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Theme

Evaluation of Gastroprotection and Antioxidant effect of Pine (*Pinus halepensis*) barks on Indomethacin induced Ulcer in Wistar rats

Presented by: KAOUACHI Aicha

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President	**Dr DJAHRA A. B.**	**M.C.A El-Oued University**
Examiner	**Dr MEDILA I.**	**M.C.A El-Oued University**
Supervisor	**Dr. DEROUICHE S.**	**M.C.B El-Oued University**

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Dédicace

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Abstract

This investigation was aimed to study the effect of aqueous extract of the bark of *Pinus halepensis* and zinc (as antioxidants) for prevention of gastric ulcer in indomethacin induced gastric mucosal injury in rats. Thirty female albino Wistar rats, weighing between 117-182g were divided into 6 groups of 5 animals each (n = 5); Group 1: normal control, Group 2: INDO ulcer rats received normal saline, Group 3:ulcer rats was treated with aqueous extract of the bark of P.halepensis (300mg/kg), Group 4: ulcer rats was treated with zinc (1mg/kg), Group 5: ulcer rats was treated with aqueous extract of the bark of P. halepensis+ zinc and Group 6 ulcer rats was treated with standard drug ranitidine for 15 days. Stomach ulcer was induced by a single oral administration of indomethacin (30 mg/kg). Various parameters as, the volume of gastric juice, total acidity, ulcer index, percentage protection, pepsin activity, hematological, biochemical and antioxidant markers were estimated. Histopathology of stomach tissues was observed. Results of qualitative analysis revealed that the aqueous extract show richness in, phenols (coumaric acid), flavonoids, terpenoids, saponins, and carbohydrates and poor in alkaloids. Total phenol and flavonoid content shows highest concentration in aqueous extract of P. halepensis (34.10mg GA EQ/gm, 3.27mg QEQ/gm). IC50 values of the plant was 34.92µg/ml. In this study, the toxicity test showed no mortality or behavioral change up to 5000 mg / kg of albino Wistar rats. In this work, obtained results show that the ulcer index and total acidity were significantly reduced ($p < 0.05$) in ulcer induced rats pre-treated with test substances. Pepsin activity was decreased significantly ($p < 0.05$) when compared with indomethacin treated rats. The alteration observed in Hematological and some biochemical parameters in ulcer rats and showed significant restoration in aqueous extract of the bark of P. halepensis and zinc or combined groups. The level of MDA and GSH were significantly decreased ($p < 0.05$) in rats treated with extract. Histopathology of gastric mucosa confirmed the gastro-protection by plant and zinc treatment. The study reveals anti-ulcer, anti-inflammatory, analgesic and antioxidant property were observed in bark aqueous extract of P. halepensis groups with a benefic effect of zinc to reduced oxidative stress and gastric ulcer induced in rat.

Kywords: *Pinus halepensis*, zinc, ulcer, stomach, oxidative stress, phenol, Wistar rats.

الملخص

الهدف من هذا العمل هو دراسة تأثير المستخلص المائي للحاء نبتة الصنوبر الحلبي Pinus halepensis وعنصرالزنك (كمضاد للاكسدة) للوقاية من قرحة المعدة التي يسببها دواء الإندوميتاسين عند الجرذان. استعملنا في هذه الدراسة ثلاثين جرذ أنثى من نوع وستار البينوس، وزنها بين 117-182 غ حيث تم تقسيمها إلى 6 مجموعات (5جرذ في كل مجموعة) على النحو التالي; المجموعة 1: جرذان شواهد نظامها الغذائي عادي ، المجموعة 2: جرذان مصابة بالقرحة (INDO) ، المجموعة 3: جرذان مصابة بالقرحة معالجة مسبقا بالمستخلص المائي للحاء الصنوبر الحلبي (300مغ/كغ)، المجموعة 4: جرذان مصابة بالقرحة معالجة مسبقا بعنصر الزنك (1مغ/كغ)، المجموعة 5: جرذان مصابة بالقرحة معالجة مسبقا بالمستخلص المائي للحاء الصنوبر الحلبي+ الزنك و المجموعة 6 : جرذان مصابة بالقرحة معالجة مسبقا بدواء (Ranitidine) لمدة 15 يومًا. تم استحثاث قرحة المعدة عن طريق تناول دواء (Indomethacin) بجرعة واحدة مقدرة ب 30 مغ/كغ عن طريق الفم. في هذه الدراسة تم تقدير معايير مختلفة مثل ، حجم عصير المعدة ، الحموضة الكلية، مؤشر القرحة ، نسبة الحماية ، نشاط البيبسين، مكونات الدم ، بعض المعايير البيوكيميائية ومعايير الاجهاد التاكسدي. مع الملاحظة الضوئية لأنسجة المعدة. أظهرت نتائج التحليل الكيفي للنبتة أن مستخلصها المائي غني بمواد الايض الثانوي مثل الفينولات (خاصة حمض الكوماريك) والفلافونويد والتربينويدات والصابونين والكربوهيدرات وخلو المستخلص من القلويدات. نتائج المحتوى الفينولي والفلافونويدات في المستخلص المائي تظهر تراكيز كبيرة في النبتة المدروسة (34.10مغ/غ بالنسبة للفينولات و 3.27مغ/غ بالنسبة للفلافونويدات). اختبار السمية لجرعات كبيرة من النبتة تصل الى 5000 ملغ / كغ لم يظهر اي وفيات او اعراض جانبية تذكر على مستوى الجرذان. من جهة اخرى، اظهرت النتائج المتحصل عليها أن مؤشرا القرحة والحموضة الكلية انخفضا بشكل معنوي ($p <0.05$) في الفئران المصابة بالقرحة و المعالجة بالنبتة و الزنك كما اظهرت ايضا انخفاض كبير لنشاط البيبسين ($p <0.05$)عند نفس المجموعات مقارنة بالفئران المصابة بالقرحة دون علاج. وقد لوحظ من خلال النتائج ايضا تغير في المعايير البيوكيميائية و مكونات الدم عند الجرذان المصابة بالقرحة لكن في المقابل اظهرت المعالجة بالنبتة والزنك تحسنا في هذه المعايير. نتائج الاجهاد التأكسدي هي الاخرى اظهرت انخفاض في مستوى MDA و GSH بشكل ملحوظ ($P <0.05$) عند الفئران المعالجة بالمستخلص المائي للحاء الصنوبر الحلبي او الزنك مقارنة بالجرذان المصابة بالقرحة. وفي الاخير اكدت نتائج التحليل النسيجي ان المعالجة بواسطة لحاء الصنوبر الحلبي او الزنك تظهر حماية كبيرة للغشاء المخاطي للمعدة مما منع الاصابة بالقرحة المعدية عند الجرذان. من خلال هذه الدراسة نستخلص ان المستخلص المائي لنبات الصنوبر الحلبي له قدرة كبيرة على حماية المعدة من التقرحات كما يتميز بخصائص علاجية اخرى من خلال انه مضاد للالتهابات ، ومسكن للالم ومضادات للأكسدة كما لوحظ التأثير المفيد لعنصرالزنك في التقليل من الإجهاد التأكسدي وقرحة المعدة عند الجرذان.

الكلمات المفتاحية: الصنوبر الحلبي ، الزنك ، القرحة ، المعدة ، الإجهاد التأكسدي ، الفينول ، الفئران Wistar.

Abbreviation list

AA: arachidonic acid

Ach: Acetyl coline

ADP: adenosine diphosphate

AMP: adenosine monophosphate

ATP: adenosine triphosphate

Ca2+: calcium

CAT: catalase

CCK: cholecystokinin

COX-1: cyclooxygenase 1

COX-2: cyclooxygenase-2

DNA: deoxyribonucleic acid

DPPH: 1,1-diphenyl-2-picryhydrazyl

EC: epicatechin

ECG: epicatechin 3-gallate

ECL: enterochromaffin-like

EDTA: ethylenediaminetetraacetate

EGC: epigallocatechin

EGCG: EGC 3-gallate

GI: gastrointestinal

GOT: Glutamic Oxaloacetate Transaminase.

GPR: gastrin-releasing peptide

GPT: Glutamic Pyruvate Transaminase.

H+: hydrogene

H2O2: hydrogen peroxide

HCL: hydrochloric Acid

HCO3- : bicarbonate

HCT: haematocrit

HGB: haemoglobin

IL-1β : interleukins 1β

IL-6 : interleukins 6

IL-8: interleukins 8

IL-10 : interleukins 10

IND: indomethacin

iNOS : Inducible nitric oxide synthase

LDH: Lactate Dehydrogenase.

LPO: lipid peroxidation

LYM: lymphocyte

MDA: Determination of malondialdehyde

MDH: Malate Dehydrogenase.

MOS: mitochondrial oxidative stress

MPTP: mitochondrial permeability transition pore

NF-jB/Rel: Nuclear Factor-κB

NF-kappaB: nuclear factor kappa B

NF-κB: nuclear factor-kappa B

NO: nitric oxide

NSAIDs: non-steroidal anti-inflammatory drugs

O2•−: superoxide

ONOO: peroxynitrite

PG: prostaglandin

PG-E2: prostaglandin E2

PGHS: prostaglandin H2 synthase

PGII: Group II pepsinogens.

PH: hydrogen potential

PPI: Proton Pump Inhibitor

RBC: red blood cell

RNS: reactive nitrogen species

ROS: reactive oxygen species

ROS: reactive oxygen species

RS: reactive species

TNF α: tumor necrosis factor

WBC: white blood cell

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Introduction

Introduction

Stomach is a remarkable organ as it secretes digestive juices that can digest the various foods we eat, but seldom digests it-self. However, one pathological condition that has given the stomach serious problem is ulcer. It is a conglomerate of heterogeneous disorders, which manifests itself as a break in the lining of the gastrointestinal mucosa bathed by acid and/or pepsin. It is a major health hazard in fact considerable number of people worldwide (Akpamu, et al. 2013).

Gastric ulcer disease is a universal condition, its prevalence is estimated at 10% of the world's adult population (Driouche et al.2017). A number of factors are responsible for this gastric ulcer disease of which 70% to 80% are due to infection of Helicobactor pylori, a spiral shaped, gram negative bacteria also oxidative damage is responsible for gastric mucosal injuries, including ulceration, erosion and haemorrhage. It increases the formation of superoxide radicals and hydrogen peroxide (H2O2), lipid peroxidation and protein oxidation, which can directly promote damage of DNA, membrane lipids, cellular proteins and organelles (Murat et al .2015).

However, the use of non-steroidal anti-inflammatory drugs (NSAIDs) accounts for approximately 25% of gastric ulcer cases with an upward trend (Tarnawski et al. 2003). Non-steroidal anti-inflammatory drugs (NSAIDs) are some of the most commonly prescribed drugs in the world (Sung et al.2017) and the most second popular medications, following antibiotics in medical and dental uses over the world (Omar et al.2017). As a result of their anti-inflammatory, analgesic, and antiplatelet effects, NSAIDs are used in clinical practice for treatment and prevention of rheumatoid arthritis, osteoarthritis, collagen disease, and ischemic cardiovascular or cerebrovascular disease (Sung et al.2017).

Indomethacin is a non-steroidal anti-inflammatory agent with antipyretic, analgesic properties and is an indole derivative designated chemically as 1-(p-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid (Karima Fadhil Ali et al.2015).

Natural products have a wide range of diversity of multi-dimensional chemical structures; in the meantime, the utility of natural products as biological function modifiers has also won considerable attention. Subsequently, they have been successfully employed in the discovery of new drugs and have exerted a far-reaching

impact on chemicobiology (Haidan et al.2016). Pine *Pinus halepensis* are a large species of trees which grew widely in temperate regions are a large species of trees which grew widely in temperate, it has been used in traditional medicine in many parts of the world.

Thus, this study was carried out to investigate the gastro-protective effect of aqueous extract of P. halepensis barks and Zinc against experimental gastric ulcer induced by indomethacin in rats.

First part

Bibliographic synthesis

Chapter I

Stomach

1. Anatomy and histology

1.1. definition and situation

The stomach is a hollow muscular organ of the digestive tract in the shape of a pocket or in the shape of an uppercase J, located in the upper part of the abdomen between the esophagus and the duodenum.In adults, it is 20 cm long, 10 to 12 cm wide, 8 to 9 mm thick, a capacity of 1.5 to 3 liters and a pH that varies between 1.5 and 5 (figure01).The core function of the human stomach is as an aid to digestion.(Nthony O'Connor*et al*, 2014) (BouchoukaRacha-Lydia, 2017).

Figure 01: Situation of the stomach (Bouchouka Racha-Lydia.2017).

1.2. anatomical description

The opening from the oesophagus into the stomach cardiaclocated near the heart. to the left of the cardiac region, the fundus. The largest part of the stomach is the body, which turns to the right. The body narrows to form the pyloricregion, which joins the small intestine.The opening between the stomach and the small intestine is the pyloric opening (figure02.a)(Seeley *et al*,2004). The two main types of glandular mucosa of the stomach, the acid-secreting oxyntic mucosa of the body and fundus and the mucus gland mucosa of the antrum. The gastric mucosa contains many deep glands (CESARE BORDI*et al*, 2000) (figure02.b). In the cardia and the pyloric region, the glands secrete mucus. In the body of the stomach, including the fundus, the glands also contain parietal (oxyntic) cells,which secrete hydrochloric acid and intrinsic factor, and chief (zymogen, peptic) cells,which secrete pepsinogens, and, enterochromaffin-like (ECL) cells, which release the paracrine agent histamine, and cells that secrete the peptide messenger somatostatin, are scattered throughout the tubular glands (Kim E. Barrett*et al*, 2010) (Vander *et al*,2001). The pyloric glands cover the gastric antrum and pylorus and contain gastrin cells (G cells), mucous cells, and other endocrine cells. Each of these cell types has evolved into a highly specialized secretory cell

that contributes to gastric secretion (John Del Valle, Andrea Todisco,2009). (figure02.c). according to (R.Greger/U. Windhorst. 1996), (CESARE BORDI et *al*,2000) and (SUSUMU ITO*et al*, 1963) the gastric cells secretion and functions of secreted is in the **Tab.01**

Table01: **The gastric cells secretion and functions of secreted**

Cell type	Substance secreted	Function of secreted
Mucosa surface cell	Mucus	Protection of lumen surface against pepsin
Mucosa neck cell	Bicarbonate	Protect the gastric mucosa from the damaging effect of acid
Parietal cell	Gastric acid HCL	Activate pepsin
	Intersect factor	Form a complex with vitamin B_{12}
enterochromaffin-like	Histamine	stimulating acid secretion from parietal cells
Chief cell	Pepsin	Digests Proteins
	gastric lipase	Digests fats
D cell	Somatostatin	inhibit acid secretion
G cell	Gastrin	Stimulate gastric acid secretion

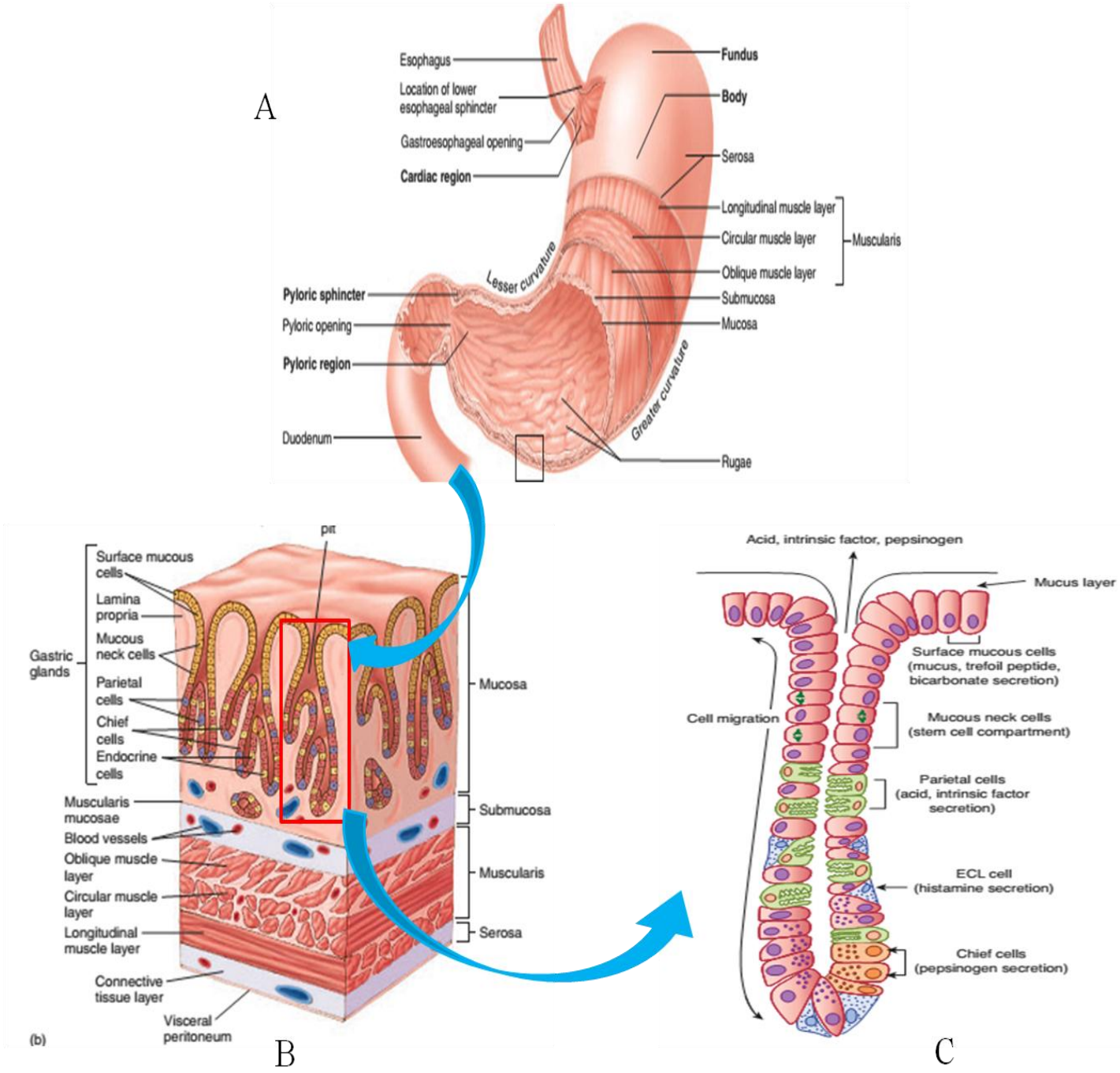

Figure 02 : Anatomy and Histology of the Stomach.(Rod Seeley,et al. 2000).

(KIM E. BARRETT et al.2010).

1.3. Gastric secretion

The fluid is secreted into the stomech called gastric juice**.** Gastric secretion represents a very complex mixture of electrolytes. water. carbohydrates. proteins. peptides. and amino acids. which are partly in solution and partly in more or less stable suspension.The large molecular materials of gastric secretion include: enzymes. mucosubstances. serum proteins. peptides and products of proteolytic degradation of gastric proteins and mucoproteins. and blood group substances. as well as various biologically active materials,the three major constituents of gastric juice are : mucus component, the enzyme component and the aqueous component. (GeorgeB.JerzyGlass,1964) (R.Greger/U. Windhorst. 1996).

1.3.1. Hydrochloric acid

1.3.1.1. Secretion

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 via channels in the mucus layer created by the relatively high intraglandular hydrostatic pressures generated during secretion, approximately 17 mm Hg.47(MITCHELL L. SCHUBERT, DAVID A. PEURA, 2008).Hydrochloric acid production and excretion showing in fig.03, where CO_2 diffuse into the cell and combined with H_2O to form H_2CO_3 which dissociate to HCO_3 and H^+. HCO_3beck to blood by extarchage with Cl^- which diffuse to gastric glad by exchange by K^+. H^+ actively transported into duct gastric gland.(Seeley−Stephens−Tate,2004).

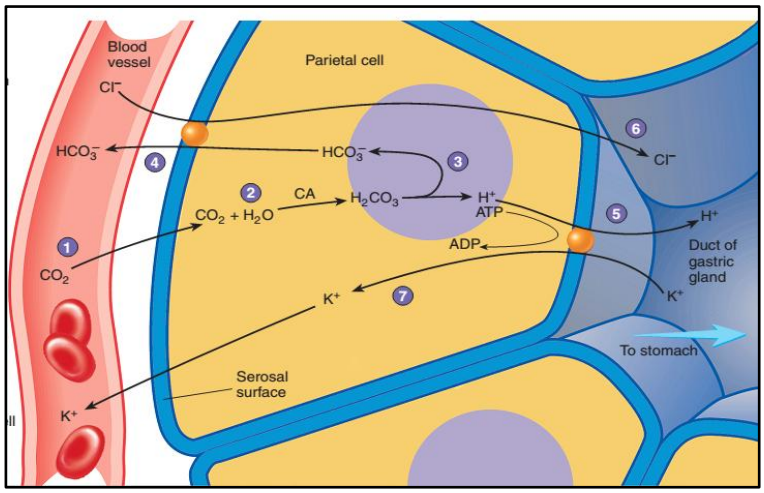

Figure 03: Hydrochloric Acid Production by Parietal Cells in the Gastric Glands of the Stomach.(Seeley−Stephens−Tate,2004).

1.3.1.1.1. Functions and regulation secretion

Gastric acid secretion can be divided into 3 phases: the cephalic, gastric, and intestinal phases.The cephalic phase of gastric acid secretion is mediated by vagal excitation stimulated by the thought, sight, smell, or taste of food.This can be elicited bysham feeding. Vagal excitation causes the release of acetylcholine, which stimulatesgastric acid and pepsin secretion(Mercer DW, Robinson EK.2008).The gastric phase of gastric acid secretion is mediated by gastric distention as foodenters the gastric lumen. HCl function is to maintain a sterile environment and to initiate the conversion of pepsinogen to pepsin.The majority of gastric acid release occurs during thisphase(Silen W,1993)(.The intestinal phase of gastric acid secretion is primarily inhibitory and begins whenfood enters the small intestine.The least amount of gastric acid is released during thisphase.Intraintestinal acid inhibits gastric acid secretion through an enterogastrone(Gregory S, Kelly ND,1997).

1.3.2.Pepsinogen

1.3.2.1. Secretion

Pepsin, by definition, is a proteolytic enzyme maximally active at a highly acid pH and inactivated in neutral or alkaline solution.The chief cells secrete pepsinogens, contained in zymogen granules. These are the precursors of the pepsins (proteases) in gastric juice. Hydrochloric acid release in the stomach stimulates the release of pepsinogen from the chief cells in the stomach. Acid secretion lowers the pH, which allows pepsinogen to unfold and cleave itself in an autocatalytic fashion, thereby generating pepsin (the active form)(John F. Reinus, Douglas Simon,2014) (John WL Fielding،Michael T. Hallissey,2005).It acts at pH 1.5–2.5 and above pH 5.4 is inactivated (figuer 04).Group I pepsinogens (PGI; old term, pepsinogen A) are expressed in chief and mucous cells of the oxyntic mucosa. Group II pepsinogens (PGII; old term,pepsinogen C), which represent 20% of total pepsin content, are expressed in oxyntic and pyloric mucosa as well as in duodenal Brunner’s glands (D. F. Magee, M.D., Ph.D,1974).

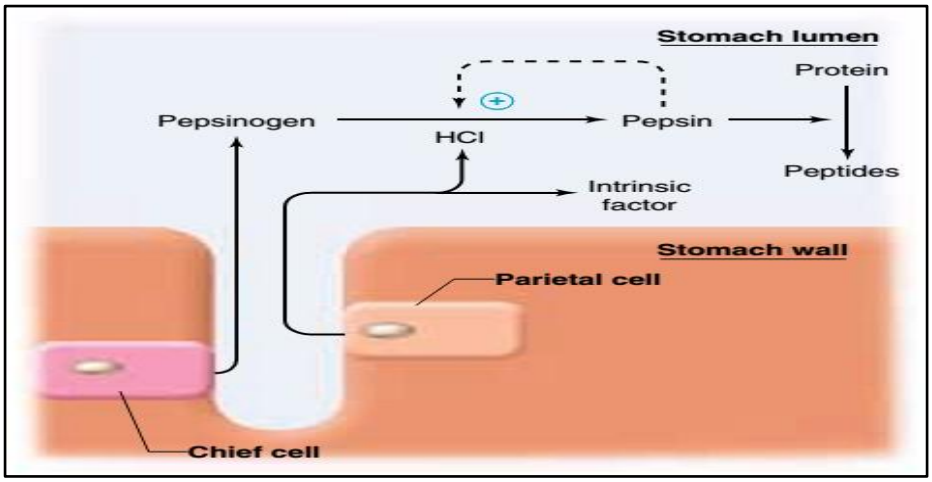

Figure: 04: Pepsin secretion (Vander et al,2011)

1.3.2.2. Function and regulation secretion

Pepsin acts on proteins, splitting them into proteoses, peptones, and polypeptides, but not to the amino acid state,Theyhavea specialaffectionforpeptidebondswhichinvolve phenylalanine,tyrosineorleucine. ACh released from intramural cholinergic neurons is the most important physiologic stimulant for pepsinogen secretion. Other stimulants include gastrin, histamine, CCK, and GRP. ((John F. Reinus, Douglas Simon,2014) (HARRY H. LE VEEN,LEONARD HALLINGER.1946)(D. F. Magee, M.D., Ph.D,1974).

1.3.3. Mucus-bicarbonate barrier

In the stomach several mucosal defence mechanisms protect the stomach against hydrochloric acid and noxious agents. The pre-epithelial protection is made up by the mucus-bicarbonate barrier. Mucus and bicarbonate, secreted by mucus cells, create a pH gradient maintaining the epithelial cell sur- face at near neutral pHThe mucus cells of the gastric epithelium produce a visco elastic gel.(figure05)(Henrik Forssell, 1988)

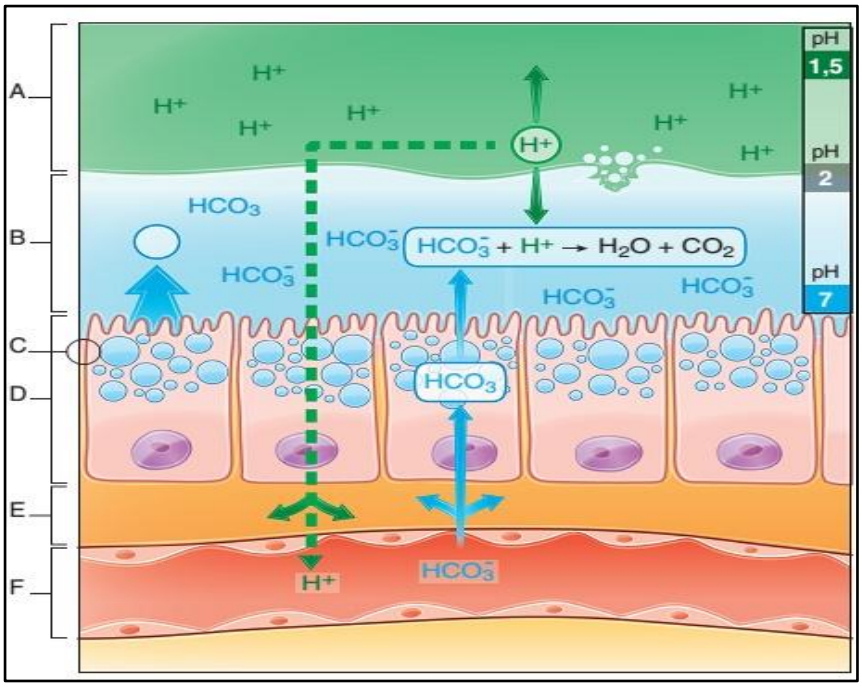

Figure 05 : The mucus-bicarbonate barrier.(Christophe Aubé et al.2014).

A – Mucus, B - Bicarbonates / phospholipides (bulle), D - Cellules épithéliales, C – Jonctions serrées, F - flux sanguin sous-épithélial (prostaglandines ++)(Christophe Aubé et al.2014).

1.3.3.1..Mucus

Mucus forms a continuoussticky water-insoluble glycoprotein gellayer over the gastric epithelium and, according to a recent study, is about 0.5 mm thick in the human stomach,Mucus is secreted to form a flexible gel adhering to the surface of the gastric mucosa (figure06)(W.D.W.REES AND L.A. TURNBERG,1982).It exists a balance between production on the one hand and degradation by pepsin and shear forces on the other. Mucus has generally been accepted as protecting the epithelium from damage by the passage of foodand also to provide a lubricant for the movement of solid material, this properties being important in preventing shear and stress forces from disrupting its integrity. (CHRISTOPHER J. SHORROCK,WYNNE D.W. REES,1988) (Allen and Garner,1980)

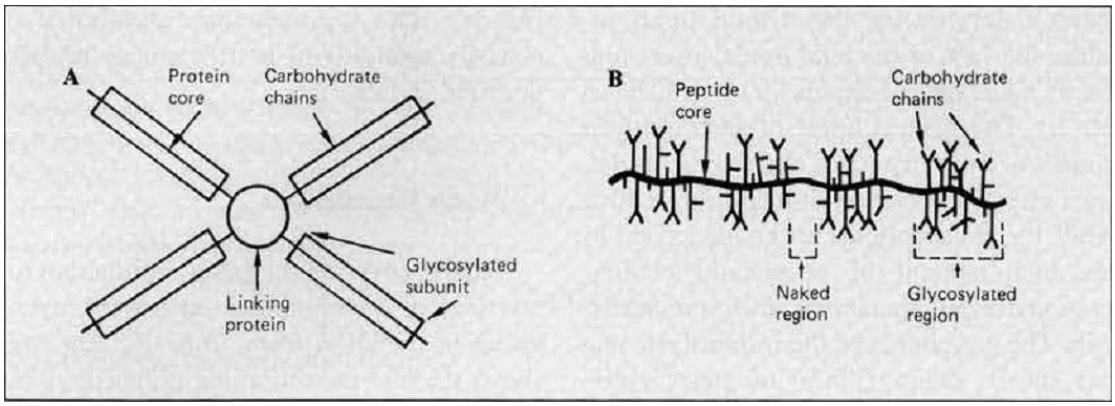

Figure 06: Proposed windmill and linear models of gastric mucus glycoprotein structure

(Bronislaw L, Slomiany, Jerzy Sarosiek, Amalia Slomiany. 1987)

1.3.3.2. Carcohydrate

Bicarbonate is secreted by surface epithelial cells of the stomach at a basal rate of about 5 to 10 percent of maxi-mal acid output. Bicarbonate ions are transported across gastric mucosa either by a metabolically dependent transcellular route involving chloridebicarbonate exchange at the apical membrane of surface epithelial cells or through intercellular channels by passivediffusion.When the parietal cell secretes H+ into the lumen it also delivers a similar number of $HCO3^-$ into the fenestrated capillary which passes its basolateral membrane and carries the latter ions to the surface epithelium. The $HCO3^-$ions then diffuse out into the surface mucus layer, neutralising any back diffusing hydrogen ions(figure 07)(Kenneth E L McColl,2011)

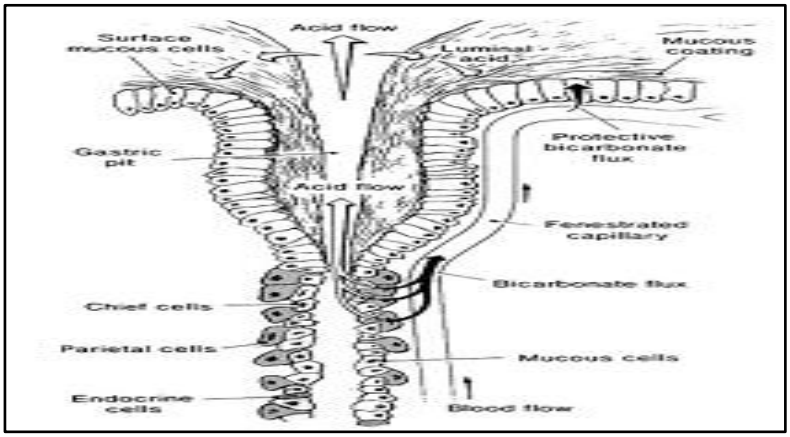

Figure 07: Schematic diagram of the mucus bicarbonate barrier of the acid secreting gastric mucos **(**Kenneth E L McColl,2011)

Reaction between H+ diffusing into the gel from the lumen ofthe stomach and HC03- secreted by the surface epithelial cells results in the formation of CO2 and water. In the acid secreting stomach, HC03- appears in the lumen as CO2 and at high secretory rates some CO2 could be reabsorbed and utilised by the parietal cells.(Figure 08) (W.D.W.REES AND L.A. TURNBERG,1982).

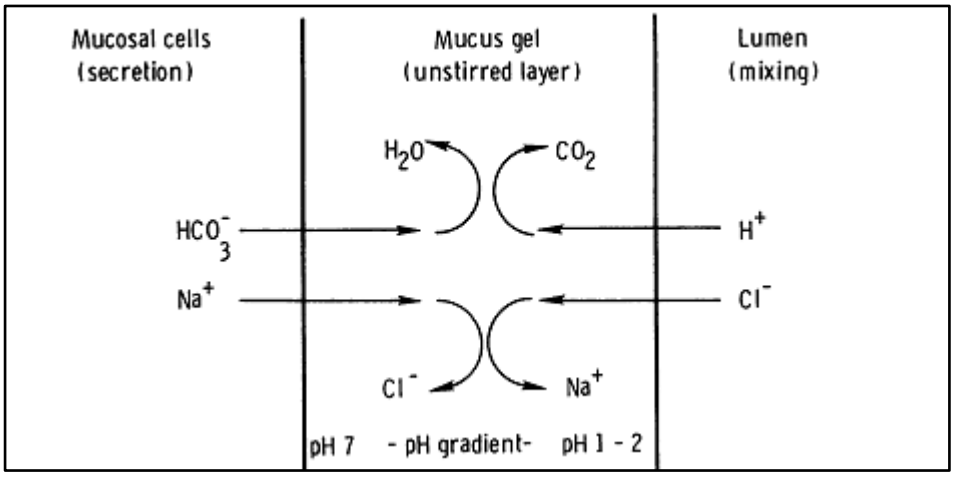

Figure 08 :A model Jor surface neutralisation within the unstirred layer of the gastric(Allen and Garner,1980)

2.Gastric diseases

2.1.Gastric ulcer disease

A gastric ulcer, also called stomach ulcer, is a deep lesion penetrating through the entire thickness of the gastrointestinal mucosa and muscularis mucosa. Ulcer disease whatever in, stomach is one of the main prevalent still unresolved medical problems that faces many patients in a wide rang of age of both sexes worldwide.(Muobarak J Tuorkey, Abdul-Aziz K K.2011)(John W. L. Fielding,Michael T. Hallissey,2005).

2.1.1 causes

Peptic ulcers fundamentally result from a breakdown in the mechanisms that normally protect the gastroduodenal mucosa from the high concentrations of acid and pepsin within the lumen, many factors directly related to impairment in mucosal defense can alter the epithelial barrier and encourage the formation of mucosal injury, the most important of which are acid secretion, bacteria and their products,non-steroidal anti-inflammatory drugs, alcohol, as well as different chemical compounds. Their effects on the gastric barrier represent important mechanisms of the pathogenesis of gastric ulcer (figure09)(Akiko Shiotani, David Y. Graham,2002).

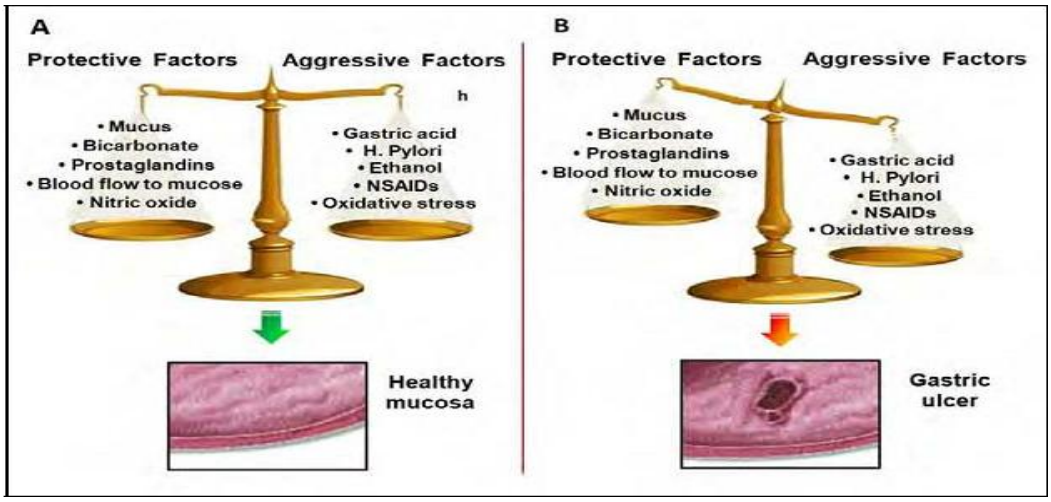

Figure 09: (A) Healthy gastric mucosa: balance between mucosal aggressive and protective factors. (B) Gastric ulcer formation: imbalance between mucosal aggressive and protective factors.

(John W. L. Fielding,Michael T. Hallissey,2005).

2.1.2. symptomes

The most common symptoms are waking at night with upper abdominal pain or upper abdominal pain that improves with eating. The pain is often described as a burning or dull ache. Other symptoms include belching, vomiting, weight loss, or poor appetite. About a third of older people have no symptoms (Subrata Roy,2016).

2.1.3.traitments

2.1.3.1 Ranitidine

Ranitidine hydrochlorideis N-{2-[[[5-[(dimethylamino)methyl]-2furanyl]methyl]thio] ethyl}-N□-methyl-2-nitro-1,1-ethenediamine (Figure10), a histamineH2 receptorantagonistwithafuranringstructure that increases its potency to inhibit gastric acid secretion inducedbyvariousstimuli (NighatShafi et al,2013).

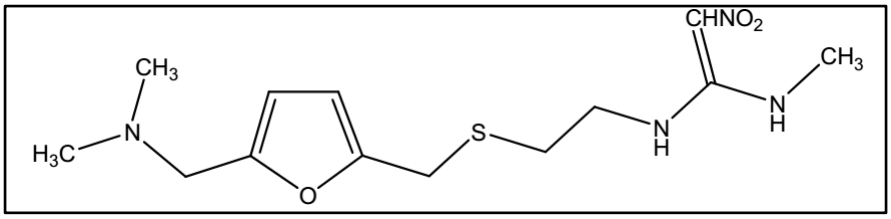

Figure 10: Ranitidine structure (Rakesh Pahwa et al.2016)

2.1.3.2. Mode of action

H2-receptor antagonists exhibit competitive inhibition at the parietal cell H2 receptor, and suppress basal and mealstimulated acid secretion. H2-receptor antagonists inhibit acid production by reversibly competing with histamine for binding to H2 receptors on the basolateral membrane of parietal cells (Rakesh Pahwa et al.2016)

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Chapter II

Indomethacin

& Oxidative stress

1. Definition

Indomethacin, or 2-(1-[(4-chlorophenyl) carbonyl]-5-methoxy-2-methyl-1H-indol-3-yl) acetic acid, is a nonsteroidal anti-inflammatory drug (NSAID) commonly used as a prescription medication to reduce fever, pain, stiffness, and swelling (Figure11). It works by inhibiting the production of prostaglandins, moleculesknown to cause thesesymptoms. (Fig.11) (SamanehAbyariMiyandoab and Mirzaagha Babazadeh,2015).

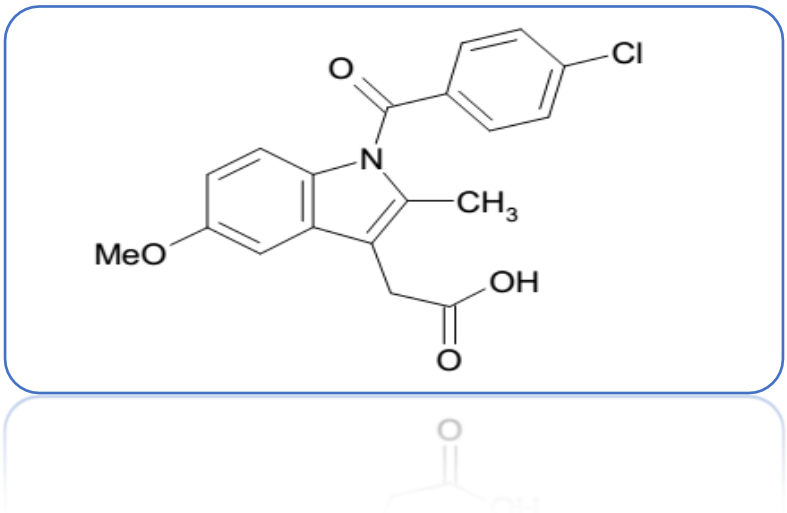


Figure 11. The structure of indomethacin

(SamanehAbyariMiyandoab and Mirzaagha Babazadeh,2015).

1.2.clinicals effects of Indomethacin

The clinical effects usually may be digestive disorders with intensities stemming from stomach pain discomfort to ulcers, people whose use of Indomethacincould be described as chronic degrees of gastric ulcers (mostly asymptomatic). Furthermore, renal failure and hyperkalaemia, cardiac failure, epistaxis, and bronchospasm are among the dangerous complications that have been reported following the administration of Indomethacin (MaryamSoleimanpour et al. 2016).

1.3. Indomethacin and gastric damage

NSAIDs inhibit the COX pathway of prostaglandin synthesis. This represents the basis of anti-inflammatory action but is also responsible for the development of side effects in the gastrointestinal tract. Inhibition of prostaglandin synthesis can exertinjurious actions on the gastric and duodenal mucosa as it abrogates a number of prostaglandin dependent defence mechanisms (Halter, Schmassmann, Peskar,et al . 2001).

1.3.1 Mechanisms of indomethacin induced gastric ulcer

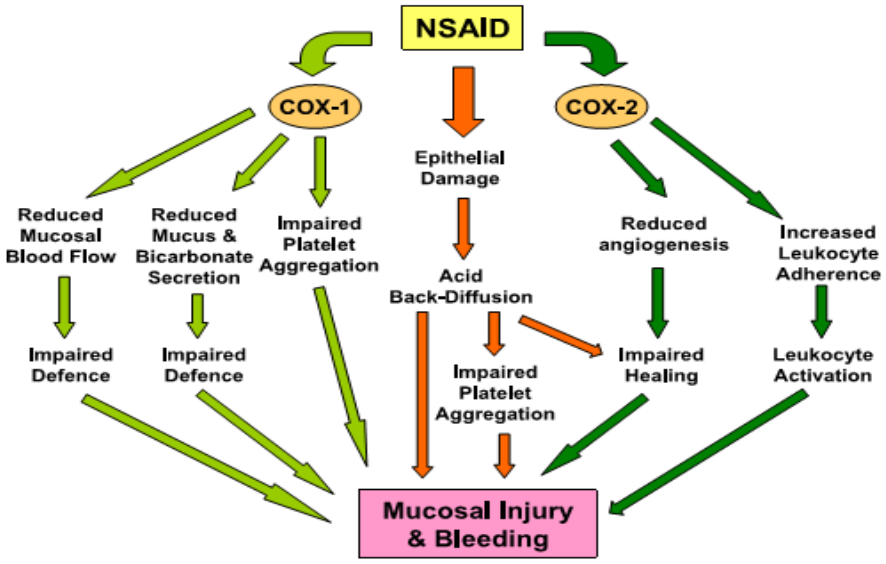


Figure 12: Mechanisms of indomethacin induced gastric ulcer

1.3.1.1 Prostaglandin inhibition

Cyclooxygenase (COX) or prostaglandin H2 synthase (PGHS) is the enzyme that catalyses the first two steps in the biosynthesis of the prostaglandins (PGs) from the substrate arachidonic acid (AA). The biosynthetic activity of both isoforms can be inhibited by indomethacin, this inhibition is due by competing with the substrate AA for the active site of the enzyme. Inhibition of prostaglandin synthesis thus plays a key role in induction ofthe gastric and duodenal mucosa injury (J. R. Vane,Y.S. Bakhle1, and R. M. Botting. 1998).

1.3.1.1.1 prostaglandin roles

PGs are involved in the regulation of various gastric functions that contribute to gastric protection., PGE2 increased mucus and HCO3- secretion, and inhibited acid secretion, respectively. The inhibitory effect of PGE2 on acid secretion was mediated in two ways by EP3 receptors, directly by inhibiting acid secretion at the parietal cells and indirectly by inhibiting histamine release at enterochromaffin-like (ECL) cells (Koji Takeuchi, Kikuko Amagase. 2017).

1.3.1.2 Topical actions of NSAIDs on the gastric epithelium

Topical application of NSAIDs increases gastrointestinal permeability allowing luminal aggressive factors access to the mucosa“back diffusionof hydrogen ion”. NSAIDs are weak organic acids. In the acidic milieu of the stomach, they are converted into more lipid soluble

unionised acids that penetrate into the gastric epithelial cells. There, at neutral pH, they are reionised and trapped withinthecellcausinglocalinjury (Halter,Schmassmann,Peskaret al.2001) (ROBERTT. SCHOEN, M.D., F.A.c.P., RONALD J. VENDER, M.D.1989).

1.4.Indomethacin and duodenal damage

NSAIDs were absorbed into the enterocytes, and uncouples the mitochondrial oxidative phosphorylation. This uncoupling causes dysfunction of the tight intracellular junctions and increases the intestinal permeability. Through the mucosal barrier whose permeability was increased, the enterocytes is exposed to luminal aggressive contents such as bile acids, hydroeolytic/proteolytic enzymes, resulting in neutrophil chemotaxis with activation of neutrophils causing nonspecific inflammation and ulcerations (Hirofumi Matsui et al. 2011).

1.5.Indomethacin and liver disorder

NSAIDs rarely cause hepatic damage, and any hepatic effects are usually reversible. NSAIDs with more potential for hepatic problems include droxicam, clometacin, and sulindac (Licata A.2010)

1.6.Indomethacin and kidney disorder

NSAIDs usage can cause a variable degree of renal dysfunction, ranging from a reversible impairment of glomerular filtration rate to irreversible renal damage(IppokratisPountos et al.2011).

2.Stress oxydative

2.1. definition

In biological systems, oxidative stress is the consequence of aimbalance between the production of free radicals and destruction byAnti-freeing defensessystems.Free radicals can causesignificant damage to cell structure and metabolism by degradingmany targets: proteins, lipids and nucleic acids. free radicalsare a particular form of chemical species (atoms or molecules) thathave a single (or unmatched) electron (Hamadi Nacereddine,2010).

2.2. Free radicals

Free radicals are molecules or molecular fragments containing unpaired electrons in atomic or molecular orbitals. One of the most important groups of these free radicals comes from the metabolism of oxygen and is called the reactive oxygen species (ROS). Another group comes from nitrogen metabolism, known as reactive nitrogen species (RNS)The formation of free

radicals is a physiological process and some play important roles in molecular signaling. Under normal conditions, these free radicals are removed by the antioxidant system to maintain a balance between oxidants and antioxidants.(Xiaoling YANG,2009).

2.3. Oxidative stress origin

Free radicals are constantly produced in small quantities, some internal sources of generation of free radicals are mitochondria, xanthine oxidase, phagocytes, reactions involving iron and other transition metals, peroxisomes, Arachidonate pathways, exercise, ischaemia / reperfusion, inflammation. Some external sources of free radicals are cigarette smoke, environmental pollutant, radiations, ultraviolet light, ozone, certain drugs, pesticides, anesthetics and industrial solvents (Shiv Kumar,2011).

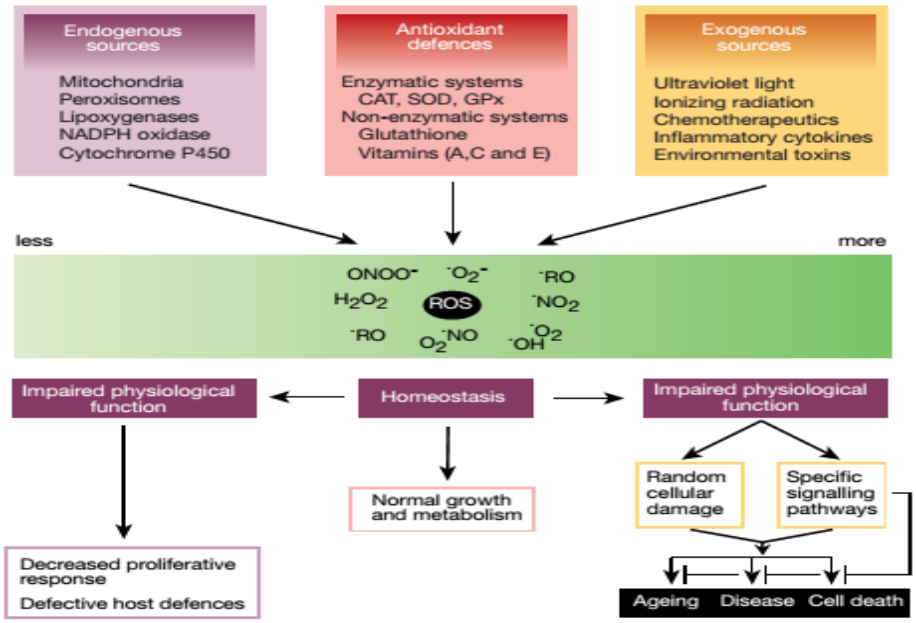


Figure 13 : The sources and cellular responses to reactive oxygenspecies (ROS) (Toren Finkel et al.2000)

2.4. Oxidative stress and Indomethacin drugs

A well-known NSAID, indomethacin (IND) was found to bind to a site adjacent to the complex I and ubiquinone of mitochondrial electron transport chain. Inactivation of mitochondrial aconitase results in the production of free iron, which in turn generates more mitochondrial •OH. Mitochondrial dysfunction, formation of the mitochondrial permeability transition pore, and generation of mitochondrial oxidative stress (MOS) is associated the oxidative stress (Krishnendu Sinha et al.2015).Gastric and duodenal ulcers represent the most common and chronic PUDs, in wish ROS-mediated increased lipid peroxidation, lowered

GSH level, and antioxidant systems are involved in the pathogenesis of almost all forms of gastric ulcer (Asima Bhattacharyy.2014).

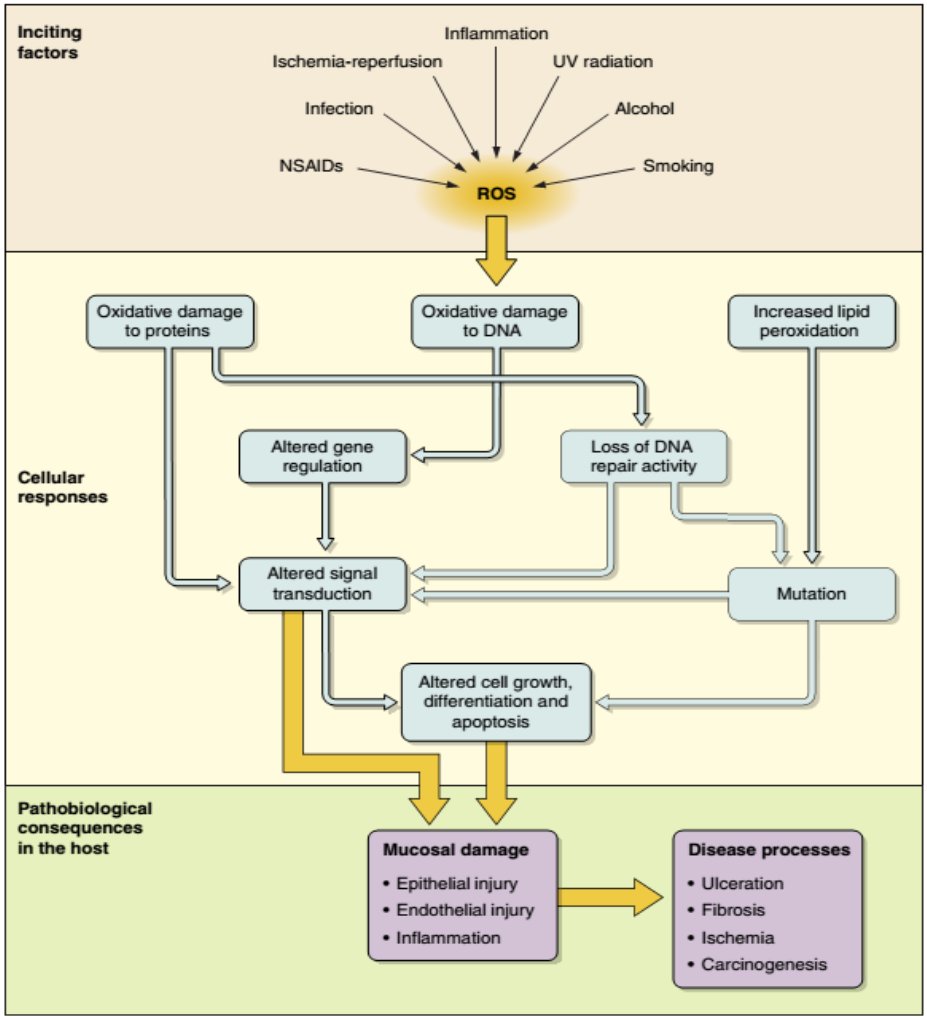


Figure 14: Schematic diagram showing the induction ofoxidative stress and its pathophysiological effects. Oxidativestress damages internal organsby causing mucosal injury (Asima Bhattacharyy.2014).

2.5. Antioxidantsystems

Continuous exposure to various types of oxidative stress from numerous sources has led the cell and the entire organism to develop defense mechanisms for protection againstreactivemetabolites (RON KOHEN et al.2002). There are various natural or synthetic antioxidant compounds. Natural antioxidants include various vitamins (vitamins A, C and E), compounds that can be synthesized by animal cells (glutathione, melatonin) or of plant origin diet (flavones, resveratrol and other polyphenols, carotenoids / lycopene). and other compounds with enzymatic catalytic activities (ebselen mimicking glutathione peroxidase

activity, synthetic complexes manganese mimicking superoxide dismutase or catalase activity) (E. Descamps.2006).

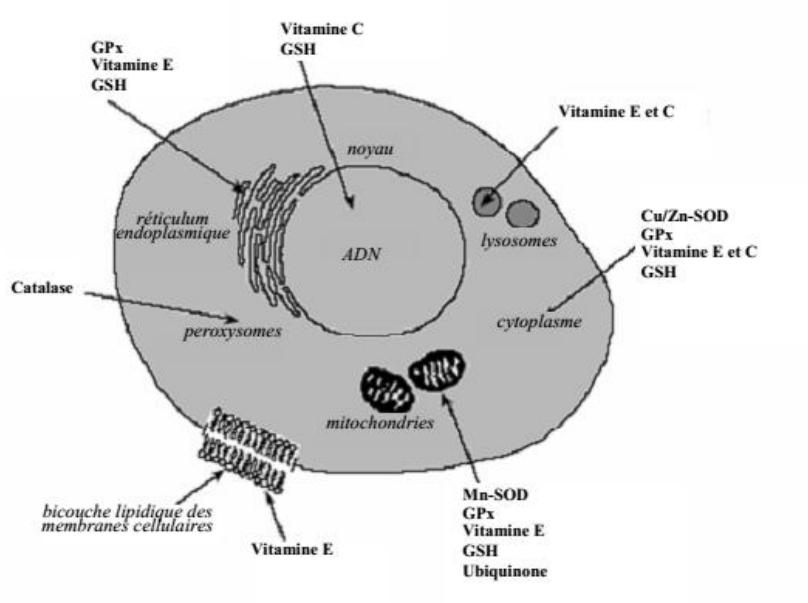


Figure 15: function levels of antioxidant systems (Blandine GARAIT. 2006).

2.5.1. Zinc as anti-oxidant agent

Zincisanintracellular signalling moleculeanditplaysanimportant role in cell-mediatedimmune functionsandoxidativestress.Zincis alsoananti-inflammatoryagent.Theseuniqueproperties ofzinc mayhavesignificanttherapeutic benefitsin several diseasesin humans (AnandaS. Prasad.2009).

The role of zinc in the antioxidant defense system has been widely investigated. Studies have highlighteditsroleintheregulationofglutathioneperoxidaseandintheexpressionofmetallothionein , as well as its role as a co-factor for superoxide dismutase. Moreover, zinc competes with iron and copper in the cell membrane, inhibits the NADPH-oxidase enzyme, and reduces chronic inflammation and hyperglycaemia (Dilina do Nascimento Marreiro,2017).

Chapter III

Pinus Halepensis

1. Definition

The Aleppo pine is an evergreen and green tree of height that exceeds twenty meters. It has a tortuous trunk, covered with a thick and cracked bark. Its silver gray and smooth bark turning to reddish brown with age (AMARI Meryem and MECHOUCHE Katia.2017)

2.Taxonomy

Pinus halepensis Mill otherwise called "Dbaghessnawber" in Arabic (Abdalla et al.2014)., Scientific name given by Philip Miller in 1768. The Aleppo pine belongs to the pinaceae family (Abietacees), genus Pinus, subgenus Pinus (Eupinus), section Halepensoids, and subgroup Halepensis. Its classification is (TAIBI .2017)

Kingdom: Plantae

Ordre: Coniférale, Pinoidine,Pinale

Subkingdom: Tracheobionta.

Family: Pinaceae (Abietaceae)

Division: Spermaphytes.

Genus: Pinus.

Subdivision: Gymnospermes.

Species: *Pinus halepensis***.**Mill

Figure 16: *Pinus halepensis***.** Tree
http://texastreeplanting.tamu.edu/ViewAllTrees.aspx

3. Distribution of *Pinus halepensis*

3.1. In the world

The geographical range of Aleppo pine is limited to the Mediterranean basin and occupies more than 3.5 million hectares. The species dominates forest ecosystems in the semi-arid areas of the Mediterranean basin. In addition to its natural range, this species has been widely used in afforestation projects and programs during the 20th century (Brahim. 2015). It is well represented in the mountain ranges of the Maghreb, in Eastern Spain, in the Balearic Islands and in Provence for the western part of the Mediterranean basin. In the eastern part of the Mediterranean basin it is found mainly in southern Greece. Finally, it is also found sporadically in Italy, Corsica, Sardinia, Sicily, Croatia, Lebanon, Israel, Jordan and Cyrenaica (figure 14) (CYRILLE, 2002).

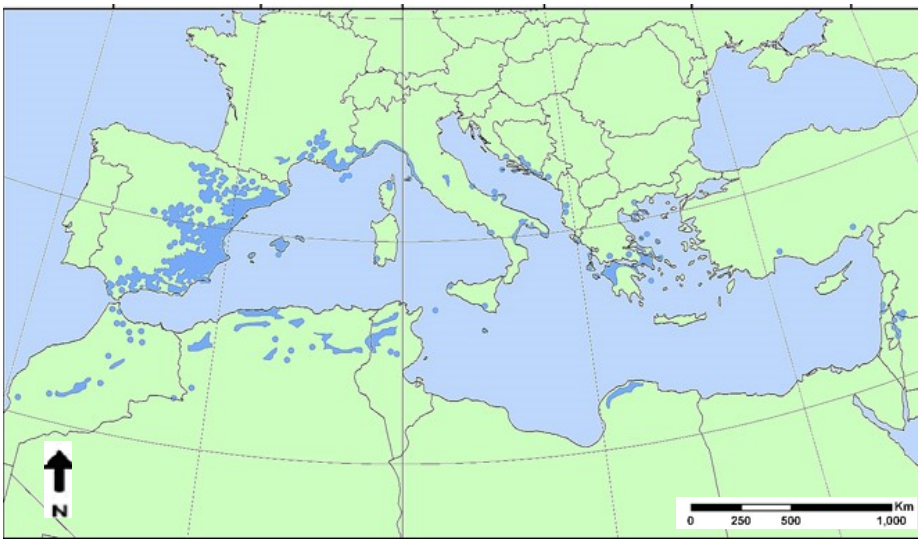


Figure 17: Distribution of *Pinus halepensis* in the world.

www.euforgen.org/species/pinus-halepensis.

3.2. In Algeria

In Algeria, it occupies 35% of the forest area. It forms settlements in the Tébessa region, the Constantinian plateaus and the Aures, the Algiers region (Medea forests), Bel Abbes, Saida and Ouarsenis, the Saharan atlas and the Djelfa region. The mountains of Ouled-Nail (figure 15).

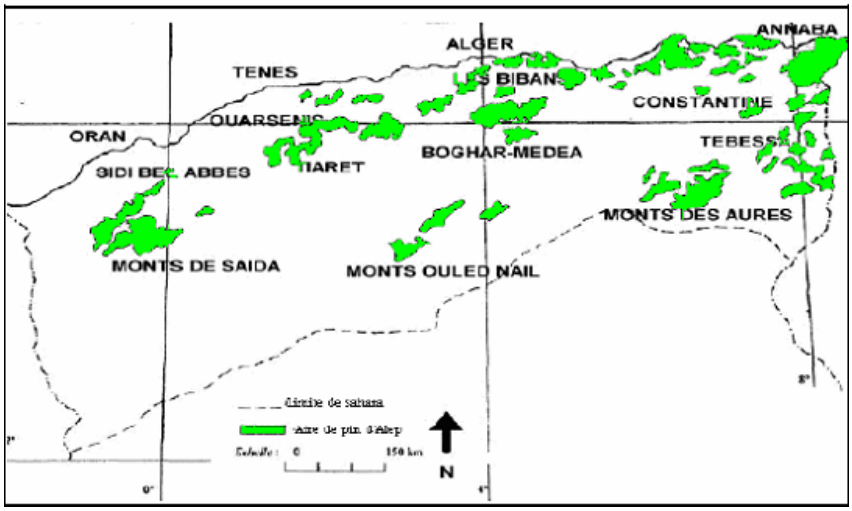


Figure 18: Distribution of *Pinus halepensis* in Algeria

(TAIBI ,2017)

4. Economic interest of the specie

Ecologically, *Pinus halepensis* is the most important forest species in many Mediterranean countries. It is generally used in reforestation programs for degraded soils, case of the "green belt" in southern Algeria, where 1 million hectares were planted with Aleppo pines more than 20 years ago (TAIBI, 2017).

Its wood is used in construction, industry, carpentry, wood and pulp, for the shoring of mines, shipbuilding and carpentry. The Aleppo pine gives about 3 kg of resin (the gem) per tree per year (Kadik, 1987). It's very resinous buds are used as balsamic and diuretic (syrups and lozenges). Norway's tar, with balsamic and antiseptic properties, is also extracted from the wood. Pine seeds are edible and used in pastry and confectionery or can be eaten raw by breaking their hulls (TAIBI,2017).

Second Part

Experimental Part

Chapter I

Materials and Methods

1. Materiels

1.1. Plant materials

In this study, pinus barks (*Pinus halepensis*) were obtained from the herbalist. These barks were powdered by mechanical grinder until a fine powder was obtained. The powders of *Pinus halepensis* are stored at room temperature in airtight containers protected from bright light until the beginning of the experiment.

Figure 19: *Pinus halepensis* barks (original photo).

1.2. Aqueous extract preparation

Aqueous and organic extracts were preparingby putting 10g of homogenized dried barks in 100 ml of distilled water at 50 C during 2h then the extract was cooled and filtered after 24h. for Aqueous extract was drying using etuve.

1.3. Animal materials

1.3.1. Animal and husbandry condition

In this study, 30 female Wister rats aged 8 weeks old and weighting 117-182g, were obtained at the Animal Service of the Pasteur Institute, Algeria. The animals were carried under the same conditions, photoperiod (12h of light/12h of black) with a relative humidity of 65.3% and an ambient temperature of (25 ± 2) C° for four weeks. Animals have free access to water and food by a standard diet (Southon et al, 1984) (Table 2). The experiment was conducted over a period of 15 days.

Table 2 : Standard diet composition **(**Southon *et al*, 1984**)**.

Raw materials	**Quantity (g / kg)**	**Percentage (%)**
Maize	326	32.6
Sacrose	326	32.6
Protein	168	16.8
Cellulose	40	4
Minerals	20	2
Vitamin	20	2
Oil	40	4

1.3.2. Ulcer induced

Pinus halepensis extracts and drugs were administered to the animals in there water for 15 days. After 15-day treatment, the animals were fasted for 24 h. Ulcer was induced by gavage using indomethacin (dissolved in 2% NaCO3) and brought to a volume of 1.0 ml with saline (pH 6.8) at a dose of 30 mg/kg on the day of sacrifice.

1.3.3. Treatment animals

After a period of adaptation, the animals were divided into sixexperimental groups of 5 animals eachas follows:

Group 1(Control groups): were normal rats received 1ml of normal saline oral.

Group 2 (IND treated groups): were gastric ulcer induced rats which received normal saline, oral for 15 days and then received single oral dose of indomethacin (IND, 30 mg/kg).

Group 3 (IND+P. hal treated rats): were rats received oral dose of aqueous extract of *Pinus halepensis* (300 mg\kg) for 15days and then received a single oral dose of IND.

Group 4(IND+Zinc treated rats): were rats received oral dose of zinc (1 mg\kg) for 15days and then received a single oral dose of IND.

Group 5(IND+P.hal+zinc): were rats received a concomitant dose of both aqueous extract of *Pinus halepensis* and zinc using same doses and routs as above and then received a single oral dose of IND.

Group 6 (IND+Rant treated rats): were rats received oral dose of Ranitidine (20 mg\kg) for 15days and then received a single oral dose of IND.

1.3.4. Sacrifice, blood and Gastric juice sampling and tissues collection

After 6 h of indomethacin administration these animals were sacrificed under slight anesthesia by chloroform (94%) by inhalation; blood samples were collected during the slaughter of animals into EDTA tube to carried FNS and dry tubes. The serum was obtained by centrifugation for 10 min at 3000 tour/min and used for biochemical analysis assays; blood sugar level measured during rat's slaughter using glucometer. Gastric juice was collected from each stomach to measure; there volumes, Ph, total acidity, pepsin and Catalase (CAT) activity. Then the stomachs were cut open along the greater curvature and washed in normal saline. Then it was laid flat and the number and degree of erosions were counted and scored. Stomach and duodenal stored at -20 C for oxidative stress and in 10% formaldehyde for histological analysis.

1.4.- Reagents and products

Sodium Chloride (Nacl), Methanol, Chloroform, Comassie Blue, Phosphoric Acid (H3PO4), Bovine Serum Albumin (BSA), Gallic Acid, Trichloroacetic Acid (TCA), Thiobarbituric Acid (TBA), Butylated Hydroxy Toluene (BHT), Chloride Hydrogen HCl, Tris, Salicylic acid, DTNB (5-5'-dithiobis2-nitrobenzoic acid), hydrogen peroxide (H2O2), GSH, FeCl3, magnesium (Mg), Fehling liquor, sulfuric acid. Aluminum chloride ($AlCl_3$)

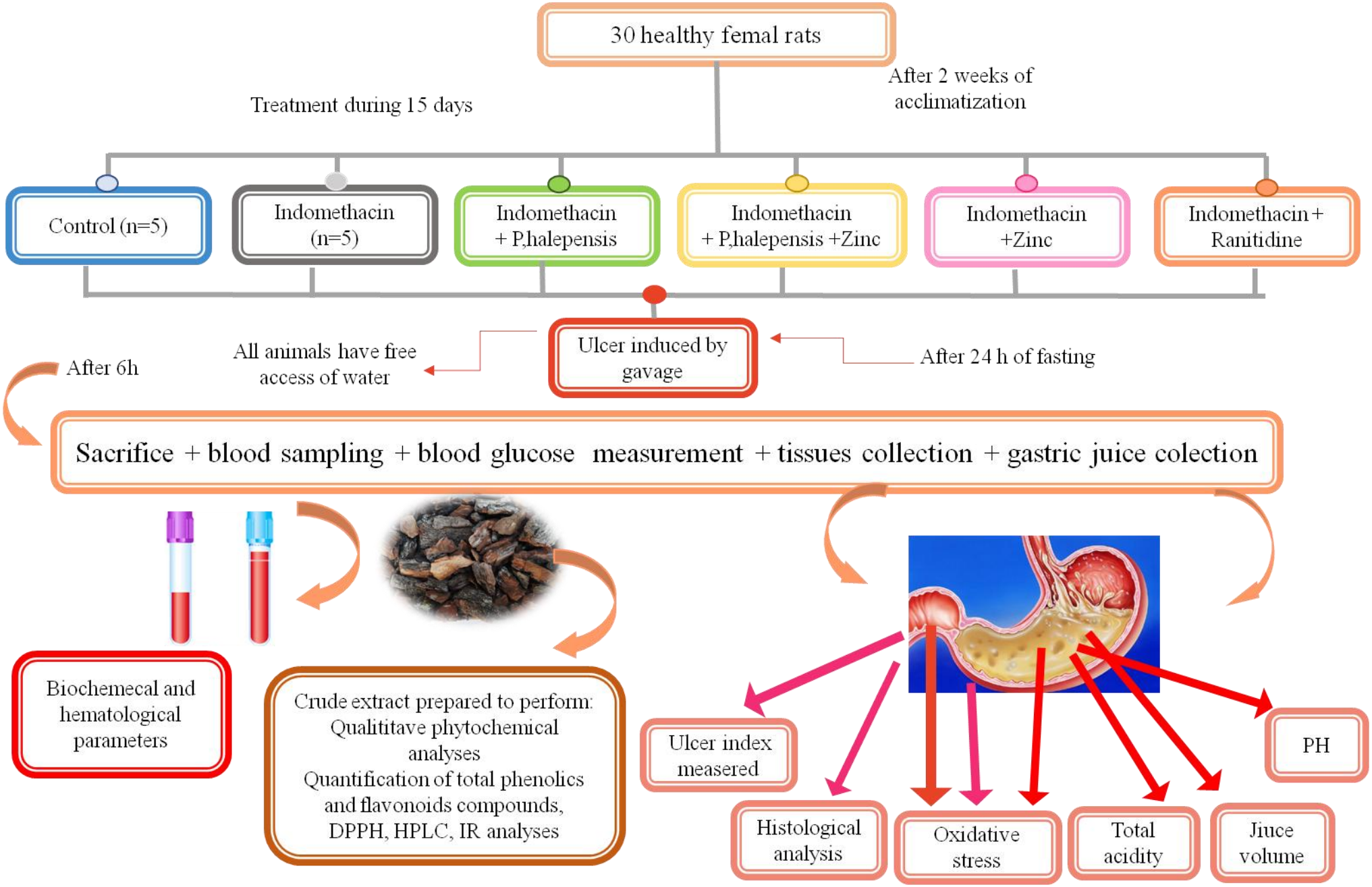


Figure 20 : Experimental design of study

2. Methods

2.1. Phytochemical analysis

According to (Ayetree Rai Basumatary.2016) seven phytochemical analysis assays were used to identify**:**

2.1.1. Alkaloids

To 2-3 ml extract with few drops Wagner's reagent were added. Formation of reddish brown precipitate indicates the presence of alkaloids

2.1.2. Carbohydrate

To 2ml of reagent was added to 2 ml of test solution and was mixed and kept a in boiling water bath for 1 min. Red precipitate formed indicates the presence of monosaccharides

2.1.3. Flavonoid

To 2-3 ml extract, few fragments of magnesium metal were added in a test tube, followed by dropwise addition of concentrate HCl. Formation of magenta colour indicated the presence of flavonoids

2.1.4. Triterpenoids

The dry crude plant extract (5 mg) was dissolved in chloroform (2 mL) and then acetic anhydride (1 mL) was added to it. Concentrated sulphuric acid (1 mL) was added to the solution. Formation of reddish violet colour shows the presence of triterpenoids.

2.1.5. Phenolic

To the 5 ml extract, few drops of natural 5% ferric chloride solution. A dark green color indicates the presence of phenolic compounds.

2.1.6. Tannin

Drops of distilled water were added to the crude plante extracts with approximately 0.25 g NaCl. The appearance of tannins was indicated when a blue green color developed after treating samples with 1 mL of ferric chloride (2%).

2.1.7. Saponins

The extract was diluted with 20 ml of distilled water and it was shaken in a graduated cylinder for 15 minutes. A 1 cm. layer of foam indicated the foam of saponins.

2.2. Total phenolic and flavonoids compounds

2.2.1. Total phenolic

Determination of the total polyphenols was carried out according to the Folin-Ciocalteu (FC) method (N. Boizot and JP Charpentier, 2006): 100 µl of artichoke extract are mixed with 500 µl of the FC reagent and 400 µl of Na 2 CO 3 at 7.5% (w / v). The mixture is stirred and incubated in the dark and at room temperature for ten minutes and the absorbance is measured at 760 nm by a UV spectrophotometer. The results are expressed in mg gallic acid equivalent / g of dry vegetable material with reference to the calibration curve of gallic acid.

Calibration curve is carried out by gallic acid at different concentrations (50-100-150-2g /ml) under the same conditions and the same steps of the assay. The results are thus expressed in milligrams of gallic acid per gram of dry extract (mg of EAG / g). All measurements are repeated 3 times.

2.2.2. Total Flavonoids

The determination of total flavonoids was carried out according to the method described by Dehpeur et al. (Dehpour A et al., 2009): 500 µl of each extract to be analysed are added to 1500 µl of 95% methanol, 100 µl of 10% (m / v) $AlCl_3$, 100 µl of 1 M sodium acetate and 2.8 ml of distilled water. The mixture is stirred and then incubated in the dark and at room temperature for 30 minutes. The blank is made by replacing the extract with 95% methanol and the absorbance is measured at 415 nm using a UV spectrophotometer. The results are expressed in mg equivalent quercetin / g of dry vegetable material with reference to the quercetin calibration curve. The quercetin calibration curve is performed by quercetin at different concentrations (10 - 20 - 30-40 µg / ml) under the same conditions and the same steps of the assay.

2.3. DPPH antioxidant test

In vitro antioxidant activity was evaluated by measuring the scavenging power of the DPPH (1,1-diphenyl-2-picryhydrazyl) radical according to the method described by (Burits and Bucar,2000), where 3ml of various concentrations (5, 10, 15, 25,50, et 60µg/ml) of *Pinus halepensis* samples were added to 75µL of methanolic solution of DPPH (1.3mg/ml). Absorbance measurements were read at 517 nm after 30 min of incubation time at room

temperature (A1). Absorbance of a blank sample containing the same amount of methanol and DPPH solution acted as the negative control (A0). The percentage inhibition [(A0-A1/A0) × 100] was plotted against the phenol content and IC50 was determined.

2.4. Hemolysis assay

Hemolysis assay was done as described by Henkelman.S. *et al.* 5mL of blood was collected from healthy volunteers in the tubes containing 5.4 mg of EDTA to prevent coagulation and centrifuged at 1000 rpm for 10 min at 40C. Plasma was removed carefully and the white buffy layer was completely removed by aspiration with a pipette with utmost care. The erythrocytes were then washed for additional three times with 1X PBS, pH 7.4 for 5 min. Washed erythrocytes were stored at 4oC and used within 6 h for the hemolysis assay. 50 uL of 10 dilutions (100 uL Erythrocytes suspension: 900 uL 1XPBS) of erythrocytes suspension was mixed with 100 uL of test samples (*P.halepensis*) (20-80ng/mL), 100 uL of 1XPBS was used as negative control and 100 uL of 1% SDS as positive controls. Reaction mixture was incubated at 37^0C water bath for 60 min. Volume of reaction mixture was made up to 1 mL by adding 850 uL of 1XPB. Finally, it was centrifuged at 300rpm for 3min and the resulting hemoglobin in supernatant was measured at 540 nm by spectrophotometer to determine the concentration of hemoglobin. Percentage haemolysis was calculated as follows

$$\text{Hemolysis inhibition } (\%) = 100 - [\text{Sample} \div \text{Control}] \times 100$$

2.5. Analgesic activity assay

A total of 6 female rats were randomly separated into three groups of four animals each and they were treated as follows: in

- Group I, rats were treated with the extract of plant at a dose of 300 mg/kg.
- Group II, rats were served as the negative control group and received distilled water (2 mL/kg)
- Group III, rats were served as the positive control group and received acetylsalicylic acid (aspirin) at a dose of 100 mg/kg.

At 60 min after extract administration, abdominal contractions were induced in the animals by intraperitoneal administration of 10 mL/kg of 0.6% of acetic acid.

The numbers of abdominal contractions over a period of 20 min following the injection of acetic acid were recorded. The degree of analgesia was calculated using the formula below:

$$Degree\ of\ analgesia = \frac{\text{Negative control} * - \text{Treated group\#}}{\text{Negative control}} \times 100$$

* was number of abdominal contraction of negative control
\# was number of abdominal contraction of treated group.

2.6. Acute toxicity test

The test was performed using healthy albino rats of Wistar strain weighing between 209 and 237 g. The animals were divided into three groups of three rats each and administered 2000 and 5000mg/kg of aqueous extract of P. halpensis orally. Animals were observed after dosing at least once during the first 30 min, periodically during the first 24 h with special attention given during the first 4 h, and daily thereafter for a total of14 consecutive days (Derouiche S.2017).

2.7. Hematological parameters analysis

The determination of hematological parameters performed using fully Auto Blood Cell Counter (ERMA) commercial reagent kits from Biomeghreb (Tunisia) using manual-analyzer.

2.8. Biochemical parameters analysis

The determination parameters were determined by using commercial reagent kits from Biomeghreb (Tunisia) using manual-analyzer.

2.8.1. Aspartate aminotransferase (GOT-ASAT)

Kinetic determination of aspartate aminotransferase activity. The reaction is initiated y adding the patient sample to the reagent. The reaction is as follows:

$$\text{2 oxoglutarate + L-Aspartate} \xrightarrow[\text{MDH}]{\text{GOT}} \text{Glutamate + oxaloacetate}$$

$$\text{Oxaloacetate + NADH + } H^+ \longrightarrow \text{Malate + } NAD^+$$

The rate of NADH concentration decrease is directly proportional to the aspartate aminotransferase activity in the sample. Wavelength: 340 nm.

GOT: Glutamic Oxaloacetate Transaminase.
MDH: Malate Dehydrogenase.

2.8.2. Alanine aminotransferase (GPT-ALAT)

Kinetic determination of Alanine aminotransferase activity. The reaction is initiated y adding the patient sample to the reagent. The reaction is as follows:

$$\text{2 oxoglutarate + L-Alanine} \xrightarrow{\text{GPT}} \text{Glutamate + Pyruvate}$$

$$\text{Pyruvate + NADH + } H^+ \xrightarrow{\text{LDH}} \text{Lactate+ } NAD^+$$

The rate of NADH concentration decrease is directly proportional to the alanine aminotransferase activity in the sample. Wavelength: 340 nm.

GPT: Glutamic Pyruvate Transaminase.
LDH: Lactate Dehydrogenase.

2.8.3. Urea

The urea is measured in kinetics according to the following reaction:

$$\text{Urea + } H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

The ammonium ions in the presence of salicylate and sodium hypochlorite react to form a green compound (Dicarboxyl indophenol) who's the intensity is proportional to the urea concentration. Wavelength: 590 nm.

2.8.4. Creatinine

Creatinine forms in an alkaline medium a complex colored with picric acid. The rate of formation of this complex proportional to the concentration of creatinine. Wavelength: 492 (490-510) nm.

2.9. Ulcer determination parameters

2.9.1. Determination of Volume and pH of Gastric Juice

The animals were sacrificed, stomach was dissected out, and the gastric juice collected was centrifuged for 5 min at 2000 rpm. The volume of the supernatant was expressed as ml and pH was measured using pH meter.

2.9.2 Determination of preventive index

Determination of Ulcer Score procedure as described by (M.D.Ayoola et al. 2016) was followed:

Ulcer index is measured or registered using the following scores involving the number and severity of ulcers in table 03

Table 03: Ulcer index measured

Score	Description
0.0	normal colored stomach
0.5	red coloration
1.0	spot ulcers,
1.5	hemorrhagic streaks,
2.0	ulcers with area >3 but ≤5mm
3.0	ulcers > 5mm,

The ulcer index and percentage protection given by the following equation:

Ulcer index (UI) = UN + US + UP / 10,

UI = ulcer index,

UN = average number of ulcers per animal,

US = average of severity score,

UP = percentage of animals with ulcer.

$$\textbf{Protection \%} = \frac{\textbf{Ulcer Index (control)} - \textbf{Ulcer Index (test)}}{\textbf{Ulcer Index (contrrol)}}$$

2.9.3. Determination of Total acidity of Gastric Juice:

Total acidity described by (Neeru V.et al.2012). Supernatant of gastric juice was taken and diluted 10 times and a few drops of phenolphthalein were added to the solution. Titration was

done using 0.025 M NaOH solutions until the color of the test solution changed to light pink, indicating pH 7.0. Volume of sodium hydroxide (NaOH) needed for titration was used in the calculation to derive the hydrogen ion concentration. The total acidity is expressed as m equiv /L using the following formula:

$$\text{Total acidity (m equiv /L)} = n \times 0.01 \times 40 \times 1000$$

Where, n = volume of NaOH quantified;

40 is the molecular weight of NaOH.

0.025 is normality of NaOH.

1000 is the factor represented in liter.

2.9.4. Determination of Pepsin Activity

Pepsin activity was described by (Neeru V.et al.2012). Centrifuged (5000 g for 10 minutes) gastric juice (0.1 mL) was added to 1 mL bovine albumin (0.5% w/v in0.01 N HCl; pH 2) and incubated for 20 minutes at 37C. Aduplicate background control tube (gastric juice blank), in which 1 mL albumin was replaced with 1 mL of 0.01 N HCl, was simultaneously run. Hydrolysis was stopped by adding2 mL of 10% TCA. All of the tubes were heated in boiling water for 5 minutes, then cooled. After denaturation of the proteins by heating in a boiling water bath for5 minutes, the precipitate was removed by centrifugation (9000 g for 10 minutes). A total of 1 mL of the supernatant was mixed with 0.4 mL of 2.5 N NaOH and 0.1 ml of the Folin-Ciocalteu reagent, then the volume was adjusted to10 mL using distilled water. Absorbance was measured at700 nm. The peptic activity was calculated in terms of micrograms of tyrosine liberated per milliliter of gastric juice.

2.10. Oxidative stress parameters

2.10.1. Preparation of homogenates

About 1g of liver was homogenized in 9 ml of buffer solution of Tris buffer saline (TBS, pH=7.4). Homogenates were centrifuged at 4000xg for 20 min and the obtained supernatant was used for the determination of antioxidant activity.

2.10.2. Determination of malondialdehyde (MDA) level

MDA was measured according to the method described by SASTRE *et al.* (2000). In brief, Pipette 100 µl of sample, 400 µl of TBA reagent into the glass and screw test tubes and seal. Heat the mixture in the Marie bath at 100 ° C for 15 minutes. Then cool in a cold-water bath

for 30 minutes leaving the tubes open to allow evacuation of the gases formed during the reaction. Centrifuge at 3000 rpm for 5 minutes and read the absorbance of the supernatant at 532 nm using a spectrophotometer. TBARS concentration was determined using the MDA molecular extinction coefficient (å = 1.53 10^5 $M^{-1}.Cm^{-1}$). The results were expressed in µmol / mg proteins.

2.10.3. Determination of reduced glutathione (GSH) level

GSH concentration was performed with the method described by Ellman. based on the development of a yellow color when DTNB is added to compounds containing sulfhydryl groups. In brief, 0.8 mL of tissue homogenate was added to 0.2 mL of 0.25% sulphosalylic acid and tubes were centrifuged at 2500 g for 15 min. Supernatant (0.5 mL) was mixed with 0.025 mL of 0.01 M DTNB and 1 mL TBS (pH 7.4). Finally, absorbance at 412 nm was recorded. Total GSH content was expressed as nmol GSH/mg proteins.

2.10.4. Determination of catalase activity

The assay of the catalase enzyme activity consists in measuring the catalase-induced loss of H_2O_2 contained in the sample by the measurement of H_2O_2 absorbance at 560 nm using spectrophotometer according to the Aebi, 1984 method.

2.11. Histopathological study of stomach tissues

After rats sacrificed, stomach tissues were removed and immersed in fixative (solution 36% formaldehyde) intel the time of slices preparation. Whish dehydrated in ascending graded series of ethanol, cleaned with toluene, immersed in paraffin, and colored with hematoxylin and eosin. Histopathological evaluation was performed with light microscope.

2.12. High preformation liquid chromatography analyses

High Performance Liquid Chromatography (HPLC) Analysis: An HPLC system was used with a detector at λ = 360 nm, the column used is of length 25cm and diameter 4.6 mm, the phase stationary C18 and the mobile phase acetonitrile / water acid (60; 40;v / v). Flow 1 ml / min at 25 ° C; pressure 8.5-8.6; with time 20 min. (JOHNSON., And PEPPERMAN., 1995; ELSAYED., And PRASHER., 2013)

2.13. Infrared spectroscopy analyses

IR analyses was done in department of Process Engineering and Petrochemicals labs by direct reading

2.12. Statistical analysis

Our statistical study is carried out by the Minitab software using (Student t test) to compere means among our different experimental groups; Differences were considered statically significant at $p<0.05$.

Chapter II

Results & Discussion

1. Results

1.1. In vitro essays of *Pinus halepensis*

1.1 .1. Phytochemical study

1.1.1.1.Qualitative phytochemical analysis of *Pinus h0alepensis*

Results of phytochemical essays shows that aqueous extract of *Pinus halepensis*is rich on different important chemical compounds such as flavonoids, phenols, carbohydrates, saponoside, tannins and terpenoids but our extract plant is poured from alkaloids (Table 04).

Table 04: Phytochemical essays of *Pinus halepensis* aqueous extract.

Compounds	Alkaloides	Carbohydrates	Flavonoïdes	Terpenoides	Phenols	Saponoside	Tannins
Aquatic Extract of P.halepensis	-	+	+	+	+	+	+

(+): Present (-): Absent

1.1.2. Total phenols and flavonoids concentration

Table 05: Total phenols and flavonoids concentration in *Pinus halepensis* aqueous extract.

Compounds	Polyphenols (mg of GAE/g of extract)	Flavonoids (mg QE/g of extract)
Aqueous Extract of P.halepensis	33,5 ± 33,7	3,25 ± 9,90

1.1.3. Infrared analysis of aqueous extract of *Pinus Halepinsis*

The results of infrared spectrum of the extract of *pinus Halepinsis* shows the existence of a band broad towards 3200 which indicates the existence of OH of way related and not free in our extract compound, on the other hand the peak of 1100 indicates the existence of CO function in our molecule (Figure 21).

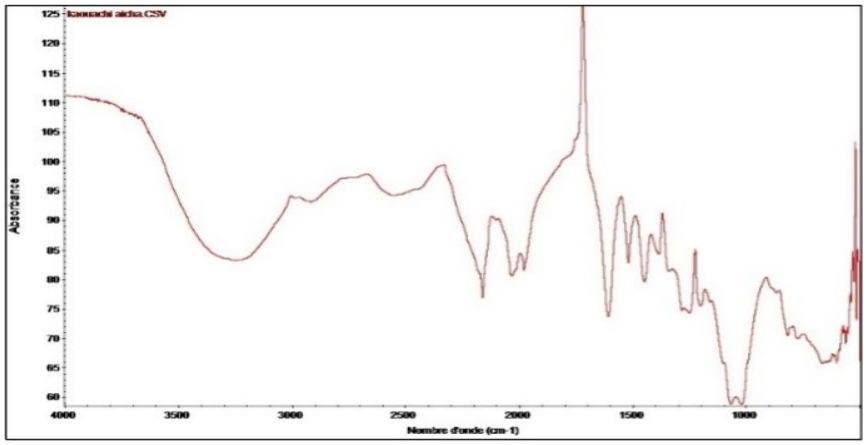


Figure 21: Infrared spectrum of P. Halepensis.

1.1.4. HPLC of aqueous extract results Pinus Halepinsis

HPLC chromatogram results of P. halepensis barks showing in fig.22 appeared a that P.halepensis bark had a deferent number of molecules detected, but we identified just one molecule which is P-coumaric acid.

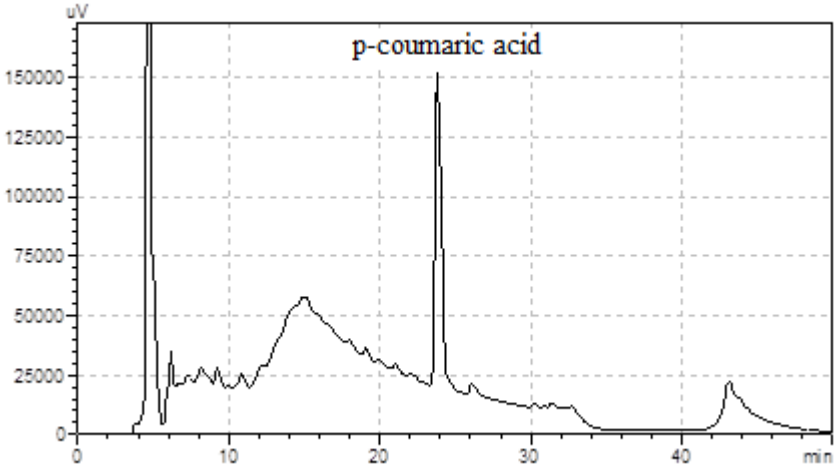


Figure 22: HPLC chromatogram of the aqueous extract of P. Halepensis

1.1.5. Antioxidant activity

1.1.5.1. DPPH radicals scavenging activity and IC_{50} value

(Figure 23) appeared the antioxidant activity of *Pinus Halepensis,* results show that the inhibition percentage of free radicals increase with the increase of extract concentration, and IC_{50} values in the DPPH radical scavenging activity assay of the extracts aqueous of P.halepensis is IC50 = 34.92µg/ml

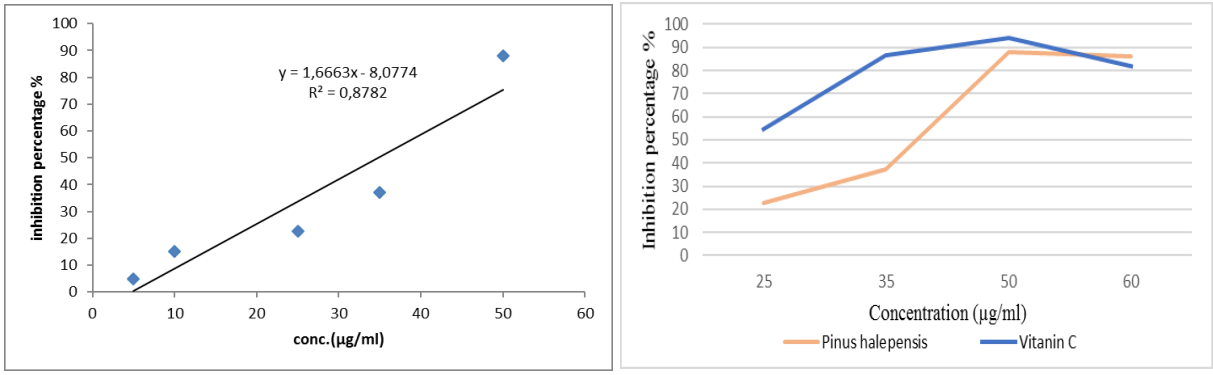


Figure 23: Inhibition percentage and IC_{50} values of *Pinus halepensis* aquatic extract

1.1.5.2. Membrane stabilizing

As shown in (Figure 24), the results of variability membrane stabilization according to deferent concentrations of aqueous extract of P.halepensis, wish have a high effect in membrane stabilization compared with SDS stander; increase of P. halepensis extract concentration (20- 40- 60µg/ml)correlated positively with the membrane stabilization.

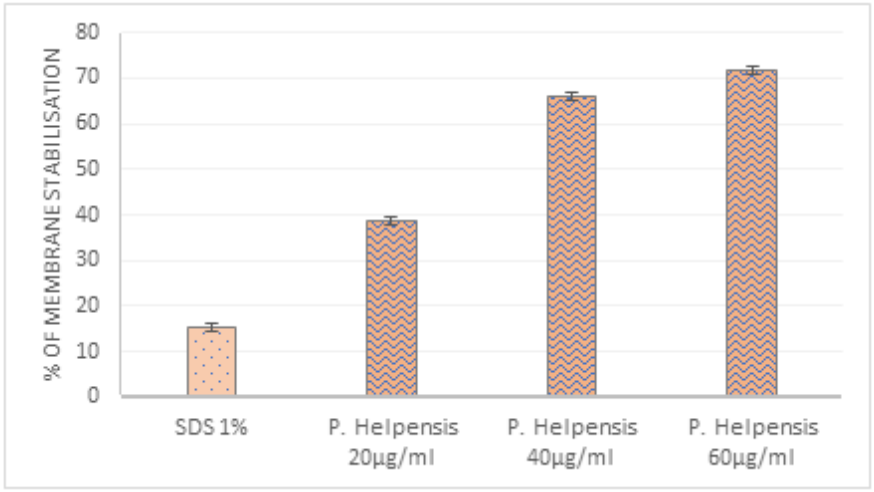


Figure 24: Membrane stabilizing effect of aqueous extract of *Pinus Helpensis.*

1.2. In vivo essays of *Pinus halepensis*

1.2.1. The analgesic activity

Result of our study concerning of analgesic activity of *Pinus halepensis*; shows the importance activity of *Pinus halepensis* to reduce analgesia compared with the best analgesic drugs Aspirin (Figure 25).

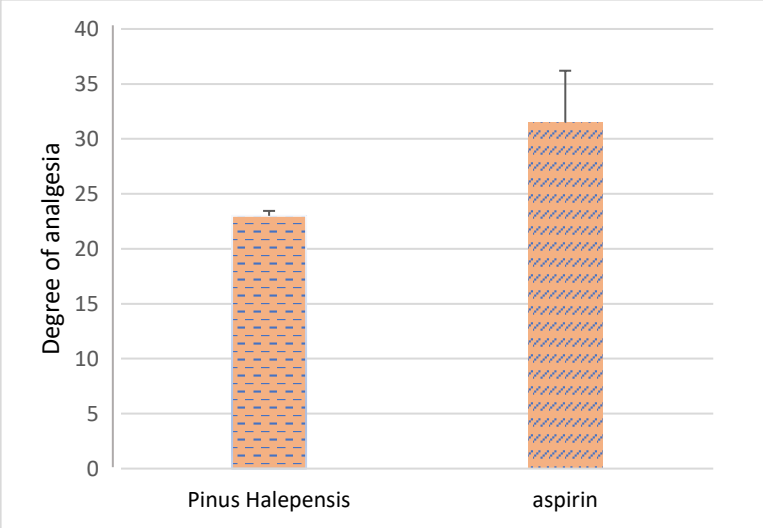


Figure 25: The analgesic *Pinus Halepensis* activity

1.2.2. Acute toxicity study

With the acute toxicity test at the limit test dose of 5000 mg/kg, neither mortality nor changes related to behavioral, autonomic, neurologic, and physical profiles were observed within the first 24 hours and during the 14-day follow-up (Table 06).

Table 06: Effect of aqueous extract of P. halepensis on physiological parameters of Wister albino rats.

Parameters	**0 h**		**3 h**		**24 h**		**Day- 7**		**Day-14**	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Death rats	0	0	0	0	0	0	0	0	0	0
Eyes	N	N	N	N	N	N	N	N	N	N
Sleep	N	N	N	N	N	N	N	N	N	N
Diarrhea	N	N	N	N	N	N	N	N	N	N

N; Normal

1.3. Hematological parameters

Hematological parameters illustrated in (Figure 26) show that, there is no effect of INDO or different treatment on RBC levels in hole the experimental rats but WBC, PLC and Lym number are decrease with very high signification ($p<0.001$) in INDO group compared to the control, also treatment with P. Halepensis+ Zn or with RANT shows a signification increase in WBC in INDO induced ulcer rats. On the other hand, P. Halepensis extract, zinc and rant treatment partial corrected the number of PLT in INDO induced ulcer animals. However, RANT drugs increase the number of LYMPHO in experimental ulcer rats

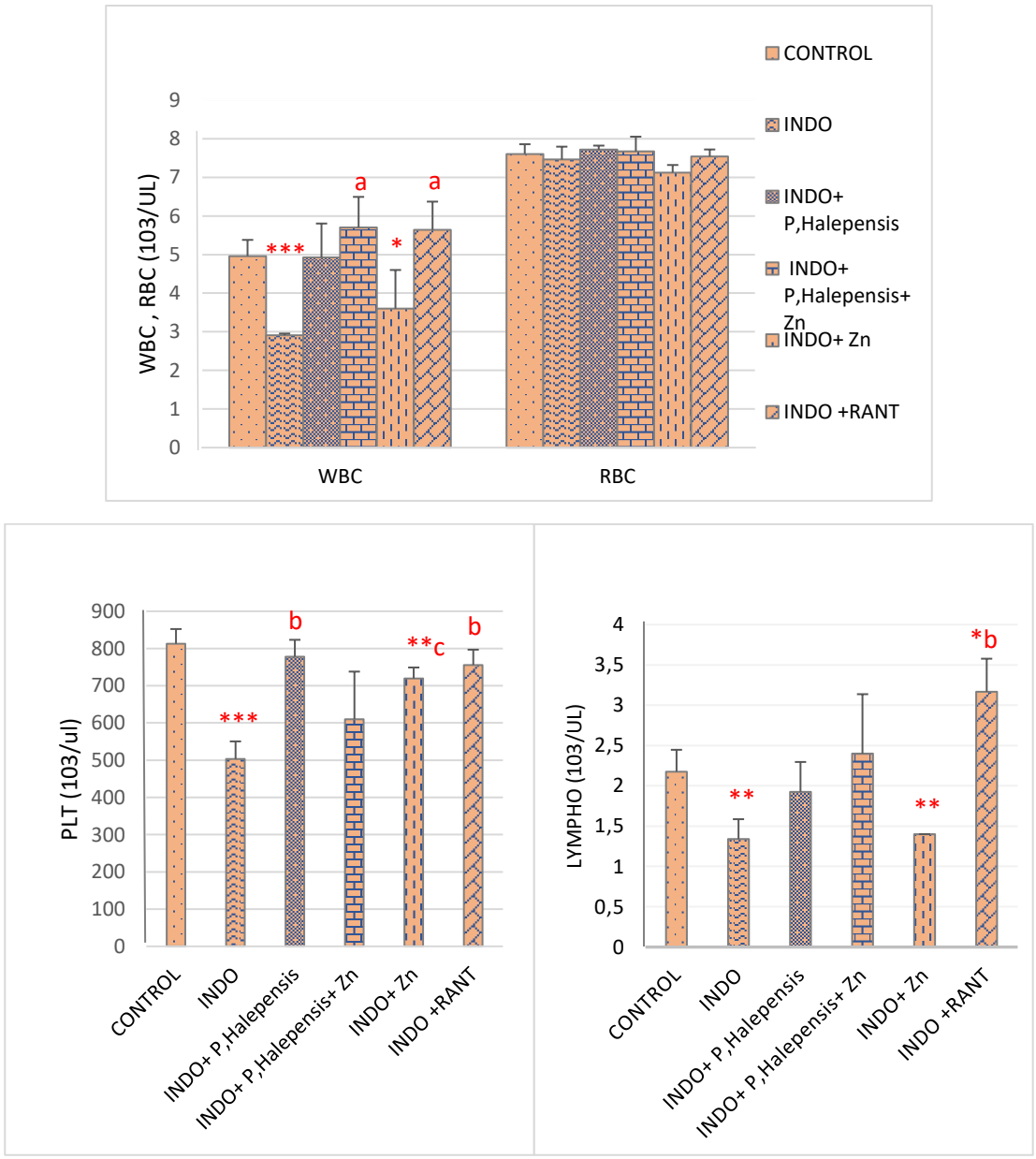


Figure 26: WBC, RBC, PLT, LYMPHO levels of control and experimental groups

Results show also a signification decrease in HGB and HCT level ($p<0.01$, $p<0.05$) respectively compared to the control. Treatment with P. Halepensis extract, zinc and rant for 15 days increase significantly the HGB level in INDO induced ulcer animals. However, RANT drugs treatment increases the level of PLC in experimental ulcer rats (Figure 27).

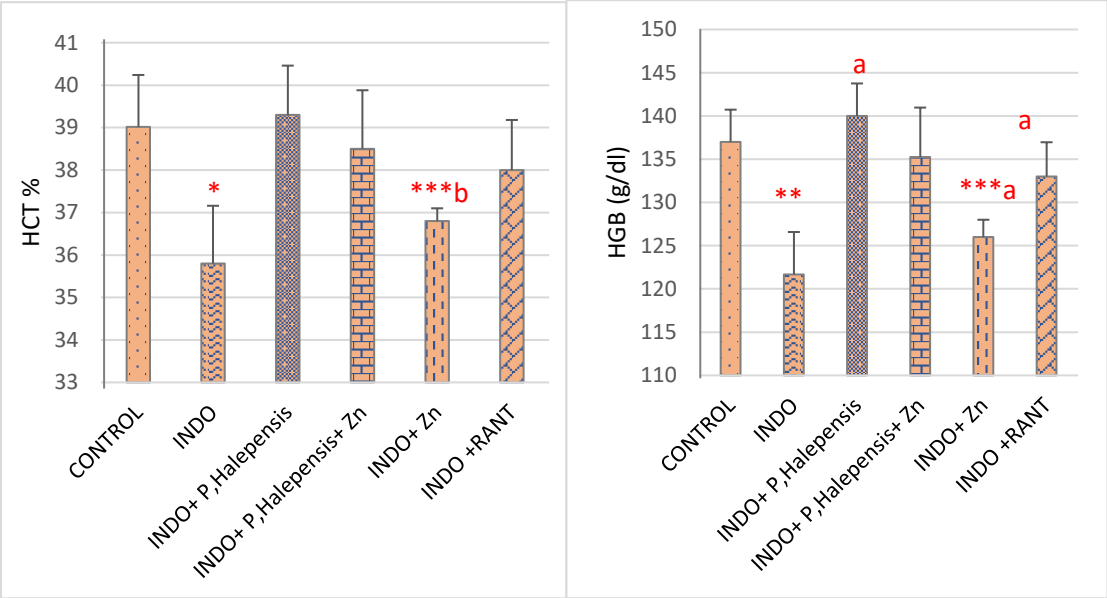


Figure 27: HCT, HGB levels of control and experimental groups.

1.4. Biochemical parameters

As for which level illustrates in (Figure 28). Below, the results of blood glucose parameters show a decrease in INDO group compared to the control. In the other side fig28. shown that blood glucose was significant increase in ulcer rats treated with P.halepensis, zinc compared to the INDO group. In fig.29 results show a very high increase in Urea level in INDO group and in the P.halepensis group compared to control.

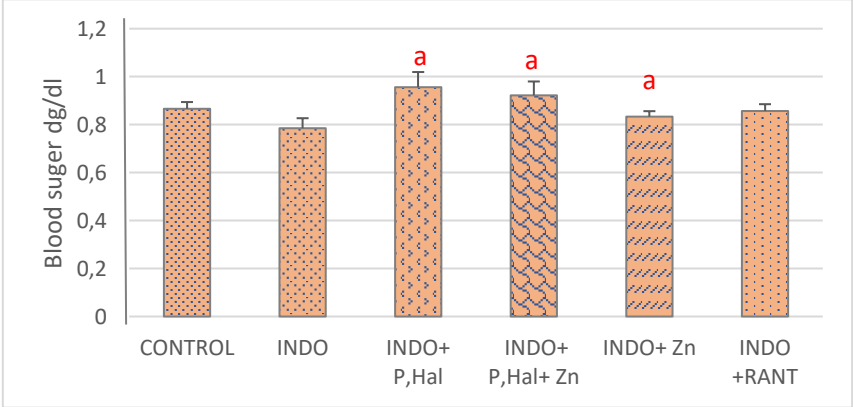


Figure 28: Blood glucose level in the control and experimental groups.

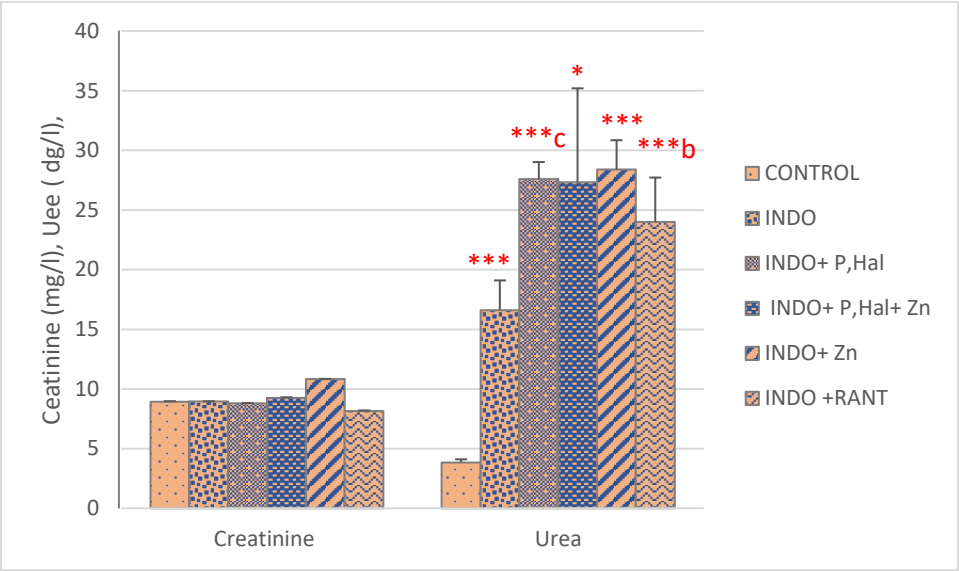


Figure 29: Creatinine and urea levels of control and experimental groups**.**

As shown in (Figure 30). the results of transaminases enzymes activities appeared significant decrease of GPT ($p<0.001$) and GOT activities ($p<0.05$) in INDO group compared to the control. Treatment with Pinus alone or with Zn and RANT increase the activity of GPT in ulcer animals compared to Drugs group and no effect of GOT activity.

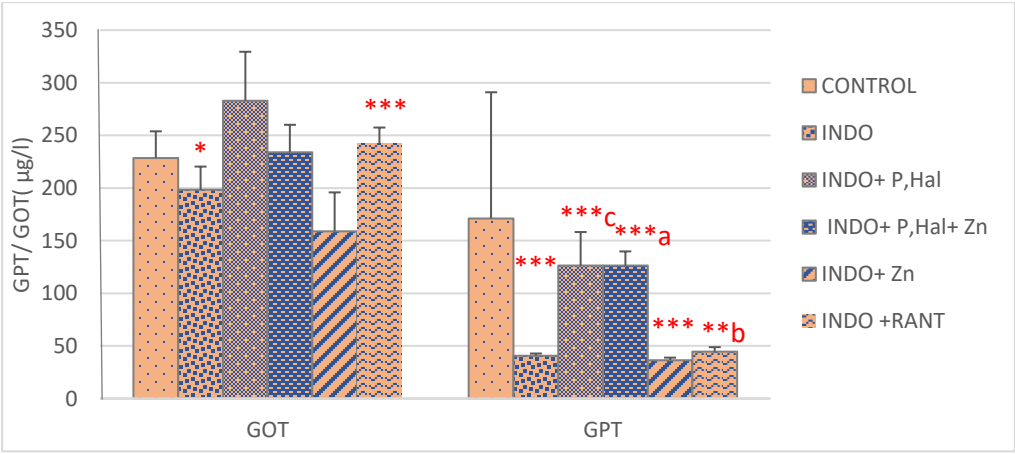


Figure 30: GPT, GOT activities in the control and experimental groups.

1.5. Ulcer determination parameters

1.5.1. Macroscopic evaluation of gastric lesions

Ulcerative indices and gastroprotection percentage were determined by measuring ulcerative lesion area. Results shown in (Figure 31). Indicated that no macroscopic in control and Indo+ P.halepensis groups, but on the contrary Indo group shows a deep and large lesion in hole stomach compared with the control, the rest of experimental groups (Indo+ P.halepensis+ Zn,Indo+ Zn, Indo+ Raniti) shows a litetls lesions in sapereted rigent of the stomach.

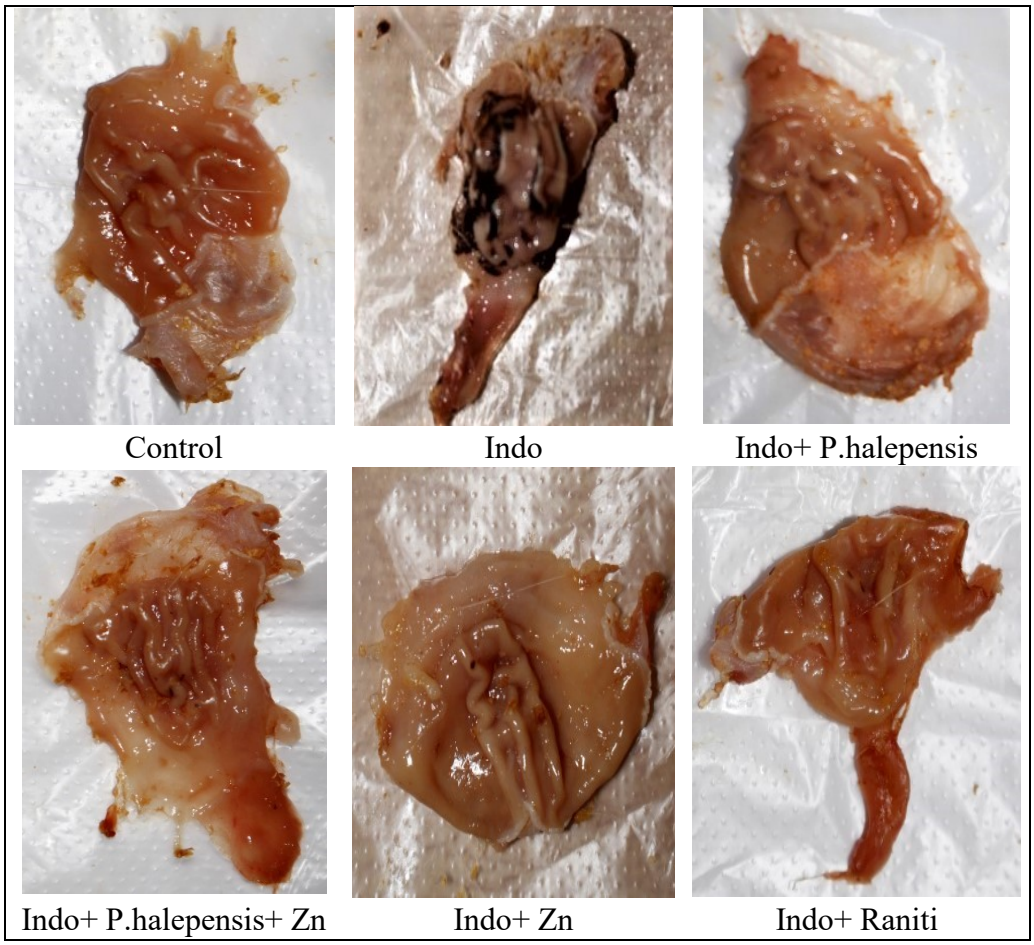


Figure 31: Macroscopic appearance of gastric mucosa in different groups

1.5.2. Determination of preventive index

1.5.2.1. Ulcer scoring

The results of Ulcer scoring show in the (Figure 32). The ulcer scoring was significantly increased in INDO group compared to the control. The results show also that treatment with plant, zinc and ranitidine decrease significantly ($p<0.001$) the Ulcer scoring compered to INDO group.

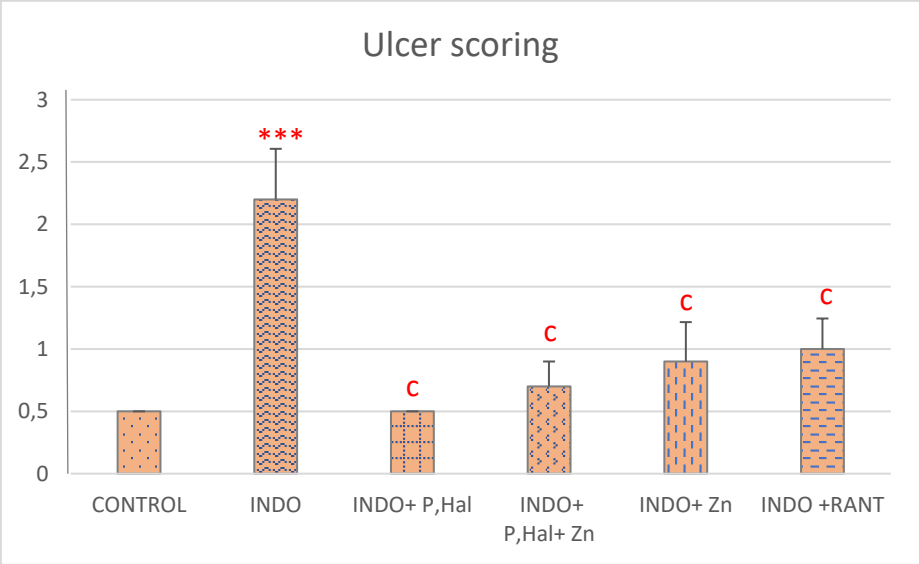


Figure 32: Ulcer scoring in the control and experimental groups

1.5.2.2. Percentage protection

Protection percentage of experimental group illustrate in (Figure 33). An high protection is appear in groupe treated with P. Halepensis with percentage 100% against stomach ulcer compared to the P.Halepensis+ Zn group(80%), Zn and Ranitidin(60%).

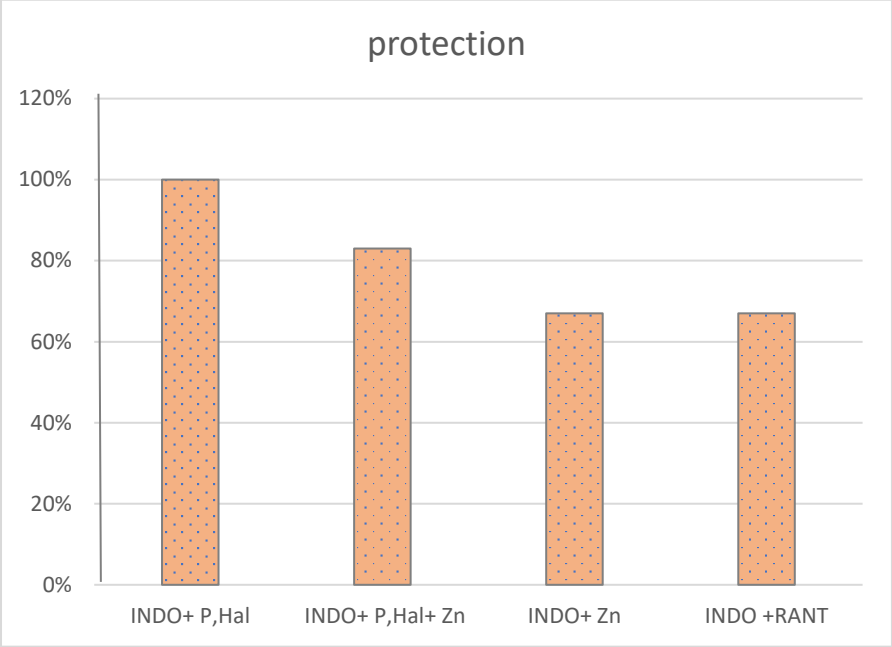


Figure 33: Percentage protection of experimental group against ulcer.

1.5.3. Determination of Volume and pH of Gastric Juice

1.5.3.1. Gastric Juice Volume

Gastric Juice Volume and pH level in the control and the experimental groups illustrate in Table 06 and Figure 34**.** Table and figure below express the gastric juice volume and pH level in control and experimental groups. Although there is a no significant deference between the volume of gastric and pH level in experimental group and the control. but the results show that there is a big decrease in juice volume in Indo, INDO+ P,Hal+ Zn and INDO +RANT and INDO+ Zn groups compared to the Control, as for INDO+ P,Hal group, the results of juice volume shows that there is an increase of volume compared to the control.

Table 07: Gastric Juice Volume in control and experimental groups

Parameters	CONTROL	INDO	INDO+ P,Hal	INDO+ P,Hal+ Zn	INDO+ Zn	INDO +RANT
Gastric Juice (Ml)	359±131	197,5±88,3	375±143	130,0 ± 60,5	51,1±22,7	152,5 ±5,8

1.5.3.2. Gastric Juice PH

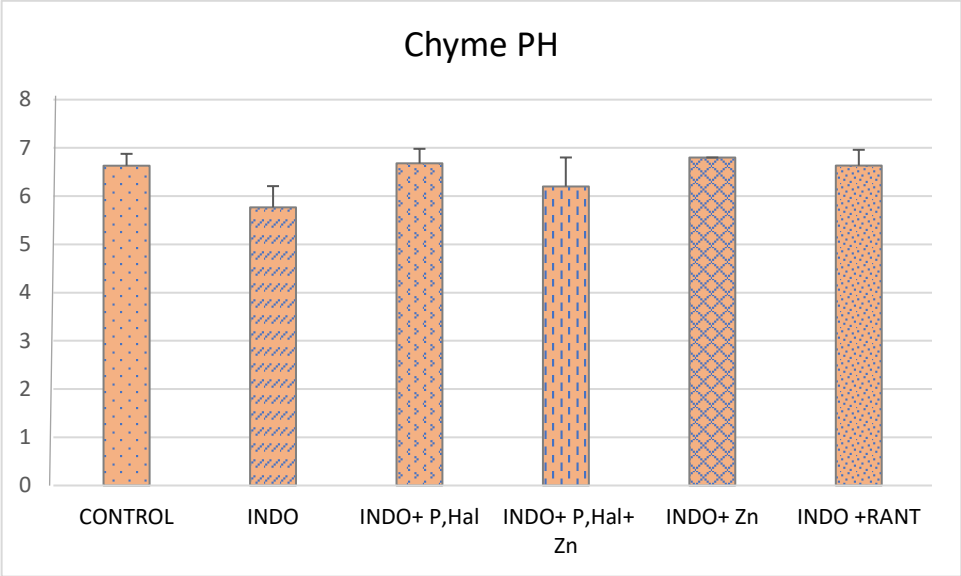


Figure 34: Gastric Juice PH of control and experimental group.

1.5.4. Determination of Total acidity of Gastric Juice

Figure 35. Show the results of total acidity. Results shows a high significant increase of the total acidity levels in INDO group compared to control. Treatment with P. Halepensis+ Zn and with Zn alone decrease the acidity levels compared to INDO group.

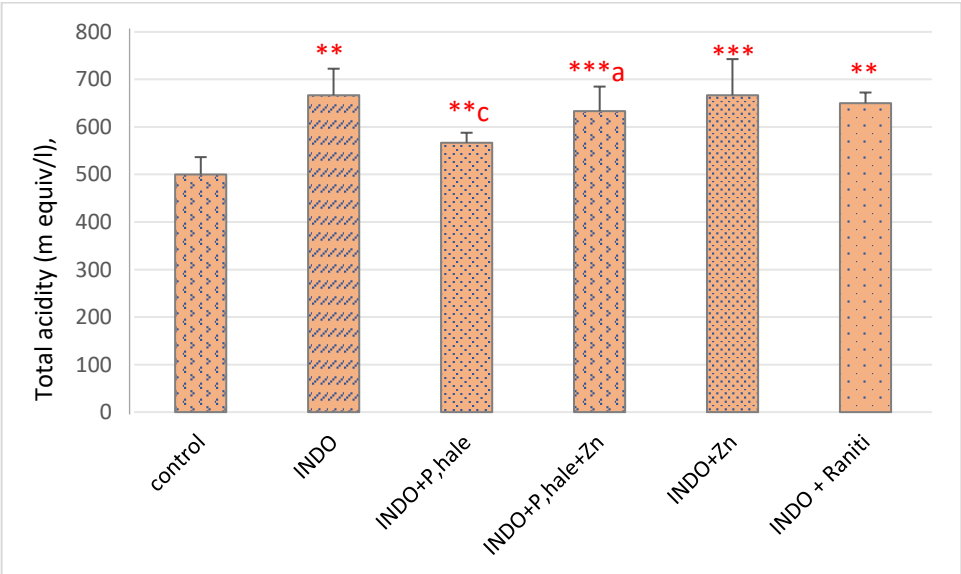


Figure 35: Total acidity levels of Gastric Juice of the control and experimental groups.

1.5.5. Determination of Pepsin activity

The results of pepsin activity illustrate in Figure 36. Which show the treatment with P.halepensis+ Zn decrease significantly ($p<0.001$) of pepsin levels compared to the INDO group, and no change activity of pepsin in the other experimental group.

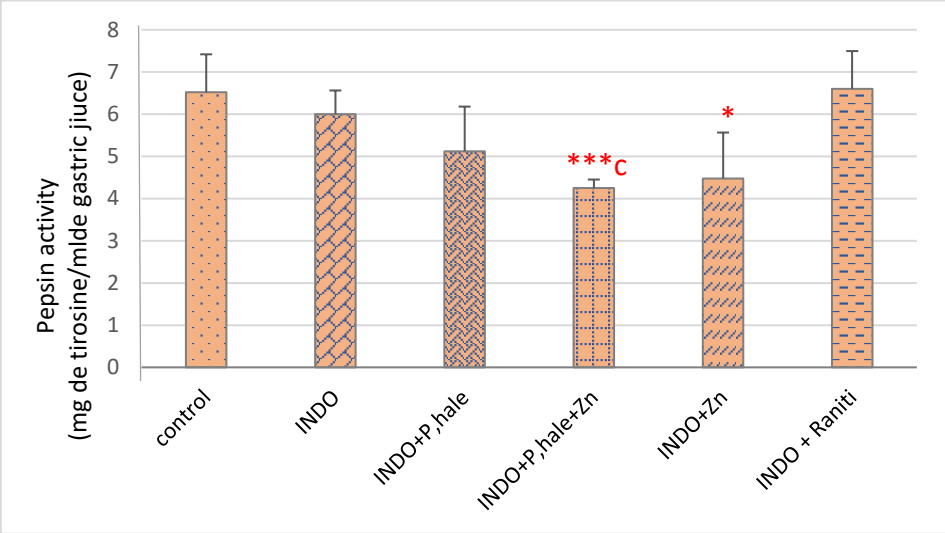


Figure 36: Pepsin activity levels in the control and experimental groups**.**

1.5.6. Oxidative stress parameters

1.5.6.1. malondialdehyde (MDA) levels

Stomach and duodenal MDA levels shows in Figure 37**.** For duodenal results, no change of the MDA level in INDO group compared to the control, but treatment P. halepensis, Zn and Ranitidine decrease the MDA level compared to the INDO induced ulcer group. For stomach MDA levels, a high signification increases in drugs group compared to the control P. halepensis alone or with zinc decrease significantly MDA level compared to the INDO drug group.

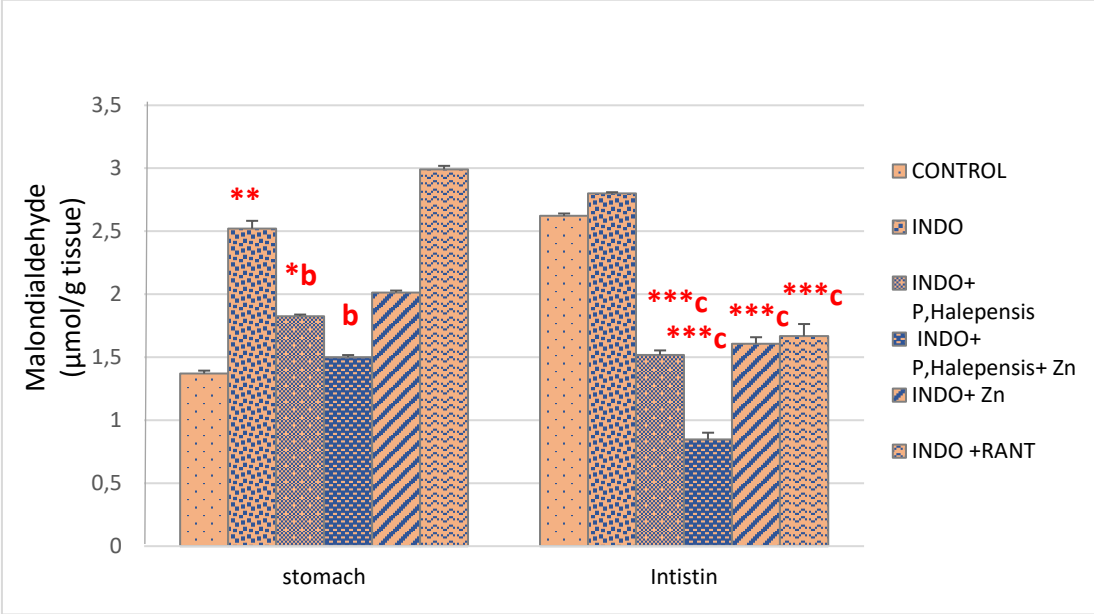


Figure 37: MDA levels in stomach and duodenal in control and experimental groups.

1.5.6.2. Reduced glutathione (GSH) level

Figure 38. Show stomach and duodenal GSH levels. In stomach, GSH levels has a very high signification increase in experimental ulcer group compared to the control, compared to the INDO group, GSH levels show a significant decrease in P. halepensis ($p<0.001$), P. halepensis + Zn ($p<0.001$), Zn ($p<0.05$) and RANT ($p<0.05$) groups. In Duodenal, no change of the GSH level in INDO group compared to the control, however, the results show an increase in GSH level in all different treatment groups.

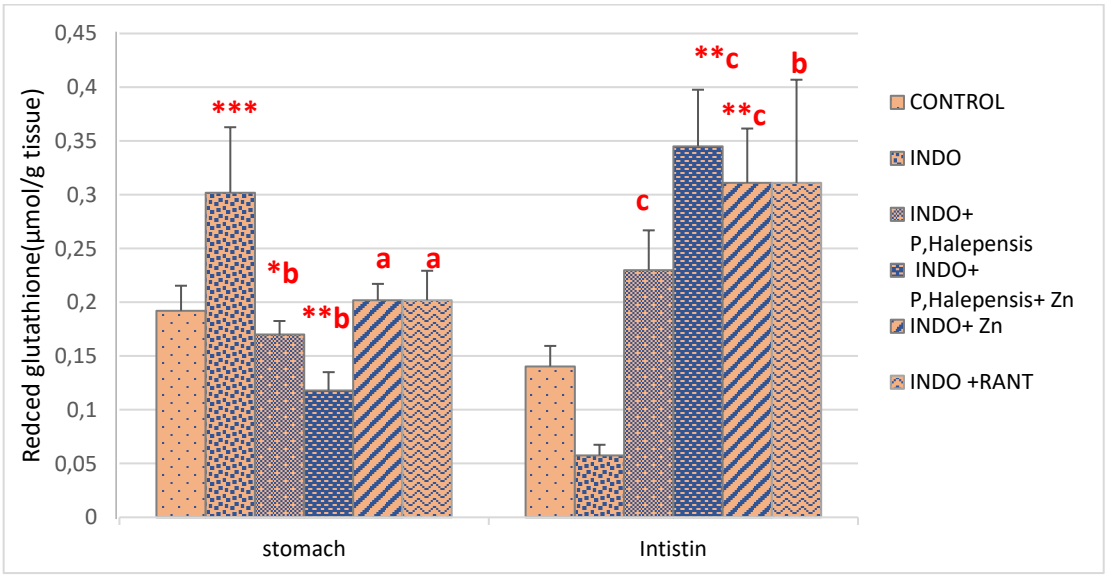


Figure 38: GSH levels in stomach and duodenal in control and experimental groups.

1.5.6.3. Catalase activity (CAT) in Gastric juice

Results of catalase enzyme activity is illustrated in Figure 39. In Gastric juice a very high signification increases of CAT activity in INDO group compared to the control. Also, the treatment rats by P. halepensis extract alone or combined with Zn significantly decrease ($p<0.01$ and $p<0.05$) the CAT activity respectively compared to INDO group.

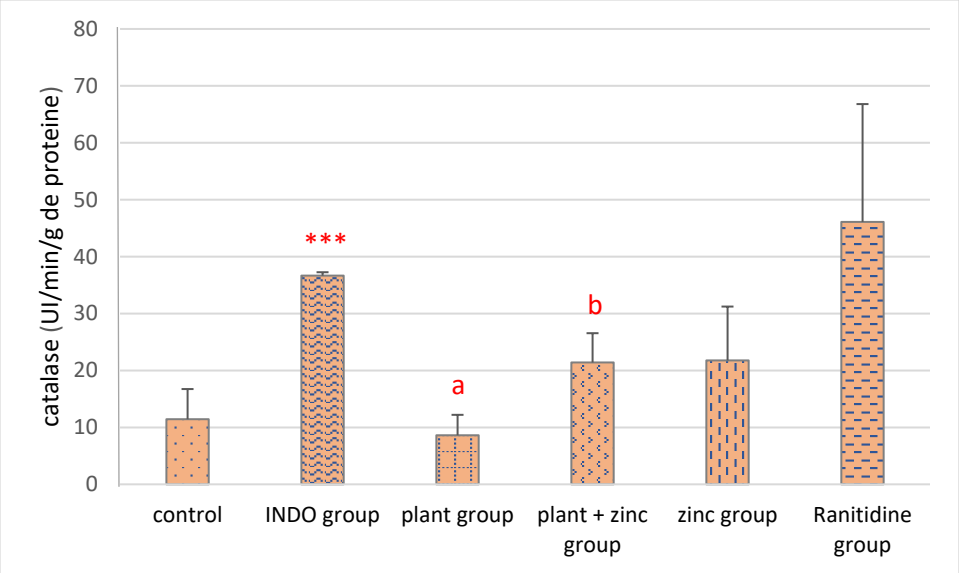
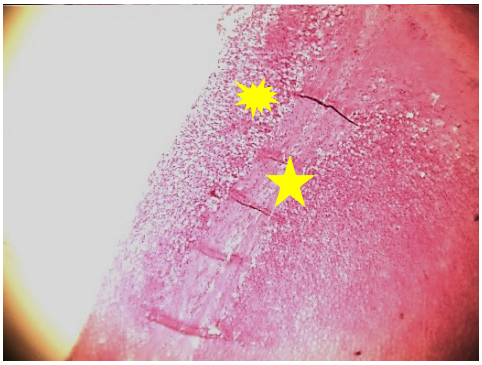


Figure 39: CAT activity in Gastric juice

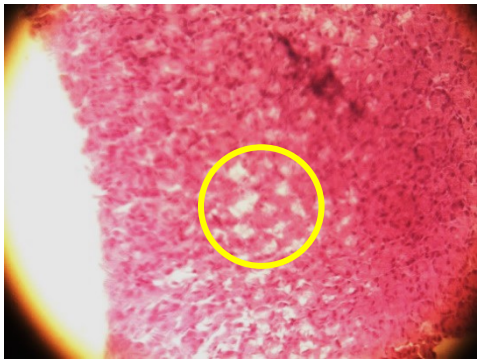
1.5.7. Stomach histological results

As shown in (Figure 40 a1,a2), results indicated normal epithelium muscular layer structurewith clear around gastric pits appearedin control group, conversely in indomethacin (INDO) group the histological results show a deep lesion in hole epithelium layer with hemorrhagic necrosis and a huge destruction in gastric pits in hole epithelium and muscular layer too appeared infig 32 b1, b2. Histological observations of the stomach body of the rats treated with P. halepensis extract show normal morphological appearance of the different components of the mucosa layer with few gastric pits lesion (Figure 40 c1,c2). In P.halepensis+ Zn and Zn group the histological results show a partial correction in morphological a deep lesion in gastric pits compared to INDO group (Figure 40 d1, d2,e1, e2). Finely, in Ranitidine treated group the results show a deep lesion in epithelium and muscular layerwith gastric pits totally destroyed compared to INDO group (Figure 40 f1, f2).

Control

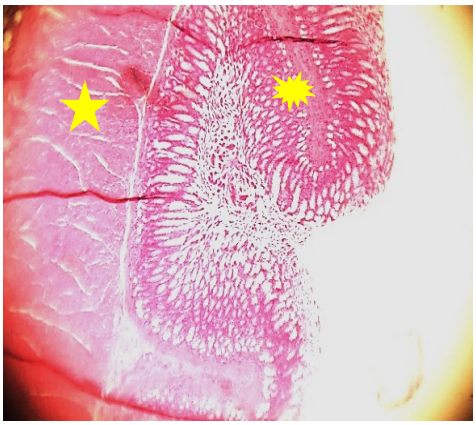


A1 × 10

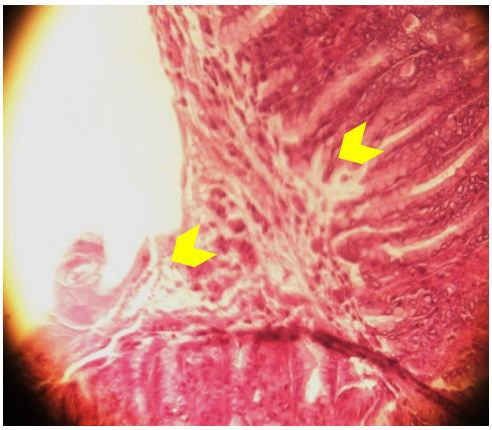


*A*2 × 40

Indomethacin group

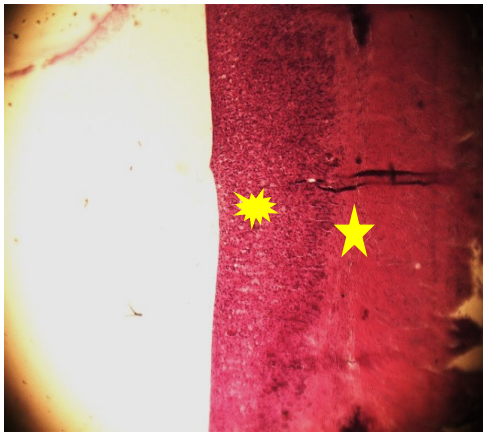


B1 × 10

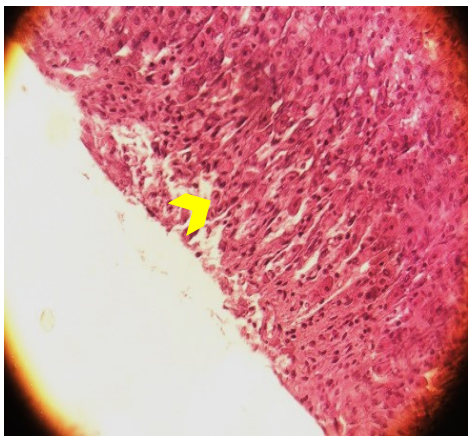


B2 × 40

Pinus halepensis group

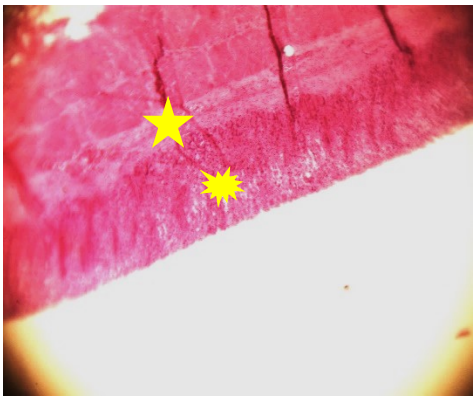


C1 × 10

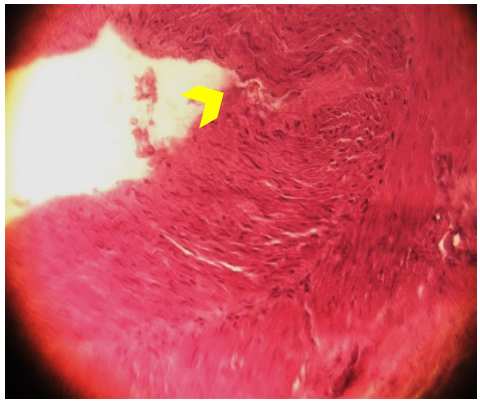


C2 × 40

Pinus halepensis + Zn group

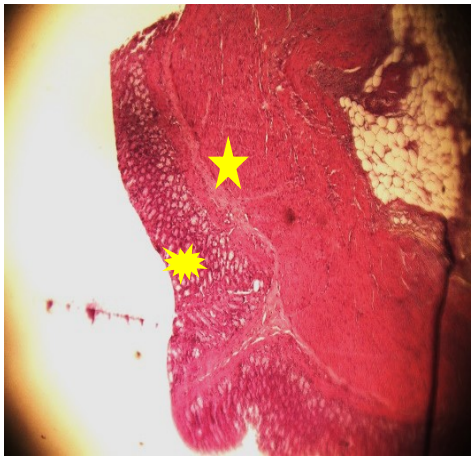


D1× 10

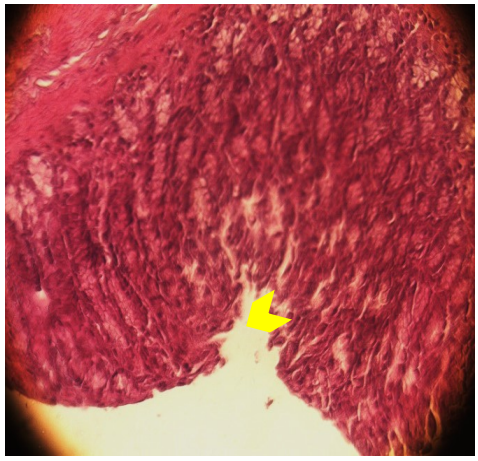


D2 × 40

Zinc group

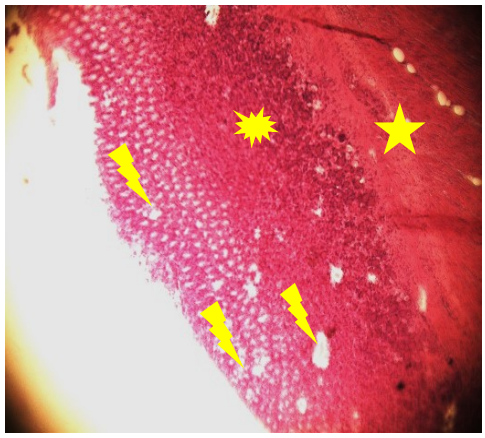


E1 × 10

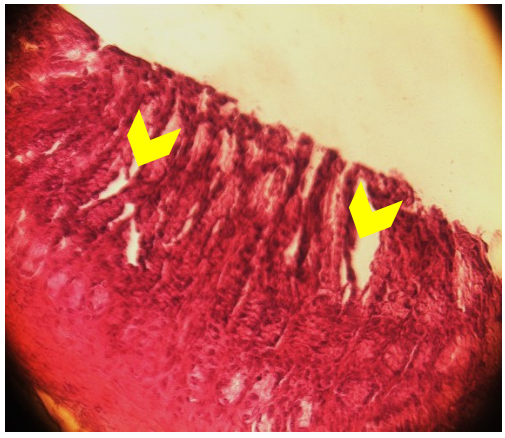


E2 × 40

Ranitidine group



F1 × 10



F2 × 40

Figure 40: Photomicrographs of stomach of all the experimental groups after treatments.

Histological examination from a control rat showing normal architecture (Figure 40; A1× 10 and A2×40); Indomethacin treated rats stomach showing some structural alteration (Figure 40 B1×100 and B2×400). Aqueous extract of P.halepensis at dose (300 mg/Kg) alone or combined with Zn showing normal appearance of stomach cells (Figure 40 C1, D1,×10 and C2, D2,×40). stomach sections of the rats administered Zinc dose (1mg/Kg) showed moderate degree of stomach damage (Figure 40 E1×10 and E2×40), the histological results of Ranitidine treated group at dose (20mg/Kg) show a deep lesion of stomach sections (Figure 40 F1×10 and F2×40).

(= epithelium cells layer, = musculair layer, = deep lesions in epithelium cells, = normal gastric pits, = gastric pits destruction).

2. Discussion

The objective of our study is determination the characteristics compound of *Pinus Halepensis*barks and evaluated their protection property associated with biological protection effect of Zinc against gastric ulcer induced by Indomethacin drugs in rats compared with Ranitidine property as gastric ulcer protector drugs.

2.1. In vitro *P. halepensis* characterization

The results of phytochemical essays **(Table 01)** chows that aqueous extract of *Pinus Halepensis*rich with different important second metabolic (flavonoids, phenols, carbohydrates, saponoside, tannins and terpenoids) but poured from alkaloids. secondary plant metabolites and have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism (Mamta Saxena et al.2013). according to (Michael Wink.2015)Terpenes show cytotoxic activities against a wide range of organisms, ranging from bacteria and fungi to insects and vertebrates and have been widely used in herbal medicine against infections, in some cases, steroids, triterpenes and saponins structurally resemble endogenous anti-inflammatory hormones, e.g., glucocorticoids.

The anti-oxidant activity results obtained from P. halepensis (fig.16), its seems to be from richness of our plant from polyphenols and flavonoids (Table 03), and the presence of Tannins in their compounds (Table 02).Plant extracts, such as flavonoids and phenolics, have raised public interest in their potential to act as antioxidants. Natural antioxidants can strengthen the endogenous antioxidant defence from ROS ravage and restore the optimal balance by neutralizing the reactive species (Jiao-Kun Li et al. 2016). The antioxidant activity of flavonoids depends upon the arrangement of functional groups about the nuclear structure. The configuration, substitution, and total number of hydroxylgroupssubstantiallyinfluenceseveralmechanisms of antioxidant activity such as radical scavenging (ShashankKumar.2013). and this is what our IR results shows (fig.15) that P.halepensis rich of linked hydroxyl groups. Tannins are a widely distributed phenolic antioxidant, present in various barks, leaves, fruits and vegetables, phenolic compounds are known to exhibit antioxidant activity by virtue of their redox property because of which they can scavenge oxygen radicals and donate hydrogen to neutralise reactive oxygen species (Debosree Ghosh.2015).

Condensedtannins, which result from the condensation of monomers of flavan-3-ol units, it has been reported to be used in treating gastric ulcers, diarrhoea, inflammations (Mohammad Asif.2015).Flavan-3-ols have been shown to behave as antioxidants via several mechanisms including the scavenging of free radicals, chelation of transitionmetals, as well as the mediation and inhibition of enzymes. These properties are due to the presence of the phenolic hydroxyl groups on the B ring in ungalloylated catechins (EC and EGC) and on the B and D rings of the galloylated catechins (ECG and EGCG) (Luís R. Silva et al.2016). according to (Hanadealawaf.2017)study the compounds Flavan-3-ol was isolated for the first time from the plantpinushalepensis barks.Coumarin (known as 1,2-benzopyrone). Antioxidant activity of phenolic compounds was correlated to their chemical structures. Structure-activity relationship of many phenolic compounds (e.g. flavonoids, phenolic acids, coumarins, tannins, etc.) has been studied. In general, free radical scavenging and antioxidant activity of these classes of compounds mainly depends on the number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules (I. Kostova et al.2011).It was found that Coumarins antioxidant efficiency was determined by the number and the position of hydroxyl group in their benzene ring. it is evident that the best scavengers of DPPH radicals are coumarins that have hydroxyl group in the position 6 or 8 in their structure.

The results shows the importance anti-inflammatory activity of P.halepensis barks with deferent concentration. According to (Luís R. Silv.2014).The anti-inflammatory of flavan-3-ols activity through their ability to scavenge NO, peroxynitrite ($ONOO^-$) and other ROS/RNS, to inhibit the translocation of NF-κB and AP-1 from the cytoplasm to nucleous and to inhibit the activity of iNOS and COX-2 was shown to inhibit the production of proinflammatory cytokines such as interleukin (IL)-1β, IL-6 and (TNF)-α and proinflammatory mediator, prostaglandin (PG)-E2, and the key rate limiting enzyme for its synthesisCOX-2. (Pedro MR.2014) demonstrated that flavan-3-ols contribute to reduce the concentration of cell oxidants because of their radical scavenging capacity, which might ameliorate inflammatory signs. For example, B2 dimers interact with components of the NF-jB/Rel family, inhibiting the NF-jB activation. In this sense, distinct dimers with similar chemical composition and structure display different capacity to attenuate inflammatory processes.Coumarin

derivatives have anti-inflammatory activity such as; Imperatorin blocks the protein expression of inducible nitric oxide synthase and cyclooxygenase-2 in lipopolysaccharide-stimulated RAW264.7. Esculetin inhibits the cyclooxygenase and lipoxygenase enzymes, also of the neutrophil dependent superoxide anion generation(K. N. Venugopalaet al.2013).So, based on this basic information about the metabolic secondary compounds of plants. the multiple characteristics of our plant *P. halepensisand* its most important activity against oxidative stress and inflammationprobably due to the presence of secondary metabolic compounds.

Figure 22 shows the analgesic activity of *Pinus Halepensis* barks, compared to analgesic drugs wish in Aspirin. Results shows that our plant have an important analgesic activity and that probably refer to their second metabolic compounds such as Rutin and Quercetin, quercetin induces an analgesiceffectamenablebyopioidreceptorantagonist. In fact, inhibition of oxidative stress, cytokine production, and opioid receptor-dependent effects seem to be major mechanisms of quercetin since they were also observed in models such as colitis, neuropathy, hepatic fibrosis(Cassia Calixto-Campos et al.2015). Analgesic effect of rutin was studied by hot plate test on swiss albino mice whereby the analgesic effect of rutin was established. Further, it was also confirmed that rutin exhibited peripheral and central anti-nociceptive activities.(AdityaGaneshpurkaretal.2016).

2.2. Hematological parameters

The results of hematological parameters, showed that no effects in RBC levels in all the experimental group compared to Control, but it showed a very high signification decrease level in WBC and LYM in INDO group. Most of the hematologic effects of NSAIDs (agranulocytosis, thrombocytopenia) are thought to result from an immune reaction and are, therefore, drug specific.(BARBARA CARDARIO et al.1991).several studies confirmed that Indomethacin induced agranulocytosis such as (JAMA.1986, Paul c. Vincent. 1986, Kamakshi V. Rao. 2014).The cause of drug-induced agranulocytosis is not fully understood, but mechanismsdirect toxicity been proposed. Direct toxicity to myeloid cells, particularly neutrophils, has been shown with medications such as indomethacin (Tesfa D et al.2010). The toxicity may be due to either the parent drug or a toxic metabolite or byproduct. The severity of neutropenia associated with these drugs is

often dose dependent, but the occurrence of reactions is still idiosyncratic. Agranulocytosis associated with direct toxicity is usually associated with a slower decline in neutrophils. Although that the mechanism is not quite clear but we can say that the toxicity of Idomethacin probably caused agranulocytosis or (decrease in WBC and LYM levels). (Pontikoglou C et al.2010).

The signification increase level of WBC in INDO+ P.halepensis + Zn group may be refer to the importance anti inflammation activity of P.halepensis barksby their different bioactive constituents. However, HCT, HGB and platetes results shows a very high decrease and no significant decrease of RBC in INDO group compared to control.Thedecreases of these parameters may be due toincrease RBC haemolysis resulting from increaseerythrocyte fragility and impairment of theantioxidant defense mechanisms. Thus, thedecreased of HGB and HCT in indomethacin induced ulcer untreated rats might be a reflectionof oxidative stress effects of indomethacin on red cellmembrane or due to bone marrow suppressiveeffect of ethanol (A. Dandare et al.2017).On the other hand, non-steroidal anti-inflammatory drugs (NSAIDs) which suppress the production of prostaglandins and thromboxanes by inactivate the cyclooxygenase enzyme, it is required for prostaglandins and thromboxane synthesis. inhibit platelet function by blocks the formation of thromboxane A2 in platelets and produced an inhibitory effect on platelet aggregation(Jyothi Y. et al.2016).These effects have beencountered and the level of this parameters wasimproved toward normalcy due to extract, zinc and ranitidine administration.It was suggested that the antioxidant potential of the extract might be responsible for preventing the destabilizing effect of indomethacin on erythrocytes membrane.

Aqueous extract of P. helpensis may have a positive impact in the hematopoietic system, which seems to have manifested in the levels of HGB, platelet andHCT. Perhaps, due to its iron content and the extract ability to improve bone marrow functions, a major site for erythropoiesis (Orhue EG et al.2008).In addition,Zinc indirectlyinfluence hemoglobin synthesis which influencesthe activity of d-aminolevulinic aciddehydratase (ALAD), an enzyme thatcatalyzes heme synthesis, which explains the increase of HGB in the group to treat by zinc**(**M Zen Rahfiludin et al.2013).

2.3. Blood glucose and serum biochemical parameters

Biochemical parameters of liver and kidney function show in fig.22 and fig.23, which appeared a significant decrease in TGO level, a very high decrease in TGP level, a very high increase in Urea level and no significant effect on Creatinine level in Indomethacin compared to control, kidney in response to indomethacin include oxidative damage and impairment of structure and function of mitochondria mediated through the production of free radicals. Indomethacin induces also impairment in structure and function of brush border membranes in the kidney mediated by free radicals and the activationof phospholipases. Sepsis and septic shock are important risk factors for acute renal failure due to alterations in glomerular hemodynamics (Walter H. Hörl.2010). Increased level of serum urea is suggestive of impaired renal function, which could be due to acute kidney disease, damage or failure. Elevation of urea level can also occur due to increased dietary protein or gastrointestinal bleeding (Daniel C. Ugwuanyi et al .2016). so probably the decease of TGO and TGP activities and increase Urea level is due to renal failure induced by indomethacin.

While in P.halapensis group the results of biochemical function of liver and kidney appeared a very high increase significant in TGP and Urea levels but without significant in Creatinine and TGO levels, Kidnye protection effect of P.halapensis barks probebly due to flavonoids, according to AchyutDahal study (2015), flavonoids prevents renal oxidative stress may include an increasing rate of GSH or by induction of its synthesis or by a scavenger effect. The results of the association group appeared amelioration the same result of aP.halepensisgroup so probably they have the same protection effect

As for Blood glucose level the results showing in fig.21, appeared a very high increase in Indomethacin group compared to control, from human studies in vivo it appears that administration of PGE, or PGE, reduces stimulated insulin levels in normal subjects. Administration of PGE, to glucose infused rats is associated not only with reduced insulin levels. but also, with increased levels of glucagon and frank hyperglycemia (MARK MORAN.1988). reported previously that treatment of pancreatic b cells with exogenous PGE2 causes a decrease in insulin secretion in vitro and in vivo and that inhibitors of endogenous prostaglandin synthesis augment glucose-induced insulin secretion in vitro and in vivo (Phuong Oanh T et al.1999).

accordin to this information the increase of blood glucose in Indomethacin group due of their inhibition of prostaglandin synthesis.

In the opposite side aqueous extract of P.halapensis group show a significant decrease in fasting blood level compared to Indomethacin group and no signification effect compared to control, Flavonoids of all sub-class have proven to have antidiabetic properties by enhancing insulin secretion via regeneration of pancreatic β-cells, enhancing insulin mediated glucose uptake by target cells, inhibiting of aldose reductase (Katarzyna Małgorzata Brodowska.2017) increasing Ca2+ uptake. Flavonoids with potent antioxidant activity were shown to be effective in management of diabetes. Antidiabetic activity of flavonoids depends on the chemical criterion (C-2-C-3 double bond and ketonic group at C-4 position on ring B) which is fundamental for the bioactivity of poly-phenol compounds. These phytomedicines play significant role in maintaining blood glucose levels, glucose uptake, insulin secretory and immuno modulatory functions (Saritha Marella.2017). so probably the hypoglecemic activity of P.halapensis barks due of their contained of flavonoids compounds. The association group appeared a decrease level in blood glucose level so probably this association can protect the increase effect in blood glucose cauded by Indomethacin.

2.4. Ulcer determination parameters

The results obtained show that indomethacin induces elevation of ulcer score, a lesion in the gastric epithelium, an increase in the acidity of gastric juice and no effect on the activity of pepsin compared with control, which causes a state of gastric ulcer in rats.

Our study agrees with several studies that Indomethacin at dose (30 mg/ kg) induced gastric ulcer such as (Uzma Saleem et al.2017) (A. G. R. Alkushi et al.2017) (Nema Ali Soliman et al.2017). our results also are Similarly to (Katary MA et al.2017) result shown that indomethacin induced aggressive factors by increase of total acidity in gastric juice. But are contrary with result of (SaheedSabiu et al.2015) show a significant decrease in pepsin activity levels and increase in gastric volume secretion in rats treated with indomethacin. Indomethacin induces its gastrointestinal toxicity via several mechanisms such as an increase in gastric acid secretion; interfere with mucosal cell regeneration via inhibition of PGE2 synthesis, production of free

radicals, reduction of gastric nitric oxide level and invasion of activated neutrophils as well as induction of gastric cells apoptosis (Katary MA et al.2017). in addition, Indomethacin is a non-selective inhibitor of cyclooxygenase (COX) 1 and 2, enzymes that participate in prostaglandin synthesis from arachidonic acid. Prostaglandins are hormone-like molecules normally found in the body, where they have a wide variety of effects, some of which lead to pain, fever, and inflammation (SamanehAbyariMiyandoab et al.2015). Cytoprotective PGs exert their protective effect on GIS mucosa by reducing gastric acid secretion (Halis Suleyman et al.2010). PGE2 increased mucus and HCO3- secretion through EP4 receptors, and inhibited acid secretion or motility through EP3 or EP1 receptors, respectively. The inhibitory effect of PGE2 on acid secretion was mediated in two ways by EP3 receptors, directly by inhibiting acid secretion at the parietal cells and indirectly by inhibiting histamine release at enterochromaffin-like (ECL) cells (Koji Takeuch.2017).

In present study, the increased pepsin activity with decrease in mucin secretion in the disease treated rats indicated altered hydrophobicity and reduced protective ability of the mucosal membrane against hemorrhagic erosions, thus resulting in tissue damage. Besides antioxidant action that protects the mucus layer and arrest ulcer progression, drugs that increase the synthesis and secretion of gastric mucus would facilitate gastric ulcer healing (Darshan Vinod Shah et al.2017).

In the other side, results of our study shows thataqueous extract of bark P.halepensis show an remarkable anti-ulcer activity induced by Indomethacin. This results it so remarkable in all levels; macroscopic, microscopic, stress and ulcer parameters. result shows no effect in gastric mucosal layer, very low ulcer scoring and with 100% protection against gastric ulcer induced by indomethacin to Indomethacin group. These results refers to their phytochemical compounds ofaqueous extract of bark P.halepensis especially; polyphenols. Where flavonoids, terpenoids, tannins, are highly gastroprotective, through different cellular and biochemical pathways (Repetto MG et al. 2002, Vale FF.2014). Flavonoids, tannins, Saponins are thought to increase mucosal prostaglandins content, decrease histamine secretion from mast cells by inhibition of histidine carboxylase. Flavonoids are among the cytoprotective active compounds for which antiulcer property has been extensively confirmed. While flavonoids are suggested to be able to stimulate the secretions of mucus, bicarbonate, and prostaglandins. Quercetin has an anti-secretory mechanism of action. It has

antihistaminic properties, therefore, decreases histamine levels, as well as preventing the release of histamine from gastric mast cells and inhibiting the gastric H+/K+ proton pump, diminishing acid gastric secretion, the gastro-protective effects of chalcones involve increasing the mucosal blood flow, stimulating the synthesis of mucus in the gastric mucosa and increasing prostaglandin levels (Mohamed Morsy and Azza El-Sheikh.2011). Saponins, which have hemolytic, immune-stimulant, and anti- inflammatory properties. It is thought that the anti-inflammatory effects of saponins reduces the risk of ulcers, by increasing defensive factors of gastric mucosa and stopping the inflammatory process, the protective effect of saponins against gastric ulcer may also be mediated by the formation of a protective mucus layer on the gastric mucosa and by selective inhibition of PGF2α (Cyril Ogbiko et al.2017). Tannins are used in medicine primarily because of their astringent properties, which are due to the fact that they react with the proteins of the layers of tissue with which they come into contact (Francesca Borrelli et al.2000). Tannins are known to "tan" the outermost layer of the mucosa and render it less permeable and more resistant to chemical and mechanical injuries (Christian E. Odo.2017). When a low concentration of tannins is applied to the mucosa, only the outermost layer is tanned, becoming less permeable and affording an increased protection to the subjacent layers against the action of bacteria, chemical irritation, and, to a certain extent, against mechanical irritation (Francesca Borrelli et al.2000). Furthermore, terpenoids have also been reported to elicit potent activity against gastric ulcers (Esther Oluwatoyin Agbaje.2017).

Our results shown also that zinc treatment partially protect gastric cell from ulcer with.Zinc is physiologically a very important metal and actas a cofactor for many enzymes. Zinc has been reportedto be useful in promoting healing of wounds and skin lesions. The efficacy of locally applied zinc on the healingof leg ulcers was assessed in a clinical study by Stromberg H.E. which showed that healing of leg ulcers isimproved by addition of zinc oxide to the local regimen. **(**H E StrömbergMagnus et al.1984).Themechanism of the Zn2+ -induced gastric protection process is not fullyexplained in this study and further studies are definitely needed todetermine whether potent gastroprotective and ulcer healingmediators such as endogenous PG and NO could be involved inthe ulcer healing action of zinc**(**W. Opoka et al.2010). In addition, in literature, antiulceractivity of other zinc salts like zinc acexamate

isreported17. Zinc sulphate had significant antiulcer activityagainst aspirin. The anti-acidseretory effect may contributeto it. There is a single report by Cho CH et al18 where zincchloride pre-treatment was found to reduce gastriculceration induced by pyloric occlusion and stressed pylorusoccluded rats (Sonali A.,2016).

The association group show the successively results; decrease in ulcer scoring which increase the percentage of protection to 80% which refer to the decrease level in total acidity and pepsin and increase the Ph level. All this improvement probably due to important role of Zinc in enzymes function and the richnessof Pine to the seconded metabolic.

Ranitidine hydrochloride, the standard drug used in this study is a H2-receptor antagonist. Its use as antiulcer drug is due to its ability to reduce acid secretion by blocking the histamine receptor type. Ranitidine is in the same class as cimetidine with the only difference that it contains furan ring in place of imidazole ring of cimetidine. Any agents with anti-histaminic property may thus have some form of antiulcer property. (Adegbolagun Temitope Adeoye et al.2016).

2.5. Oxidative Stress

In this study revealed a significant increase in MDA and GSH level in stomach of rats treated with Indomethacin group compared to control. Our results are according to the study of HakanDursun et al. shown that gastric mucosal MDA level was increased in group of rats treated by indomethacin (HakanDursun et al.2008).

Increased lipid peroxidation expresses the state of oxidative stress induced by indomethacin by several mechanisms, on the one hand, the Indomethacin inhibited the mitochondrial oxidative phosphorylation leading to the release of cytochrome c from mitochondrial inter membranous space into cytosol and to the release of ROS such as superoxide anion and H2O2. These free radicals declined the intracellular ATP concentration, leakage of Ca2+ out of mitochondria, cellular osmotic imbalance and lipid peroxidation, resulting in increased permeability and subsequent mucosal damages (Katary MA et al.2017). Oxidative stress induced generation of ROS and its overproduction is one of the prime an etiologic factor that cause gastric ulcer. As a result, some relevant biochemical marker status gets affected as peroxidation of lipids, proteins and nucleic acids, which may lead to cellular damage and cell death (Atushkumar sahoor.2017). On the other hand, MDA, is more cytotoxic to cells

affects the membrane structure and causes further destruction in the cells, accompanied by severe ulceration (Mehmet Ibrahim Turan et al .2013). Inhibitory action of indomethacin on prostaglandin synthesis coupled with free radicals formation has been opined as critical biochemical events in the pathogenesis of gastric ulceration. NSAIDs induce mucosal damage via ROS produced by recruited leukocytes. ROS-mediated mitochondrial damage as well as lipid, protein, and DNA oxidation lead to apoptosis and mucosal injur (Hidekazu Suzuki et al.2011). Oxygen derived free radicals have been implicated in the pathogenesis of a wide variety of clinical disorders and gastric damage is caused by physical, chemical and psychological factors that leads to gastric ulceration in human and experimental animals (ÖmerCoşkun et al.2004). For instance, lipid peroxidation (LPO) mediated by oxygen radicals, especially hydroxyl radicals, plays a crucial role in the development of the gastric mucosal injury induced by indomethacin (Chan Young Ock et al.2011), so that probably be the main causes of increase of MDA level in gastric homogenate. The results of CAT level show in fig32. The result of Indomethacin shows a very high increase compared to control. CAT is an enzyme scavenger that is able to remove hydrogen peroxide from the body to prevent cells from hydrogen peroxide-induced damage (RUOKUN Y et al.2015). Contrary to results of (Darshan Vinod Shah et al.2017). our results show a very high increase level in catalase activity coùpared to control. While P.halapensis group show no signification effect of catalase activity compared to control, which this is refere to the high protection of P.halapensis barks to cells damegeprobebly due to their seconde metabolic compounds.

The result of the effect of aqueous extract of P. halapensis on oxidative stress parameters appeared a high decrease of MDA in gastric homogenate compared to Indo group, and show a very high decrease in MDA level compared to control and INDOMET group in duodenal homogenate. Also reduced Glutathione (GSH) level was decreased in stomach homogenate and increased in duodenal homogenate compared to INDOMET group. The plant extract can decrease the MDA by polyphenols and flavonoids contained a hydroxyl group in their structure wish make them have a very important antioxidant activity, many flavonoids chelate free Fe and Cu that could otherwise increase ROS generation, and also reduce ROS such as $O2\cdot-$, and $HO\cdot$ (Asima Bhattacharyya et al.2014). Hydroxyl groups on this nucleus donate hydrogen and an electron to hydroxyl, peroxyl, and peroxynitrite radicals, stabilizing

them and giving rise to a relatively stable flavonoid radical. (Sofna D.S et al .2014).in our study P. halapensis decrease the reduced glutathione (GSH) level compared to drugs disease group. GSH is probably the most important antioxidant enzyme present in the cells. Therefore, other enzymes that help to generate GSH are critical to the body's ability to protect itself against oxidative stress. The functions of GSH are removal of oxygen free radical species and maintenance of membrane protein thiols (S. Gopinathan et al.2015). In addition, GSH has been shown to scavenge a wide range of reactive species (RS) including reactive oxygen species (ROS) and reactive nitrogen species (RNS). In this way, GSH can scavenge superoxide anion (O2), hydroxyl radical, and singlet oxygen directly, by donating electrons and becoming oxidized to thyil radical (GS.) (R. FRANCO et al.2007).in addition,P.halapensis treatment group show no signification effect of catalase activity compared to control, which this is refer to the high protection of aqueous extract of P.halapensis barks to cells damegeprobebly due to their second metabolic compounds.

The results obtained in this study shown also that zinc treatment ameliorate the stat of oxidative stress decreasing MDA level in stomach tissues. Zinc could inhibit oxidative stress via protectionand stabilizer of lipids and proteins. Thus, Zn protects cellular membranes and macromoleculesand preservation of sulfhydryl groups forproteins, preventing oxidation by forming strong thiolatecomplexes. Moreover, these protective and anti-apoptoticactions of zinc were shown to be mediated by the smac/DIABLO(mitochondrial protein that potentiates some forms of apoptosis)signaling in indomethacin-induced apoptosis of gastric cells **(**W. Opoka et al.2010).

The P.halepensis+ Zinc group show in amelioration in all stress oxidative parameters which led to protection against oxidative stress, this protection probably due to high effect of protection of second metabolic and the remarkable effect of zinc in ameliorate stress oxidative parameters.

2.6. Histological analysis

In this present study, Indomethacin causes a totally destruction and deep injury in gastric epithelium cells compared to control. we can explain this effect as follows: Cytokines are proteins that play an important role in the inflammatory response. The majority of the cytokines are produced by activated endothelial and epithelial cells are

able to produce these proteins, too. Cytokine expression is regulated by NF-κB and AP-1 and may be triggered by LPS, ROS and microbial species, among others. Interleukin (IL)-1B and tumor necrosis factor (TNF)-α are two of the main cytokines involved in the inflammatory response (Nayely Leyva-Lópezet al.2016). Accordin to (Katary MA et al.2017 results study, gastric ulcers are associated with inflammation, indomethacin activates NF-κB subsequently NF-κBtranslocates into the nucleus to up regulate the expression of pro-inflammatory cytokines genes such as TNF-α and cytokine-induced neutrophil chemo attractant (CINC-2α). These data were confirmed by induction of gastric tissue levels of proinflammatory cytokines such TNF-α, IL-1β and IL-6 as well as decreasing gastric level of anti-inflammatory cytokine IL-10 by indomethacin treatment Mucosal proinflammatory cytokines, such as TNF-α, IL-1β and IL-8, are considered key inducers of gastric injury induced by NSAIDs like indomethacin. Through the induction of adhesion molecules on both leukocytes and vascular endothelial cells. It was reported that IL-1β and IL-8, as well as TNF-α, can promote inflammatory response and are involved in NSAID-induced gastric mucosal injury in humans and animals (Xin Zhang et al. 2008).TNF-α, the major proinflammatory cytokine released from the migrated macrophages, NF-α, produced by macrophages, results in injury to multiple organs and causes inflammation, edema, ischemia, hemorrhage, and neutrophilic leucocytes accumulation wich plays an important role in the pathogenesis of gastric ulcers through stimulation of ICAM-1 expression on vascular endothelial cells (Sheng-Chun Dang et al .2015. TNF-a activates the cytotoxic and phagocytic activities of neutrophils, which, if directed towards endothelial cells, could destroy them (John S. Patton et al.1987).

In the other side histological results of P. halapensis shows a high protection against tissues lysis induced by Indomethacin. our in-vitro result appeard that P. halapensis barks containing different second metabolic compound which they have anti-inflammatory property such as flavonoids, according to Flavonoids appear to be important modulators of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α. However, the effect of flavonoids on intracellular signaling pathways and on other inflammatory mediators still remains to be investigated, since it would depend on the type of cells, the studied disease and the applied stimulus (Nayely Leyva-López et al.2016). In addition, flavonoids such as quercetin and luteolin, showed higher

inhibitory effect on TNF-α release than those with only one hydroxyl group in B ring (Comalada, M et al.2006), the highpretection property of our plant P. halapensis is due of inhibition of pro-inflammatory cytokines that refer to their second metabolic.

The beneficial effect of zinc at the histological level can be explained as follows: zinc and zinc-containing proteins are involved in nearly everystage of cutaneous wound repair due to their profound role in themodification of the ECM component, cell migration, proteinsynthesis and anti-inflammatory properties **(**W. Opoka et al.2010). This healingaction by zinc could also be attributable to its protective action onvisceral organs by demonstrating that zinc, exerts inhibitory effectson pro-inflammatory NF-kB activation (Verena von Bülow et al.2007). Miriam et al. reported that zinc limits iNOS-derived high output NO production in endothelial cells by inhibiting NF-κB-dependentiNOS cells and protects endothelial cells against H2O2-mediated damage via zinc-mediated increase of cellular glutathione de novo synthesis (Miriam M.Cortese et al.2011). The association of P.halepensis with Zinc give a cytoprotecting to gastric refer to their protection effect.

Conclussion and Prespectives

Conclusion and perspectives

Stomach is the third important stage in digestive system, any dysfunction that disrupts its function leads to a lack of absorption of food in the bowel. One of this major problem is Ulcer.So, the aim of this study isevaluation the gastroprotection effect of aqueous extract Pinushalepensis barks against Ulcer induced by Indomethacin.

The phytochemical analysis show the richness ofaqueous extract of P.halepensis bark from second metabolic compounds as: polyphenols, flavonoids, tannins, saponins, steroids, carbohydrate and alkaloids.

The invitro study of aqueous extract of P.halepensis appeared an important activity of their barks against inflammation and disorders induced by oxidative stress.

The invivo study ofaqueous extractof P.halepensis barks show up their important analgesic activity, andappeared that aqueous extract P.halepensis bark had no toxicity effect even with high concentrations.

Haematologicalanalysis concluded that ofrats treated by aqueous extract P.halepensis barks may have a positive impact in the hematopoietic system.

As for biochemicalparameters,aqueous extract of P.halepensis barks show the ability to protect from kidney failure.

Aqueous extract of Pinushalepensis barks give remarkable results in gastric protection against ulcer, in macroscopic level which amount to 100% this high protection was confirmed by decrease the level ofpH and total acidity in gastric juice.

The high protection ability of aqueous extract of P.halepensis barks not limited to macroscopic level but extended to microscopic level which shows their high protection in cells stabilisation against gastric injuryinduced by oxidative stress that our pinus had a remarkable decrease in MDA and increase in GSH and CAT levels.

The association of Zinc and aqueous extract of Pinushalepensis barks do not give better results than results of P.halepensis only.

For future studies, we hope that studies will focus more on detect all compounds ofPinushalepensis bark, especially the effective ones that give this amazing result to more testing them in this pathology, and why not work on the extraction of effective and potentdrug through these compounds.

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Annex

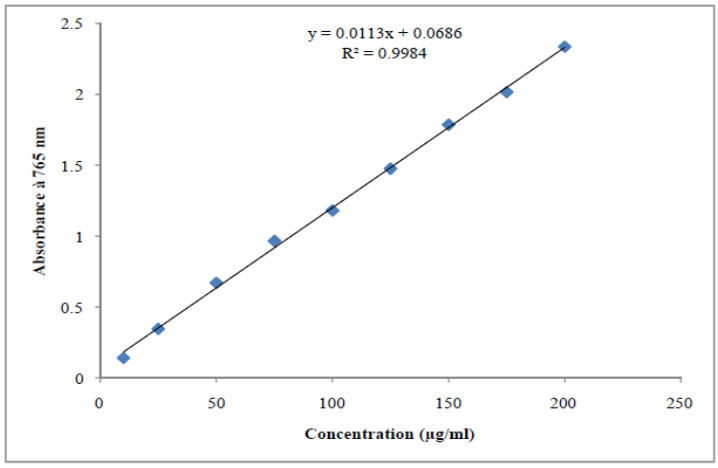

Figure: Calibration curve for Gallic acid determination of polyphenols.

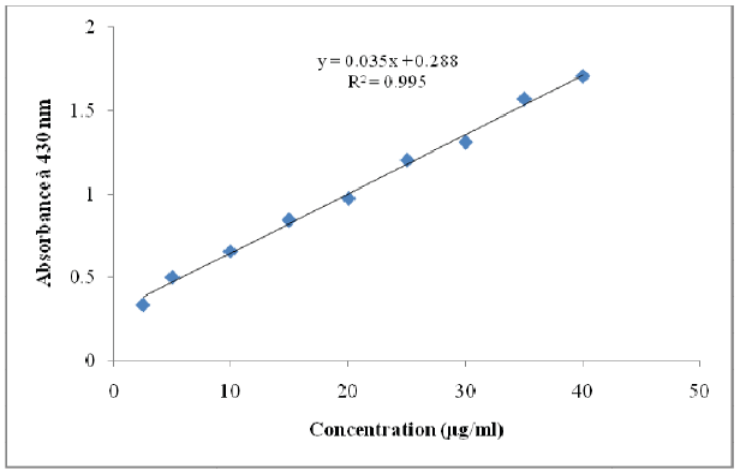

Figure: Calibration curve for Gallic acid and Quercetin for determination of total flavonoids

Table: HPLC chromatogram of flavonoids standard.

المركب	tr	المعادلة	R2
Gallic acid	5.29	y = 54681x	R² = 0.9956
Chlorogenic acid	13.392	y = 21665x	R² = 0.9853
vanilic acid	15.531	y = 65077x	R² = 0.9921
Caffiec acid	16.277	y = 84066x	R² = 0.9974
vanilin	21.46	y = 58930x	R² = 0.9966
p-Coumaric acid	23.817	y = 49495x	R² = 0.9961
Rutin	28.37	y = 28144x	R² = 0.9869
Narginig	34.788	y = 19379x	R² = 0.9968
Quercetin	45.047	y = 45378x	R² = 0.9962

Lamda =268nm