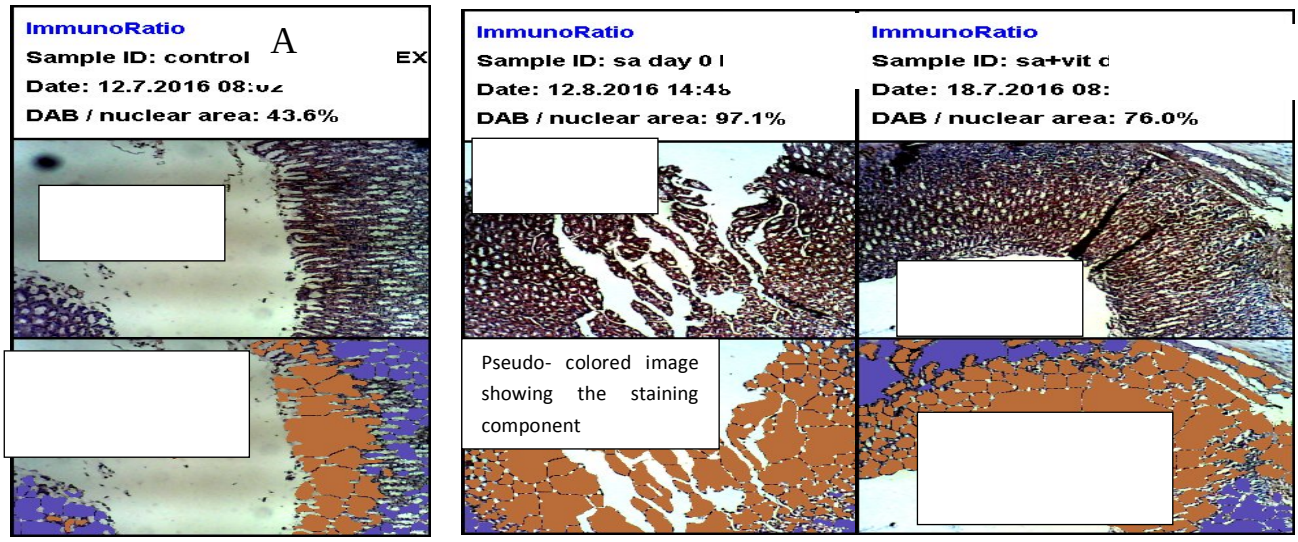


Figure 4.15: Effect KV, VE and Zn on Ki-67 expression in the gastric mucosa of the animal exposed to sodium arsenite**.**

All values are expressed as mean±SEM a – significant as compared with the control, b – signififcant as compared

Zn > KV = Control >VE >SA



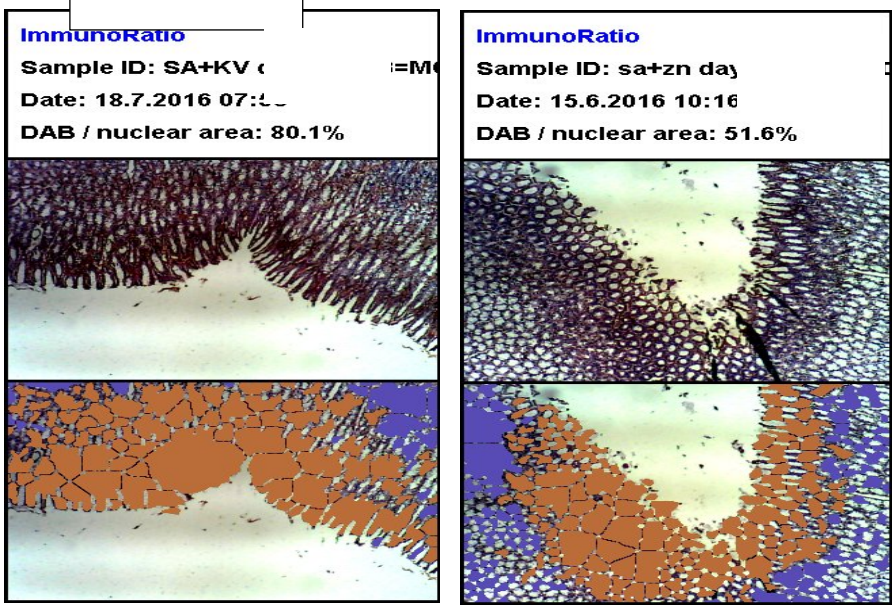


Plate 4.5. Effect KV, VE and Zn on P53 expression in the gastric mucosa of the animal exposed to sodium arsenite**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of Ki-67, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H&

E counterstain.**A**: **control** (mild expression) B: **SA exposed** (strong expression) C: **SA+ VE** (moderate expression) **D: SA+ KV** (moderate expression) **E: SA+ Zn** (mild expression). **ZN<VIT E=KV<CONTROL<SA ALONE**

30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression

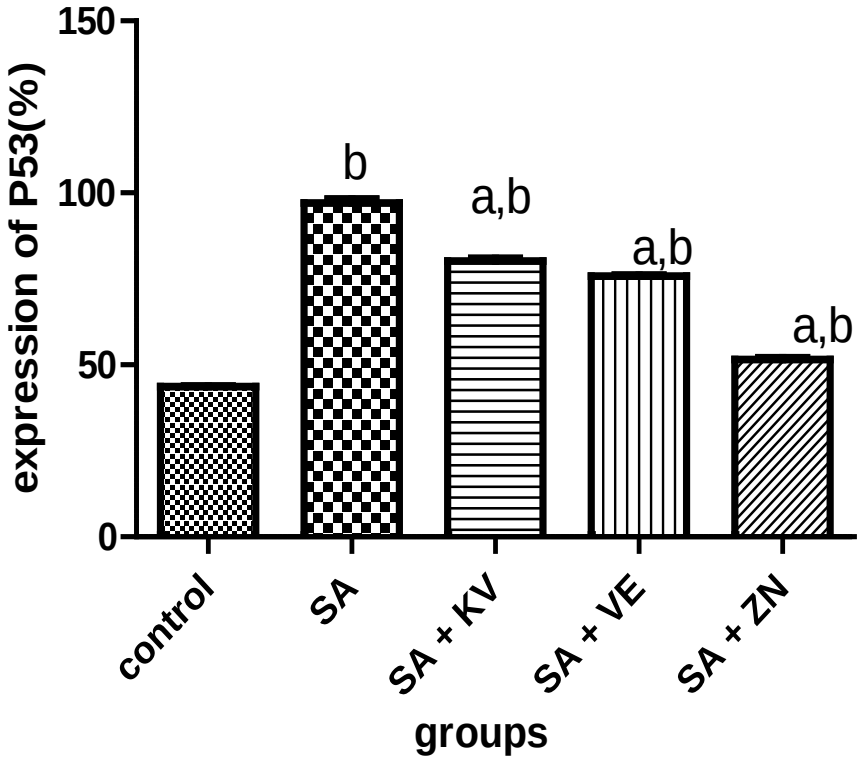


Figure 4.16: Effect of KV, VE, and ZN on P53 on sodium arsenite exposed animals. All value are expressed as mean ±SD a- significant as compared with control, b – significant as compared with SA exposed ZN<VIT E=KV<CONTROL<SA ALONE

PHASE FOUR

4.4.0. EFFECT OF VITAMIN E ON HEMATOLOGICAL PARAMETERS IN SODIUM ARSENITE EXPOSED STOMACH IN EXPERIMENTAL GASTRIC ULCER

4.4.1. EFFECT OF VITAMIN E ON PACKED CELL VOLUME IN SA- EXPOSED IN EXPERIMENTAL GASTRIC ULCER

From figure 4.17, exposure to SA+AA significantly decrease the PCV from day 7 to day and significantly reduced as compared with the AA and control on day 7- day 21 , $p< 0.05$. Treatment with VE signififcantly increased the PCV on day 3, day 14 and day 21 as compared with the control, AA and SA +AA, $p < 0.05$.

4.4.2. EFFECT OF VITAMIN E ON HEMOGLOBIN LEVEL IN SA EXPOSED IN EXPERIMENTAL GASTRIC ULCER

In control, the hemoglobin level was 16.56±0.13g/dl,17.18 ± 0.32g/dl, 16.36 ± 0.26g/dl, 18.66 ± 0.14g/dl in the control. In the AA, there was significant decrease on day 3 as compared with the control. On day 7 – 21, there was significant increase in the hemoglobin level.

In SA exposed, the hemoglobin level was signififcantly decrease from day 7- 21 as compared with AA and the control, $p<0.05$. Treatment with VE significantly increase the Hb level as compared with the control and SA, $p<0.05$ as shown in table 4.1.

4.4.3. EFFECT OF VITAMIN E ON RED BLOOD CELL COUNT (RBC) IN SA EXPOSED IN EXPERIMENTAL GASTRIC ULCER

The RBC in the AA was significantly reduced in the AA group on day 3 and day 7 post ulcer induction as compared with the control group. On day 14 and day 21, there was a significant

increase as compared with the control. In the SA + AA, the RBC significantly decreased on day 3 and day 21 as compared with the AA and control, $p<0.05$. Treatment with VE significantly increase the RBC on day 3, 7, 14 as compared with the control, AA and the SA+ AA, $p<0.05$ as shown in table 4.2.

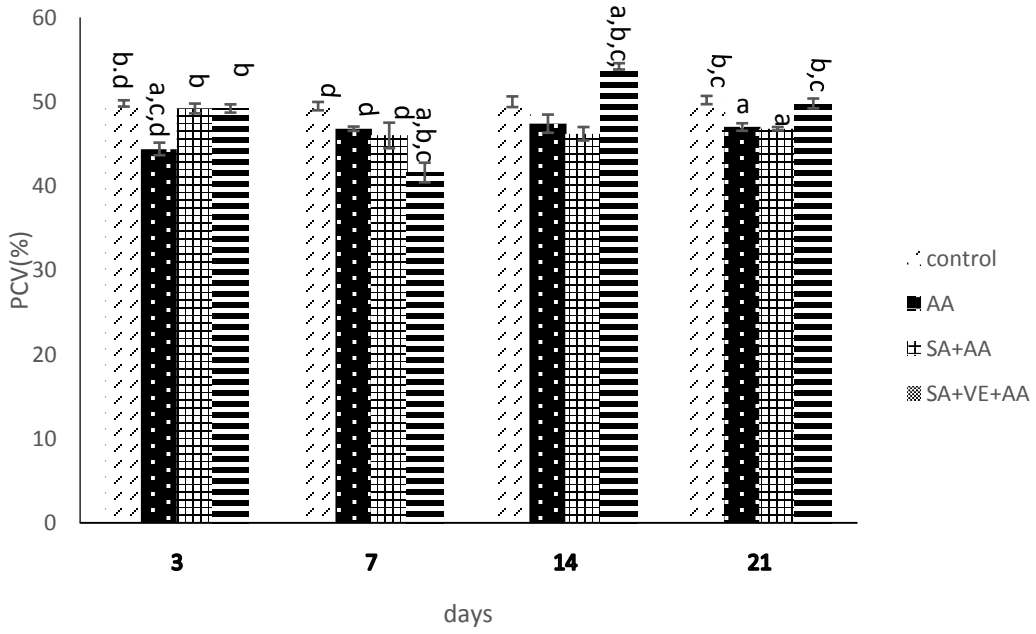


Figure 4.17. The effect of vitamin E on the packed cell volume in sodium exposed in experimental gastric ulcer on day 3, 7, 14, 21 post ulcer induction. All values are expressed as mean±SEM, $p\leq0.05$. a- significant as compared with control, b- signififcant as compared with AA, c- significant as compared with SA +AA, d – significant as compared with SA+VE + AA

Table 4.1. Effect of vitamin E on hemoglobin level in sodium arsenite exposed in experimental gastric ulcer

Grouping		**DAYS**		
Hemoglobin content (g/dl)	**3**	**7**	**14**	**21**
Control	**16.6 ± 0.1**	**17.2 ± 0.3**	**16.4 ± 0.3**	**18.7 ± 0.1**
AA	**14.8 ±0.2****	**15.6 ± 0.1****	**18.8 ± 0.4****	**17.6±0.0****

SA+AA	**16.5± 0.2**	**13.9 ± 0.4$^{\#\#}$**	**15.4± 0.3$^{\#\#**}$**	**15.5±0.7##**
SA+AA+ VE	**16.4 ± 0.2**	**15.3 ±0.50$^{\#\#}$**	**18.2 ± 0.1$^{\#\#}$**	**16.6 ± 0.2**

All values are expressed as mean±SEM, p≤0.05. **- Statistically significant as compared with control, ##- statistically significant as compared with acetic induced group.

Table 4.2. Effect of vitamin E on RBC count in sodium arsenite exposed animals in experimental gastric ulcer

Red blood cell count	**(cu/mm) Day 3**	**Day 7**	**Day 14**	**Day 21**
Control	**728000.0 ±29342.3^{b,c}**	**814000.0 ± 56697.8^{b,c,d}**	**814000.0 ± 56697.8^{b,c}**	**795200.0 ±40241.0^{a,bc}**
AA	**683000.0 ±12185.1^{a,c,d}**	**538000.0 ±41086.0^{a,c,d}**	**668000.0 ± 7985.9^{a,d}**	**900000.0 ± 30278.3 ^{a,c,d}**
SA + AA	**504000.0 ±11641.4^{a,b,d}**	**646000.0 ±200360.3^{a,b}**	**688000.0 ± 7985.9^{a,d}**	**432000.0 ± 7688.9^{a,b,d}**
SA+ VE +AA	**818000.0 ±56620.7^{a,b,c}**	**662000.0 ±100180.1**	**868000.0 ± 12783.7**	**742000.0 ± 23282.8^{b,c}**

All values are expressed as mean±SEM, p≤0.05

a – signififcant as compared with control

b – significant as compared with AA

c – significant as compared with the SA +AA

d – significant as compared with the SA + VE +AA

Table 4.3. Effect of vitamin E on total white blood cell count (TWBC) in SA exposed in experimental gastric ulcer

Groups TWBCcount cells/mm^3	**Day 3**	**Day 7**	**Day 14**	**Day 21**
Control	**6560.0 ± 238.0**	**6560.0 ± 238.0**	**6560.0 ± 238.0**	**6560.0 ± 238.0**
AA	**7150.0 ± 171.5^{a}**	**6640.0 ± 379.6**	**7660.0 ± 217.8^{a,}**	**4840.0 ± 177.5^{b}**
SA+AA	**6760.0± 159.7**	**7020.0 ± 249. 4^{a,c}**	**7780.0 ± 193.1**	**8100.0 ± 260.3^{a,b,c}**
SA+AA +VIT E	**6640.0 ± 74.70^{b}**	**5640.0 ± 75.0^{a,b,c}**	**7080.0 ± 58.21^{c}**	**5920.0 ± 458.1^{b,c}**

All values are expressed as mean±SEM, p≤0.05. a - Statistically significant as compared with control, b - statistically significant as compared with acetic induced group.

4.4.4. EFFECT OF VITAMIN E ON TOTAL WHITE BLOOD CELL COUNT.

The total white blood cell count in the AA was significantly increased on day 3 as compared with the control, $p < 0.05$. It was significantly decreased on day 7 and day 21 as compared with control. In the SA +AA, there was significant increased from day 7 – day 21 as compared with the AA and control, $p < 0.05$. Treatment with VE significantly decreased the TWBC on day 7 and day 21 as compared with SA and the control, $p<0.05$ as shown in table 4.3.

4.4.5. EFFECT OF VITAMIN E ON NEUTROPHIL-LYMPHOCYTE IN SODIUM ARSENITE EXPOSED IN EXPERIMENTAL GASTRIC ULCER

From the table 4.5, the neutrophil-lymphocyte ratio (NLR) was 1.3 ± 0.1,1.0 ± 0.1, 1.1 ± 0.1, 1.1 ± 0.1 in the control, on the on day 3, 7, 14, 21. It was significantly increased on day 3 and day 7 as compared with the control, $p< 0.05$. The NLR significantly reduced on day 14 and day 21 as compared with the control.

SA exposure significantly increased the NLR from day 3, 7 and 14 as compared with the control and the AA. It significantly decrease on day 21,but significantly increased as compared with the AA and the control.

VE treatment significantly reduced the NLR especially on day 14 and day 21 as compared the control, AA, SA +AA, $p<0.05$ as shown in table 4.4.

4.4.6. EFFECT OF VITAMIN E ON NEUTROPHIL COUNT IN SODIUM ARSENITE EXPOSED IN EXPERIMENTAL GASTRIC ULCER

The percentage of neutrophil was significantly reduced on day 3 and day 7 in the AA group as compared with the control. On day 14 and 21, it was significantly increased as compared with the control. In the SA +AA, there was significantly increase in the neutrophil count as compared with the control and AA on day 3 and 14, $p< 0.05$. In the treated with VE, there was significant decrease in the neutrophil count as compared with the control, SA + AA, AA on the day 14 and day 21, $p<0.05$ as shown in table 4.5.

Table 4.4. Effect of VE on Neutrophil-Lymphocyte(NLR) in SA- exposed in experimental gastric ulcer

Neutrophil/lymphocyte ratio	**DAYS**			
	3	**7**	**14**	**21**
Control	**1.3 ± 0.1**	**1.0 ± 0.1**	**1.1 ±0.1**	**1.1 ±0.1**
AA	**3.3 ± 0.3$^{a, c,d}$**	**2.3 ± 0.1^{a}**	**0.3 ±0.0$^{a, c, d}$**	**0. 3 ± 0.1$^{a, c}$**
SA+AA	**6.6 ±0.4^{a,b,d}**	**2.1 ± 0.2^{a,d}**	**1.4 ±0.2^{a,b,d}**	**0.4 ± 0.3**
SA+ VE + AA	**3. 4 ±0.3$^{a, c}$**	**1.6 ±0.1 $^{b, c}$**	**1.1 ±0. 1^{b,c}**	**0. 1 ±0.2 ^{a,b,c}**

All values are expressed as Mean±SEM, $P<0.05$.a - significant as compared with the control, b- significant as compared with the AA, c - significant as compared with the AA, d- significant as compared with the SA +VE +AA

Table 4.5. Effect of vitamin E on Neutrophil count (%) in sodium arsenite exposed in experimental gastric ulcer

Grouping (%)	Day 3	Day 7	Day 14	Day 21
Control	**32.4 ± 1.0**	**31.4 ± 2.1**	**32.6± 3.2**	**29.4 ± 0.1**
AA	**6.5 ±0.2[a]**	**8.0 ± 0.5 [a]**	**49.6 ± 2.5[a]**	**40.2 ±0.9[a]**
SA+VE+ AA	**48.0 ± 1.4[b]**	**38.4 ± 2.5[b]**	**22.0 ±1.1[b]**	**6.6 ±1.1[b]**
SA+AA	**46.8 ±0.8[b]**	**28.2±1.0[b]**	**44.0 ±0.5**	**27.0±3.1[b]**

All values are expressed as mean±SEM, $p<0.05$. a - Statistically significant as compared with control, b - statistically significant as compared with AA.

Table 4.6. Effect of vitamin E on lymphocyte count in sodium arsenite exposed animals in experimental gastric ulcer

Grouping **Lymphocyte count(%)**	**Day 3**	**Day 7**	**Day 14**	**Day 21**
Control	**25.8 ± 0. 2**	**30.8 ± 3.2**	**31.8 ± 2.9**	**28.2 ±2.8**
AA	**24.4 ± 0.9**	**15.8 ± 1.2[a]**	**15.0 ± 0.5 [a]**	**17.6 ± 0.4 [a]**
SA+ VE +AA	**12.8 ± 0.8 [b]**	**18.8 ± 1.9**	**17.0 ± 2.1**	**17.0 ± 1.4**
SA+AA	**7.2 ± 0.5[b]**	**17.6 ± 1.2**	**36.2 ± 0.6[b]**	**23.6 ± 1.7[b]**

All values are expressed as mean±SEM, $p \leq 0.05$. a - Statistically significant as compared with control, b - statistically significant as compared with acetic induced group

4.4.7. EFFECT OF VITAMIN E ON LYMPHOCYTE COUNT IN SODIUM ARSENITE EXPOSED RATS IN EXPERIMENTAL GASTRIC ULCER

The percentage of lymphocyte was significantly reduced in AA on day 7 – 21 as compared with the control, $p<0.05$. In the SA – exposed, the lymphocyte count was significantly increased on day 14 and 21 as compared with the AA, and the control. SA+ VE significantly reduced on day 14 and day 21 as compared with the SA and the control, $p<0.05$ as shown in table 4.6.

4.4.8. EFFECT OF VITAMIN E ON MONOCYTE COUNT IN SODIUM ARSENITE EXPOSED RATS

The percentage of monocyte was significantly increased on day 3 and day 7 as compared with the control, the count significantly reduced ion day 14 and day 21 as compared with the control. Exposure to SA +AA was significantly increased on day 7 and day 21 as compared with the control, and AA. Administration with the VE significantly decrease the monocyte count on day 3, 7, 14, 21 as compared with the SA, AA and the control, $p <0.05$ as shown in table 4.8.

4.4.9. EFFECT OF VITAMIN E ON EOSINOPHIL COUNT IN SODIUM ARSENITE EXPOSED RATS IN EXPERIEMENTALLY INDUCED GASTRIC ULCER

The percentage of eosinophil was significantly increased increased in the AA on day 3 and day 7 as compared control, $p< 0.05$. It was significantly reduced on day 14 and 21.

Exposure to SA +AA significantly increase the eosinophil count on day 14 and day 21 as compared with the control and AA, $p<0.05$. VE treatment significantly decrease the eosinophil count on day 3,14, and 21 as compared with the SA +AA, AA and the control, $p< 0.05$ as shown in table 4.8.

Table 4.7. The effect of vitamin E on monocyte count in sodium arsenite exposed in experimental gastric ulcer

Grouping Monocyte (%)	**Day 3**	**Day 7**	**Day 14**	**Day 21**
Control	**5.9 ± 0. 4**	**6.8 ± 0. 4**	**7.5 ± 0.5**	**6.4 ± 0.8**
AA	**7.4 ± 1.2**	**12.6 ± 0.9[a]**	**9.2 ± 0.4[a, c, d]**	**8.2 ± 1.0**
SA+AA	**6.6 ± 0. 8[a, c]**	**16.6 ± 1.4[a,b, d]**	**4.4 ±0.8[a, b, d]**	**27.4 ± 2.4[a,b,d]**
SA+VE +AA	**1.4 ± 0.4[a, b, c]**	**6.0 ± 0.0[b,c]**	**7.2 ± 0.5[b, c]**	**5.4 ± 1.2[b,c]**

All values are expressed as mean±SEM, $p\leq0.05$. a - Statistically significant as compared with control, b - statistically significant as compared with AA, c- significant as compared with the SA+AA, d- significant as compared with the SA +VE +AA

Table 4.8. Effect of vitamin E on eosinophil count (%) in the sodium arsenite exposed in experimental gastric ulcer.

Grouping(%)	**Day 3**	**Day 7**	**Day 14**	**Day 21**
Control	29.2 ± 1.2	29.2 ±1.2	29.2± 1.2	29.2±1.239

AA	55.0 ± 3.1[a, c, d]	67.6±0.9[a, c,d]	28.4±1.3[a, c,d]	28.8±1.9 [c, d]
SA+AA	32.0 ±1.4 [b, d]	29.6 ± 1.7 [b,]	58.0 ± 2.2[b]	60.2 ± 2.4[b]
SA+VE +AA	21.2 ± 0.5[b]	49.6± 2.0[b]	13.4 ± 0.6[b]	29.8± 4.0

All values are expressed as mean±SEM, p <0.05. a - Statistically significant as compared with control, b - statistically significant as compared with acetic induced group, c- significant as compared with SA +AA, significant as compared with the SA+VE+AA

4.5.0: EFFECT OF VITAMIN E, KOLAVIRON ON SODIUM ARSENITE EXPOSED RATS IN EXPERIMENTAL GASTRIC ULCER

4.5.1 EFFECT OF KOLAVIRON, VITAMIN E ON PROTEIN LEVEL IN SODIUM ARSENITE EXPOSED IN EXPERIMENTAL GASTRIC ULCER

Protein level in AA group significantly decreased on day 3 and increased significantly on day 7- 21 as compared with SA. In the SA exposed, the protein level eas significantly decreased on day 3 as compared with the control, $p < 0.05$. It significantly increased on day 7- 21 but significantly decreased as compared with the control, AA.

In SA+ VE, the protein level was significantly increased on day 3- day 21 as compared with the SA +AA, $p < 0.05$ while in the VE + AA, the protein was significantly increased on day 21 as compared with the AA, control and SA +AA.

In SA+ KV+AA, there wwas significant increase in the protein level on day 14 and day 21 as compared with the control, AA and SA +AA, $p< 0.05$. In KV + AA, there was significant increase on day 3-21 as compared with the SA+AA, and on day 21, it was significantly increased as compared with the AA and control, $p < 0.05$ as shown in figure 4.18.

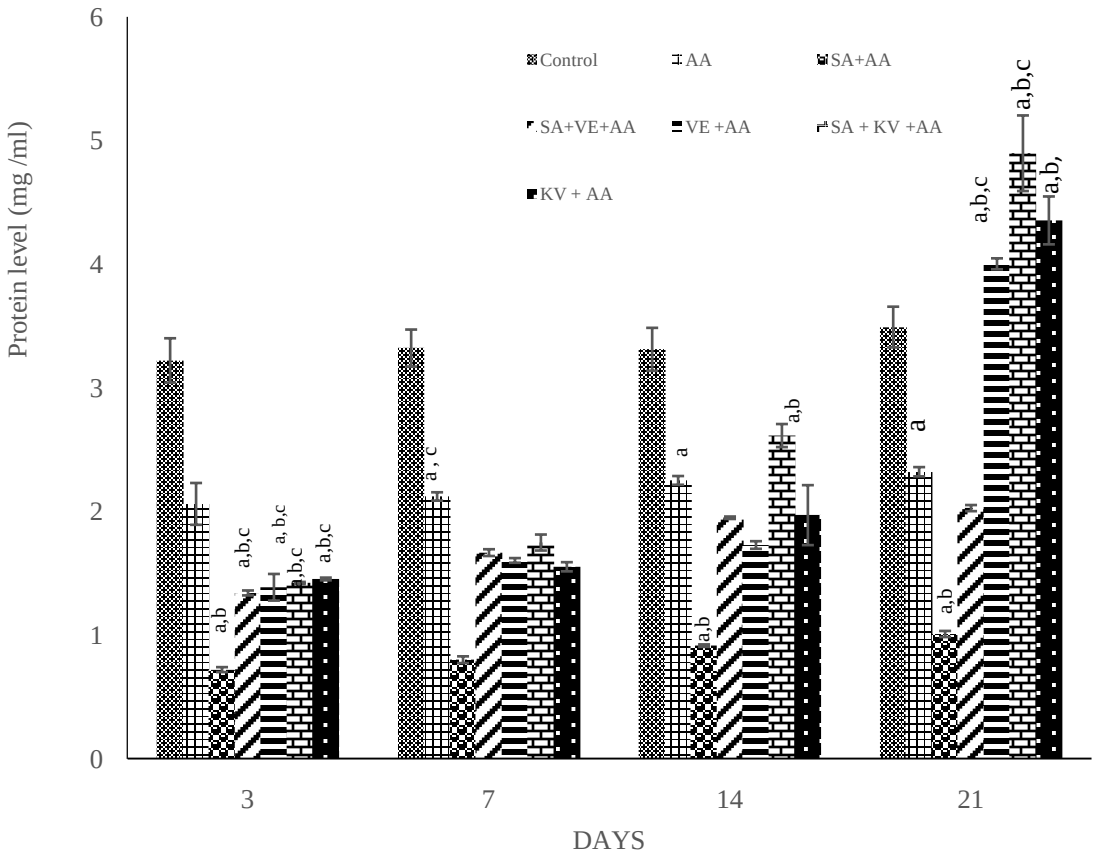


Figure 4.18. Effect of Kolavironand vitamin E on protein level in the mucosa of the stomach in experimental gastric ulcer.all values are expressed as mean±SEM, $p \leq 0.05$, a - significant as compared with normal control, b signififcant as compared with AA, c – significant as compared with SA+AA,d- significant as compared with the SA+VE + AA , rate of increase in protein level = SA+KV+AA>KV+AA> SA+VE+AA >VE+AA>AA >SA+AA

4.5.2. EFFECT OF VITAMIN E, KOLAVIRONON CATALASE ACTIVITY IN SODIUM ARSENITE EXPOSED IN EXPERIMENTAL GASTRIC ULCER

In the AA group, there was significant increase in the catalase activity on day 3, 7, 14, and 21 as compared with the control. In SA+AA, the catalase activities was significantly reduced as compared with the AA and control as shown in Table 4.10

In the SA+VE + AA, there very significant increase in the catalase level on day3, 7, 14, and 21, as compared to AA, SA + AA. In VE+ AA group, was significantly decreased on day 3 and day 7 as compared with the SA + VE +AA. It was significantly increased on day 14 and 21 as compared with the SA + AA, SA+ VE, AA and control.

KV treatments significantly increase the CAT activities as compared with the control,SA+ AA and AA on day 3, 7, 14 and 21 but signififcantly reduced as compared with SA +VE +AA, VE +AA, $p<0.05$ as shown in table 4.9.

Table 4.9. Effect of kolaviron, vitamin E on Catalase in the sodium exposed in experimental gastric ulcer

GROUPING	DAY 3	DAY 7	DAY 14	DAY 21
Control	**246.0±5.0**	**246.0 ± 5.0**	**246.0 ±5.0**	**246.0 ± 5.0**
AA	**516.0 ±0.4** [a, c]	**472.0 ±5.4** [a, c]	**401.0 ±5.6** [a, c]	**38.0 ±8.5** [a, c]
SA+AA	**430.0 ±37.4** [a, b]	**359.0 ±4.3** [a, b]	**342.0 ±5.2** [a, b]	**324.0 ± 3.0** [a, b]
SA+VE +AA	**1124.0 ±0.6** [a, b,c]	**1019.0 ±15.3** [a, b, c]	**858.0 ± 8.3** [a, b, c]	**811.0 ±30.0** [a, b, c]
VE+AA	**359.1 ± 66.5** [a, b,c]	**260.8 ± 44.0**	**779.9 ± 128.3** [a, b]	**1087.3 ±132.2** [a, b]
SA+KV+AA	**634.0 ±177.4** [a, b, c]	**372.9±89.1** [a, b,c]	**348.0 ± 45.8**	**520.0 ±344.5** [a, b,c]
KV+AA	**694.0 ±43.8** [a, b,c]	**208.4±32.0**	**1643.0 ±153.4**	**302.1±100.5**

All values are expressed as mean±SEM, P< 0.05. Rate of increase = VIT E+ACIU> SA+VTI E+ ACIU>SA+KV +ACIU>KV+ACIU>CONTROL>ACIU>SA+ACIU a -significant as compared with the control. b- significant as compared with AA, c- significant as compared with the SA+AA, d- significant as compared with the SA+KV+AA.

4.5.3. EFFECT OF VITAMIN E, KOLAVIRON ON MALONDIALDEHYDE (MDA) IN SODIUM ARSENITE EXPOSED IN EXPERIMENTAL GASTRIC ULCER.

SA exposure significantly increase the MDA level on day 3, 7, 14, and 21 as compared with the control and AA. VE treatments significantly decreased in the MDA level as compared with SA +AA, AA and control. KV treatment significantly decrease the MDA level as compared with the SA + AA, AA, SA + VE, $p < 0.05$ as shown in table 4.10.

4.5.4. EFFECT OF VITAMIN E AND KOLAVIRON ON SUPEROXIDE DIMUTASE (SOD) IN SODIUM ARSENITE EXPOSED IN EXPERIMENTAL GASTRIC ULCER

The SOD in the SA+AA was significantly decreased as compared with the control and AA group on day 3- 21, $P<0.05$. Administration with VE significantly increased the SOD activities in the SA +VE+ AA and VE +AA on day 3, day 14 and day 21 as compared with the SA+AA, AA and it was significantly increased as compared with the control on day 21.

KV treatment significantly increased the SOD in the SA+KV +AA and KV+ AA as compared with the SA+ AA and AA on day 3- 21, $p<0.05$. However, there was significant increase in SA +KV +AA on day 21 as compared with the control as shown in table 4.11.

4.6.0. EFFECT OF KOLAVIRON, VITAMIN E ON ULCER AREA OF SODIUM ARSENITE EXPOSED IN EXPERIMENTAL GASTRIC ULCER

From plate 6 , the ulcer area was significantly increase in AA as compared with the control with intact mucosa layer on day 3 and it significantly decrease on day 7 to day 21,$p < 0.05$.

Exposure to SA +AA significantly increase the ulcer area on day 3, day 14 and day 21 as compared with the AA and the control. In the VE treatments, it was significantly decreased on day 7 -21 as compared with the SA+AA and AA, $p<0.05$.

Administration with KV significantly decreased the ulcer area from day 7-day 21 as compared with SA+VE< SA+AA and AA, $p<0.05$ as shown in plate 4.6.

Table 4.10. Effect of vitamin E on malondialdehyde (MDA) in SA exposed in experimental gastric ulcer

GROUPING	Day 3 (U/mg protein)	Day 7 (U/mg protein	Day14 (U/mg protein	Day 21 (U/mg protein
Control	$4.7x10^{-6}$ ±1.6 $x10^{-7}$	$4.7\ x10^{-6}$ ± 1.6 $x10^{-7}$	$4.7\ x10^{-6}$ ± 1.6 $x10^{-7}$	$4.7\ x10^{-6}$ ± 1.6 $x10^{-7}$
AA	$1.9\ x10^{-5}$ ±2.3 x $10^{-7\ a,c}$	$1.4x10^{-5}$± 5.1 $x10^{-7a,c}$	$1.3\ x10^{-5}$ ± 2.7 $x10^{-7a,c}$	$4.5\ x10^{-6}$ ± $2.1x10^{-6a,c}$
SA+ AA	$6.8\ x10^{-3}$ ± $3.7\ x10^{-6a,b}$	$1.1\ x10^{-4}$ ±1.2 x $10^{-6a,b}$	$8.7\ x10^{-5}$ ± 3.5 $x10^{-6a,b}$	$7.1\ x10^{-5}$ ± 3.9x $10^{-6a,b,c}$
SA+AA+ VE	$3.2\ x10^{-5}$ ± $3.9x\ 10^{-7}$	$2.7\ x10^{-5}$ ± 7.6 $x10^{-7a,b,c}$	$2.1\ x10^{-5}$ ±6.1 $x10^{-7a,b,c}$	$1.7\ x10^{-5}$± 4.3 x $10^{-7a,b,c}$
VE+AA	$7.0\ \times10^{-6}$ ± 1.1 $\times10^{-6\ a,b,c}$	$5.4\ \times10^{-6}$± $6.8\times10^{-8\ a,b,c}$	$3.9\ \times10^{-6}$ $\pm4.8\times10^{-7\ a,b,c}$	$4.6\ \times10^{-6}$ ± 4.8 $\times10^{-8\ a,b,c}$
SA+KV+AA	$4.9\ \times10^{-6}$ ± 3.4 $\times10^{-7\ a,b,c}$	$6.0\ \times10^{-6}$ ± 8.0 $\times10^{-7\ a,b,c}$	$4.8\ \times10^{-6}$± 9.2×10^{-7}	$3.3\ \times10^{-6}$ ± 7.3 $\times10^{-7\ a,b,c}$
KV+AA	$6.0\ \times10^{-6}$ ± $6.2\ \times10^{-7\ a,b,c}$	$6.6\ \times10^{-6}$ ± 3.3 $\times10^{-6}$ **	$4.1\ \times10^{-6}$ ± $3.6\times10^{-8\ a,b,c}$	$4.0\ \times10^{-6}$± 9.0 $\times10^{7\ a,b,c}$

All values are expressed as mean±SEM, P<0.05 a- significant as compared with control, b – significant as compared with the AA, c – significant as compared with the SA +AA. Rate of lipid peroxidation reduction = SA+KV+AA < KV+AA < VIT E+AA < SA+VIT +AA = control< AA < SA+AA

Table 4.11. Effect of kolaviron and vitamin E on SOD activity in sodium arsenite exposed in experimental gastric ulcer

GROUPING	DAY 3 (U/mg protein)	DAY 7 (U/mg protein)	DAY 14(U/mg protein)	DAY 21(U/mg protein)
Control	1343.0 ± 242.5	1209.0 ±267.3	1093.0 ±49.2	804±143.4
AA	840.0 ±26.3[a, c]	807.2±11.6[a, c]	764.0±18.6[a, c]	780.0 ±12.3[a, c]
SA+AA	595.0 ±3.9[a, b]	594.0 ±4.4[a, b]	570.0 ±19.0[a, b]	194.0±2.5[a, b]
SA+VE + AA	1343.0 ±14.8[b, c]	976.0 ±45.4[c]	1196.0 ±124.8 [a, b, c]	1132.0 ±62.7 [a,b, c]
VE+AA	1104.0 ±43.0 [b c]	100.0 ± 65.6 [a, c]	941.0 ± 59.8 [a, b, c]	1076.0 ±149.3 [a, b,c]
SA+KV+AA	1013.0 ± 4.0 [b, c]	1232.0 ± 66.1 [b, c]	927.0 ± 59.8 [a, b, c]	1131.0 ±149.3 a,b, c
KV+AA	996.0 ± 49.9 [b, c]	852.0 ± 52.2 [c]	1129.0 ± 55.9 [a, b,c]	772.0 ± 143.3

All values are expressed as mean±SEM, P<0.05, a- significant as compared with control, b- significant as compared with the AA, c –significant as compared with the SA +AA

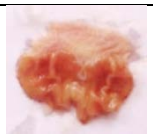
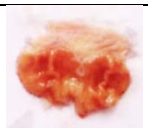
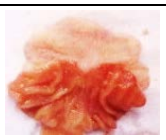
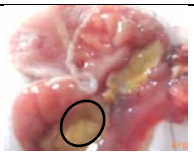
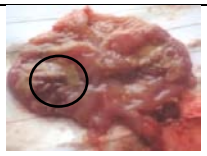
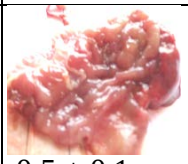
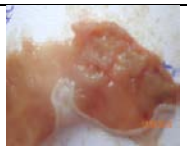
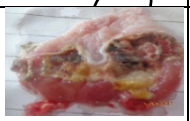
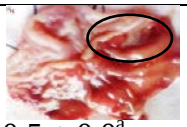
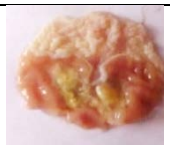
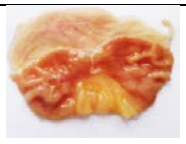
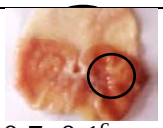
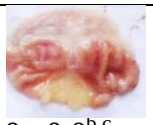
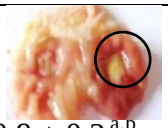
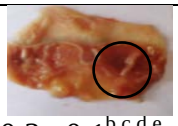
GROUPING	3DAYS	7DAYS	14DAYS	21DAYS
Control	 0.0 ± 0.0	 0.0 ± 0.0	 0.0 ± 0.0	 0.0 ±0.0
AA	 0.7 ± 0.0	 0.6 ±0.1	 0.5 ± 0.1	 0.3 ± 0.0
SA+AA	 1.4±0.6[a]	 0.8 ±0.0 [a,b]	 0.2 ± 0.1[a,b]	 0.5 ± 0.0[a]
SA+VE+AA	 1.2 ±0.2 [a]	 0.9 ±0.2[a,b]	 0.7±0.2 [a]	 0.0 ±0.0 [a, b]
VE+AA	 1.1±0.1[a]	 0.7 ± 0.0[a]	 0.5 ± 0.1	 0.1 ±0.0 [a,b]
SA+KV + AA	 1.1 ±0.8[c]	 0.7±0.1[c]	 0.3 ±0.2[b,c]	 0.0 ±0.0[b,c]
KV+AA	 0.9 ± 0.2 [a,b]	 0.3 ± 0.1[b,c]	 0.3 ±0.1[b,c,d,e]	 0.0 ±0.0 [b,c]

Plate 4.6.Effect of KV, VE treatment in SA+AA in experimental gastric ulcer. All values are mean ± SEM, $p<0.05$, a-significant as compared with control , b –significant as compared with the AA, c-significant as compared with the SA +AA, rate of healing= SA+ KV+AA+KV+AA >SA+VE+AA>VE >AA>SA +AA.

4.6.1. EFFECT OF KOLAVIRON,VITAMIN E ON H^+ - K^+ -ATPase IN SODIUM ARSENITE EXPOSED IN EXPERIMENTAL GASTRIC ULCER

Sodium arsenite exposure significantly decrease the H^+ - K^+ATPase activities on day 3-day 21 as compared with the AA. KV treatment significantly decrease the $H^{+-}K^+$ATPase activities as compared with the AA on day 3-day 21, $p<0.05$. VE treatments significantly decrease the H^+ - K^+ATPase activities as compared with the AA as shown in table 4.12.

4.6.2. EFFECT OF KOLAVIRON AND VITAMIN E ON THE NITRITE CONCENTRATION IN SODIUM ARSENITE EXPOSED IN EXPERIMENTAL GASTRIC ULCER

The nitrite concentration was significantly decrease in the AA on day 3, 14 and 21, although there was significant increase on day 7. Exposure to sodium arsenite (SA+AA) significantly decrease the nitrite concentration on day 7- day 21 but significantly increase as compared with the AA on day 3, 14 nd 21. Administration of KV significantly increase the nitrite concentration on day 14 and 21 as compared with the AA and SA+AA, $p<0.05$. It was significantly increase on day 21 as compared with the VE treatment, $p<0.05$.

VE significantly increase the nitrite concentration on day 7-21 as compared with the control, AA, SA +AA, $P<0.05$ as shown figure 4.19.

4.6.3. EFFECT OF VITAMIN E AND KOLAVIRON ON THE EPITHELIAL LAYER OF GASTRIC MUCOSA EXPOSED TO SODIUM ARSENITE IN EXPERIMENTAL GASTRIC ULCER

Histological photomicrographs showed moderate to mild ulceration with infilteration of few inflammatory cells in the AA from day 3- 21. Exposure to sodium arsenite extensive and complete erosion with necrosis on day 3, 7 and with appearance of abnormal cells on day 14 and severe hemorrhage on day 21 as compared with the AA group.

In the group administered with SA+ KV +AA, there was focal necrosis in the gastric mucosal and intense inflammation as compared with the SA+ AA on day 3.On day 7(as shown in plate 4.7), there was moderate ulceration with stromal inflammation which became mild ulceration with stromal edema as compared with the SA+AA on day 14.In the KV+AA, there was moderate ulceration from day 7-14 and mild ulceration on day 21 with an initial necrosis on day 3 as compared with the SA+KV+AA.

In the group administered with SA+VE + AA, there was mucosal necrosis with hemorrhage and intense inflammation and this become a moderate ulceration with intense inflammation on day 7(plate 8), 14 and 21as compared with the SA +AA as in plate 4.7- 4.10.

4.6.4. EFFECT OF VITAMIN E AND KOLAVIRON ON THE EXPRESSION OF PECAM-1(CD31) IN SA EXPOSED IN EXPERIMENTAL GASTRIC ULCER

In the group exposed to SA+AA, there was a mild expressionas compared with the AA group. In SA + VE +AA significantly in the PECAM-1 expression on day 3 as compared with the SA+AA and the AA. There was moderate expression on day 7 -14 and strong expression on day 21(88%) as shown in plate 4.11-4.14 and in figure 4.20.

In the VE + AA shows a moderate expression on day 3- day 14 as compared with AA group and SA +AA group. On day 21, there was mild expression as compared with SA + AA, AA and SA +VE +AA. SA+KV group showed a mild expression on day 3(41.3%) as compared with the control, SA +AA, SA +VE +AA, and VE + AA. On day 7-21 (60.0%, 66.3%.64.9%), there was a moderate expression as compared with other groups.KV + AA showed a moderate expression on day 3(71.2%) and there was significant increase in the expression on day 7((83%) as compared with the SA + AA and AA group. There was significant decrease in the expression on day 14 and significant increase on day 21 as compared with the control, VE treated groups, $p<0.05$ as shown in figure 4.11- 4.14 and figure 4.20. Rate of expression =VE>KV>Control>SA+ACIU

Table 4.12. Effect of Kolairon, vitamin E on H^+ - K^+ ATPase in SA-exposed in acetic acid induced ulcer**.**

Groups	Day 3	Day 7	Day 14	Day 21
AA	**4.5 ± 0.8**	**4.5 ±0.8**	**4.5 ± 0.8**	**4.5 ±0.8**
SA+AA	**1.9 ± 0.3**	**1.4 ±0.26**	**1.3 ± 0.3**	**2.7±0.6**
SA+KV+AA	**2.6 ± 0.5[a.b]**	**2.5 ±0.5[a.b]**	**2.8 ±0.6 [a,b]**	**3.0 ±0.4 [a]**
KV+AA	**3.0±0.7 [a,b]**	**3.4 ±0.7**	**3.2 ± 0.4**	**2.0 ±0.4[a,b]**
SA+VE+AA	**4.4±1.0**	**5.5 ± 0.2**	**2.6 ± 0.8[a]**	**2.1 ±0.8[a,b]**
VE+AA	**2.0 ± 0.7[a]**	**1.7±0.4**	**2.2 ±0.7[a]**	**3.2 ±0.9**

All values are mean ± SEM,a- significant as compared with the AA, b- significant as compared with SA +AA, $p<0.05$, SA+AA< KV<VITE<AA

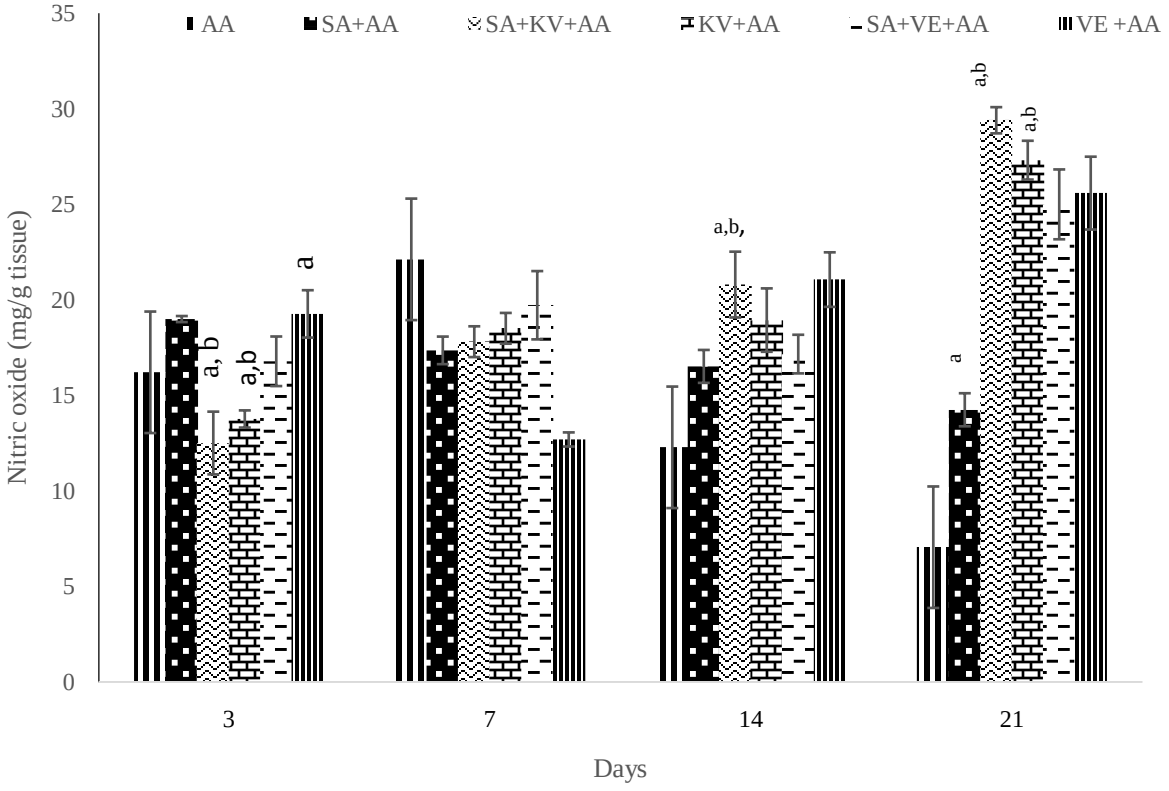


Figure 4.19: Effect of Kolaviron, vitamin E on nitric oxide in sodium arsenite in acetic induced ulcer. All values are mean±SEM,a -significant as compared with the AA and b-significant as compared with SA+ AA, $P<0.05$.

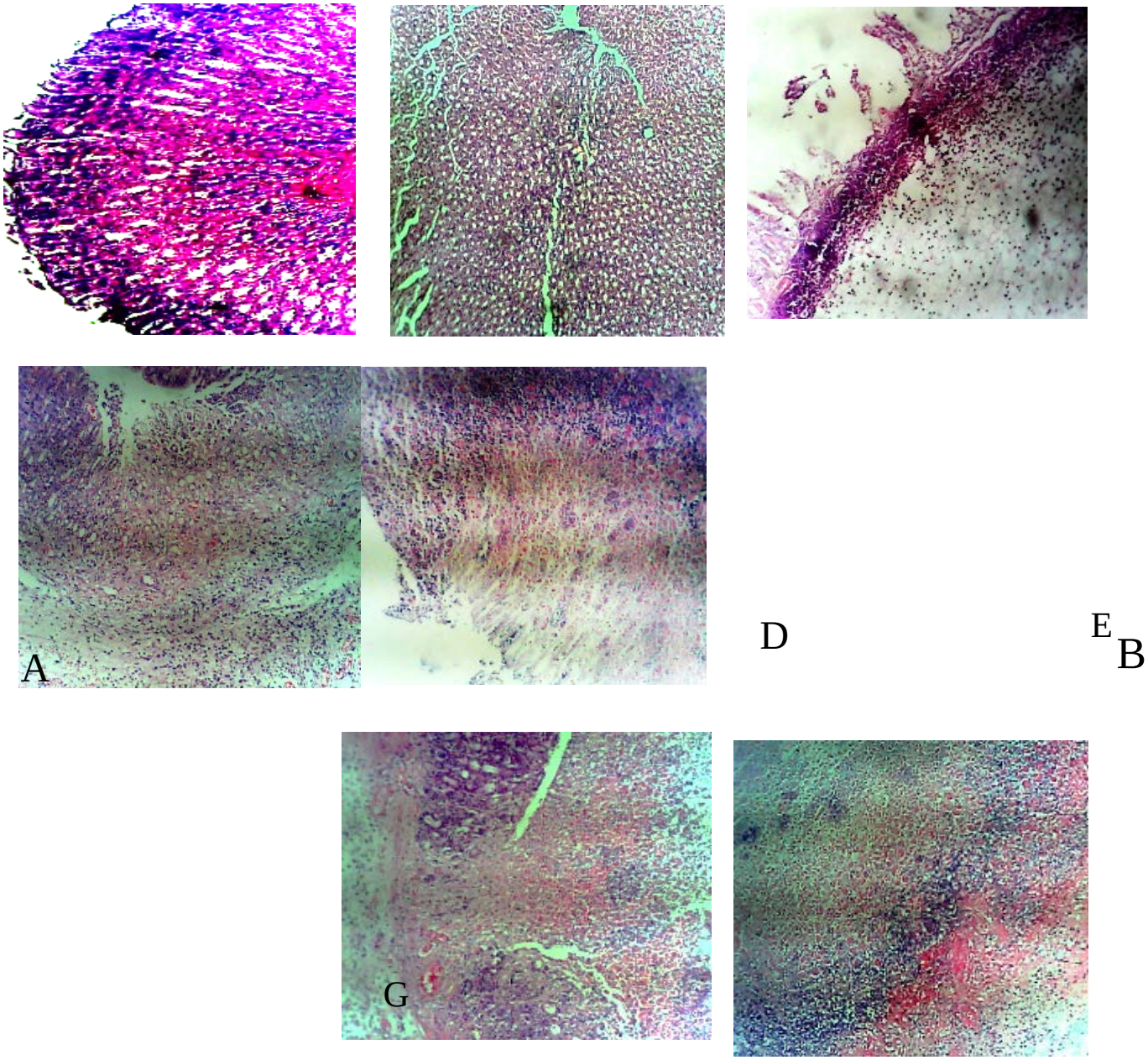
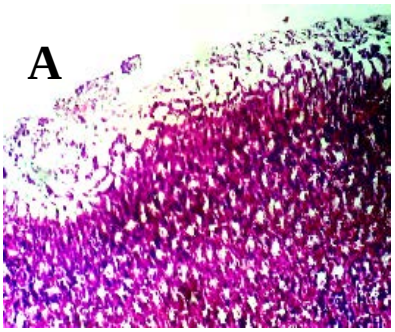


Plate 4.7. Photomicrograph of sodium exposure and treatment with KV, VE on day 3post ulcer induction (H and E X100). **A**. Control showing intact epithelium and no visible lesion. **B**. Ulcerative control group(AA), showing moderate ulceation and necrosis of glands, moderate aggregation of inflammatory cells in submucosa. **C**. Sodium arsenite exposed (SA+AA) showing complete and extensive mucosal erosion with increased inflammatory cells. **D.**SA+ KV+AA(kolaviron treated) group with mmoderate erosion, necrosis and infilteration of inflammatory cells,hyperplasia**E.** KV +AA group showing moderate erosions of surface epithelium, moderate aggregation of inflammatory cells at the base of the mucosa and submucosa. **F.** SA+VE+AA group with moderate erosion and necrosis of galnds, marked infiltration of inflammatory cells with focal hemorrhage, hyperplasia was aslo seen.**G.** VE +AA group showing focal hemorrhage, necrosis with moderate ulceration.



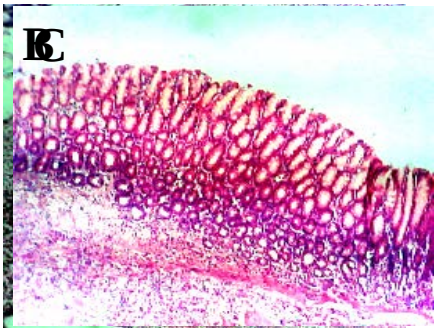
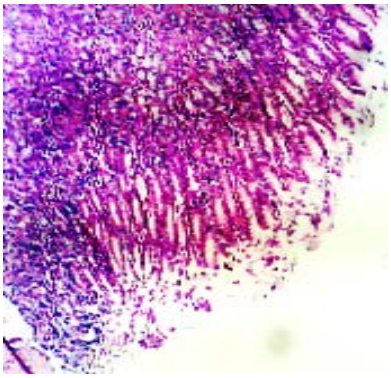
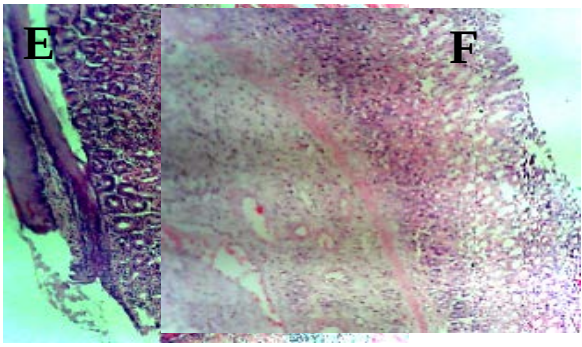
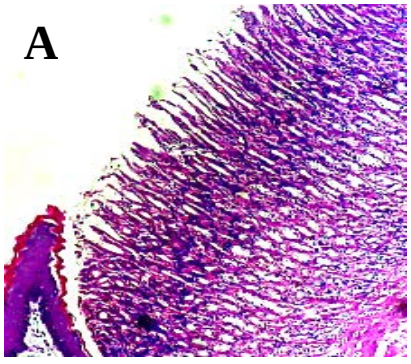
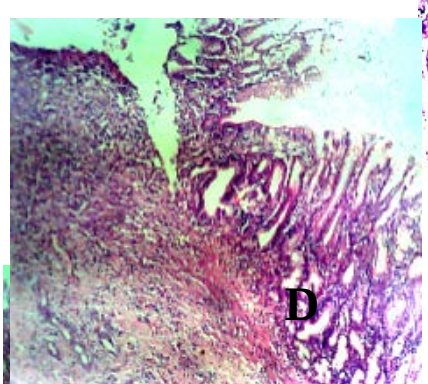


Plate 4.8: Photomicrograph of sodium exposed and treatment with KV, VE treatment day 7 post ulcer induction (H and E X100). **A**. Ulcerative control group(AA), showing mild ulceration with few inflammatory cells. **B.** Sodium arsenite exposed (SA+AA) showing hyperplasia with necrosis and aggregate inflammatory cells. **C.** SA+ KV+AA(kolaviron treated) group mild ulceration **D.** KV +AA group showing mild erosions of surface epithelium with infilteration of inflammatory cells. **E.** SA+VE+AA group with moderate erosion with infilteration of inflammatory cells.**F.** VE +AA group showing moderate ulceration with infilteration of inflammatory cells.









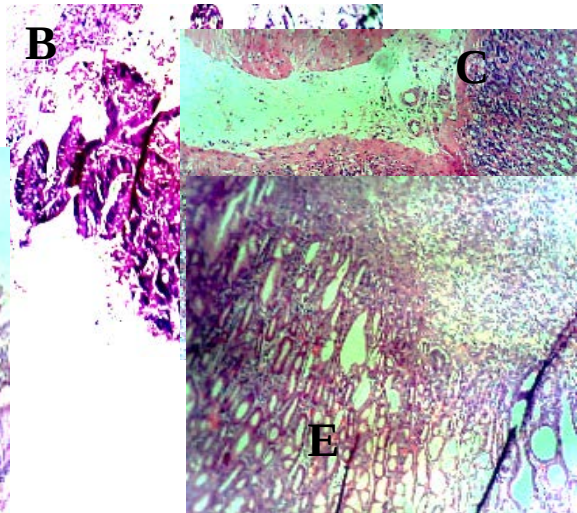


Plate 4. 9. Photomicrograph of sodium exposed and treatment with KV, VE on day 14 post ulcer induction(H and E X100). **A**. Ulcerative control group (AA), showing mild ulceration with few inflammatory cells. **B.** Sodium arsenite exposed (SA+AA) showing hyperplasia with necrosis and abnormal glands(atypical). **C.** SA+ KV+AA(kolaviron treated) group molderate ulceration with inflammatory cells **D.** KV +AA group showing mild erosions of surface epithelium with infilteration of inflammatory of cells**E.** SA+VE+AA group with moderate erosion with infilteration of inflammatory cells.**F.** VE +AA group showing moderate ulceration with infilteration of inflammatory cells.

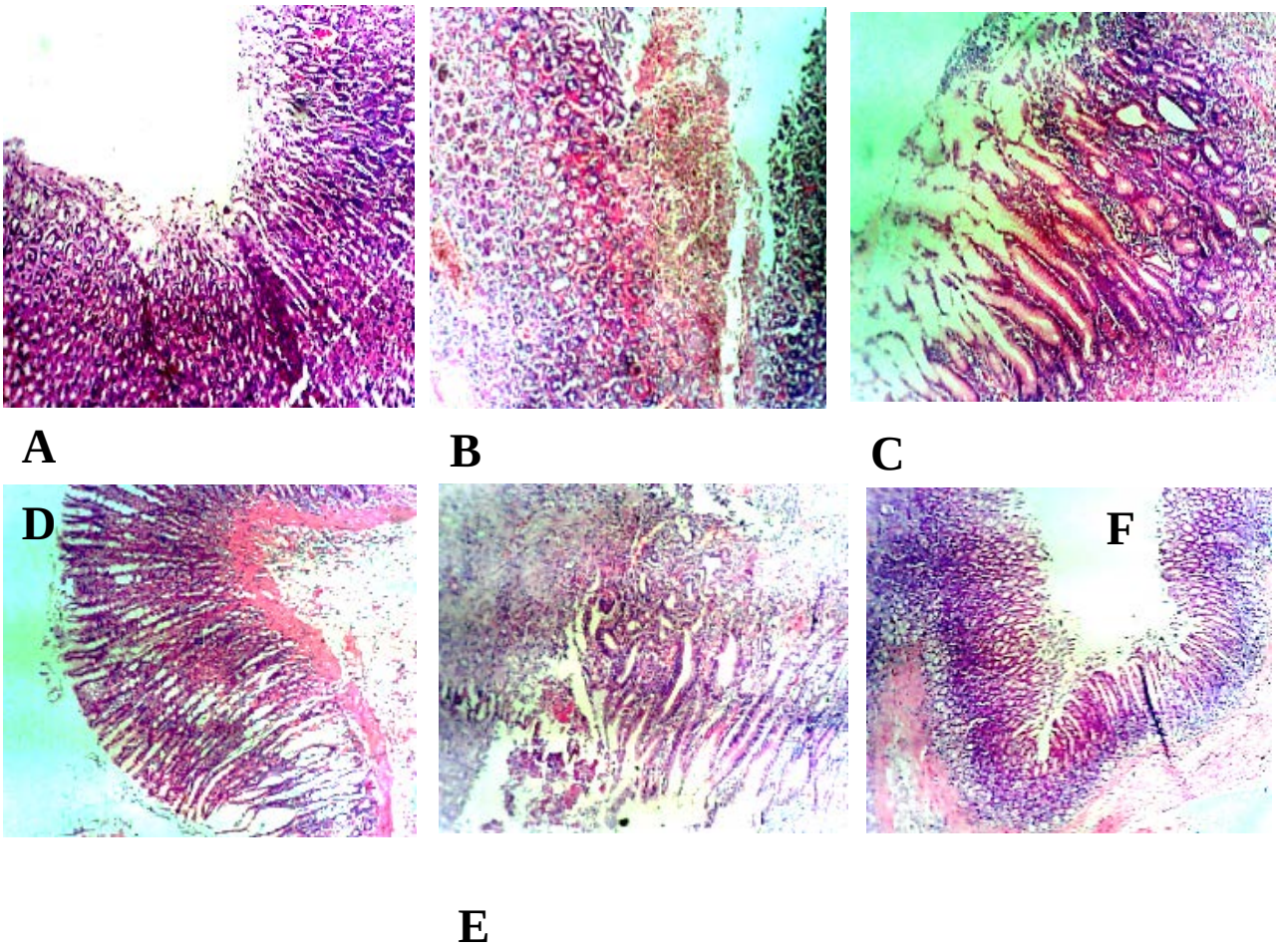
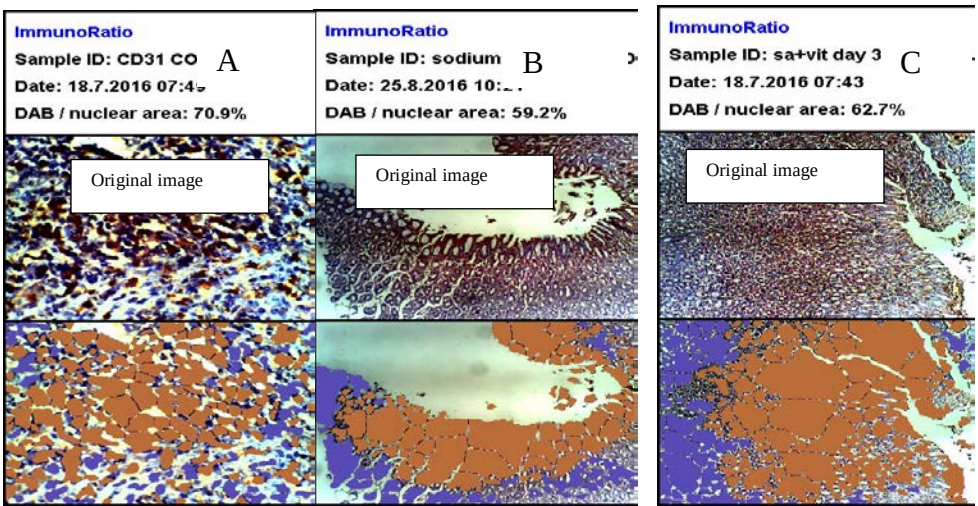


Plate 4. 10. Photomicrograph of sodium exposed and treatment with KV, VE after acetic acid induced gastric ulcer on day 21(H and E X100). **A**. Ulcerative control group (AA), showing mild ulceration with few inflammatory cells. **B.** Sodium arsenite exposed (SA+AA) showing hyperplasia with necrosis and aggregate inflammatory cells. **C.** SA+ KV+AA(kolaviron treated) group mild ulceration with interglandular erosion with inflammatory cells **D.** KV +AA group showing mild erosions of surface epithelium with inilf. **E.** SA+VE+AA group with moderate erosion with infilteration of inflammatory cells, multi focal hemorrhage.**F.** VE +AA group showing mild ulceration with infilteration of few inflammatory cells.



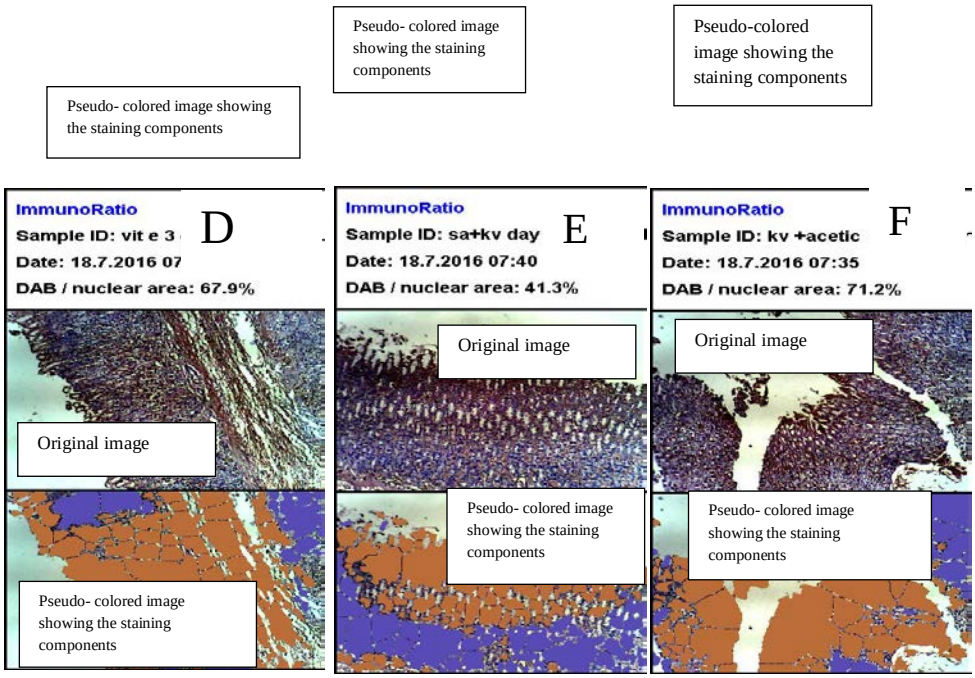
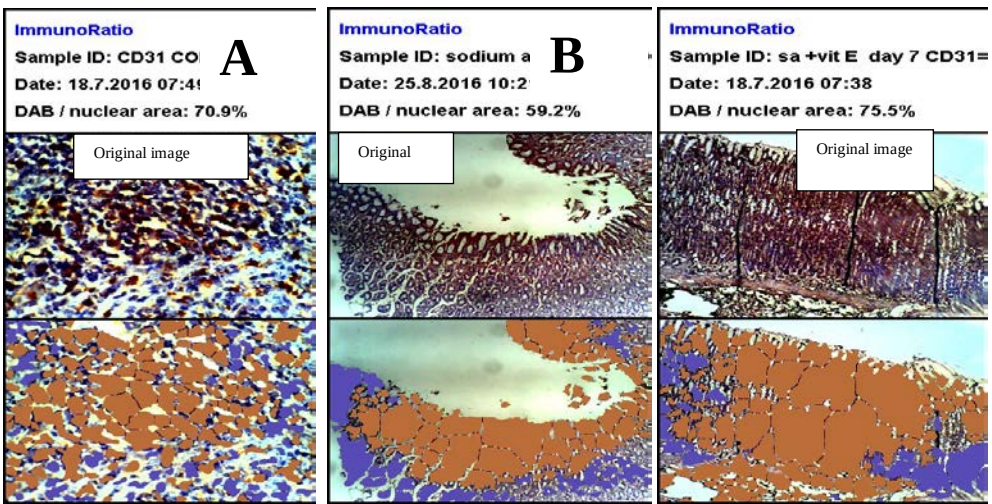


Plate 4. 11: Effect of KV, VE on CD31 expression in SA exposed on day 3 post ulcer induction**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of PECAM- pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **control** (moderate expression) B: **SA exposed** (mild expression) C: **SA+ VE** (moderate expression) **D: VE + AA (**moderate expression) **E: SA +KV+AA**(mild expression) **F: KV +AA**(moderate expression)**.** 30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression



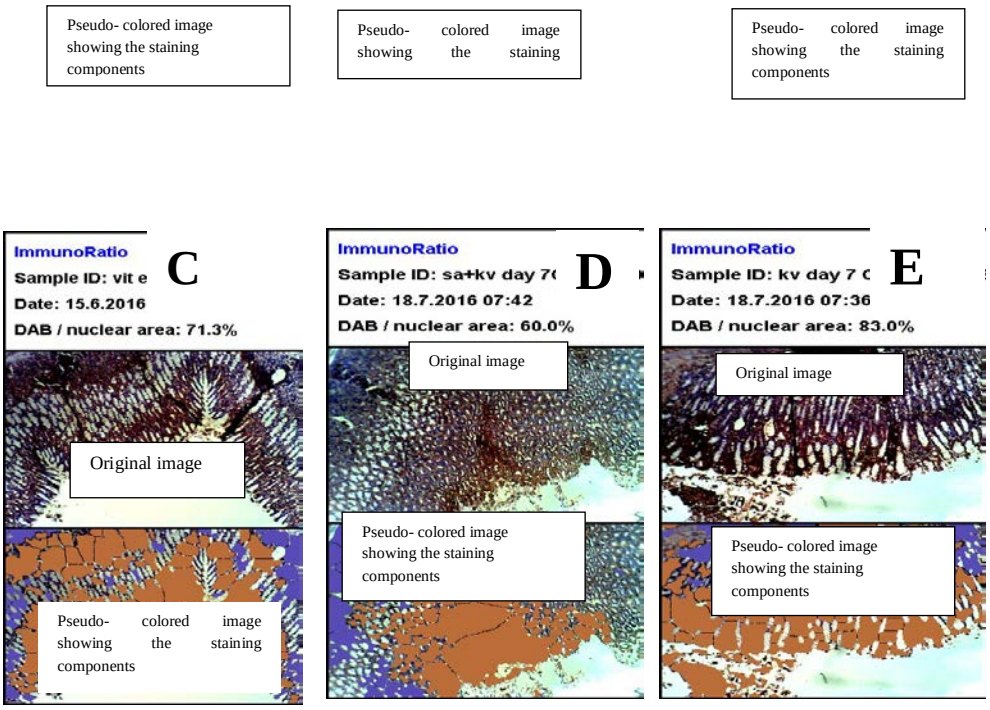
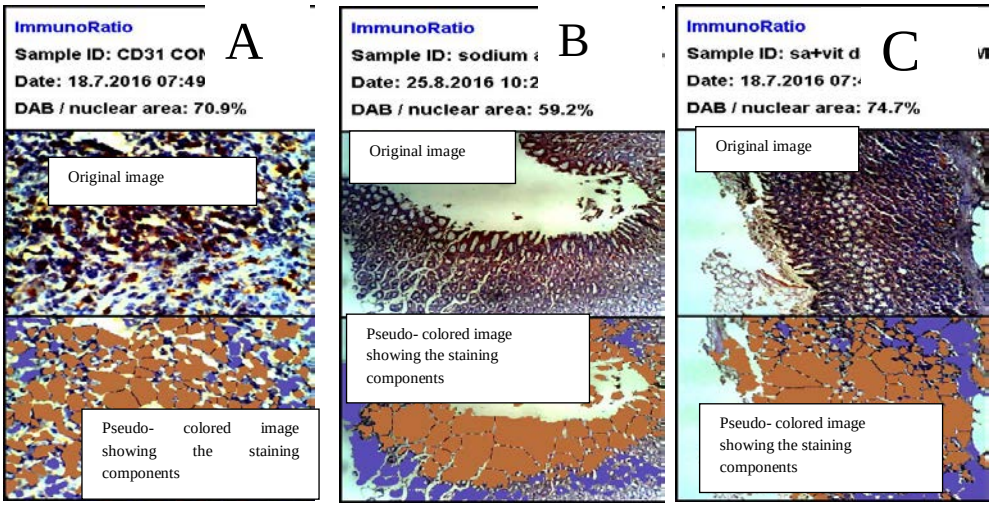


Plate 4.12: Effect of KV, VE on CD31 expression in SA exposed on day 7 post ulcer induction**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of PECAM- pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **control** (modrate expression) B: **SA exposed**(mild expression) C: **SA+ VE** (moderate expression) **D:VE + AA** (moderate expression) **E: SA +KV+AA**(moderate expression) **F: KV +AA**(moderate expression).30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression



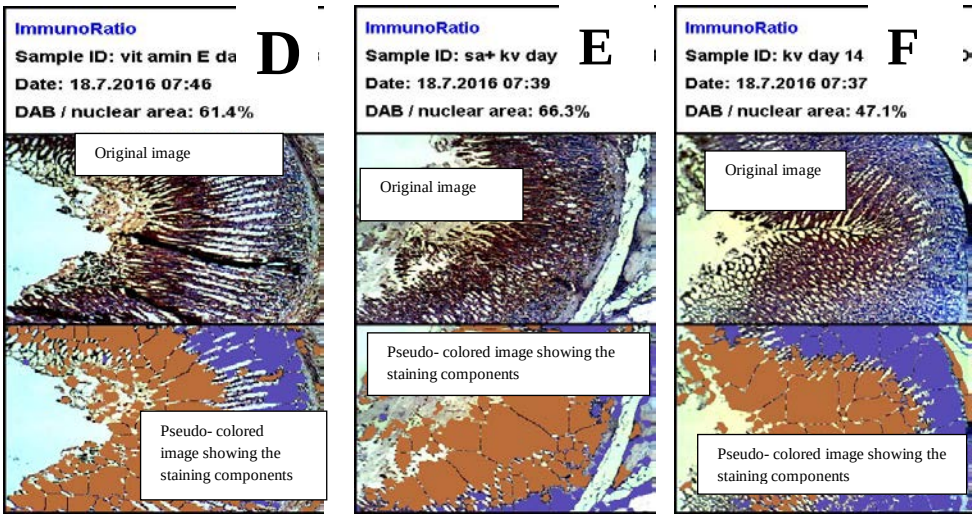
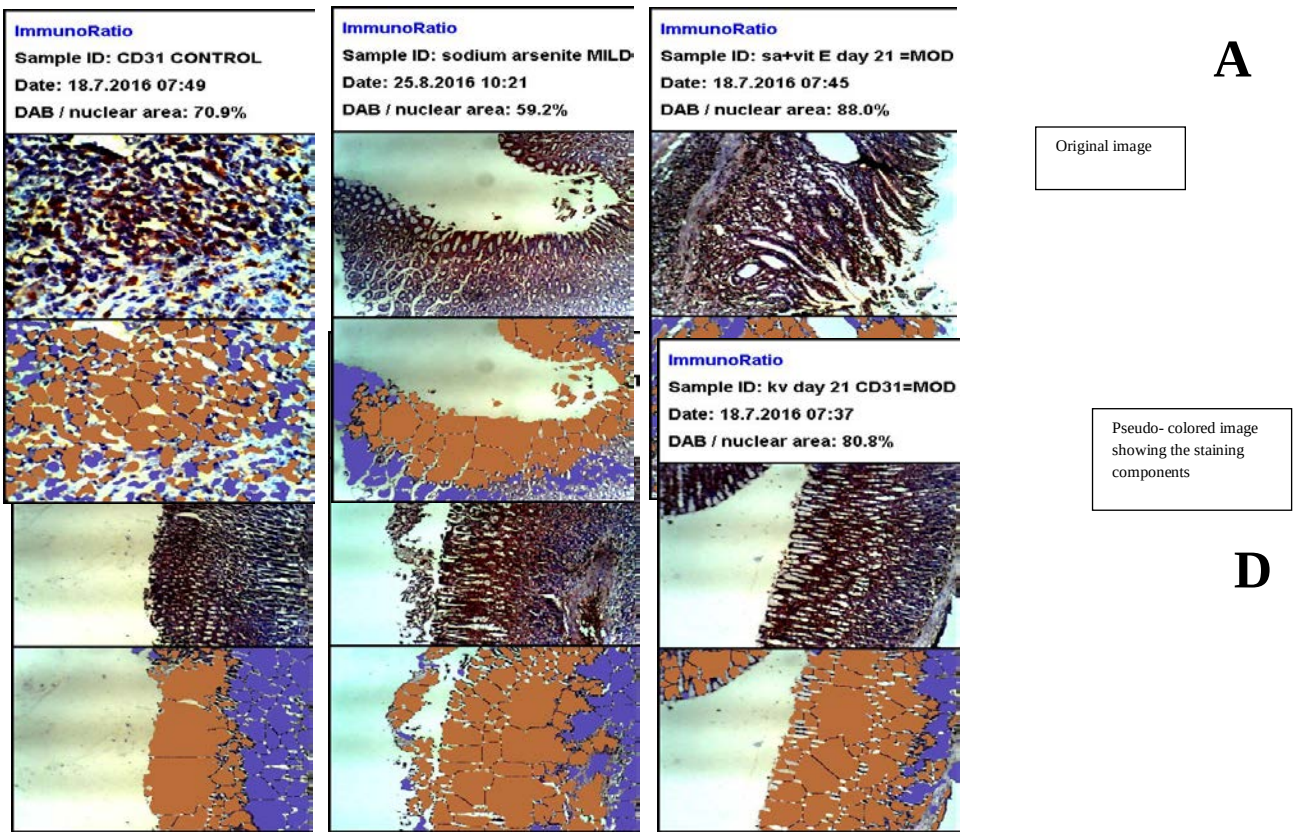


Plate 4.13: Effect of KV, VE on PECAM-1 expression in SA exposed on day 14 post ulcer induction**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of PECAM- pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain.. **A**: **control** (modrate expression) B: **SA exposed**(mild expression) C: **SA+ VE** (moderate expression) **D:VE + AA** (moderate expression) **E: SA +KV+AA**(moderate expression) **F: KV +AA**(mild expression)

30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression



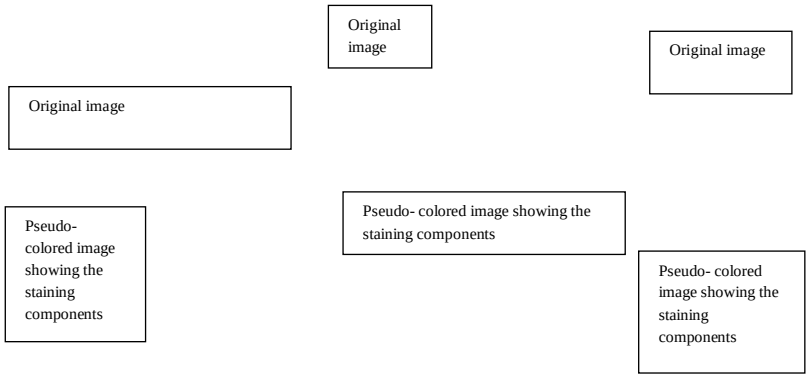
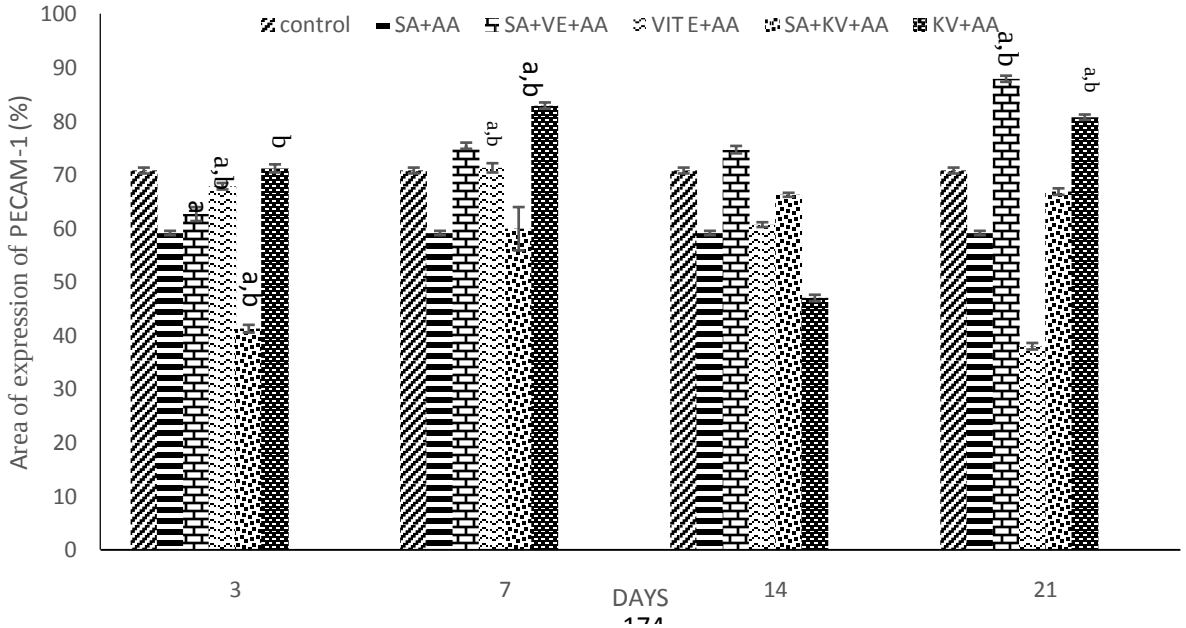


Plate 4.14: Effect of KV, VE on PECAM-1 expression in the SA exposed on day 21 post ulcer induction**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of PECAM-1, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **control** (mild expression) B: **SA exposed** (mild expression) C: **SA+ VE**(strong expression) **D:VE + AA** (moderate expression) **E: SA +KV+AA**(moderateexpression) **F: KV +AA**(mild expression)

30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression



4.6.5. EFFECT OF KOLAVIRON, VITAMIN E ON EXPRESSION OF KI67 IN SODIUM ARSENITE EXPOSED POST ULCER INDUCTION

Ki-67 expression was mildly expressed in the AA(control) as compared with the SA group. In the SA +VE+AA, there was mild expression(44.7%) on day 3 and day 14(49.1%) as compared with the control (59.5%) and SA+AA(55.6%). On day 14, it significantly increased to moderate expression(75.2%) and strong expression in day 21(87.9%) as compared with the control and SA +AA group.

In the VE +AA, the expression of Ki67 was mild on day 3(42.2%) and day 7(52.8%) as compared with the control and the SA +AA. The expression on day 14 and day 21 was not statistically different as compared with control and the SA +AA as shown in plate 4.15-4.18.

In the SA+KV group, there was mild expression on day 3 as copared with the control and the SA +AA group, $p<0.05$. There was significant increase on day 7-21 (strong to moderate expression) as compared with the SA +AA, control(AA) as shown in plate 4. 15 –4.18.

The rate of expression of Ki67 =SA+KV+ACIU>KV+ACIU>SA+VIT E +ACIU>VIT E +ACIU>CONTROL>SA+ACIU

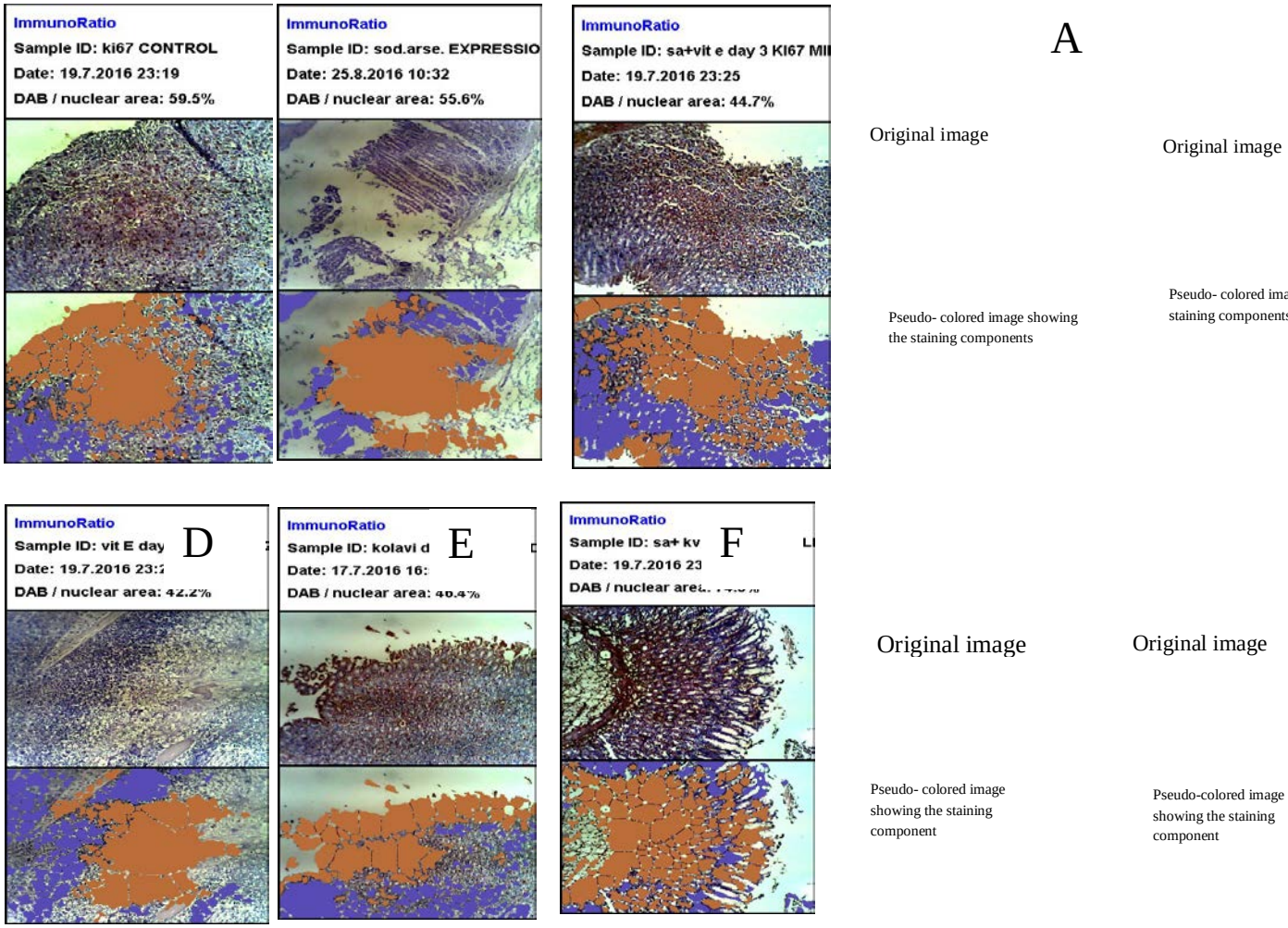


Plate 4. 15. Effect of KV, VE on Ki-67 expression in sodium arsenite exposed on day 3 post ulcer induction**.** **Original image:** shows the photomicrograph of immunostained tissue showing the expression of ki-67, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour

deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **control** (mild expression) B: **SA exposed** (mild expression) C: **SA+ VE**(mild expression) **D:VE + AA** (mild expression) **E: SA +KV+AA**(mild expression) **F: KV +AA**(moderate expression). 30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression

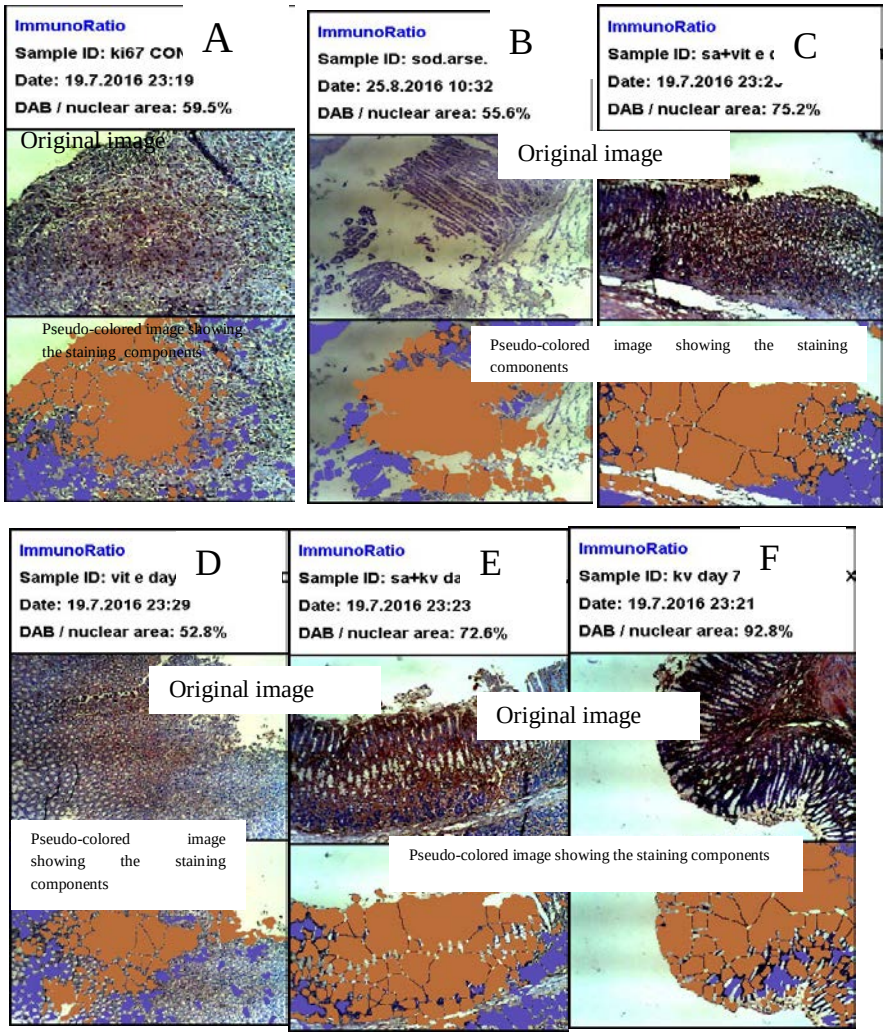


Plate 4.16. Effect of KV, VE on Ki-67 expressionin sodium arsenite exposed on day 7 post ulcer induction**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of Ki-67, pseudo

colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **control** (mild expression) B: **SA exposed** (mild expression) C: **SA+ VE**(mild expression) **D:VE + AA** (mild expression) **E: SA +KV+AA**(moderate expression) **F: KV +AA**(strong expression), 30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression

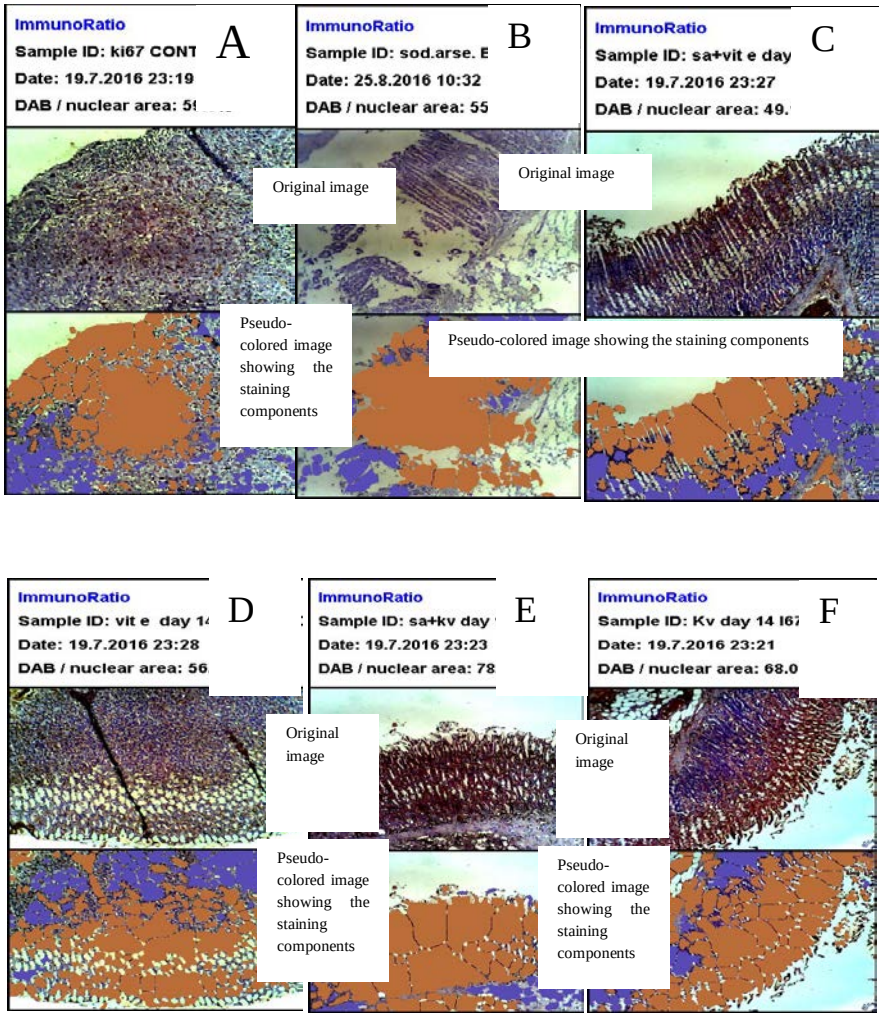


Plate 4. 17. Effect of KV, VE on Ki-67 expression in sodium arsenite exposed on day 14 post ulcer induction**.Original image:** shows the photomicrograph of immunostained tissue showing the expression of Ki-67, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **control** (mild expression) B: **SA exposed** (mild expression) C: **SA+ VE**(mild expression) **D: VE + AA** (mild expression) **E: SA +KV+AA**(moderate expression) **F: KV +AA**(moderate expression).30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression

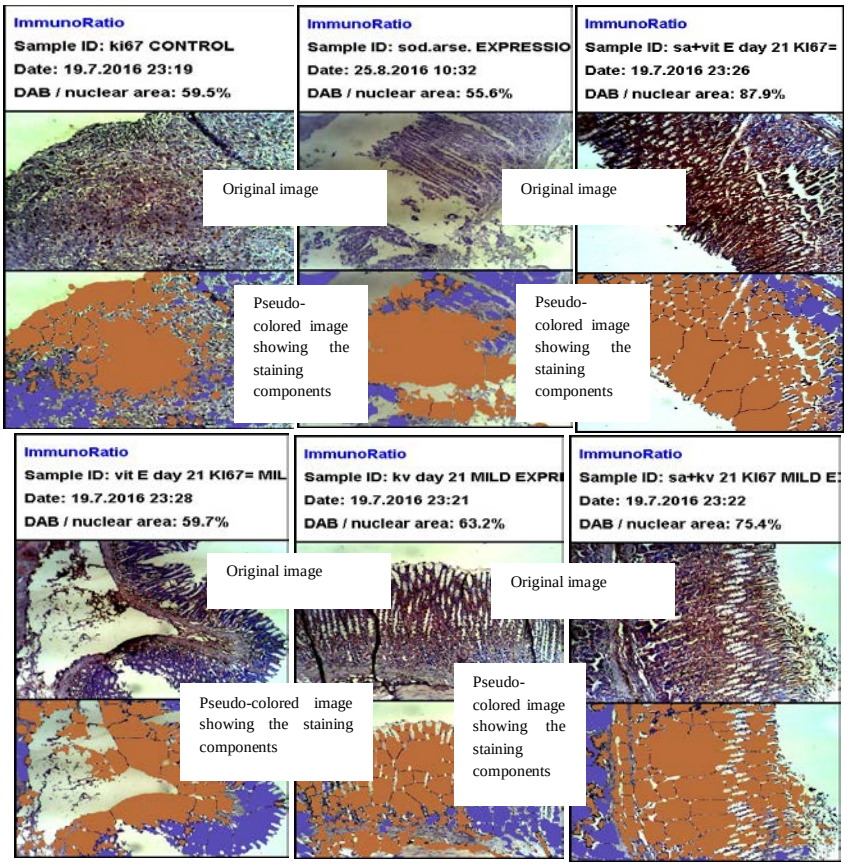


Plate 4.18.Effect of KV, VE on Ki-67 expressionin sodium arsenite exposed on day 21 post ulcer induction**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of Ki-67, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **control** (mild expression) B: **SA exposed** (mild expression) C: **SA+ VE**(moderate expression) **D: VE + AA** (mild expression) **E: SA +KV+AA**(moderate expression) **F: KV +AA**(moderate expression).30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression

4.6.6. EFFECT OF KOLAVIRON AND VITAMIN E ON P53 EXPRESSION IN SODIUM ARSENITE EXPOSED POST ULCER INDUCTION

There was significant increase in the P53 expression in the sodium arsenite exposed as compared with the control, $p<0.05$. VE treatment significantly decrease the expression of P53 on day 3(48.5%) and increase moderately on day and 14 and significantly increase on day 21 , however significantly reduced as compared with SA +AA group, $p <0.05$.

In the SA+KV +AA and KV +AA, there was moderate and mild expression of P53 as compared with the SA +AA group as shown in plate 4.19 -4.22.

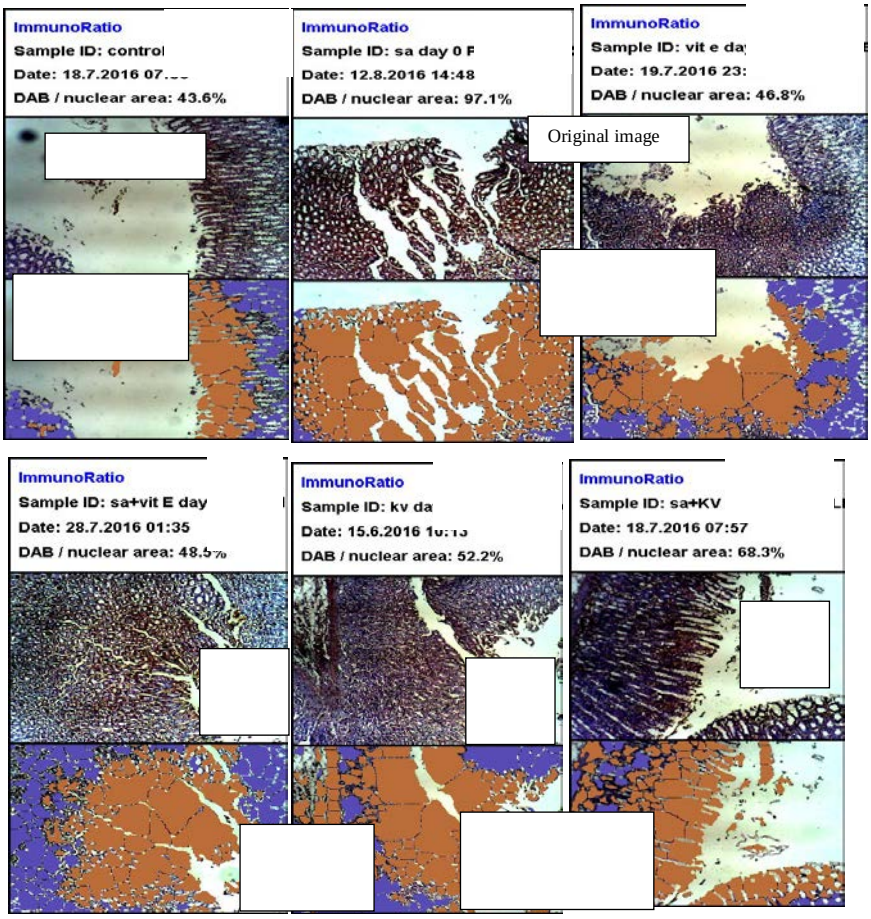


Plate 4.19: Effect of KV, VE on P53 expression in sodium arsenite exposed on day 3 post ulcer induction**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of P53, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **control** (mild expression) B: **SA exposed** (strong expression) C: **SA+ VE**(mild expression) **D: VE + AA** (mild expression) **E: KV +AA**(mild expression) **F:SA +KV+AA**(moderate expression30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression

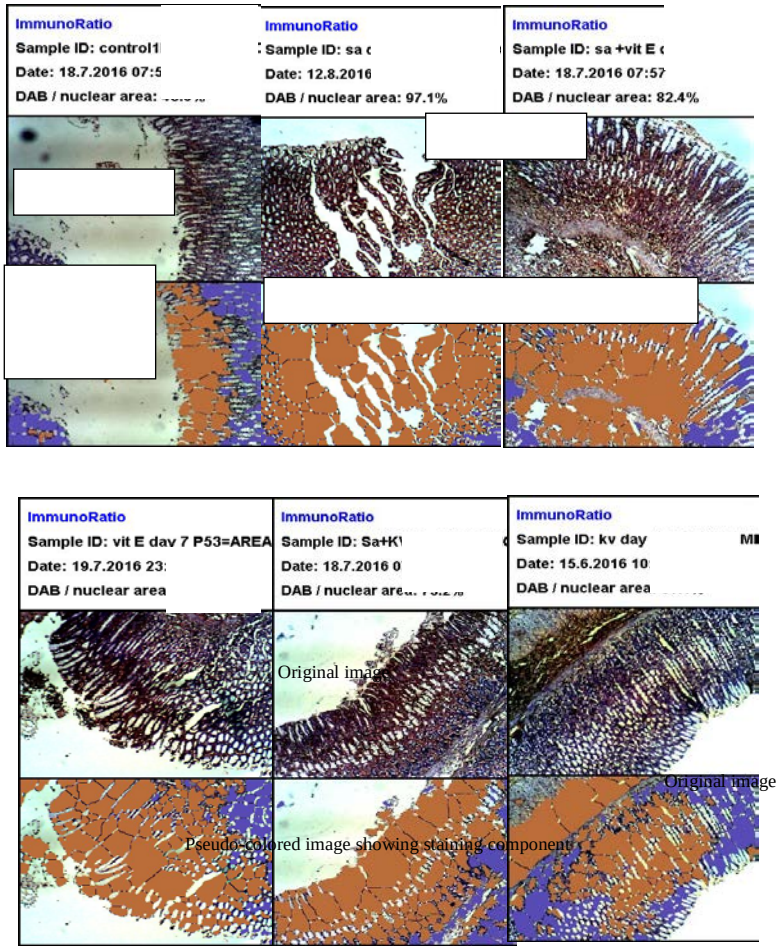
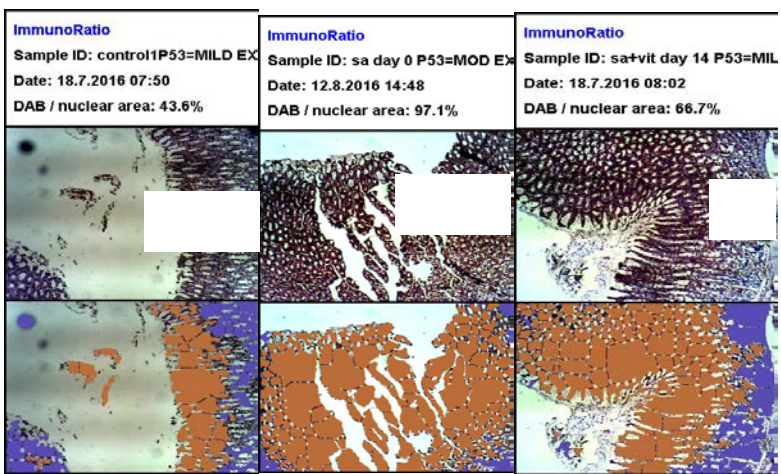


Plate 4.20: Effect of KV, VE on P53 expression inin sodium arsenite exposed on day 7 post ulcer induction**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of P53, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **control** (mild expression) B: **SA exposed** (strong expression) C: **SA+ VE**(moderateexpression) **D: VE + AA** (moderate expression) **E: SA +KV+AA**(moderate expression) **F: KV +AA**(mild expression).30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression



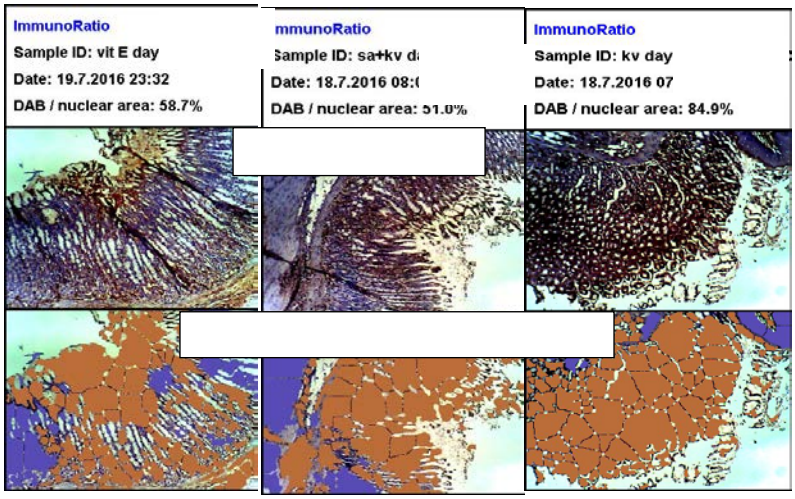
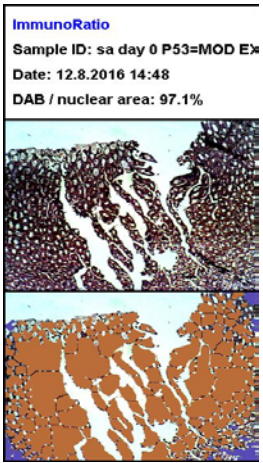


Plate 4. 21. Effect of KV, VE on P53 expressioinsodium arsenite exposed on day 14 post ulcer induction**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of P53, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **control** (mild expression) B: **SA exposed** (strong expression) C: **SA+ VE**(moderate expression) **D: VE + AA** (moderate expression) **E: SA +KV+AA**(mild expression) **F: KV +AA**(moderate expression).30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression



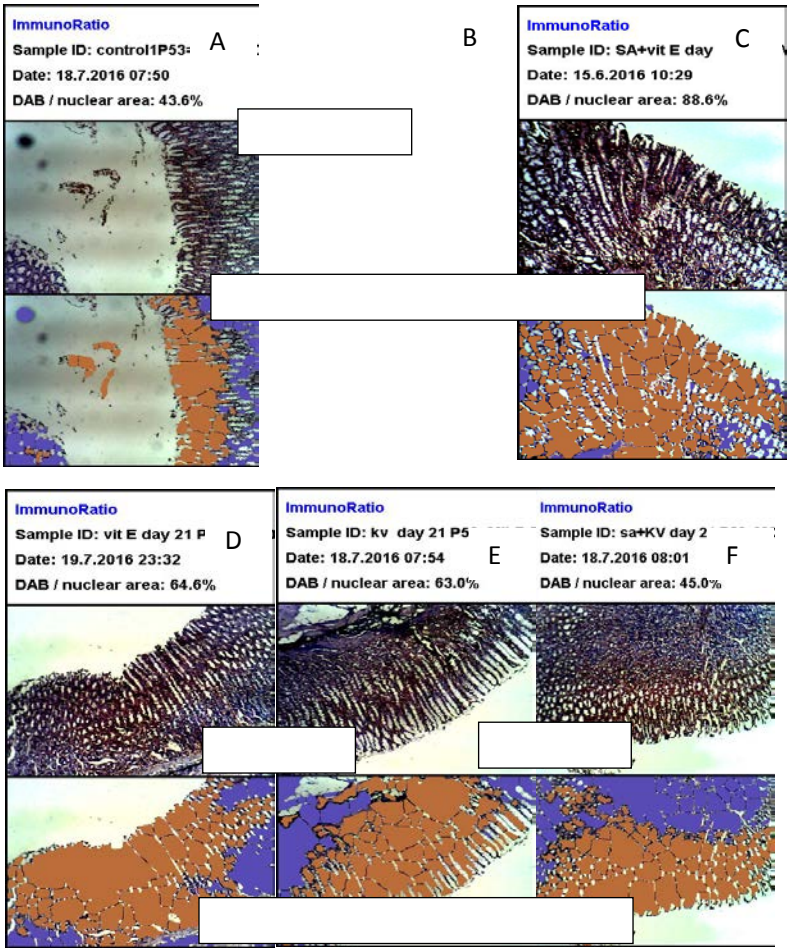


Plate 4.22: Effect of KV, VE on P53 expression in sodium arsenite exposed on day 21 post ulcer induction**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of P53, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **control** (mild expression) B: **SA exposed** (strong expression) C: **SA+ VE**(moderate expression) **D: VE + AA** (moderate expression)**E:) KV +AA (**moderate expression) **F: SA +KV+AA**(mild expression).30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression

PHASE V

4.7.0. EFFECT OF ZINC TREATMENT ON SODIUM ARSENITE EXPOSED POST ULCER INDUCTION

4.7.1. THE EFFECT OF ZINC ON TOTAL GASTRIC ACIIDITY SECRETION IN SODIUM ARSENITE EXPOSED POST ULCER INDUCTION

Total gastric acidity was significantly increased in the AA on day 3 as compared with the control on day 3, it significantly reduced on day 7 -21 as compared with the control. In SA +AA. There was significant decrease in the total gastric acidity from day 3- 21. In SA +AA, the total gastric acidity was significantly reduced from day 3-21 as compared AA and control. SA+ZN+AA sifgnificantly reduced the total gastric acidity as compared with the control, SA +AA, AA as shown in table 4.13.

Table 4.13.Effect of Sodium Arsenite and Zinc on total gastric acidity post ulcer induction

Grouping	**Day3(mEq/L)**	**Day 7 (mEq/L)**	**Day14 (mEq/L)**	**Day21 (mEq/L)**
Control	**1.3 ± 0.0**	**1.3 ± 0.0**	**1.3 ± 0.0**	**1.3 ± 0.0**
AA	**2.7 ±0.0*** **	**1.4 ±0.2*** **	**1.1 ±0.1*** **	**0.2 ± 0.0*** **
SA+AA	**0.1 ± 0.0** **	0.2 ±0.0**	**0.1** ±0.0**	**0.0** ±0.1**
SA+Aa+ZN	0.0 ±0.0**	0.0 ±0.0**	0.0±0.0	0.0±0.0**

All **values are mean ±SEM,P≤0.05,*** ** significant as compared with the control and ** ** significant as compared with AA**

4.7.2. THE EFFECT OF SODIUM ARSENITE AND ZINC ON ULCER AREA IN EXPERIMENTAL GASTRIC ULCER

In the AA group, there was significant increase in ulcer area on day 3 and this significantly reduced on day 7 to day 21. SA exposure significantly increase the ulcer area on day 3, day7 and day 21 as compared with the AA. Zn treatment significantly reduced the ulcer area oon day 3- day 21 as compared with the SA +AA, AA, $p < 0.05$ as shown in table 4.14.

4.7.3. EFFECT OF ZINC TREATMENT ON HISTOLOGY OF SODIUM ARSENITE EXPOSED MUCOSA POST ULCER INDUCTION

The histological staining reveal that there was severe and complete ulceration with necrosis, intense sub mucosa edema in SA +AA. Epithelial infiltration with some areas of atypical cells and glandular hyperplasia on day 3. There was mass cellular distruption with multi focal ulceration with hemorrhage from day 7- day 21.

In AA treatment, there was moderate cellular and glandular hyperplasia with mucosal ulceration on day 3, there was mild mucosal ulceration with moderate cellular /focal hyperplasia with infiltration of white cells/lymphocytes on day 3- 21 as compared with the AA.

In SA+AA+ZN treatment, there was complete ulceration with severe necrosis on day 3, with stroma edema with infiltration of inflammatory cells and necrosis on day 7-14. There was moderate to mild interglandular inflammation as compared with the AA and SA +AA as shown in Plate 4.23 and the summary as seen in table 4.16.

4.7.4. EFFECT OF ZINC ON EXPRESSION OF PECAM-1 OF SODIUM ARSENITE EXPOSED STOMACH POST ULCER INDUCTION

In the AA, PECAM-1 (CD31) expression significantly increase on day 3, on day 7 and 14, there was significant moderate expression and on day 21, mild expression was observed (as shown in plate 4.24).

In the SA+AA, there was mild expression of CD31 on day 3, 14 and 21 as compared with the SA+AAgroup (as shown in plate 4.25). Treatment with Zn significantly increased the expression as compared with the SA+AA, and AA group as shown in plate 4.26.

Table 4.14: Effect of Zinc on ulcer area in sodium arsenite exposed stomach post ulcer induction.

GROUPS	3days(cm^2)	7days(cm^2)	14days(cm^2)	21days(cm^2)
AA	0.7 ±0.00	0.5±0.0	0.7±0.3	0.1±0.0
SA+AA	1.0 ±0.1***	0.7±0.0***	0.3± 0.0***	0.7±0.0***
SA+Zn+AA	0.6 ±0.0**	0.5±0.0**	0.5 ±0.1***	0.4±0.0**

All values are mean ±SEM, P<0.05, *** significant as compared with AA, ** - significant as compared with the SA+AA.

TABLE 4.15: qualitative result showing effect of sodium arsenite and zinc treatment on the gastric mucosa (H&E stains of the stomach section)

	Day 3	Day 7	Day 14	Day 21
AA	Mucosal ulceration Moderate cellular Glandular hyperplasia	Mild Mucosal ulceration Moderate cellular /focal hyperplasia Infiltration of white cells/lymphocytes	Surface epithelial erosion ,infiltration by inflammatory cells and stroma inflammation ,mild necrosis	Mild ulceration
SA+AA	Complete mucosa ulceration extending through the lamina propria with Severe / extensive cellular necrosis. Intense sub mucosa edema Epithelial infiltration with some areas of atypical cells and glandular hyperplasia	Mass cellular disruption/ diffrentiation Multi foci ulceration and necrosis	Severe cellular necrosis with hyperplasia ,intense infiltration and appearance of atypical cells	Moderate cellular necrosis with hemorrhage.presence of atypical cells
SA+AA +ZN	Complete ulceration with severe necrosis	Stroma edema with infiltration and necrosis	Intense infiltration and moderate ulceration	Moderate to mild interglandular inflammation

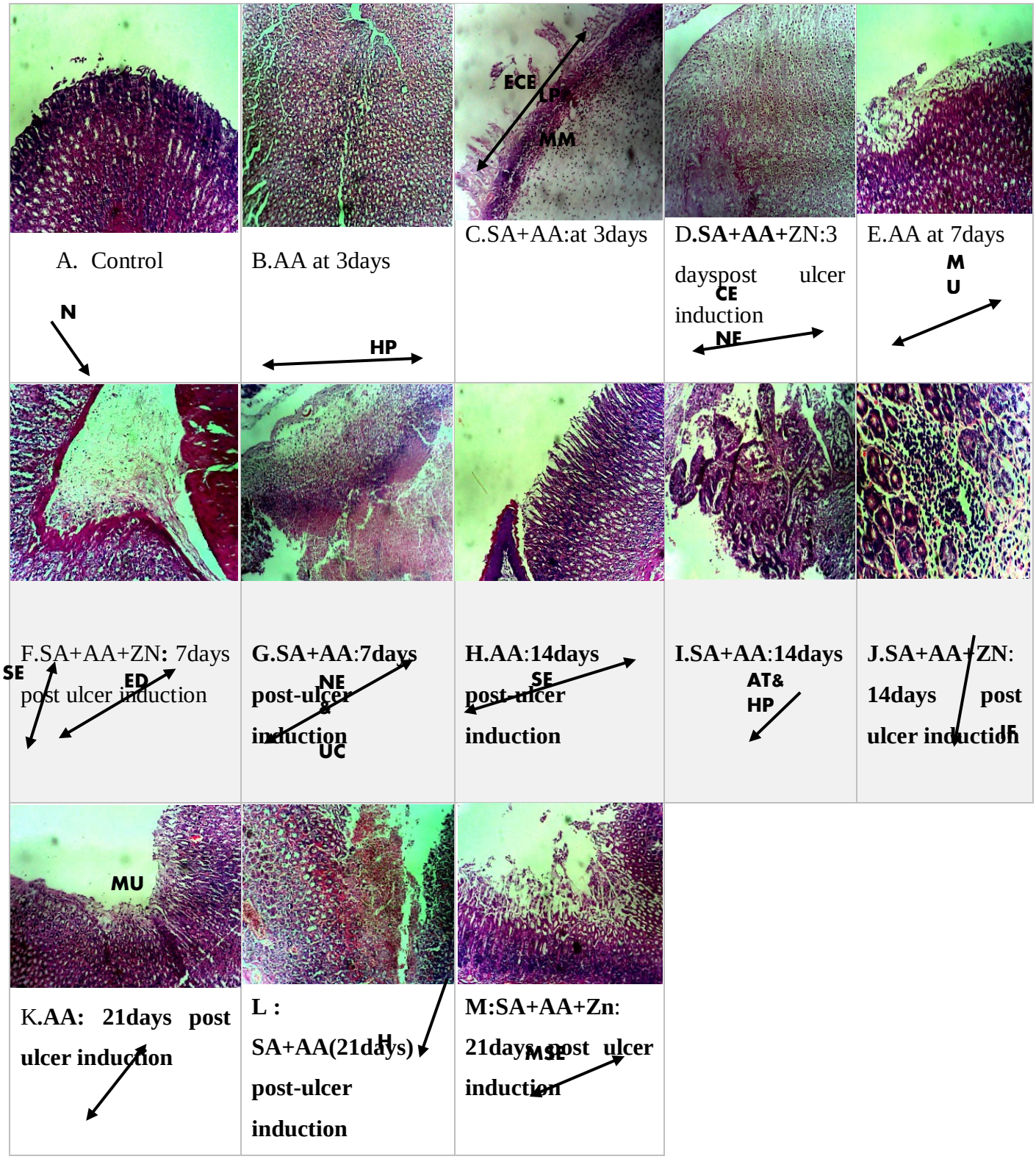


Plate 4.23 :The histology of stomach exposed to SA and treated with the Zn MU: Mild ulceration, AT: atypical cells, H:hemorrhage, MSE: mild surface erosion/ulceration, IF: infiltration of inflammatory cells, ED: edema, NE: Necrosis, HP: hyperplasia, ECE: extensive and complete erosion/ulceration, CE: complete erosion /ulceration, MM:

muscularis mucosae, LP: lamina propria, N :neck of gastric glands, SA+ AA(sodium arsenite exposed +acetic induced ulcer). AA-acetic induced group, SA+ZN+AA-sodium arsenite exposed +acetic induced ulcer +zinc

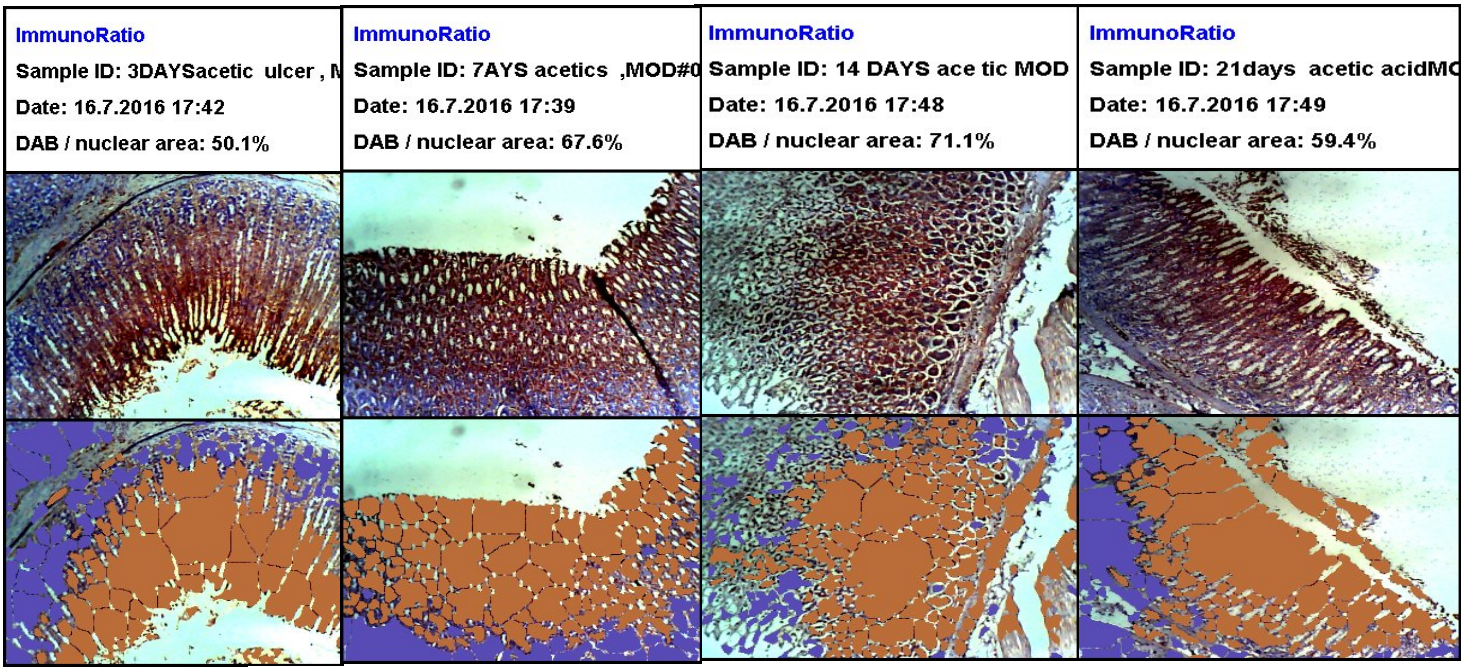


Plate 4.24. Ef...A on PECAM-1 expres... e gastric mucosa of the a... duced with acetic acid induced ulceration on day 3- day 21**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of PECAM-1, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) ...econvolution alogrithms separating the counterst... the DAB precipitate (area of ...e colour- H& E counterstain. **A**: **AA day 3**(mil... **AA day 7** (strong expression) C: **AA day 14**(moderate expression) **D: AA day 21** (mild expression)**.** 30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression

Original image

Original image

Pseudo-colored image showing the staining component

Pseudo-colored image showing the staining component

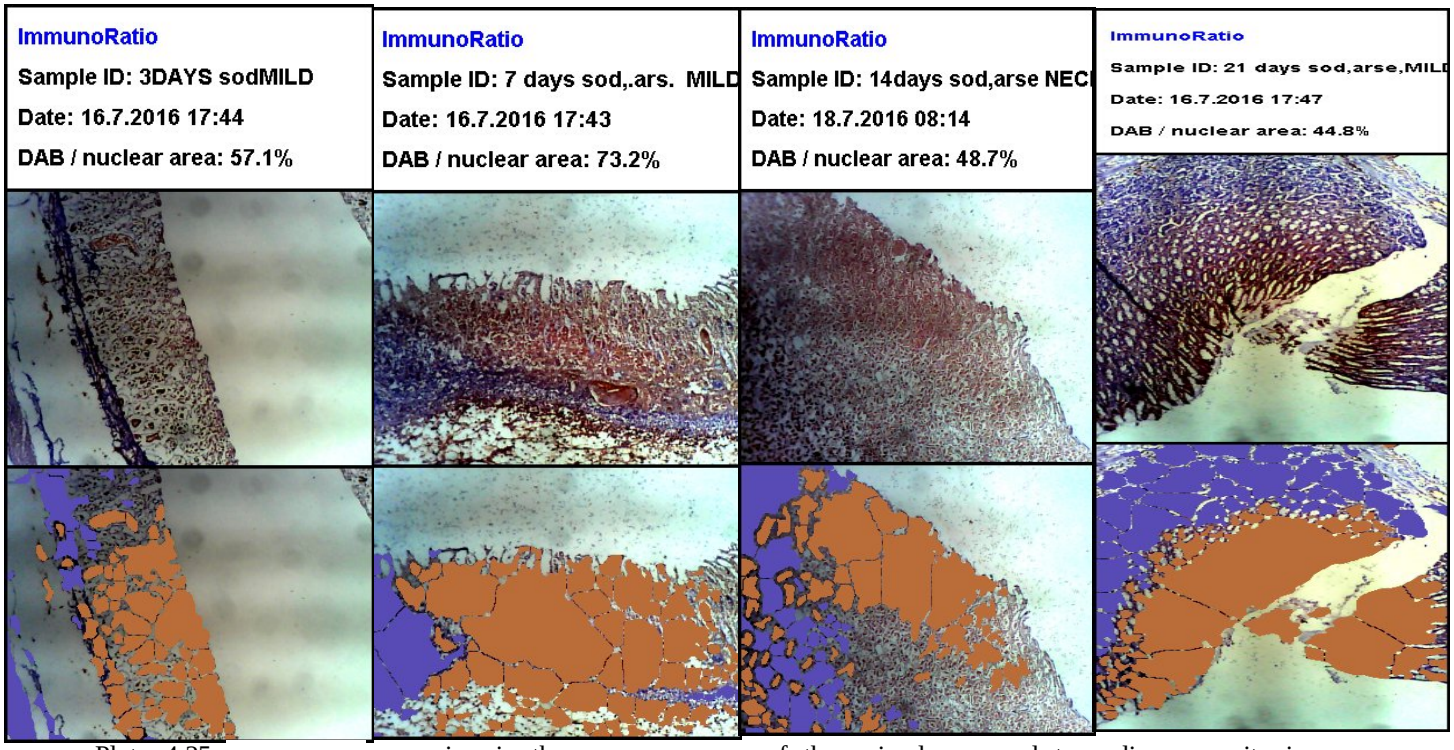


Plate 4.25: expression in the a of the animals sodium arsenite in experimenta er on day 3- day 2 **age:** shows the pho n of immunostained tissue showing the expression of PECAM-1, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **SA +AA day 3** (mild expression) B: SA +**AA day 7** (n **AA day 14** (moderate expressio **ay 21** (mild expression)**.** 30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - s

Original image

Original image

Pseudo- colored image showing staining component

Pseudo-colored image showing staining component

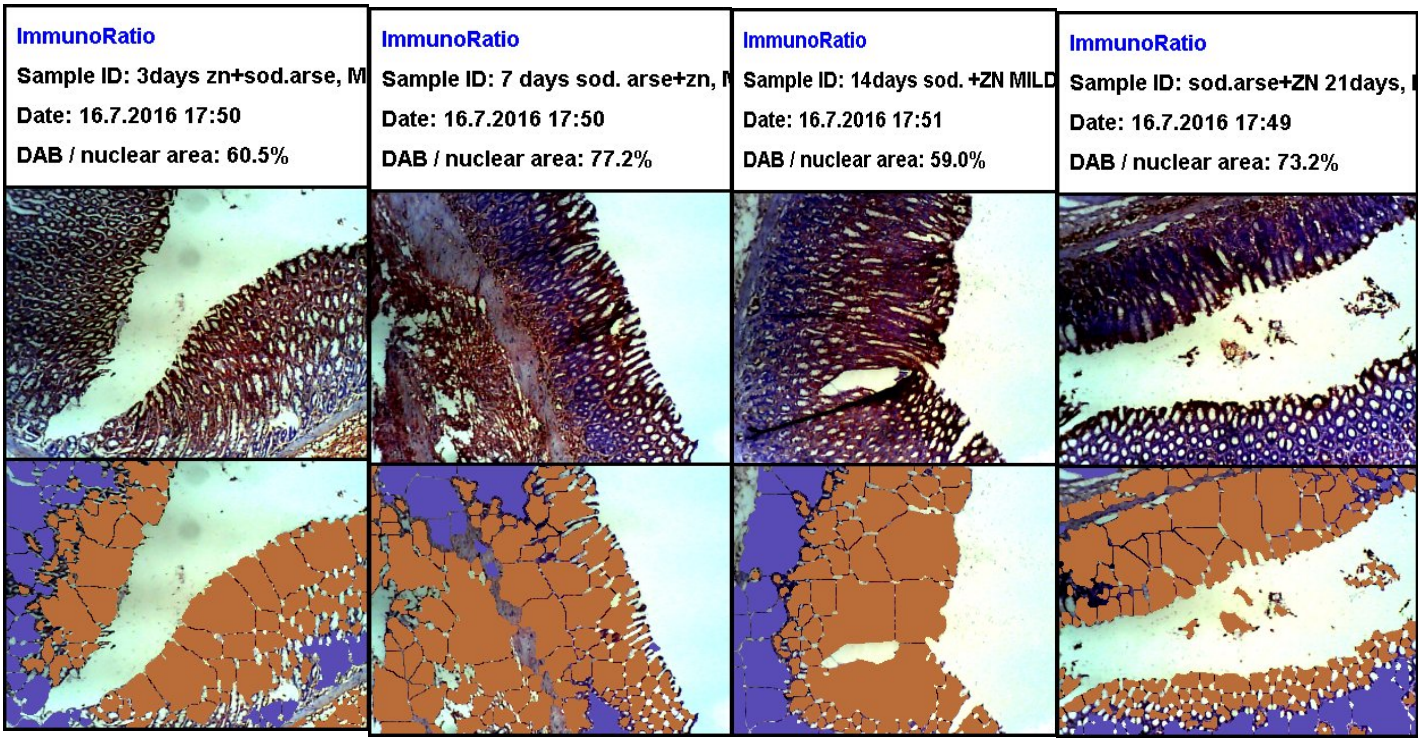


Plate 4.26: PECAM ɔression in the gastric mucosa of the animals exposed to sc arsenite and treated with zinc in experimental gastric ulcer on day 3- day **Original image:** show photomicrograph of immunostained of PECAM-1, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- A + Zn+ **AA day 3**(moderate expression) B: SA +Zn+ **AA day 7** (moderate expression) C: SA+Zn +**AA day 14** (mild expression) **D: SA+Zn + AA day 21** (moderate expression)**.** 30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression

4.7.5. EFFECT OF ZINC ON EXPRESSION OF FACTOR VIII(FACTOR 8) OF SODIUM ARSENITE EXPOSED STOMACH IN EXPERIMENTAL GASTRIC ULCER

Factor VIII expression in the gastric mucosa sections following immunohistochemical staining was significant increased in the AA group on 3 – 21(moderate to strong expression) (plate 4.27). In SA +AA exposed, there was significant decrease in the factor VIII expression as compared with the AA group on day 3, 7 and 21 with moderate expression on day 14(plate 4.28). Treatment with Zn significantly increase the expression of the factor VIII from day 3-21 as compared with the SA +AA, $p<0.05$, (as shown in plate 4.29).

4.7.6. EFFECT OF ZINC ON EXPRESSION OF KI67 OF SODIUM ARSENITE EXPOSED STOMACH IN POST ULCER INDUCTION

The intensity of Ki67 expression in the gastric mucosa sections following immune histochemical staining in AA was significantly reduced on day and significantly increase opn day 21 but the expression was mild as shown in plate 4.30.

In the SA+AA group, there was significant reduction in ki-67 expression as compared with the AA group on day 3- 21(plate 4.31). Treatment with Zn significantly increase the expression of ki-67 on day 3 (moderate expression) as compared with the AA and SA +AA. There was mild expression on day 7- 21 as shown in plate 4.32.

4.7.7. EFFECT OF ZINC ON EXPRESSION OF EGFR OF SODIUM ARSENITE EXPOSED STOMACH POST ULCER INDUCTION

EGFR expression was significantly high on day 3 and reduces on day 7 and significantly increases on day 14 - day 21 in the AA group (as shown in plate 4. 33). In the SA+AA, there was increase in percentage expression in EGFR on day 3 and reduced on day 7-day 14 and increase on day 21(as shown in plate 4.34) .There was significant reduction as compared with the AA group, $p<0.05$. In the SA+Zn+AA group, there was significant increase in the expression of EGFR on day 3-21 as compared with the AA and SA +AA, $p <0.05$ (as shown in plate 4.35).

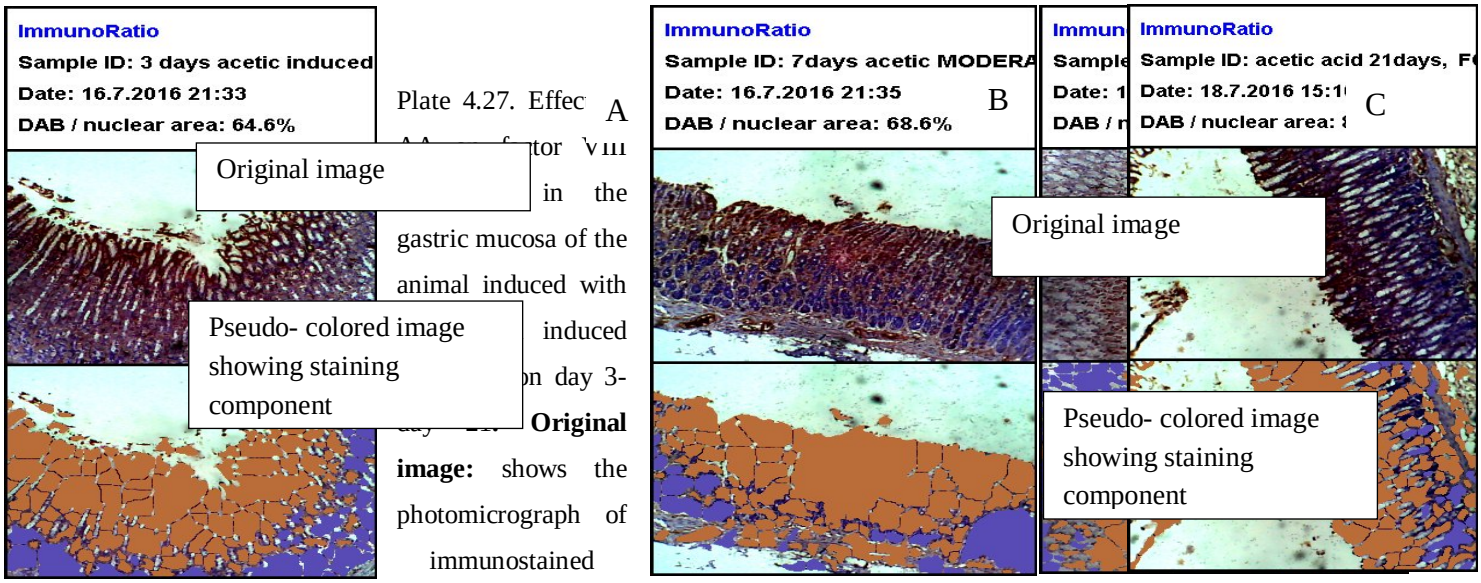


Plate 4.27. Effec A ctor VIII in the gastric mucosa of the animal induced with induced n day 3- **Original image:** shows the photomicrograph of immunostained tissue showing the expression of factor VIII, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **AA day 3**(moderate expression) B: **AA day 7** (moderate expression) C: **AA day 14** (moderate expression) **D: AA day 21** (strong expression)**.** 30-59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression

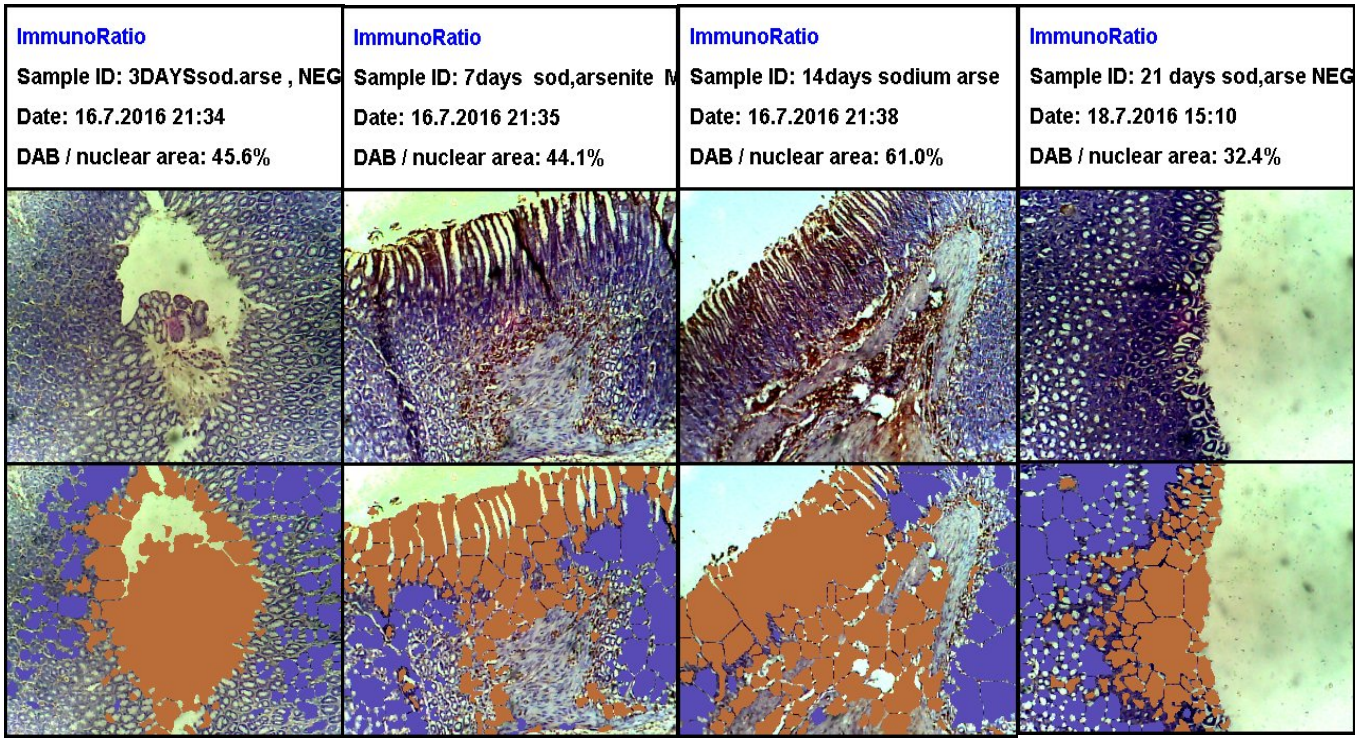


Plate 4.28. Facto VIII expression in the gastric isa of the animals ex to sodium arsenite in experimental gast er on day 3- day 21**. Origi age:** shows the photo graph of immunostained tissue showing the expression of Factor VIII, pseudo colored image: show the percentage of positively stained nuclear are deconvolution alogrithms separating t the DAB pr lue colour- H& E counterstain. **A**: **SA +AA day 3**(mild expression) B: SA +**AA day 7** (moderate expression) C**: SA**+**AA day 14** (moderate expression) **D: SA+ AA day 21** (mild expression)**.** 30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression

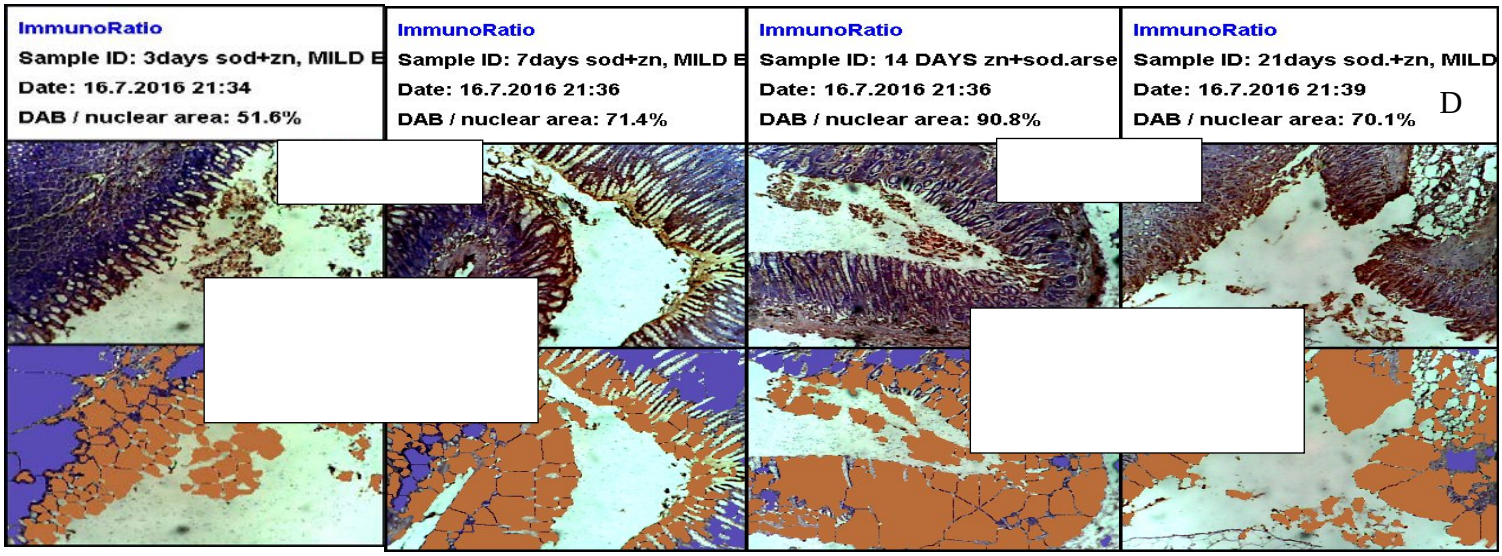
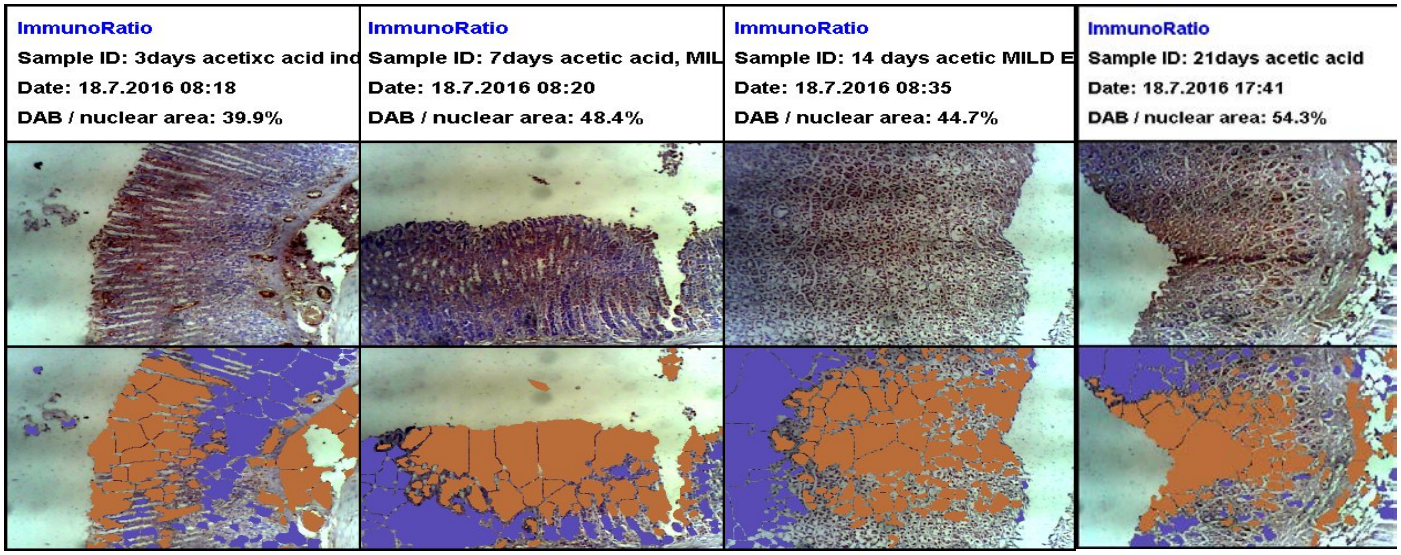


Plate 4.29. Factor VIII expression in the gastric mucosa of the animals exposed to sodium arsenite and treated with Zn in experimental gastric ulcer on day 3- day 21**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of factor VIII, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **SA +ZN+AA day 3**(mild expression) B: **SA +ZN+AA day 7** (moderate expression) C**: SA+ZN**+ **AA day 14** (strong expression) **D: SA+ZN+ AA day 21** (moderate expression)**.** 30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong express



A B C D

Plate 4.30. Ki-67 e[redacted] on in the gastric mucosa of the animals induced gastric ulcer with acetic acid on day 3-day 21**. Original image:** [redacted] ograph of immunostained tissue showing the expression of Ki-67, pseudo colored [redacted] of positively stained nuclear area (lal[redacted] colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **AA day 3**(mild expression) B: **AA day 7** (mild expression) C**: AA day 14** (mild expression) **D: AA day 21** (mild expression)**.** 30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - s[redacted]

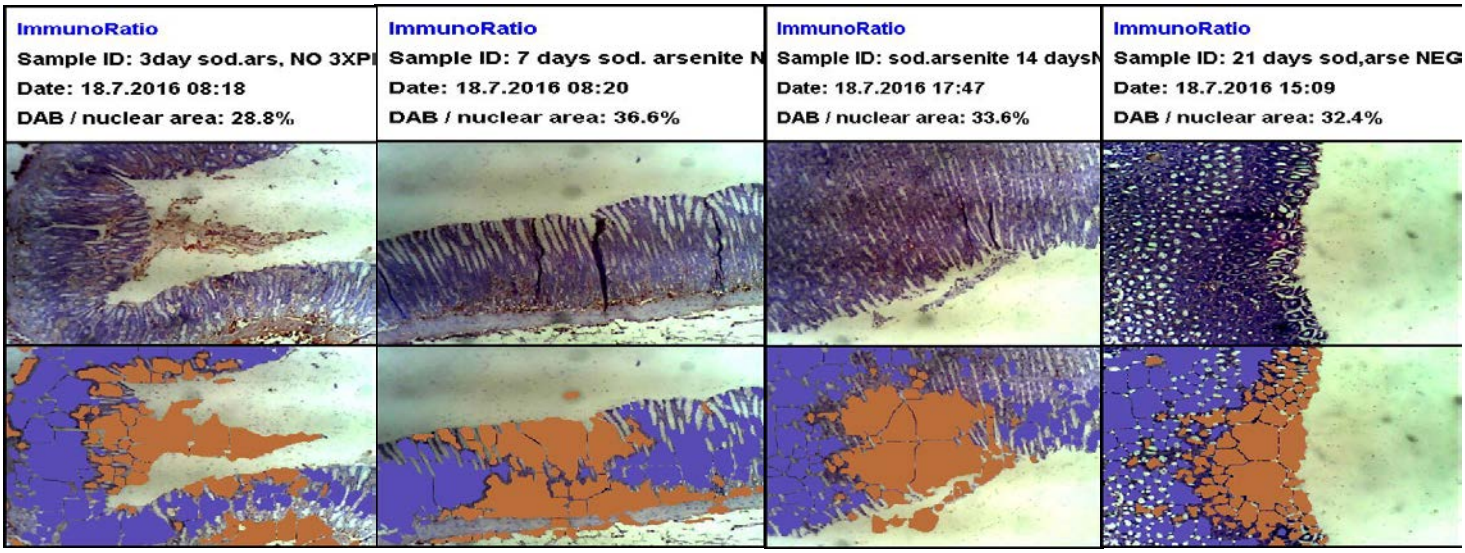


Plate 4.31: Ki-67 expression in the gastric mucosa of the animals exposed to sodium arsenite in experimental gastric ulcer on day 3- day 21**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of K d image: show the percentage of positively stained nuclear area (labeling index) using colou grithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **SA +AA day 3**(mild ex **A day 7** (mild expression) C**: SA**+ **AA day 14** (mild expression) **D: SA+ AA day 21** (mild expression) 30-59% -mild expressio on, 80-100% - strong expression

Original image

Original image

Pseudo-colored image showing staining component

Pseudo-colored image showing staining component

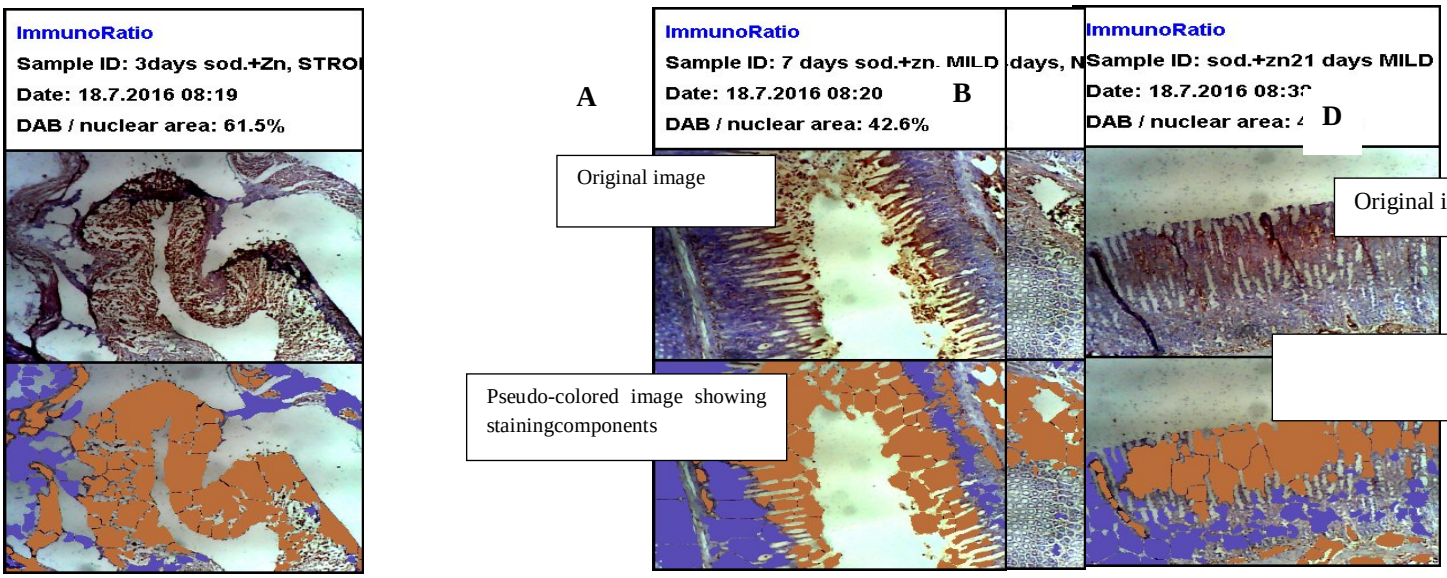


Plate 4.32: Ki-67 expression in the gastric mucosa of the animals exposed to sodium arsenite and treated with zinc in experimental gastric ulcer on day 3- day 21**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of Ki-67, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **SA +AA +ZN day 3**(moderate expression) B: **SA +Zn+ AA day 7** (mild expression) C**: SA**+**ZN+AA day 14** (mild expression) **D: SA++ZN+AA day 21** (mild expression)**.** 30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression

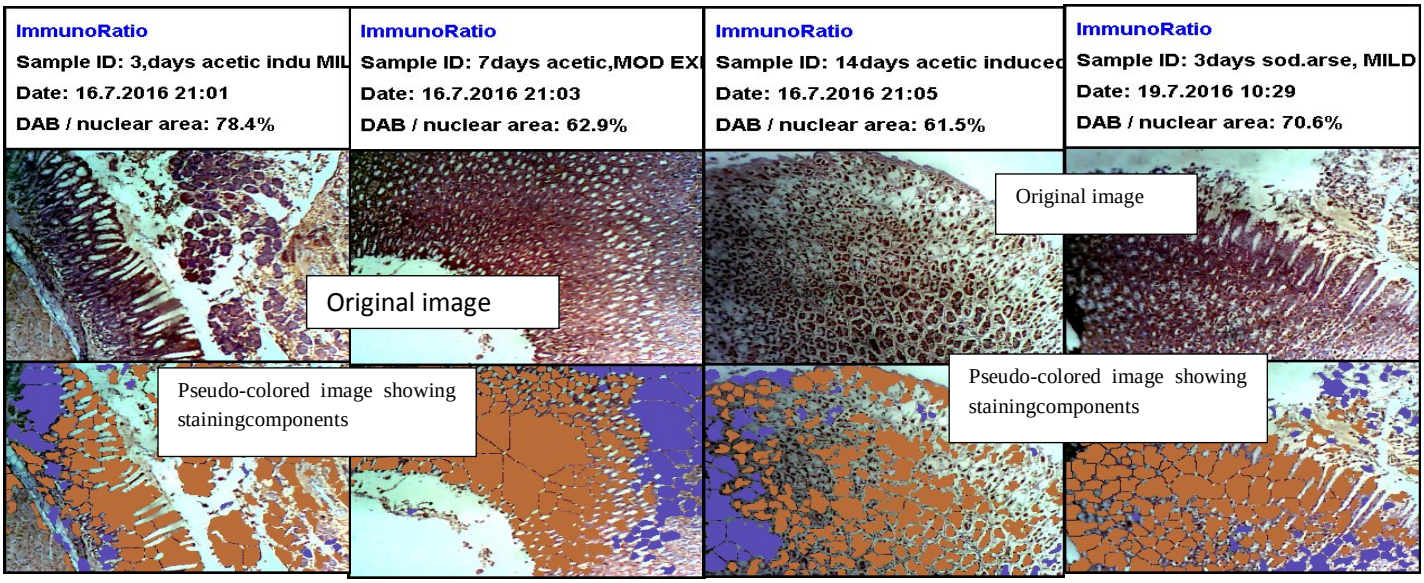


Plate 4.33: Effect of AA on EGFR expression in the gastric mucosa of the animals on day 3- day 21**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of EGFR, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **AA day 3**(moderate expression) B: **AA day 7** (moderate expression) C**: AA day 14** (moderate expression) **D: AA day 21** (moderate expression)**.** 30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression.

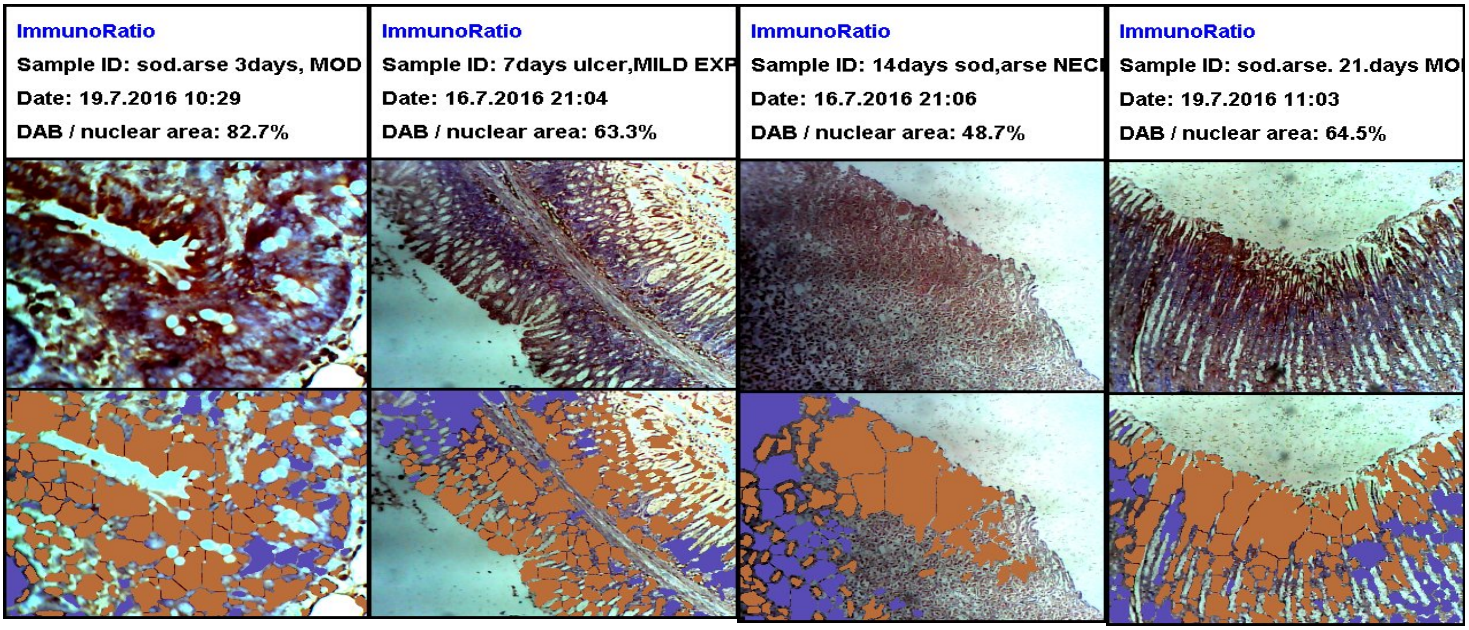


Plate 4.34: EGFR expression in the gastric mucosa of the animals exposed to sodium arsenite on day 3- day 21 in experimental gastric ulcer**. Original image:** shows the photomicrograph of immunostained tissue showing the expressio mage: show the percentage of positively st (labeling index) usi grithms separating the counterstain. Brown recipitate (area of expression), blue colour- H& E counterstain. **A**: SA+**AA day 3**(strong expression) B: **SA+AA day 7** (moderate expression) C**: SA+AA day 14** (mildexpression) **D: SA+ AA day 21** (moderate expression)**.** 30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression

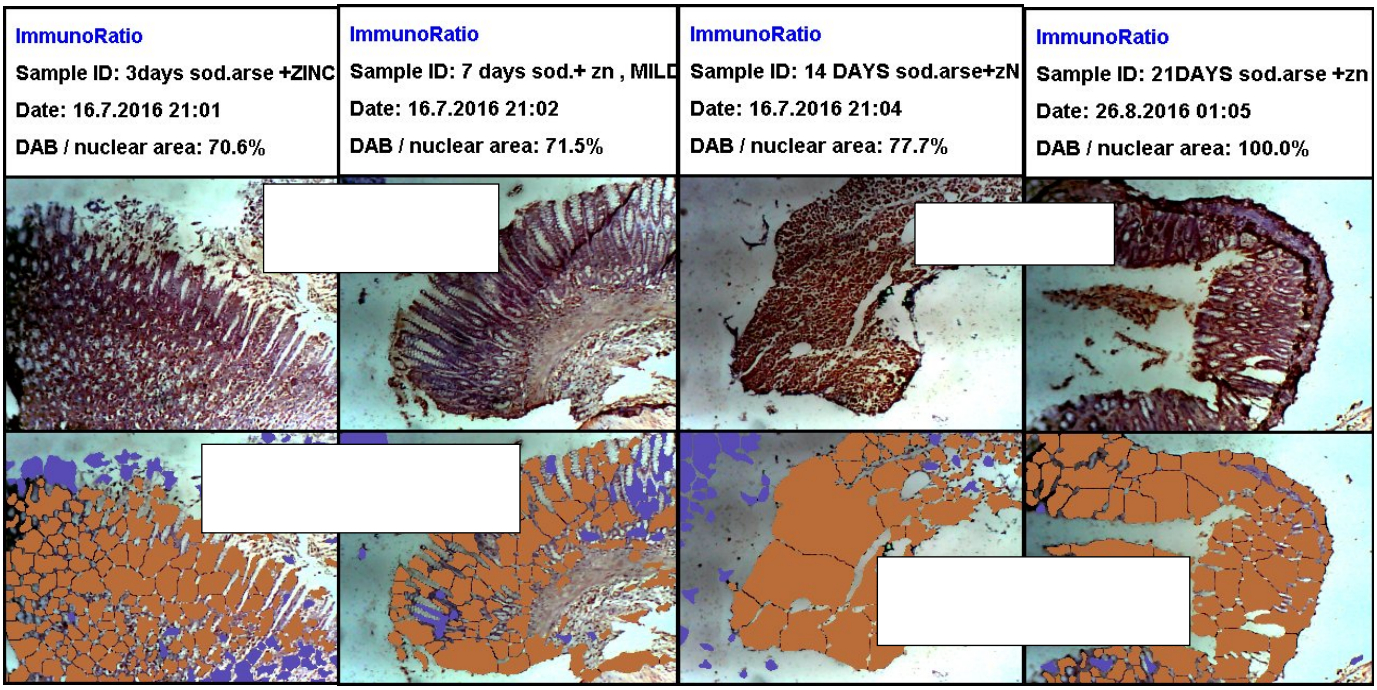


Plate 4.35: EGFR expression in the gastric mucosa of the animals exposed to sodium arsenite and treated with Zn on day 3- day 21 in experimental gastric ulcer**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of EGFR, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: SA+Zn+**AA day 3**(moderate expression) B: **SA+Zn+AA day 7** (moderate expression) C**: SA+ Zn+AA day 14** (moderate expression) **D: SA+Zn+AA day 21** (strong expression)**.** 30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression

4.7.8. EFFECT OF ZINC ON EXPRESSION OF P53 ON SODIUM ARSENITE EXPOSED STOMACH IN EXPERIMENTAL GASTRIC ULCER

The expression of P53 in the gastric mucosa sections following immunohistochemical staining was significantly decrease in the AA group from day3- 7 and moderately expressed on day 7, 14, and day 21(plate 4.36). Exposure to SA+AA significantly increase the rate of expression from day 3-21 as compared with the AA group (as shown in plate 4.37). Administration with Zn significantly decrease the expression of P53 in the stomach mucosa as shown in plate 4.38.

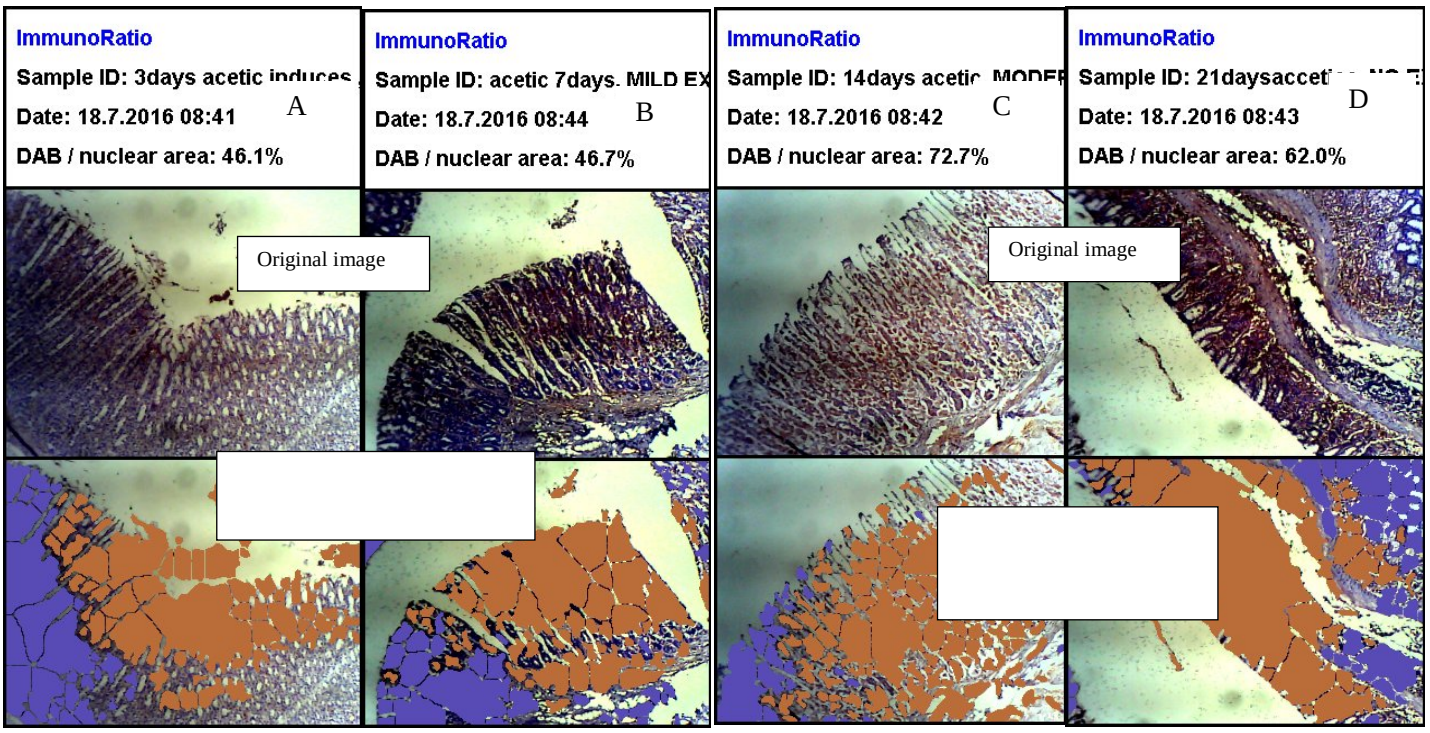


Plate 4.36. Effect of AA on P53 expression in the gastric mucosa of the animals exposed post ulcer induction on day 3- day 21. **Original image:** shows the photomicrograph of immunostained tissue showing the expression of P53, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: **AA day 3**(mild expression) B: **AA day 7** (mildexpression) C**:AA day 14** (moderate expression) **D: AA day 21** (moderate expression)**.** 30- 59% -mild expression, 60-79 %- moderate expression, 80-100%

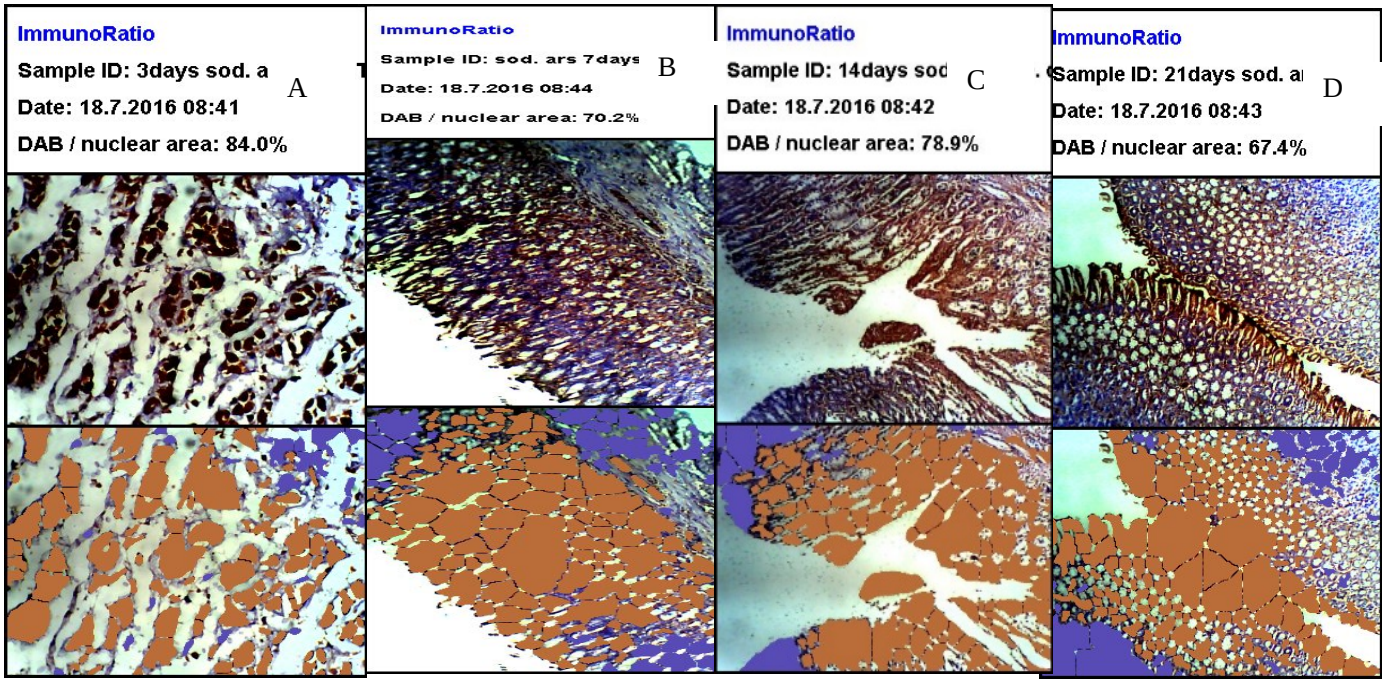


Plate 4.37: P53 expression in the gastric mucosa of the animals exposed to sodium arsenite on day 3- day 21 in experimental gastric ulcer**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of P53, pseudo colored image: show the percentage of positively stained nu g index) using colour deconvolut ing the counterstain. Brown colour- the (area of expression), blue colour- H& E counterstain. A: SA+**AA day 3**(strong expression) B: **SA+AA day 7** (moderate expression) C**: SA+AA day 14** (moderate expression) **D: SA + AA day 21** (moderate expression)**.** 30- 59% - mild expression, 60-79 %- moderate expression, 80-100% - strong expression

Original image

Original image

Pseudo-colored image showing stainingcomponents

Pseudo-colored image showing stainingcomponents

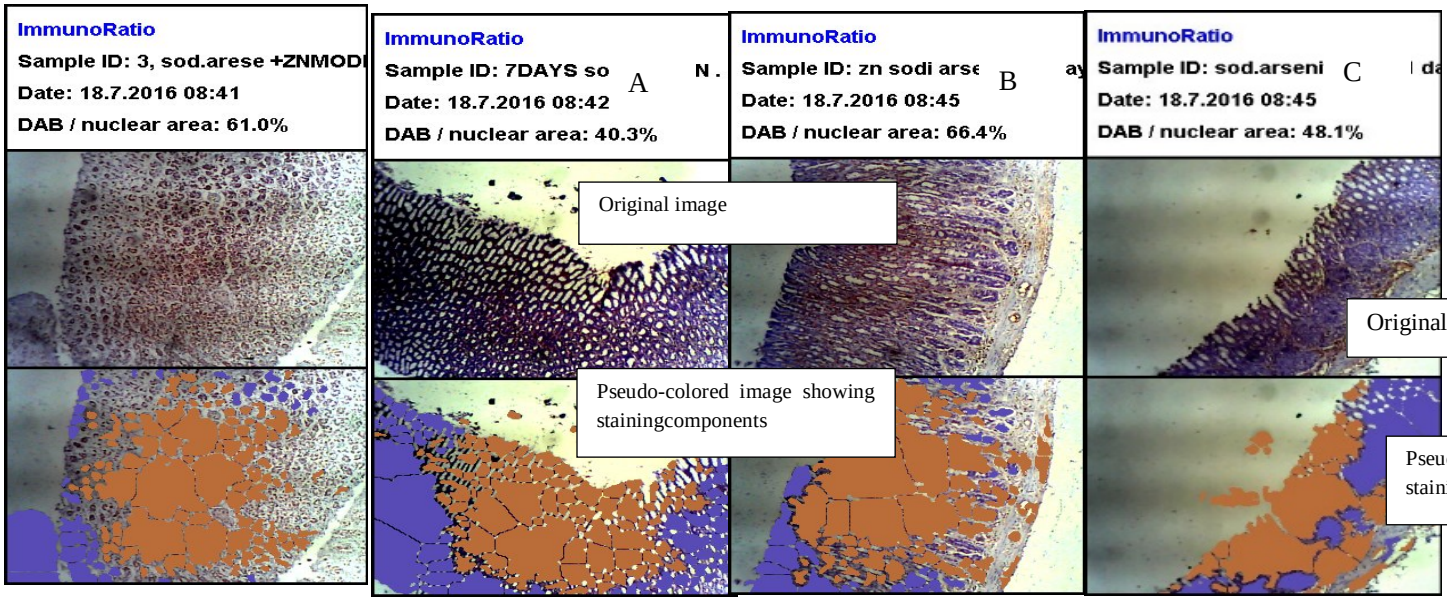


Plate 4.38. P53 expression in the gastric mucosa of the animals exposed to sodium arsenite and treated with Zn on day 3- day 21 in experimental gastric ulcer**. Original image:** shows the photomicrograph of immunostained tissue showing the expression of P53, pseudo colored image: show the percentage of positively stained nuclear area (labeling index) using colour deconvolution alogrithms separating the counterstain. Brown colour- the DAB precipitate (area of expression), blue colour- H& E counterstain. **A**: SA+Zn+**AA day 3**(moderate expression) B: **SA+Zn+AA day 7** (mild expression) C**: SA+ Zn+AA day 14** (moderate expression) **D: SA+Zn+AA day 21** (mild expression)**.** 30- 59% -mild expression, 60-79 %- moderate expression, 80-100% - strong expression

CHAPTER FIVE

DISCUSSION

5.1.0: TOXICITY OF SODIUM ARSENITE ON EXPERIMENTAL INDUCED GASTRIC ULCER

Studies have shown that toxic trace element such as arsenic acid affects the gastrointestinal tract(GIT)epithelium before proceeding to its other targets organs(Vasquez *et al.,* 2015) hence its toxicity on the GIT cannot be underestimated. There has not been detailed reports on its effectson both normal states and disease states of the gastrointestinal tract. In this study, we dealt with the effect of sodium arsenite exposure on chronic experimentally induced gastric ulcer. It was observed that sodium arsenite increases the ulcer formation and delayed the process of gastric ulcer healing on day 3 –day 21.

Arsenic acid is an environmental and occupational toxins that human are exposed to especially via drinking of contaminated water and food (especially food cooked with contanminated water)(ESPA,2005a). Exposure to sodium arsenite orally represents the major route of exposure. Previous reports suggested that action of the arsenic acid starts in the gastrointestinaltract epithelial cells, modulating their chemical reaction by increasing oxidative reactions;this indicates its high risk of causing inflammation/damages the GIt mucosa (Saxena *et al*., 2009, Calatayud *et al.*, 2013).

The luminal application of acetic acid in the rat's stomach induced a chronic experimental gastric ulcer which resembles the type of human peptic ulcer both interm of ulcer formation,healing and histology(Okabe and Amagase,2005). This makes a right and good choice of chronic gastric ulcer model,its process of gastric ulcer induction is via increase in volume of luminal acid and mucosal necrosis. Gastric ulcer is caused by an imbalance between the defensive mucosal factors (like glycoprotein proliferation, and antioxidants such as catalase, superoxide dismutase and glutathione level) and the offensive factors such (like increased gastric acid secretion, pepsin secretion, lipid peroxidation) (Prabha *et al.*, 2009). Application of acetic acid caused the ulcer in the mucosa of the stomach which was further increase with the administration of both 5mg/kg and 10mg/kg of sodium arsenite on day 3-day 21. In the Phase I of this study, Sodium arsenite increased the ulcer area in the stomach mucosa as compared with the acetic acid induced ulcer group. The mechanism of action of arsenic acid is usually via increased in oxidative stress. Oxidative stress is one of the etiology

of gastric ulcer (Tandon, 2004). It is therefore suggested the ulceration was increased by increased oxidative stress caused by arsenite in the stomach mucosa.

Total gastric acidity was initially increased in the acetic acid induced ulcer group initially on day 3 and reduced progressively on day 7-day 21 . In sodium arsenite exposed group, the total gastric acidity was significantly reduced with a significant increase in the ulcer area as observed on day 3-day 21.Studies have shown that the treatment modality for gastric ulcers thereby involves mucosal defence and reduction of acid secretion(Wallace,2005). In this study, the decrease in the total gastric acidity observed in the sodium arsenite does not improve/reduce ulcer formation but rather increase the ulcer formation. It has been evaluated that arsenic is usually transformed in the acidic phase of the stomach (Calatayud *et al.,* 2013, Zhao *et al.,* 2014), there is enhancement of redox reaction of arsenic acid. This helps to induce its toxicity in the stomach mucosa (Vazquez *et al.,* 2015). Arsenic acid is also known for damaging mitochondria of cell, DNA and increases cell death as well (Jiang *et al.,* 2009, Chen *et al.,* 2004, Kirkpatrick *et al.,* 2003, Huang *et al*., 1999). It is therefore suggested that sodium arsenite increases ulceration by increased oxidative reaction and damaging gastric acid secreting cells.

5.1.2.PHASE II: EFFECT OF SODIUM ARSENITE EXPOSURE ON ULCER HEALING IN EXPERIMENTAL GASTRIC ULCER

In phase II of this study , there was marked delay in the ulcer healing process in the sodium arsenite exposed as compared with the acetic acid induced ulcer on day 3-day 21 by increasing the ulcer area, reduced total gastric acidity,decreased mucosal thickness, increased ulcer depth and decreased parietal cell density.

As stated in Phase I,that oxidative reaction coupled with suggested damaged of secreting gastric cells contributes to the delay in ulcer healing process It has been evaluated that gastric acid in the lumen can cause digestion of a newly formed granulation tissue, thereby inhibiting and causing delay of gastric ulcers(Kazumori *et al.,* 2007). The mucosal barrier has been established as key component of the GIT protective mechanism against toxicants (Allen and Flemstrom, 2005), it is the first line of defense formed by mucus gel, bicarbonate and surfactant phospholipid which cover the mucosal surface (Lichtenberger, 1999; Allen and Flemström, 2005). The thickness of the mucosa is very important in the healing rate of ulcer apart from the gel, increase in the thickness causes a dynamic balance between its secretion and its erosion mechanically by shear forces of the digestive process and by proteolytic

degradation especially from luminal pepsin in the stomach (Penissi and Piezzi, 1999; Repetto and Llesuy, 2002, Allen and Flemström, 2005). Therefore, sodium arsenite reduces the mucosal thickness which contribute to delay in the ulcer healing rate. Similarly, alteration of the cyto-architecture of gastric mucosa with increased intensive mucosal edema, inflammation and ulceration via increase in the relative stomach weight suggests that sodium arsenite also delayed ulcer healing. Midwood *et al.,* 2004 suggested that a prolong inflammation leads to tissue damage.

Increase in the ulcer depth also contribute to the delay in the healing of ulceration in the sodium arsenite group. Increase in the ulcer depth has been attributed with offensive factors and environmental factors such as smoking, alcohol, hyperacidity etc (Hasebe *et al.,* 1998). This study suggests that sodium arsenite increased the ulcer depth which contribute to delay healing.

To also evaluate the quality of ulcer healing process, it is very important to also investigate the regeneration of parietal cells. A further investigation was carried out to examine the parietal cell density. In this study, the parietal cell density was initially low at the onset of ulcer formation and there was gradual increase in the density as healing proceeds to the day 21 of observation as seen in the acetic induced ulcer group. In the sodium arsenite, there was difference as the parietal cell density reduced from onset of healing process with no appearance of parietal cells on day 14, and a sudden appearance of parietal cells on day 21 of healing process. In acetic induced ulcer model, it has been observed that in the study by Nakano *et al.,* 1995, there is a significant increase in the regeneration of parietal cells, with budding of cystic glandular tubules with subsequent differentiation and appearance normal fundic mucosa with parietal cells identified within few days to months of ulcer healing process (Nakano *et al.,*1995). This can be seen in the in acetic induced ulcer group which showed a significant increase in parietal cell density as healing progresses while the sodium arsenite exposed does not coincide, it is therefore suggested that sodium arsenite could inhibit the process of differentiation and regeneration of parietal cells as it can cause damage in the DNA and mitochondria of the cell. Absence of parietal cells on day 14 indicates or suggest that there could be destruction of parietal cell. Itseffect on parietal cells also supports the decrease observed in the total gastric acidity in the phase I and II of this study.

5.1.3. PHASE III: EFFECT OF KOLAVIRON, ZINC SULPHATE AND VITAMIN E ONTHE GASTRIC MUCOSA EXPOSED TO SODIUM ARSENITE

Different intereventionwas used in PhaseIII; (antioxidant such as kolaviron, vitaminE, micronutrient (zinc), their effects on H^+/K^+ATPase, nitrite concentration, antioxidants and markers such as CD31, Ki67 and P53 was evaluated in normal stomach.The antioxidants treatment have been shown to promote inhibition of sodium arsenite induced oxidative stress by scavenging the ROS and promoting the antioxidant network. It was therefore suggested that zinc and Kolaviron could be more effective in inhibiting the effect of sodium arsenite by increasing the nitrite concentration which is very important in angiogenesis, CD31 which is also important in angiogenesis and increased regeneration (Ki67), reduced apoptotic index in the gastric tissue. Administration of vitamin E reduces the lipid peroxidation and also increases the antioxidant enyzmes such as SOD, catalase and protein level. Vitamin E enhances Ki67 as well but not high as compared found with the zinc and the kolaviron treatments.CD31 was well expressed in the vitamin E treatments as compared with kolaviron treatments. In some studies, it was suggested that alpha tocopherol (vitamin E) can play pivotal role in antioxidant induced angiogenesis(Daghini *et al.,* 2007,Burns *et al.,* 2013).This can be seen in vitamin E treatment in this study

The histology revealed that vitamin E moderately reduced the ulcerformation while treatment with kolaviron and zinc showed a mild ulceration.

Kolaviron significantly reduced the H+-K+ATPase in the stomach tissue and increase the catalase significantly as compared with the vit E and the zinc administered group. Kolaviron inhibits the sodium arsenite gastrotoxicity by enhancing nitrite level, CD31, Ki67 and reduced P53 more as compared with the vitamin E group. This study suggested kolavironinhibits sodium arsenite gastrotoxicity by improving the process of regenerationg of new cells via increased Ki67 apart from its ability to reduce the activity of proton pump,increasing antioxidant network in the stomach of the animals.

However, increased P53 production in vitamin E treatment indicates an increased DNA damage. Studies have shown that the use of topical vitamin E increased the number of p53 – positive foci and this reflects an increase in DNA damage (Burns *et al.,* 2013). It is suggested that vitamin E might have also exerted pro-oxidant effect by increasing tocopheroxyl radical and contributing increase to DNA damage. Therefore, strongly expressed P53 in vitamin E suggest that it might posses weak anti apoptotic properties.

Zinc has been suggested as a primary requirement for cell growth and cell proliferation (Tynga *et al.,* 2012;Jones *et al.,* 2013) as it is a requirement by several growth enzymes.This

study however suggests that zinc and kolavironare effective in inhibiting the toxicity of sodium arsenite via proliferative mechanism by increasing Ki67. Ki67 is a well recognized nuclear antigen present in the proliferating cells), CD31 for angiogenesis (improving the blood flow).

5.1.4. PHASE IV: EFFECT OF SODIUM ARSENITE AND VITAMIN E ON HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS

Intervention with vitamin E on sodium arsente showed a significant reduction in the ulcer formation as shown in the Phase IV. Vitamin E improves the rate of healing in the presence of sodium arsenite toxicity by reducing the ulcer area from day 3 to day 21. It is however noted that vitamin E is a very important antioxidant that scavenges free radical oxygen species. In etiology of ulcer, the oxidative stress is also amajor culprit in ulcer formation. From studies, it was observed that vitamin E has a proactive protection on emerging metal poisoning and also inhibits the actions of sodium arsenite .i.e has a sustainable curative values on afflicted individual. (Flora *et al.,* 2008).

Improving hemodynamic is another paramount method in improving healing process, as hypoxia causes damage to the gastric mucosa (Konturek *et al.,* 2005; Wallace, 2000). The delivery of oxygen to the mucosa of the stomach does not depend only on the blood flow but on the oxygen content of the arterial blood flowing to the mucosa. In re-epithelization of erosive and ulcerative defect, hemoglobin have shown its efficacy of multimodality therapy and accelerating healing of ulcers (Guerrin *et al.,* 1976; Elizavetina *et al.,* 1989). Hemoglobin is an iron –containing and oxygen metalloprotein in the red blood cells of all vertebrates (Maton *et al.,* 1993). It carries oxygen from the respiratory organs to the rest of the body where it releases the oxygen to burn nutrients to provide energy to power the functions of the organism, and collects the resultant carbon dioxide to bring it back to the respiratory organs to be dispensed from the organism (Dominguez *et al.,* 1981). In mammals,it makes up about 97% of the red blood cells dry content, and around 35% of the total content including water (Weed *et al.,* 1963). In this study, there was initial reduction in both hemoglobin content and PCV which later increased as the process of healing proceed in the acetic induced and sodium arsenite + vitamin E treatment(SA+VE+ AA) while in the group exposed to sodium arsenite (SA+AA) showed a continuous reduction in the hemoglobin content and PCV.

It is therefore suggested that decrease in the PCV and hemoglobin content will also contribute to the slowdown or delay in healing process of ulcer. The red blood count in the

SA+AAdecreases from the onset of the healing process to end of 21days which also contribute to the slowdown in the ulcer healing process. It has been observed in some studies that a decrease in hemoglobin level could be due to binding of inorganic arsenic acid binding to the α- chain of the hemoglobin chain causing accumulation in the red blood cell which thereby lead to damage of red blood cell. The destruction of the red blood cell resulting in to anemia in the system (Meiling *et al.*,2004, Blair *et al.,* 1990).Exposure of red cells to arsenic cause death of cells after few hours due to the depletion of ATP by uncoupling the formation of ATP and loss of the integrity of the membrane. In this study, it is suggested that these factors could be why arsenite delay the rate of ulcer healing. In the group administered with vitamin E, it was found that theVitamin E (alpha-tocopherol) enhances the hemoglobin level/ concentration, PCV and the red blood cell. Vitamin E is fat soluble and a natural antioxidant that helps to prevent the propagation of free radicals reaction (Packer, 1994, Kappus and Diplock, 1992). Vitamin E is a membrane stabilizer that protects crititcal cellular processes against damage by ROS and from the result of the study, vitamin E was able to mop up the oxidative stress caused by arsenic acid in the red blood cell and enhances the red blood cellcount, hemoglobin level and the PCV, hence improve ulcer healing process.

The white blood cells count increases in the sodium arsenite exposed while in the acetic induced ulcer group shows an initial increase which later reduced on day 7 and further increase from day 14 – day 21. This suggest a high immune response after induction and could serve as a booster for the healing process. Such immune response provide an explanation for the significant increase in white blood cells in the rats (Paul *et al.,* 2005) and it has been observed that the process of tissue repair requires a biological response whereby the body's cellular defence mechanisms are recruited and present at the damaged area with accompanying vascular and neural responses (Mann *et al.,* 1995). In the SA+AA, it was suggested that there was elevation of white blood cells from day 7- day 21 is as result of stress generated by sodium arsenite. In the group treated with vitamin E shows an initial increase in the white blood cell count which reduced at the end of 21days. This therefore explains that vitamin E was able to reverse the effect of sodium arsenite on the white blood cells count. The increase in the monocyte, neutrophil, lymphocyte and the eosinophil in the SA+AA group suggested that the stress generated in the gastric mucosa of the stomach was high. In the AA the increase was not as high as found in the SA+ AA towards the end of 21 days post ulcer induction.In SA+AA exposed, it is suggested that the uncurbed inflammatory responses seen

also contribute to delay ulcer healing process. Vitamin E was able to correct the toxicity of sodium arsenite in the blood system.

The neutrophil –lymphocyte ratio is very important indicator in inflammatory status and it is a prognostic tool /indicator in determining primary malignancy (Halazun *et al.,* 2008). It was said to be an independent predictor of various clinical problems ranging from cardioivascular events to cancer which suggested that peripheral leukocyte counts and NLR may be diagnostically useful(Muhmmed Suliman *et al,* 2010; Ubukata *et al,* 2010). NLR increased significantly in the SA+AA and reduces as healing proceed to day 21, the increase was significantly higher than the AA, and lower in SA+VE+AA. As healing proceed, AA and SA +VE+AA maintained a lower NLR which cause a faster improvement in ulcer healing.

In gastric oxidative stress, imbalance between offensive factors and defensive factors in the stomach which plays a pivotal role in gastric hemorrhage and ulcer formation (Hung, 2005). It has observed that over production ofROS is one of the major pathogenic factors that results in oxidative destruction which include lipid peroxidation, protein oxidation and DNA damage which proceed to cell death. They are referred to as second messengers which can activate diverse redox –sensitive signaling transduction cascades including mitogen – activated protein kinases and downstreams transcription factors like NF-AB and AP-1 which regulates expression of several pro-inflammatory genes, all these factors actually increase the elaboration of chemical and humoral mediator of tissue inflammation and damages /injury (Ali and Harty, 2009). In this study, with exposure to sodium arsenic, there was increase in lipid peroxidation, with lower enzymatic antioxidant (catalase, SOD) with a lower protein level. This implicates the role of oxidative stress in the toxicity of the sodium arsenite in the stomach mucosa. From several studies, formation of reactive oxygen species like nitrogen species has been one of the mechanism of action of arsenic generally (Hughes and Kitchin, 2006; Kitchin and Ahmad, 2003; Kitchin and Conolly, 2010; Lantz and Hays, 2006; Shi *et al.*, 2004). Arsenic has been said to induce oxidative stress (reactive oxygen species) by different mechanisms such as genotoxicity, signal transduction, cell proliferation, and inhibition of DNA repair, etc. There are increased in the oxidation as shown in the group exposed to sodium arsenite and VE supplement has been shown to mititgate the toxicity of sodium arsenite (Sudha and Mathanghi, 2012). Vitamin E was able to reverse the toxicity as antioxidant enzymes are more enhanced in the gastric mucosa and lower lipid peroxidation index (MDA) and high protein level was observed. This explains that ulcer healing process was more enhanced with vitamin E supplement.

5.1.5. EFFECT OF SODIUM ARSENITE AND VITAMIN E ON H^+ -K^+-ATPASE AND NITRITE CONCENTRATION

The gastric ATPase is a very important intrinsic membrane protein found or localized in the membrane of the parietal cells. It is a key enzyme that is associated with final step of acid secretion by catalyzing electro neutral exchange of luminal K^+ for cytoplasmic H+ and externally coupled with ATP hydrolysis. It however consists of α and β- subunits which are encoded by separate genes. The α subunit is the catalytic subunit responsible for ion exchange, while the β- subunit is heavily glycosylated and necessary delivery of α- subunit to the plasma membranes(Shin *et al.*,1997,Helander *et al.*,1993). It is however been demonstrated that several intracellular signals involving H_2, M_2 and gastrin receptors stimulates H+/K+ ATPASE activities to secrete acid and decrease in its activities is responsible for decrease in gastric acid secretion(Sachs *et al.*, 1995).

In this study, there was a significant decrease in the H+/K+ ATPase, in the SA+VE+AA, and VE +AA, as compared with the control. In the SA+VE+AA , there was initial reduction in the pumps level which significantly increase on day 7 and as healing process proceed , the pump level decrease till the completion of the ulcer healing process and likewise in the VE+AA. From this study, it was observed that sodium arsenite caused a reduction in the pump level which caused low basal gastric secretion and this implicate that the ability of arsenic acid to cause inflammation might probably be not be via hyperacidity. Arsenic also known for damaging the mitochondria, DNA damage, apoptotic cell death (Jiang *et al.*, 2009, Chen *et al.*, 2004, Kirkpatrick *et al.*, 2003, Huang *et al.*, 1999). One of the ways arsenic mode of action is by interaction with the phosphate which result in the process known as “arsenolysis” which involved formation of esters linkages with its hydroxyl group which is unstable and this bond can easily dissociates, coupling with adenosine 5 triphosphate in the cell to arsenic anhydrides which is unstable and this can result in ATP diminish (Dixon,1997, Duodroff *et al*,1947). It is therefore suggested that sodium arsenite might cause diminish of H^+-K^+ATPase by the utilizing the ATP involved in the process formation HCL. It has also been demonstrated that ROS formed by arsenic is one of the most studied mode of action involved in several of the proposed processes including genotoxicity, signal transduction, cell proliferation, and inhibition of DNA repair. It may occur during oxidation of arsenite to arsenate (Del Razo *et al.*, 2001). Studies had shown that arsenite toxicity in the

gastrointestinal tract is by altering the gut microbiome community at the abundance level and disturbance of metabolic profiles at the function level (Lu *et al.,* 2014).
Vitamin E supplement showed an increased in the pump level initially which reduced as the healing process proceed to the completion of the healing process. This explains the ability of vitamin E can mop up the ROS created by oxidative process caused by arsenite.

In the ulcer healing process, nitric oxide (NO) is said to be an important bioactive agent and signaling molecule that helps to mediates a variety of actions such as vasodilatation, neurotransmission, host defense, and iron metabolism (Stefans *et al.,* 1998). It has been demonstrated that the enodtheliial cells can generate nitric oxide from L- arginine in a reaction catalyzed by enzyme nitric oxide synthase (Bredt and Synder, 1990). It has a beneficial effects on the gastric mucosa defense by maintaining the gastrointestinal blood flow and inhibiting adherence and activation (release of reactive oxygen metabolites and proteases (Berg *et al.,* 2004). Assay has been carried out by Ignarro *et al.,* 1987 that nitrite determination using diazotization reaction can be used to determine NO_2 level in experimental sample. In this study, the nitrite concentration was significantly high on day 3 post ulcer induction in SA+VE and VE+AA, this explains that vitamin E enhance nitric oxide via increase in nitrite concentration and this increase indicates that blood flow is highly maintained and reactive oxygen species was inhibited, this however increase the ulcer healing process. In the ulcer healing process , it has been demonstrated that there are appearance of granulation tissue and angiogenesis(angiogenic microvessel),mRNA in gastric ulcer has been well established after 3days, this however indicate that with high nitric oxide level increased which enhances the afore mentioned activities in the ulcer healing processes. The nitrite concentration reduced significantly on day 7 in the SA+VE+AA and VE+AA groups and increases at the completion of ulcer healing process. In the SA exposed group, there was reduced nitrite concentration as compared with the control,SA+VE+AA and VE+AA. Decrease in the nitric oxide indicate the sodium arsenite inhibits nitric oxide production by endothelial cell and slow down or inhibit the process of ulcer healing process. It has been observed that arsenite generate nitric oxide production by endogenous NO synthase , the NO is however used up by flavin enzymes such as NAD(P)H oxidase and NO synthase to generate free radical species with arsenite exposure(Flora *at al.*,2008).

5.2.0. EFFECT OF KOLAVIRON ON ULCER FORMATION IN SODIUM ARSENITE EXPOSED IN EXPERIMENTAL GASTRIC ULCER

The use of herbal has been suggested to be a better option suitable for treatment of arsenic poisoning (Flora *et al.*, 2008). Kolaviron is defatted ethanol extract from the seeds of *Garcinia Kola* (Iwu *et al.*, 1990, Olaleye *et al.*, 2000). Ulcer area in the group exposed with sodium arsenite and treated with the kolaviron showed a significant reduction on day 3 which reduced on the subsequent days, while in sodium arsenite exposed group showed a significant increase in ulcer area, as compared with those treated with the kolaviron. In the acetic induced ulcer treated with the kolaviron showed a significant increase in ulcer area on day 3 and was significantly reduced on the other days as the healing process proceed. The ulcer area was much reduced in the acetic induced ulcer and kolaviron treated than in the sodium arsenite exposed + acetic acid induced ulcer treated with kolaviron. Kolaviron has been demonstrated to significantly attenuate gastrointestinal injuries induced by several ulcerogen such as indomethacin, ethanol, HCL-ethanol mixture(Ibironke *et al.*, 1997,Olaleye ,2005, Olaleye and Farombi, 2006). In this study, kolaviron inhibit the toxicity of sodium arsenite on gastric mucosa by reducing the ulcer area, this contribute to ulcer healing.

5.2.1. EFFECT OF SODIUM ARSENITE AND KOLAVIRON ON H^+-K^+ ATPASEACTIVITIES

Kolaviron showed a significant reduction in the H^+-K^+ ATPase /pump both in the group exposed to sodium arsenite, induced with acetic acid. The gastric H^+-K^+ ATPase /pump gastric is α, β-heterodimeric enzyme and had been shown its action cause gastric acid secretion, due to the action of the ATP-dependent hydrogen – potassium exchanger. It is however the final step of acid secretion, any inhibitor of the pump would be more effective in suppressing the gastric acid secretion other than a receptor antagonist (Shin *et al.* 2009, Fellenius *et al.*,1981, Sachs *et al.*,1976).

In this study, kolaviron reduced the pump level in the group exposed to sodium arsenite better than the group induced with acetic acid. In the sodium arsenite exposed, there was a very low H^+ -K^+ ATPase level as compared with other group. This indicate sodium arsenite reduced the gastric H+,K+ ATPase, this reduction could be as result of damage to the parietal cells,and also diminshing of ATP in process of damage to cell of the gastric mucosa as observed in previous phase in this study. This suggests that kolaviron suppresses the activity of H+-K+ ATPase. In sodium arsenite exposed with kolaviron treated showed at the Kolaviron at the same time repair the damage caused by sodium arsenite as shown in all the other parameters that enhances healing.

5.2.2. EFFECT OF SODIUM ARSENITE AND KOLAVIRON ON NITRITE CONCENTRATION

In the sodium arsenite with kolaviron treatment showed a significant increase in the nitrite concentration on day 3 and this reduces on the other subsequent days and in the acetic acid induced ulcer with kolaviron treatment showed an initial low nitrite concentration on day 3 and day 7, this significantly increase on day 14 and day 21. In the sodium arsenite alone, there was a low nitrite concentration as compared with the other groups. In the ulcer healing process, nitric oxide (NO) is said to be an important bioactive agent and signaling molecule that helps to mediates a variety of actions such as vasodilatation, neurotransmission, host defense, and iron metabolism(Stefams *et al.,* 1998). It has been demonstrated that the enodtheliial cells can generate nitric oxide from L- arginine in a reaction catalyzed by enzyme nitric oxide synthase(Bredt and Synder,1990). It has a beneficial effects on the gastric mucosa defense by maintaining the gastrointestinal blood flow and inhibiting adherence and activation (release of reactive oxygen metabolites and proteases)(Berg *et al.,* 2004).

Therefore, with increase in the nitrite concentration in the group treated with kolaviron indicate the upregulation of nitric oxide and also inhibit the sodium arsenite toxicity on gastric mucosa. . Decrease in the nitrite concentration indicate the sodium arsenite inhibits nitric oxide production by endothelial cell and slow down or inhibit the process of ulcer healing process. It has been observed that arsenite generate nitric oxide production by endogenous NO synthase , the NO is however used up by flavin enzymes such as NAD(P)H oxidase and NO synthase to generate free radical species with arsenite exposure(Flora *at al.,*2008). Also,kolaviron increases the nitric oxide, It has been documented that phytochemical constituents of *Garcinia Kola* seeds which include saponins,tannins,flavonoids,proteins,glycosides,reducing sugar, starch, sterols and triterpenoids,with flavonoids predominating . it has been suggested that flavonoids,one of the possible bioactive agents responsible for antiulcerogenic and antinflammatory properties of Kolaviron(Alarcon De la Lastra *et al.,* 1994, Izzo *et al,* 1994, Kim *et al.,* 2004, Esimone *et al.,* 2007,Landberg *et al.,* 2011).

5.2.3. EFFECT OF SODIUM ARSENITE AND KOLAVIRON ON BIOCHEMICAL PARAMETERS

Kolaviron treatment on sodium arsenite exposure showed a great increase in the protein level, decrease in the lipid peroxidation (MDA), increase in catalase, SOD which indicate that kolaviron inhibits sodium arsenite toxicity, and also enhance healing process in the gastric mucosa. It has been reveal that *Garcinia Kola* extract prevented lipid peroxidation and increasing the enzymatic anti-oxidants and reducing lipid peroxidation index (Adaramoye,2009). Flavonoids have been shown to be responsible for the anti ulcerogenic properties.it is however known that its anti-inflammatory activities has been complemented by their ability to activate Nrf2 which increases anti-oxidant defenses(González-Gallego *et al.,* 2007). This study showed that kolaviron ameliorates the activity of sodium arsenite and also possess antiulcer effect as well.

5.3.0. EFFECT OF SODIUM ARSENITE, KOLAVIRON AND VITAMIN E ON EXPRESSION OF HEALING FACTORS SUCH AS CD31, KI67 AND P53

In this study, intervention with kolaviron showed a significant expression of CD31 in the gastric mucosa. There was an initial mild expression which increases progressively towards the end of 21 days. CD31 is very important in angiogenesis, it is however observed that kolaviron enhances CD31 expression, increasing improving the ulcer healing processes well as inhibiting the toxicity of sodium arsenite on the ulcer process. In the SA+KV +AA, there was moderate expression and this indicates that kolaviron was able to enhance expression of CD31 and inhibit the toxicity of the sodium arsenite. In the KV +AA, angiogenesis was also enhanced in the gastric mucosa, improving the ulcer healing rate.

Also, Ki67 was also moderately expressed on day 7 and strongly expressed day 14 in group administered with SA+KV+AA which indicates that kolaviron enhances proliferation of cells and also inhibit sodium arsenite effect in the gastric mucosa. The Ki67 was more expressed than CD31 and this suggested that Kolaviron exhibited proliferative properties more than angiogenic properties. Ki67 was more expressed in the Kolaviron as compared with the vitamin e treated group.

P53 was mildly expressed in the gastric mucosa of SA+KV+AA. P53 is very important in the apoptosis.Apoptosis is an important and potent factor in gastrointestinal epithelial turn over in ulcer healing process (Nagano *et al*, 2005). As discussed previously, arsenic has been known to induce apoptosis in sveveral tissues it is exposed to, by decreasing cell viability, accompanied by apoptotic DNA fragmentation, chromatin condensation, and caspase activation. Kolaviron was able to reduce the expression of P53 (indicator of cell damage due

apoptosis). From documentation by Olaleye and Cho, 2010, Kolaviron inhibits the indomethacin induced apoptosis in the intact rat stomach and in the gastric mucosal cell line. This suggest kolaviron to be anti-apoptotic in its activities and enhances ulcer healing process, and the same time inhibiting the toxicity of sodium arsenite.

Vitamin E was able to enhance expression of CD31 in the gastric mucosa of both SA+ VE+ AA and VE + AA as comapared with the kolaviron. It is also suggested that it enhances angiogenesis in the gastric mucosa.CD31 was much more expressed in the vitamin E treatment as compared with the Kolaviron treatment. Vitamin E seems to be in enhancing angiogenesis in the gastric mucosa of animals exposed to sodium arsenite.

Ki67 was mildly expressed on day 3 and day 21 in the groups treated with SA+VE+AA there was moderate expression on day 7 and 21, it also indicate vitamin E might support cell proliferation in ulcer healing and not strongly enhanced proliferation as found in the kolaviron. It is suggested to have weak proliferative properties.

P53 was also strongly expressed in all the vitamin E treated group on day7 and day 21. It also suggest vitamin E to also possess a weak anti -apoptotic substance that can inhibit the toxicity of the sodium arsenite**.**

5.4.0. PHASE V: EFFECT OF SODIUM ARSENITE AND ZINC ON TOTAL GASTRIC ACID SECRETION, ULCER AREA.

Zinc is one of the trace element and a metal that is a cofactor for enzymes (Sonali *et al.*, 2012). It is a micronutrient for humans, animals and plants (Shah and Sachdev, 2001; Das and Das, 2012). The functions of zinc are numerous categorized in to three categories; the regulatory, catalytic and structural roles (Stipanuk, 2006). It is involved in numerous aspects of cellular metabolism and is required for the catalytic activity of several enzymes (Anony. 2001). Zinc is not an antioxidant but can limit oxidant-induced damages in several ways (Disilvestro, 2000), some of which include protection against vitamin E depletion (Noh and Koo, 1998). It can stabilize the membrane structure (Bray and Bettger, 1990) and restriction of endogenous free radical production (Disilvestro, 2000).

In this study, zinc decreased the basal gastric acid secretion from onset of ulcer formation, healing process to the completion of the process. From day 3, there was significant decrease in the gastric secretion than the SA+AA group. This indicate that zinc possess an anti-secretory

effect of gastric acid. It has been investigated that zinc possesses a potential rolein the proliferation and generation of the protective barrier, namely the mucous gel layer at the surface of the stomach (Watnabe *et al.,* 1995). These studies suggested that the reduction in acid secretion observed over time which could be related to an increase in the thickness of the gel layer. The ulcer area was significantly decreased in the SA+AA+zinc group as compared with the SA+AA, and AA groups. It has been investigated that zinc sulphate possesss anti-ulcer effect and can limit oxidant –induced damages by restricting endogenous free radicals. It plays a major in the wound healing (Lansdown *et al.,* 2007, Dilsvestro 2000).

5.4.1. EFFECT OF ZINC ON HISTOLOGY OF THE STOMACH IN SODIUM ARSENITE EXPOSED IN EXPERIMENTAL GASTRIC ULCER

In the SA+AA, there was a complete mucosa ulceration extending through the lamina propria with severe / extensive cellular necrosis, Intense sub mucosa edema with epithelial infiltration and and glandular hyperplasia, appearance of atypical cells in some area on Day 3 and on the day 7 there was multi foci ulceration with necrosis , mass cellular disruption/ differentiation and this proceed to day 14 with severe necrosis and hyperplasia , intense infiltration and presence of atypical cells while on day 21 there was moderate cellular ,necrosis with foci hemorrhage and presence of atypical cells. Arsenite has been demonstrated to cause disruption in the cell by alteration in the signal transduction/cascade and imbalance in antioxidant which inturns triggers cellular apoptosis in cell, by H_2O_2 ,a mediator that act to transfer cytochrome c in to cytosol and activation of proteases and degradation in the cell. On the other hand, arsenic-mediated cytotoxicity which is thought to be due to high accumulation of this metalloid in the cells (Dong, 2002, Flora *et al.,* 2008). This confirms the ability of sodium arsenite to slow down the ulcer healing process. In the group treated with zinc, there was complete ulceration with severe necrosis on day 3, stroma edema with infiltration and necrosis on day 7, intense infiltration and moderate ulceration on day 14 and on day 21 mild ulceration with moderate infiltration of inflammatory cells. This indicates that zinc could intercept the actions of sodium arsenite, as it has been shown that zinc sulphate possess an antiulcer effect and very active in restricting endogenous free radical species. Anderson in 1995 suggested that zinc maintains the integrity of the skin and mucosa membranes, and it was also observed that most patients with chronic leg ulcer have been observed to have abnormal zinc metabolism and low serum zinc(Wilkinson and Hawke,1998) which is required for protein synthesis cell growth and wound healing (Lansdown *et al.*, 2007).

5.4.2. PHASE V: EFFECT OF ZINC ON MOLECULAR FACTORS OF ULCER HEALING IN SODIUM ARSENITE EXPOSED IN EXPERIMENTAL GASTRIC ULCER

Healing of ulcer is a very complex process and a genetically repair process after injury, attempting a restitution towards integrity, intense cell proliferation to fill this deep mucosal defect and to reconstruct gastric glands within the scar is also important (Tarnawski *et al.,* 1991; Tarnawski, 1993; Tarnawski *et al.,* 1995; Tarnawski *et al.,* 1997,2005). This is however achieved by proliferation of cells from the ulcer margin and their migration onto the granulation tissue to cover (re-epithelialise) the ulcer base. In addition, the poorly differentiated cells from the base of the ulcer margin sprout into the granulation tissue forming tubules, which undergo transformation into gastric glands (Wright *et al.,* 1990; Podolsky, 1994). Several growth factors are required for these process (Tarnaswki, 2005).

5.4.3. EXPRESSION OF CD31

The endothelial cells plays a major role in the control of coagulation , thrombolysis, vascular tone,permeability, inflammation and tissue repair, they expressed markers such as CD31,CD34, vonWillebrand on normal endothelium (Cines et al. 1998; Mu¨ ller et al. 2002a, Pusztaszeri *et al.,* 2006). The CD31 is a member of immunoglobulin family, is about 130 kDa transmembrane glycoprotein also known as PECAM-1(platelet adhesion molecule -1). It can be found on surfaces of platelet, monocyte macrophages and neutrophils .it is very important in the adhesion cascade between EC and inflammatory cell during inflammation hereby facilitating or enhancing leucocyte migration between Ec during angiogenesis.it is a recently recognized angiogenic marker (DeLisser *et al.,* 1997; Matsumura *et al.,* 1997; Zhou *et al.,* 1999). This present study on the interplays of angiogenesis to the healing of gastric ulcer induced by acetic acid, exposure to sodium arsenite and zinc supplement, taking to account the expression on the different days. These result showed that there was an increase in the activation of angiogenesis by increasing the CD31 moderately on day 3 – day 21 of the healing process in the AA. In the sodium arsenite exposed, there was decreased in expression of CD31 from the onset to completion of the healing process ,except on day 7 which showed a moderate expression while with zinc supplement, there was moderate expression from day 3 to day 14 and mild expression on day 21. Angiogenic responses are switched on at the early stage of ulcer healing (Tarnaswki, 2005), this can be seen in the acetic acid induced ulcer group. The importance of angiogenesis in gastroduodenal ulcer healing has been extensively

studied. For instance, stimulation of angiogenesis in granulation tissues has been shown to dramatically accelerate the healing of experimental duodenal ulcer in rats (Folkman *et al.*, 1991). In the sodium arsenite exposed, the healing process was slow down as result of decrease in angiogenesis as a result of a decrease in the CD31 expression. It is however observed in this study zinc enhances angiogenesis by increasing moderately the CD31 expression, thereby increasing the angiogenesis process in the ulcer healing and also inhibiting the effect of sodium arsenite at the same time.

5.4.4. EXPRESSION OF FACTOR 8

The Factor VIII is a blood clotting protein that is produced by the liver, endothelia and some epithelia cells (Toole *et al.*, 1984). In cellular injury such as inflammation and malignancy, factor VIII is activated in response to this cellular (Truett *et al.*, 1985). In this study, the healing of gastric ulcers induced by acetic acid, sodium arsenite exposed and sodium arsenite with zinc treatment was evaluated. There was an increase in expression of factor VIII with respect to days of healing in the acetic induced group. In sodium arsenite, there was mild expression to negative expression with respect to days of healing. This however indicates that sodium arsenite inhibit the expression of factor VIII which is also essential for healing process while in the zinc treated, there was moderate to strong expression of factor VIII. Treatment with zinc showed effectiveness inenhancing the angiogenesis as compared with the AA and SA.

5.4.5. EXPRESSION OF KI-67

Ki-67 is a well-recognized nuclear antigen present in the proliferating cells, is usually expressed in all stages of cell cycle except the G0 and early part of G1 (Weidner *etal.*, 1994,Young –Eun Joo *et al.*, 2006). A dynamic balance between epithelia cell proliferation and apoptosis is essential for maintaining the normal mucosal integrity (Moss *et al.*, 1996). Alterations in the balance of epithelial cell proliferation and apoptosis (Correa and Miller, 1998) contribute to gastric ulcerogenesis or even carcinogenesis (Que and Gores, 1996; Moss, 1998)

In this study, expression of Ki-67 in acetic acid induced ulcer was mild from the onset and became moderately expressed towards the completion of the ulcer healing and this explains that there was proliferation in the healing process. In the sodium arsenite exposed group, there

was negative expression to a very mild of Ki67 expression from day 3- day 21. This explains that arsenite can inhibit cell proliferation but rather can enhance tumor cell proliferation in a very chronic inflammation in the stomach mucosa. It has been demonstrated that arsenic can increase expression of gene responsible of positive regulation of cell cycle and simultaneous expression of negative regulation genes which could be excessive, hence leads to cytoxicity of arsenite as well leads to malignancy in some cases (Chen *et al.,* 1996).

In the group treated with zinc, it showed an initial moderate expression at the onset of the healing process, which became mild towards the end of day 21. From studies, it has been observed that zinc could reverse the effect of arsenic acid, and it has been observed that arsenite in combination with zinc reduced damage of peripheral vessel (Gebel,1998). It is therefore suggests that zinc was able to inhibit the gastro-toxic effect of sodium arsenite by increasing the expression of Ki-67

5.4.6. EXPRESSION OF EGFR

In the healing zone on the ulcer margin, there is de-differentiation and expression of epidermal growth factor receptor (Tarnawski,2000,Tarnawski,1993). This is usually initiated within 3days after ulcer formation, as it is essential for ulcer healing. Stimulus for increased epithelial cell proliferation in the mucosa of the ulcer margin is most likely initiated by EGF and/or TGF-α (Poulsen, 1987). The EGF mediates its biological effects on target enterocytes via binding to a specific 170-kDa membrane-bound glycoprotein receptor, the EGF receptor. The EGF receptor has been found localized in the foetal and adult gastrointestinal tract, liver, and pancreas (Forgue-Lafitte *et al.,* 1984). Binding of the EGF receptor activates the intrinsic tyrosine kinase, which then leads to a complex cascade of cellular events that ultimately result in DNA synthesis and cellular growth (Barnard *et al.,* 1995). Chronic administration of EGF produces a significant increase in mucosal DNA, RNA, and protein content (Johnson and Guthrie, 1980). This proliferative action of EGF is believed to contribute to the normal maintenance of mucosal integrity and functions within the gastrointestinal tract (Playford and Wright, 1996). It has been shown that EGF-R is beneficial in pathophysiological processes in the gastrointestinal tract by either reducing injury(Konturek *et al.,* 1988; Konturek *et al.,* 1995) or accelerating repair (Skov-Olsen *et al.,* 1986), capable of providing protection against a variety of gastric insults, both acid dependent (Konturek *et al.,* 1988; Konturek *et al.,* 1992) and acid independent (Konturek *et al.,* 1992; Konturek *et al.,* 1995). Its importance of EGF in ulcer healing is thereby demonstrated by a marked increase in its receptors and EGF-

producing cells in experimental gastric ulcers in rats induced by acetic acid(Skov-Olsen *et al.,* 1986; Tarnawski *et al.,* 1992) or cryoprobe (Alison *et al.,* 1995).

There was a strong expression of EGFQ R in the acetic acid induced ulcer group on the day 3 and which became strong on day 7 - day 21 in the AA .This indicate that there was upregulation of EGFR and probably the EGF producing cell which help to enhances the healing at ulcer margin in the gastric mucosa. In the sodium arsenite exposed, there was a strong expression on day 3 which became mild from the day 14 and moderate on day 21 of the healing process. This explain that sodium arsenite deregulate the EGFR production by its gastro-toxic effect. The zinc treated showed moderate expression on day 3 –day 14 and strong expression on day 21. This suggest zinc to be important in upregulating EGFR and also increases proliferation which helps to accelerate the healing process. Zinc deficiency has been suggested to lead to decrease in formation of connective tissue repair and alteration of wound healing process. Expression of EGFR was much more enhanced in this study than expression of Ki67 by zinc supplement in this study.

5.4.7. EXPRESSION OF P53

P53 is an oncogenetic repressor or tumor suppressor seen in many cancers and some chronic inflammation, its anti- cancer function has been realized by triggering apoptosis (Xu *et al.,*2001). P53 is .the most striking example of a gene that control normal cell proliferation, cellular function such as DNA repair, differentiation, genomic plasticity,and programmed cell death (Harris, 1993, Greenblat*et al.,* 1995). In this study, acetic acid induced ulcer group showed a negative express all through the different days of observation (mild expression from day3 - day21). In sodium arsenite exposed, there was strong expression on day 3 –day 14 and it became moderate on day 21. In the zinc treated, there was an initial moderate expression on day 3 and on day 7 and 14 negative expression and on day 21 a mild expression. This explain that sodium arsenite increases P53 which indicate sodium arsenite induced apoptosis in the gastric mucosa of the stomach. Apoptosis is programmed cell death is envisaged as eliminating cells with DNA damage or growth dysregulation that could become precursors of malignant clones. In this way it complements growth arrest and DNA repair as mechanisms to preserve the genetic integrity of tissues (Christopher, 1999). Abnormalities in P53 expression represent the most common molecular change not only in cancer, but also in precancerous gastric lesions, including gastric dysplasia (Sasano *et al.,* 1993). An increased wild-type P53 expression may also represent a cellular response to DNA damage (Shiao *et al.,* 1994). ; P53is

part of a pathway invoked upon DNA damage in mammalian cells through the induction of G and mitotic arrest (Blattener *et al.,* 1994, Cross*et al.*, 1995).

Sodium arsenite has been demonstrated to induce an increase in P53 protein levels which might be activated in response to DNA damage, replicative defects or might be due to inhibition of DNA ligase activity (Salazar*et al.,* 1997*)*. Zinc showed a decrease in the P53 level which indicate that zinc could interrupt the activity of sodium arsenite on the gastric mucosa. It has been reported that zinc is a highly charged metal that can inhibit arsenic induced apoptosis in neuronal cell line, by manifesting antioxidant properties inside the cell , especially inducing synthesis of metal binding protein such as metallothionen which can scavenge six arsenic moieties per molecule(Toyama et al. 2002), may also act to stabilize the cytoskeleton, preventing arsenic-induced disruption of cell membranes (Milton *et al.,* 2004).

Also, it can probably inhibit the attack of arsenic on mitochondrial in releasing cytochrome c and its damaging effect on DNA structure(Pelicano *et al.,* 2002; Shen *et al.*,2003). Previous studies stated it is possible for zinc to compete with heavy metals for binding sites without inducing oxidative stress by retaining the structure of enzyme allosteric sites and this form of treatment can be regarded as been compeptitive and targeted as most of the heavy metals also adopt the same pattern/pathway of bioactivation(Falana *et al.,* 2013).It is therefore suggested that zinc activities in this study might be via the same mechanism in the gastric mucosa

5.5.0. PROPOSED MECHANISM OF SODIUM ARSENITE TOXICITY ON GASTRIC MUCOSA AND ON HEALING OF ULCER

It is postulated that sodium arsenite have toxic effect in the gastric mucosa and also aggravate ulcer incidence, this could be responsible for the increase in ulcer indices obtained in the gastric mucosa in the stomach. This is further enhanced by increased inflammatory property, and immune response shown by increase neutrophil-lymphocyte ratio, increased white blood cell count and decrease in hemoglobin content, RBC count. The above factors resulted into oxidative stress i.e. increased oxidative factors system and increase lipid peroxidation. This process is then ac+companied by decrease expression of molecular factors of healing responsible for the proliferative and antigenic phases of ulcer healing and increase in the apoptosis.

With intervention of vitamin E, zinc and kolaviron limit the toxic activity of sodium arsenite. It is therefore postulated that sodium arsenite cause toxicity via lipid peroxidation-dependent

mechanism, could also undergoes alteration in apoptosis, damage to DNA and cytotoxicity , mechanism still under study.

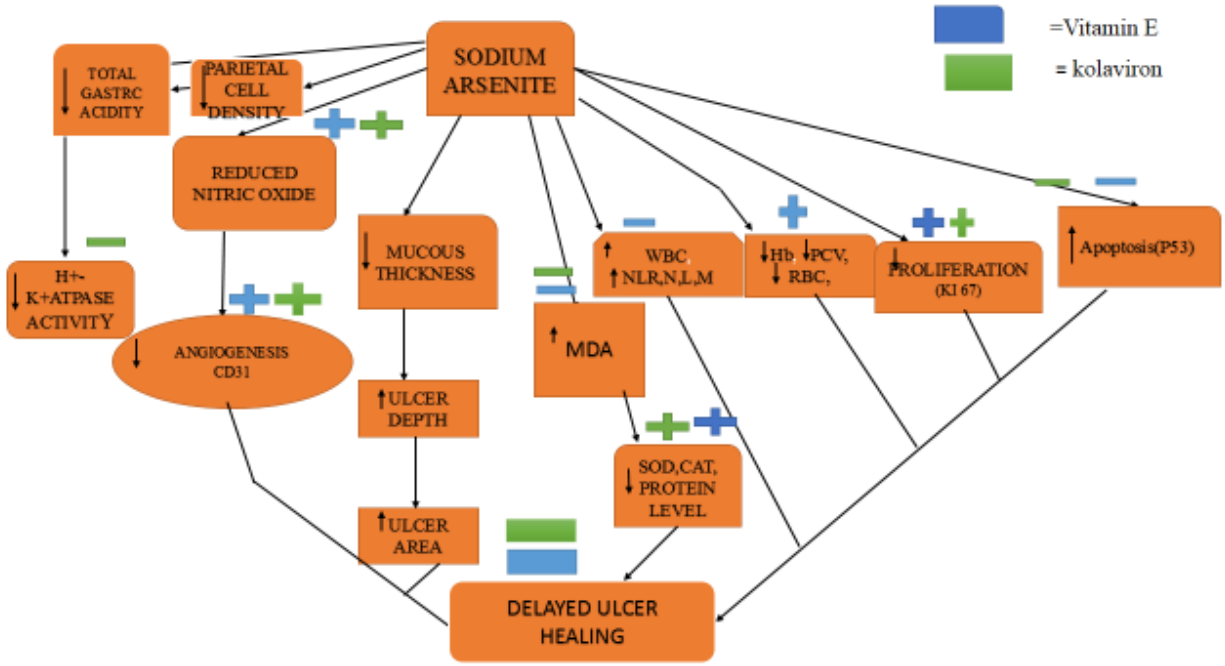


Figure.5.1. **Schematic diagram of proposed mechanism of sodium arsenite and intervention on ulcer healing**

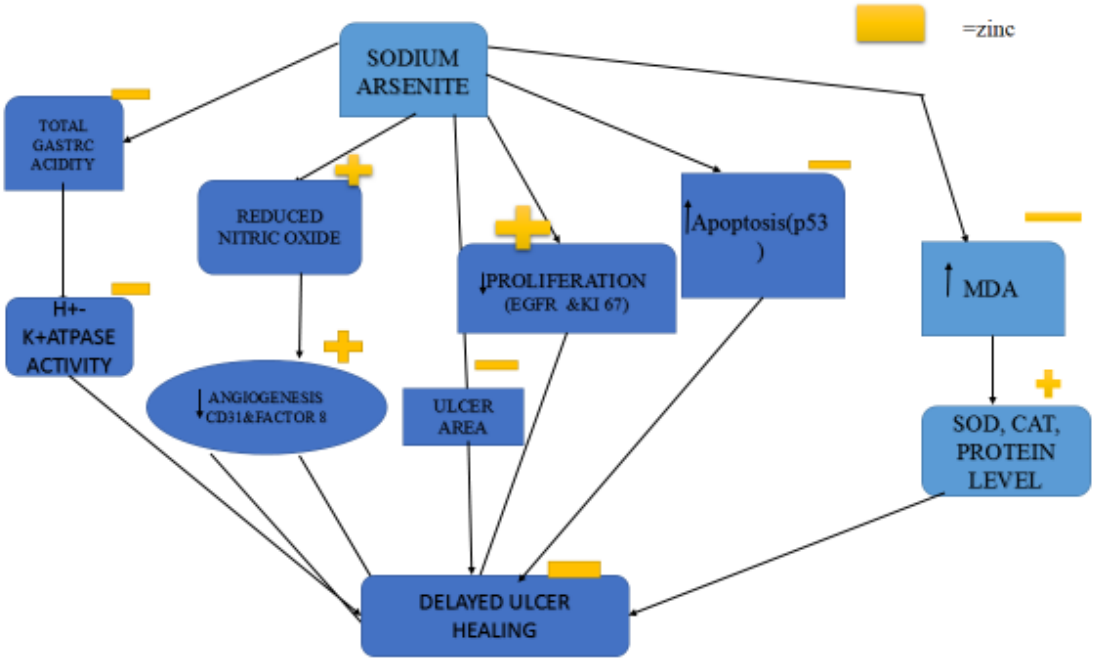


Figure 5.2. **Schematic diagram of proposed mechanism of sodium arsenite and intervention on ulcer healing**

5.6.0. Summary of findings

This study provided evidence the that sodium arsenite first transformation starts in the GIT and its exposure cause a serious damage to gastric mucosa reducing the defense mechanism, which answer the question sodium arsenite(a heavy metal) toxicity is not limited to other target organs as liver, rather cause serious damage to the GIT.

Its toxicity on experimental gastric ulcer caused a serious delay in the process of healing by increase in the ulcer area and depth, reduction in mucosal thickness and damage to the secretory cell of the stomach (evidence of its ability to destroy secretory cell/parietal cells in the stomach of the rats).Reduction in gastric acidity and H^+-K^+-ATPase observed indicate that its mechanism of toxicity does not depend on increase in gastric acid secretion rather exert a direct toxic effect on muocsa and epithelial cells of the stomach.

One of the mechanisms of sodium arsenite is lipid peroxidation dependent; suggesting that oxidative stress plays an important role in the sodium arsenite toxicity,

Sodium arsenite also cause damages to the red blood cellby reducing thudy also elucidate some of the mechanism by which sodium arsenite exert its toxicity on the healing of ulcer, which include alteration in proliferation and angiogenesis, increase ulcer formation with increased oxidative stress, as well as increased apoptosis in the gastric mucosa.

Antioxidants(especially kolaviron ,vitamin E and zinc) was shown toreverse the mechanisms of sodium arsenite in delaying the ulcer healing processes by upregulating the factors necessary for healing and reducing detrimental activites of arsenic acid.

Zinc reversed the gastro-toxicity and the delayed induced in ulcer by sodium arsenite by followings

i. Decreasing the ulcer area and increasing the activities of NO, increasing the angiogenic process by increasing factor VIII and PECAM-1 in the stomach mucosa.
ii. Upregulating the expression of ki-67 and EGFR in the injured tissue which indicate an increase in the proliferative process
iii. Also, reduction in P53 expression suggested the zinc ability to inhibit the apoptotic activites induced by sodium arsenite

Kolaviron also reverse the effect of sodium arsenite by

i. Reducing the ulcer area and increasing the nitric oxide activities in the injured tissue
ii. Increases the activites of antioxidant and reduced the lipid peroxidation
iii. Repaired the inflamed tissue and reverse the delayed ulcer healing by increasing the ki-67 expression which is important in proliferation.
iv. Also reduced the P53 expression thereby reversing the apoptosis induced by sodium arsenite

Vitamin E reversed the delayed in ulcer healing induced by sodium arsenite by reducing lipid peroxidation ,increasing antioxidant activites, increasing the process of angiogenesis but seems not to be a strong antiapoptotic agent.

Therefore the rate of reversal of delayed induced in ulcer healing by sodium arsenite = Zinc > kolaviron > vitamin E.

5.6.1. Conclusion

Findings in this study revealed thatSodium arsenite delayed gastric ulcer healing in rats via oxidative stress, inflammation, alteration in proliferative and apoptotic activities and impaired angiogenesis in the stomach. However, intervention of *Kolaviron*,zinc, and vitamin Ereversed the mechanism of sodium arsenite induced delayed in experimental gastric ulcer.

5.6.2. Recommendations

Exposure to arsenic acid via drinking water still remains unavoidable, as it is naturally part of most of the sources of water and its increase is as a result of anthropogenic activites,hence becomes a key concern in the communities.The presence of arsenic acid in the body system increase the risk of ulceration in a normal gastrointestinal tract and in diseases state (cause relapse/delay of the healing process). However, it is recommended that incorporation of antioxidants(Zinc&kolaviron) in diets especially those of plants source (kolaviron)will help to reduce the gastrotoxicity of arsenic acid.

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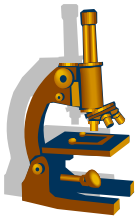
APPENDIX



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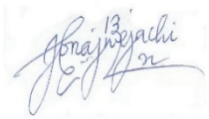
28th August, 2016

TO WHOM IT MAY CONCERN

I humbly wish to certify that the immunohistochemical studies of his /her research project was carried out in my laboratory between December, 2015 and March, 2016.

Kindly accord him/her all necessary assistance, please.

Yours Faithfully,



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