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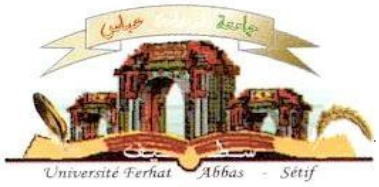

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THESIS

Presented by

Roumaissa Ounis

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TOPIC

Pharmacological activities of *Thymelaea hirsuta* L. extracts: *in vitro* and *in vivo* studies

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بسم الله الرحمن الرحيم

(وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا)

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Hind Amira, Hassiba Benabdallah, Walid Maamache, Fatima Benchikh, **Roumaissa Ounis** and Smain Amira. Scavenging and redox potential activities of hydro-methanolic extract from *Achillea odorata* L. areal parts. The First national Seminar on Green Chemistry and Natural Products (GCNP'22), University of Echahid Hamma Lakhdar, El-Oued.

Hind Amira, Hassiba Benabdallah, Walid Maamache, Fatima Benchikh, **Roumaissa Ounis** and Smain Amira. Scavenging and redox potential activities of DEfrom *Achillea odorata* L. areal parts. 1er séminaire national sur l'évaluation des activités biologiques des plantes médicinales et docking Moléculaire (SNABPMDM-1), université Mostafa Benbou laid Batna 2.

Mamache Walid, Benabdallah Hassiba, Benchikh Fatima, Amira Hind, Bencheikh Amor, **Ounis Roumaissa**, Kaoudoune Chahrazed, Amira Smain. *In vitro* antioxidant activity of aqueous extract of *Senecio perralderianus* Cos. National seminar on phytotherapy and pharmacognosy (NSPP- 2023). University Setif 1.

ملخص

تنتمي نبتة *Thymelaea hirsuta* (L). Endl إلى عائلة Thymeleaceae ، هي شجيرة دائمة الخضرة زهرية. تم إجراء دراسة إثنوبوتانية تستكشف الاستخدامات التقليدية لـ *T. hirsuta* يليها تقييم في المختبر للأنشطة المضادة للأكسدة والمضادة للإنزيمات. بالإضافة إلى ذلك، تمت دراسة تأثيرها على إفراغ المعدة والعبور بالأمعاء وحماية المعدة المتقرحة. أظهرت النتائج أن *T. hirsuta* مستخدمة بشكل شائع في الطب الشعبي، خاصة في علاج الاضطرابات الجلدية والتنفسية واضطرابات الجهاز الهضمي وبشكل خاص في علاج القرحة المعدية. في دراسة مقارنة للمستخلص المغلى (DE) ومستخلص المائي والميثانولي (HME) ومستخلص الميثانول (ME) ، أظهرت النتائج زيادة في محتوى الفينول الكلي والفلافونويد والدباغ على التوالي. بينت نتائج التحليل الكيميائي للمستخلصات المختارة (DE, HME) باستخدام HPLC-DADوفرة حمض السينناميك الترانس، الفانيلين، وحمض الكلوروجينيك. أظهر كل من المستخلصين نشاطًا مضادًا عاليًا من خلال اختبارات DPPH, phenanthroline, استخلاب الحديد وتبييض β-carotene على التوالي. كما أظهر كلا المستخلصين نشاطًا مثبطًا ضد إنزيم الأستيل كولينيستيراز (AChE) و إنزيم البيوتيريل كولينيستيراز (BChE). كما أظهر كل من DE و HME تثبيطًا للألفا أميلاز بقيم IC_{50} تبلغ 0.77±52.80 و 0.95±43.71 في 100 ميكروغرام / مل على التوالي. و الألفا جلوكوزيداز بقيم IC_{50} تبلغ 0.95±69.72 و 0.85±53.36 ميكروغرام / مل على التوالي. أبدى كلا المستخلصين تثبيطًا ضعيفا لانزيم تايروزيناز. في الدراسات التي أجريت داخل الكائن الحي، أظهرت مستخلصات النبات التي تمت دراستها أنها آمنة عند الجرعات التي تم اختبارها. كما لوحظ أن معاملة الفئران بكل من DE أو HME قد أدى إلى تأخير معنوي في الإفراغ المعدي باعتماد مسارالبروستاغلوندين. بينما أدت الى تسريع العبور المعوي بإعتماد كلا المساراين الفيزيولوجيين الوظيفيين، مسارالبروستاغلوندين و أكسيد الأزوت(NO). من جهة أخرى، أدت معالجة الفئران بجرعات متزايدة من DE أوHME إلى مستوى عالٍ من الحماية ضد القرحة المعدية المحدثة بواسطة الإيثانول، خاصة في حالة HME التي تتجاوز تأثير حماية الرانيتيدين كعقار مرجعي. للمستخلصين آليات عمل متنوعة بينما تشترك في مسار NO، بالإضافة إلى ذلك فقد أظهرا قدرة على تعزيز مستويات الغلوتاثيون (GSH) والبروتين و نشاط الكاتالاز، و/أو تثبيط أكسدة الدهون.

كلمات مفتاحية : *Thymelaea hirsuta* ، مضاد الأكسدة، مضاد للانزيمات، إفراغ المعدة، العبور المعوي، القرحة المعدية.

Abstract

Thymelaea hirsuta (L). Endl (*T. hirsuta*) belongs to Thymeleaceae family. It is an evergreen perennial flowering shrub. An ethnobotanical study was conducted to investigate the traditional uses of *T. hirsuta,* followed by an *in vitro* assessment of the antioxidant and the anti-enzymatic activities. Additionally, an *In vivo* investigation explored its effects on gastric emptying, intestinal transit, and gastric protection. *T. hirsuta* emerged as a common employed remedy, for dermatological, respiratory and digestive tract disorders, particularly in the treatment of gastric ulcers. In a comparative study of DE(DE), hydromethanol extract (HME) and methanol extract (ME), the results showed a respective increase in total phenolic, flavonoids and tannins contents. The chemical profile of the chosen extracts (DE and HME) revealed by HPLC-DAD analysis showed the abondance of trans-cinnamic acid, vanillin and chlorogenic acid. In series of *In vitro* experiments, both extracts exhibited respectively a high antioxidant activity through DPPH, phenanthroline, ferrous ion chelating, and β-carotene bleaching assays. The plant extracts inhibited moderately AChE and BChE. Additionnaly, DE and HME showed a significant inhibitory effect on α-amylase with an IC_{50} values of 52.80 ± 0.77 and 43.71 ± 0.95 at 100 µg/mL, respectively, and α-glucosidase with IC_{50} values of 69.72 ± 0.95 and 53.36 ± 0.85 µg/mL, respectively. Both extracts demonstrated a weak tyrosinase inhibition. In terms of *In vivo* study, the plant extracts demonstrated a favorable safety profile at the tested doses. Notably, both DE and HME induced a significant delay in gastric emptying in mice involving prostaglandin pathway, while accelerating intestinal transit and involving both prostaglandin and Nitric oxide (NO) pathways. In the other hand, the treatment of rats with increasing doses of DE or HME resulted in a high level of protection against ethanol-induced ulcer in the stomach, particularly in the case of HME surpassing the protective effect of ranitidine as a refference drug. The extracts had a divergent mechanism of action while sharing the NO pathway. Furthermore, they enhanced glutathione (GSH) and protein levels, catalase activity and/or inhibited lipid peroxidation.

Key words: *Thymelaea hirsuta*, antioxidant, anti-enzymatic, gastric emptying, intestinal transit, gastric ulcer.

Résumé

Thymelaea hirsuta (L). Endl (*T. hirsuta*) appartient à la famille des Thymeleaceae. C'est un arbuste à fleurs vivace à feuillage persistant. Une étude ethnobotanique a été menée pour investiguer les utilisations traditionnelles de *T. hirsuta*, suivie d'une évaluation *in vitro* de l'activité antioxydante et anti-enzymatique. De plus, une investigation *In vivo* a exploré ses effets sur la vidange gastrique, le transit intestinal et la protection gastrique. *T. hirsuta* est apparu comme un remède couramment utilisé pour les troubles dermatologiques, respiratoires et du tractus digestif, particulièrement dans le traitement des ulcères gastriques. Dans une étude comparative des extraits décocté (DE), hydro-méthanolique (HME) et méthanolique (ME), les résultats ont montré une augmentation respective du contenu total en polyphénols, flavonoïdes et tanins. Le profil chimique des extraits choisis (DE et HME) a révélé par l'analyse HPLC-DAD a montré l'abondance d'acide trans-cinnamique, de vanilline et d'acide chlorogénique. Dans une série d'expériences *in vitro*, les deux extraits ont présenté respectivement une forte activité antioxydante à travers les essais de DPPH, phénanthroline, de chélation des ions ferreux et de blanchiment du β-carotène. Les extraits végétaux ont modérément inhibé l'acétylcholinestérase (AChE) et la butyrylcholinestérase (BChE). De plus, DE et HME ont montré des effets inhibiteurs significatifs sur l'α-amylase avec des valeurs de IC_{50} de 52,80 ± 0,77 et 43,71 ± 0,95 à 100 µg/mL, respectivement. Et l'α-glucosidase avec des valeurs de IC50 de 69,72 ± 0,95 et 53,36 ± 0,85 µg/mL, respectivement. Les deux extraits ont démontré une faible inhibition de la tyrosinase. En termes d'étude *In vivo*, les extraits végétaux ont présenté un profil de sécurité favorable aux doses testées. Notamment, à la fois DE et HME ont induit un retard significatif de la vidange gastrique chez les souris impliquant la voie des prostaglandines, tout en accélérant le transit intestinal et impliquant à la fois les voies des prostaglandines et de l'oxyde nitrique (NO). D'autre part, le traitement des rats avec des doses croissantes de DE ou HME a entraîné un niveau élevé de protection contre les ulcères induits par l'éthanol dans l'estomac, notamment dans le cas de HME dépassant l'effet protecteur de la ranitidine comme médicament de référence. Les extraits avaient des mécanismes d'action divergents tout en partageant la voie du NO. De plus, ils ont augmenté les taux de glutathion (GSH), de protéines, et de l'activité de la catalase et/ou inhibé la peroxydation lipidique

Mots-clés: *Thymelaea hirsuta*, antioxydant, antienzymatique, vidange gastrique, transit intestinal, ulcère gastrique.

List of abbreviations

ABTs	2.2′-Azino-bis (3-ethylbenzenothiazoline -6- sulfonic acid)
AD	Alzheimer disease
Ach	Acetylcholine
APC	Antigen presenting cell
BHT	Butylated hydroxytoluene
Bch	Butyryl choline
CAT	Catalase
CMC	Carboxymethyl cellulose
CNS	Central nervous system
COX	Cyclooxygenase
DAD	Diode Array Detection
DM	Diabetes Millitus
DU	Duodenal ulcer
DPPH	2, 2-diphenyl-1-picryl- hydrazyl
DTNB	5, 5' -dithio-bis (2-nitrobenzoic acid)
DW	Dried weight
FRAP	Ferric reducing antioxidant power assay
FCR	Folin ciocalteu reagent
GAE	Gallic acid equivalent
GI	Gastrointestinal
GPX	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfure oxidized
GU	Gastric Ulcer

HE	Hematoxylin
HPLC	High-performed liquid chromatography
IBD	Inflammatory bowel disease
IC_{50}	Inhibitory concentration for 50% of activity
IL	Interleukin
iNOS	inducible NOS
LPO	Lipid peroxidation
LPS	Lipopolysaccharide
МΦ	Macrophage
MPO	Myeloperoxidase
MMP	Matrix metalloproteinase
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
Nos	Nitric oxide synthase
NSAIDs	Non-steroidal anti-inflammatory drugs
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate buffered saline
PUD	Peptic Ulcer Disease
QE	Quercetin equivalent
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxyde dismutase
TAE	Tanic acid equivalent
TBA	Thiobarbutiric acid

TCA	Trichloro-acetic acid
TFC	Total flavonoid content
TNBS	Trinitrobenzene sulfonic acid
TNF	Tumor necrosis factor
TPC	Total polyphenol content
TTC	Total tannins content
WHO	World Health Organization

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INTRODUCTION

Medicinal plants have been used by primitive communities for medicinal purposes from ancient times (Hamilton *et al*., 2004). The biological properties of medicinal plants have attracted increasing attention in recent years in an effort to determine and evaluate their therapeutic potential, as well as to identify the main bioactive compounds and potential synergies (Martins *et al*., 2014; Uysal *et al*., 2023). In Algeria, Morocco, and Tunisia, a medicinal plant called *Thymelaea hirsuta (T. hirsuta)* is referred to as "Methnane." (Jamila et al., 2014). Belonging to the Thymelaeaceae family, a group of 50 genera of flowering plants (Galicia-Herbada, 2006). *T. hirsuta* has historically been used to treat inflammation, hypertension, respiratory issues and recently for degenerative conditions related to oxidative stress (Le Floc'h, 1983; Jamila *et al*., 2014; Miara *et al*., 2019). Oxidative stress is a factor in a number of degenerative conditions, including diabetes, GI disorders, brin dysfunction, cancer, cardiovascular disease, and immune system deterioration (Halliwell, 2007; Valko *et al*., 2007; Mohammed *et al*., 2022). Chemically, it is defined as a relative excess of reactive oxygen species (ROS) when compared to antioxidants (Lushchak, 2014).

Diabetes mellitus (DM), a metabolic condition characterized by increased blood glucose levels, has long been a serious public health concern. The enzymes α-glucosidase and α-amylase play critical functions in managing blood glucose levels in the human body (Van de Laar, 2008). The inhibition of these enzymes is critical in the regulation of blood glucose levels in pathological situations such as diabetes (Li *et al*., 2005).

Alzheimer's disease (AD) is a frequent neurological problem in humans that is characterized by cognitive dysfunction, behavioral instability, and limits in performance of ordinary routine activities. Acetylcholine (ACh) and butyrylcholine (BCh) are neurotransmitters involved in memory acquisition and storage. They are in charge of impulse transmission across the brain synapse, but their levels decline in AD (Ahmad *et al*., 2016). In order to restore the activities of ACh and BCh at the neural synapse, it is necessary to block the enzymes acetylcholinesterase (AChE) and buturylcholinesterase (BChE), which are responsible for the lowered levels of these neurotransmitters (Ullah *et al*., 2016).

In the context of GI pathology, PUD is among the main problems of the GITwich have a potentially serious complications; bleeding, perforation, pénétration and obstruction with a high risk of mortality (Milosavljevic *et al*., 2011). It generally occurs in the stomach or proximal duodenum, less commonly, it affects the oesophagus or Meckel’s diverticulum (Dunlap et Patteson, 2019). This pathology is a multifactorial process. Where, an ambalance between the aggressive factors (acid, pepsin, NSAIDs, *H. pylori*) and protective and /or reparative mechanismes (bicarbonate, blood flow, mucus, cell junctions apical resistance, restitution,

mucoid cap, proliferation, growth factors) is the main cause of it induction (Casalnuovo *et al*., 2022).

Actually, the treatment of peptic ulcer is based mainly on anti-secretory medications (histamine H2 receptor antagonists, proton pump inhibitors) and cytoprotectors such as sucralfate and salts bismuth (Lauret *et al*., 2015). While, phytotherapy marks a broad spectrum in ulcer healing; in experimental models of gastric ulcer, natural products including plants and their extracted components have been extensively employed (Djanaev *et al*., 2022).

A variety of digestive diseases, such as gastroparesis, functional dyspepsia, and gastroesophageal reflux disease, have been associated with GI motility disorders (Camilleri, 2014). The movement of the stomach's contents into the duodenum for additional processing and absorption is known as gastric emptying. The volume and composition of the meal, the availability of hormones and neurotransmitters, and individual variations in digestive physiology are a few of the variables that control this process. On the other hand, the flow of food through the small and large intestines, where it is further broken down and absorbed into the bloodstream, is referred to as intestinal transit. The consistency and fiber content of the food, as well as individual variations in gut bacteria and digestive performance, all play a role in controlling this process (Serra *et al*., 2013). Several studies demonstrated the effect of plant extracts on GI motility disorders by enhancement of gastric emptying and intestinal transit (Kimura et Sumiyoshi, 2011)

In light of the significance of these medical conditions and their widespread impact, our research is dedicated to finding natural treatments. We recognize the importance of addressing these health challenges and the limitations of conventional therapies. Through our work, we aim to contribute to safer and more sustainable healthcare solutions. To achieve this goal, we have chosen to conduct our investigations through the following steps:

- ✓ An ethnobotanical study were conducted to investigate the traditionnal uses of *T. hirsuta* medicinal plant in order to obtain a forecast of its usage.
- ✓ Extraction of plant extracts.
- ✓ *In vitro* studies :
  - ➢ Determination and quantification of *T. hirsuta* phytochemical content.
  - ➢ Evaluation of the antioxidant activity of plant extracts.
  - ➢ Evaluation of the antienzymatic activity of plant extracts.
- ✓ *In vivo* studies:
  - ➢ Evaluation of the acute oral toxicity of plant extracts in rats.

- Effect of plant extracts on gastric emptying in mice and the possible mechanism underlying.
- Effect of plant extracts on intestinal transit in mice and the possible mechanism underlying.
- Effect of plant extracts on ethanol-induced gastric ulceration in rats and possible mechanim involved.
- Effect of plant extracts on macroscopic and histopathological appearance in rat gastric mucosa.
- Determination of mucus content in gastric mucosa.
- Evaluation of the *In vivo* antioxidant activity of plant extracts in gastric homogenate.

LITTERATURE

1 The gastro intestinal tract (GIT)

1.1 Anatomy and function of the gastro-intestinal tract

The GIT consists of a system of organs, that share constitutional similarities, and are variously structured as cylinders, spheroids, or intermediate shapes, with the pancreatic and biliary ducts acting as important side-branches. Food digestion and transportation are the two main functions of the GIT (Gregersen et Kassab., 1996). The GIT comprises the mouth, pharynx, esophagus, stomach, belonging to the upper GI system, small intestine, large intestine, rectum, and anus are part of the lower GIT (Menizibeya, 2018).

The shape and mechanical characteristics of the various segments vary greatly; the oesophagus primarily functions to swiftly carry food boluses from the mouth to the stomach, where food is stored for a period of time while also being broken down into smaller components. The GI sphincters separate the GIT into compartments. Moreover, the intestine also plays a crucial role in immunological processes (Gregersen *et al*., 1996).

1.1.1 Stomach

1.1.1.1 Anatomy of the stomach

a) Gross anatomy

The stomach comprises 5 segments; the cardia, fundus, corpus, antrum, and pylorus (Figure 1). The cardia is the closest point where the stomach and oesophagus connect. The fundus is located to the left of the cardia. The main portion of the stomach, the corpus, is located beneath the fundus. The convex left section of the stomach is the major side, or greater curvature. The right concave portion is the lesser curvature, or minor side. The more distal section in stomach is known as the antrum. The pylorus is a sphincter made of smooth muscle that links the duodenum and stomach (Barrett, 2014).

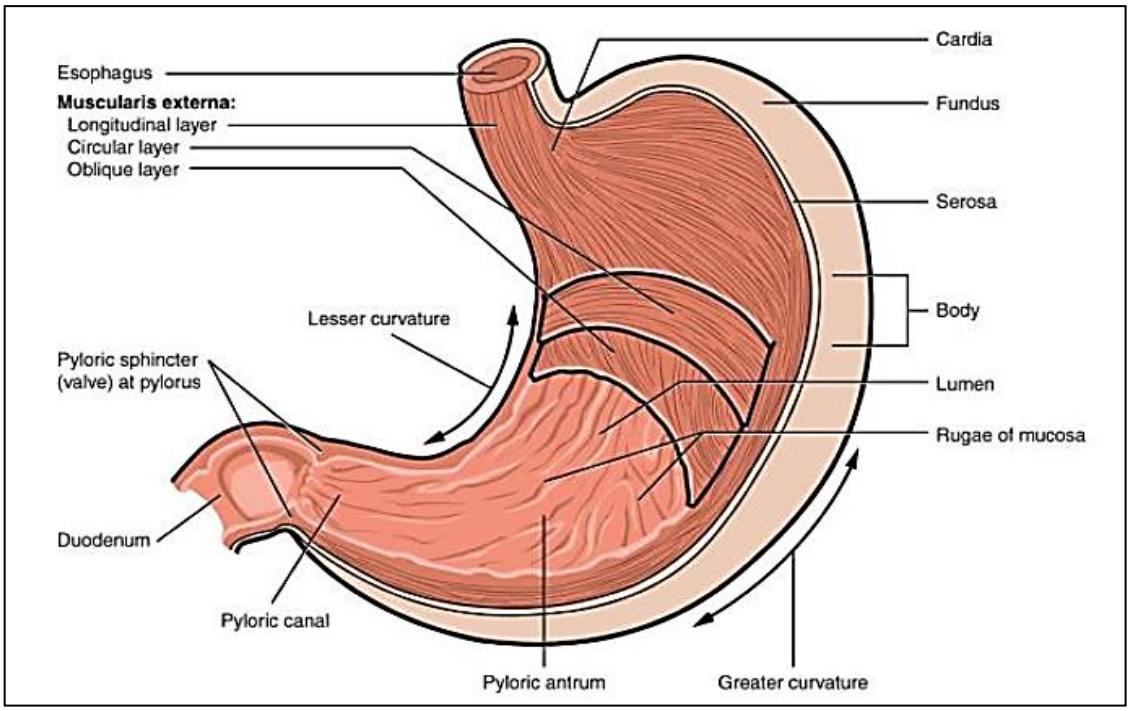

Figure 1. Anatomy of the stomach (Barrett, 2014)

b) Microscopic anatomy

The stomach's mucosal lining is characterized by a simple columnar epithelium that lines the stomach evenly. The gastric pits, which are made up of surface mucous cells, branch out into long, branched, tubular glands that give the gastric mucosa, also known as the gastric foveolae, a leafy appearance. From the top down, each gland contains unique areas: the gastric pit, isthmus, neck, and base (Figure 2). The stomach can be split into three glandular regions, each of which contains a different type of cell. These sections include the cardia, which contains cardiac glands, the fundus and body, which include oxyntic glands, and the pyloric region, which contains antral glands (Barrett, 2014).

Cardiac glands are mostly made up of mucous cells, with a few dispersed parietal cells, undifferentiated cells in the neck, and the majority of endocrine cells in the gland's base, cardiac glands and are mostly responsible for producing mucus, these gland's expression differs amongst ethnic populations (Huang, 2011). Oxyntic glands are found in the fundus and body of the stomach, the Greek word oxynein, which means "acid-forming," inspired the name of these acid-producing glands. The key cell types include the surface epithelial cells, mucous cells found in the GPs, isthmus, and neck, parietal cells, which are densely concentrated in the neck and emit hydrochloric acid (HCl) and intrinsic factor, basal chief (zymogenic) cells, which release pepsinogen, and enterochromaffin-like (ECL) cells which produce histamine as a potent

stimulant for parietal cell acid synthesis. The antral mucosa differs from the fundus/body mucosa in a higher percentage of gastrin-secreting G cells and fewer cells that produce acid (Wilson et Sterenson, 2019).

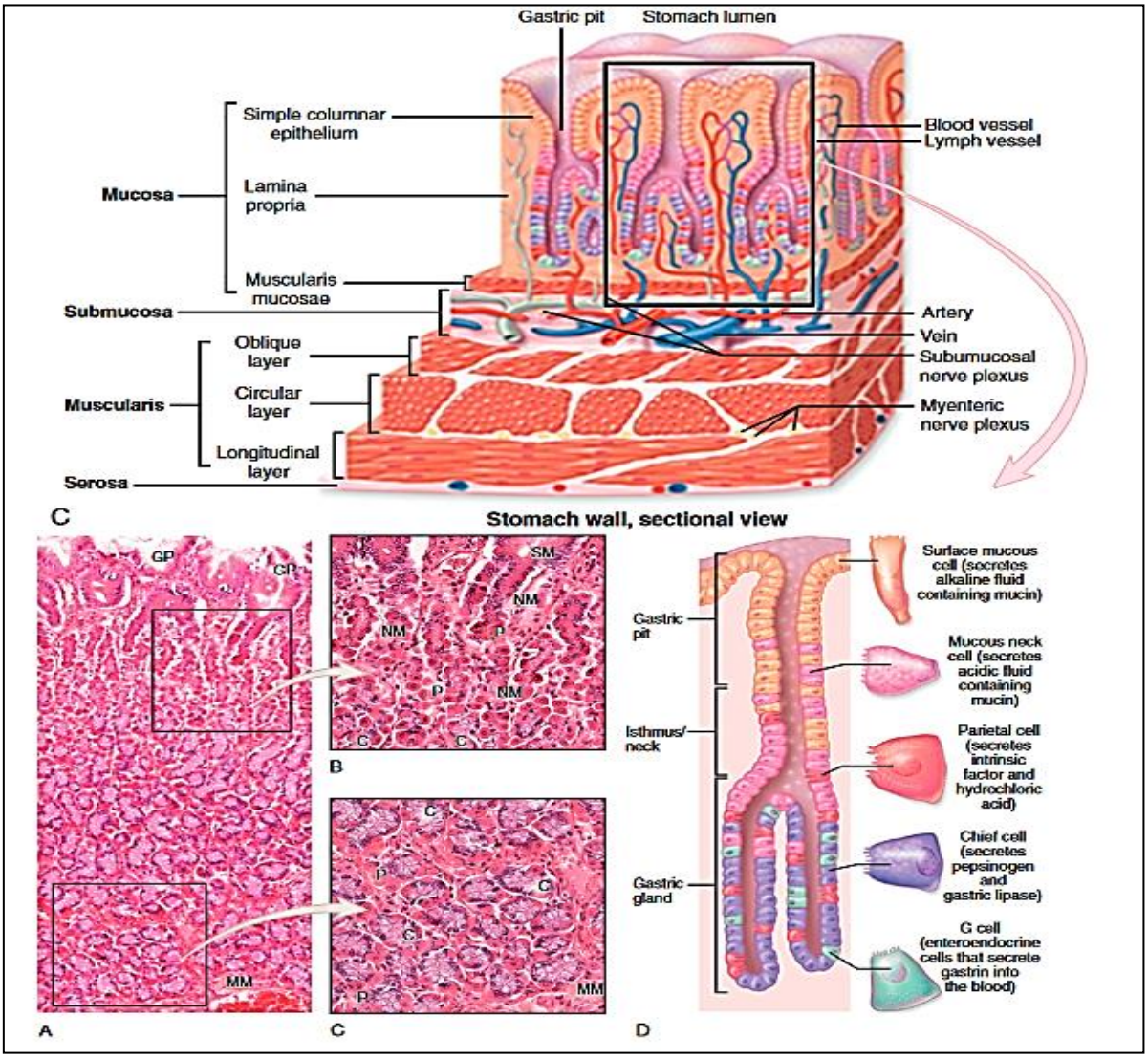

Figure 2. Microanatomy of Gastric Glands: Layers and Cell Types (Mescher, 2016).

(A) The long, coiled GGs penetrate the complete thickness of the mucosa, from the gastric pits (GP) to the muscularis mucosae (MM). (B) In the neck of a gastric gland, below the surface mucous cells (SM) lining the gastric pit, are small mucous neck cells (NM), scattered individually or clustered among parietal cells (P) and stem cells that give rise to all epithelial cells of the glands. The numerous parietal cells are large distinctive cells often bulging from the tubules, with central nuclei surrounded by intensely eosinophilic cytoplasm with unusual ultrastructure. Chief cells (C) begin to appear in the neck region. Around these tubular glands are various cells and microvasculature in connective tissue. (C) Near the MM, the bases of these glands contain fewer parietal cells (P) but many more zymogenic chief cells (C). Chief cells are found in clusters, with basal nuclei and basophilic cytoplasm. From their apical ends chief cells secrete pepsinogen, the zymogen precursor for the major protease pepsin. Zymogen granules are often removed or stain poorly in routine preparations. (Both x200; H&E stain) (D) Diagram showing general morphology and functions of major gastric gland cells.

1.1.1.2 Physiology of stomach

a) Gastric acid secretions

The physiological secretion of gastric acid is controled by three stimulating pathways, two inhibitory pathways, and several regulators. Ach, which is released by cholinergic cells from the vagal trunks, histamine, which is released by ECL cells, and gastrin, which is released by G cells, are the three stimulating pathways in charge of acid production in the stomach (Schubert *et al*., 1993; Schubert *et al.*, 2008). The two inhibitory pathways by extrinsic signals (1) somatostatin, released by D cells and (2) prostaglandins (E and I) (Saffouri *et al*., 1984).

b) Mucus secretion

The mucus-producing columnar cells, also known as "mucous neck cells," which cover the whole luminal surface and extend below into the glands, are the most prevalent epithelial cells. These cells generate bicarbonate-rich mucus, which plays a crucial role in shielding the epithelium from acid and other chemical irritants and coating and lubricating the stomach surface. It is composed of disulfide-bonded glycoprotein subunits that combine to create a water-insoluble gel that is impermeable to H+ ions. Prostaglandins increase production, luminal acid and vagal activity serve as stimulants. Bicarbonate is additionally produced by parietal cells. These epithelial barrier cells are extremely adhering due to restrict junctions between them (Daniels et allum., 2005).

c) Pepsin secretion

In the oxyntic region of the stomach, chief cells release pepsinogen. Low stomach pH transforms pepsinogen into pepsin (its active protease state), while pH levels above 4 render it inactive. It is released in response to stimulation from gastrin, cholecystokinin, and Ach. Pepsin has two main functions: mucolytic action and protease activity (Samloff., 1981; Allen *et al*., 1988).

d) Hormone secretion

Gastrin, a peptide that plays a key role in regulating acid secretion and gastric motility, is the main hormone released from the gastric epithelium (Daniels et allum., 2005).

e) Other Secretions

Other enzymes secreted by gastric epithelial cells include gelatinase and an acid-resistant lipase which hydrolyzes medium- and short-chain fatty acid triglycerides to produce glycerol and free fatty acids (Daniels et allum., 2005).

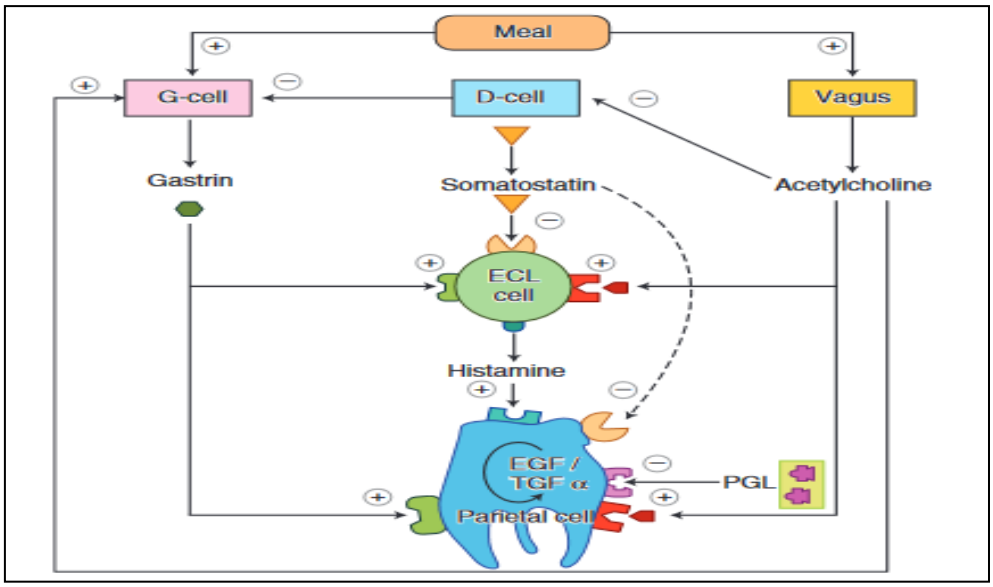

Figure 3. Physiology control of acid secretion (Landa *et al*., 2019).

PGL: prostaglandins, **EGF**: epidermal growth factor, **TGFα** : transforming growth factor alpha

1.1.1.3 Innervation of the stomach

Both parasympathetic and sympathetic nerves innervate the stomach. The larger splanchnic nerve and celiac plexus are used by sympathetic nerves to communicate pain. Afferent signals from the two anterior and posterior vagal trunks that descend laterally via the diaphragm's esophageal hiatus and are attached to the muscularis of the esophagus are indicative of parasympathetic innervation. The posterior wall of the stomach is innervated by the right vagal trunk, which travels posteriorly between the aorta and the esophagus, generates a celiac branch, and continues into the lesser curvature of the stomach. The criminal nerve of Grassi is a branch of the stomach-esophageal junction. It is necessary that it is found during a truncal vagotomy since it is suspected to be associated to recurrent symptoms. The anterior nerve of Latarjet, which passes across the anterior lesser curvature of the stomach and innervates the pylorus and the anterior wall of the stomach, emerges from the left vagal trunk as it passes anteriorly through the esophagus (Soybel, 2005).

1.1.2 Intestine

1.1.2.1 Smal intestine

The longest part of the GI tract is the small intestine. It is in charge of nutrition absorption, preserving water and electrolyte balance, creating an immunologic barrier, and secreting endocrine hormones, it measures around 7 meters in length from the pylorus to the ileocecal valve (ICV). The duodenum, jejunum, and ileum are the three segments that constitute this

organ. The jejunum and ileum are intraperitoneal structures, whereas the majority of the duodenum is situated in the retroperitoneum. The small intestine's lumen contains a sophisticated arrangement of structures that promotes nutrition absorption. Each structure is in charge of extending the intestine's surface area to improve nutrition absorption and digestion. The final result is an increase in surface area of 600 to 1000 times, or 250 to 400 m^2. Every 3 to 6 days, the small intestine's epithelium is replaced, and a range of factors can affect this process.

The innervation of the small intestine is controlled by two distinct and separate systems. The central nervous system (CNS) gives rise to the autonomic nervous system (ANS). The ENS is a specialized nervous system present only in the GI tract. This system is made up of neurons that are embedded in the intestinal wall and react to both local and general stimulation. The ENS is connected to the CNS via sympathetic and parasympathetic nerve fibers, which can modify the ENS's activity in response to external stimuli. The ENS also controles its own activity in response to intrinsic stimuli. Additionally, the intestinal wall contains sensory neurons that relay information to the ENS, sympathetic nervous system, spinal cord, and brainstem (Campbell *et al*., 2019).

1.1.2.2 Colon and rectum

The colon is a tubular organ that is around 30 to 40 cm long at birth and 150 cm long in adults. This makes it roughly one-fourth the length of the small intestine. The colon starts at the Ileocecal valve and terminates distally at the anal margin. It is divided into four segments: the cecum and vermiform appendix, the ascending, transverse, and descending portions of the colon, rectum, and the anal canal. The colon's diameter is largest in the cecum (7.5 cm) and narrowest in the sigmoid (2.5 cm), before ballooning in the rectum close to the anal canal (Bass et wershil., 2016).

1.2 Histology and cellular organisation of the GIT

The typical structure of the wall of the GIT consists of four layers; the mucosa, submucosa, muscle and serosa (Figure 4). The mucosa is the innermost layer supported by the submucosa, wich is a layer of connective tissue. The muscularis externa is the muscle wall surrounding the submucosa, the adventitia or serosa is the outermost layer of the GI tube. The mucosa is mainly composed of a layer of smooth muscle cells called the muscularis mucosae that forms the boundary between the mucosa and the submucosa, a thin layer of loose connective tissue called the lamina propria, and an innermost layer of epithelial cells. The mucosal and submucosal layers of the small intestine and colon are arranged into ridges known

as plicae circulares that enhance the absorptive surface area by around three times. Additionally, the mucosa's tiny fingerlike extensions called villi that extend into the lumen double the absorptive surface area. Each villus has a core that contains arterioles that ascend to the tip without branching, split at the tip to form a network of capillaries, and then descend along the edges of the villus in a fountain-like pattern in close proximity to the basement membrane and epithelial cells (Shen, 2009).

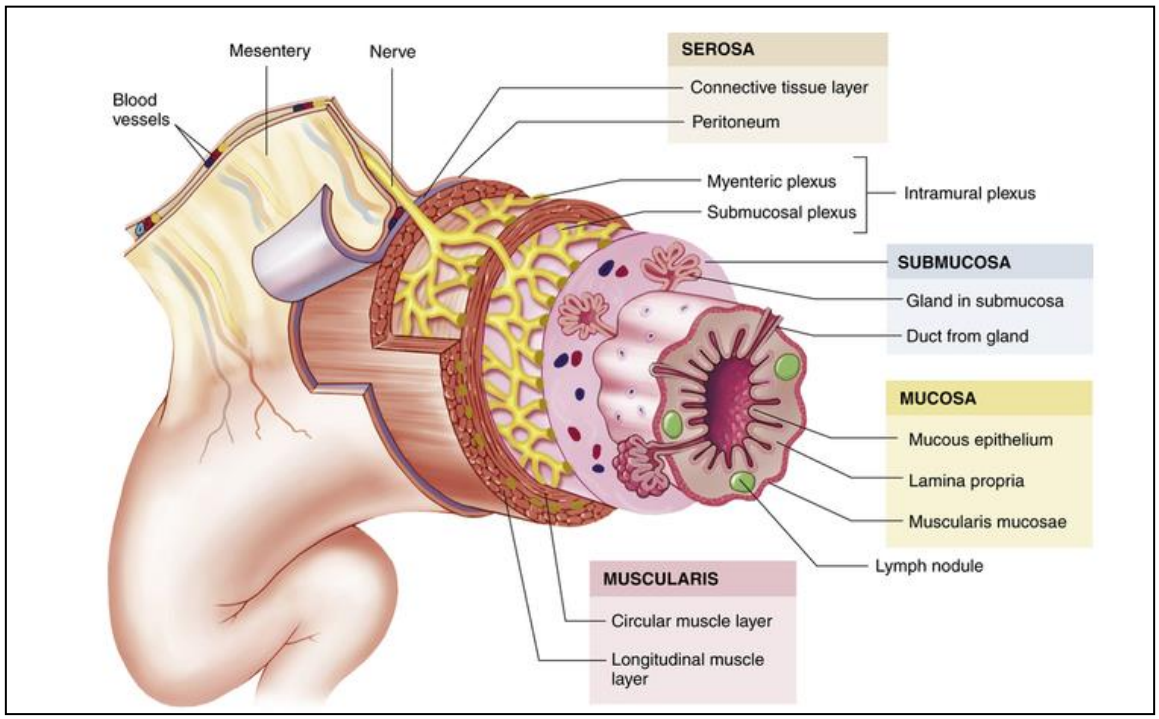

Figure 4. Structural complexity of the GIT layers (Patton *et al.*, 2013)

The wall of the GITis made up of four layers with a network of nerves between the layers. Shown here is a generalized diagram of a segment of the GI tract. Note that the serosa is continuous with a fold of serous membrane called a *mesentery*. Note also that digestive glands may empty their products into the lumen of the GITby way of ducts.

1.3 Gastrointestinal Circulation

The GITcirculation can be divided into arterial blood and venous blood circulation, much like any other part of the circulatory system. However, other writers classify the blood circulation of the GIT at the organ level (Jansson *et al.*, 2016; Granger *et al.*, 2015) The circulation of the GITis a part of the larger splanchnic circulation, which is made up of parallel circulations of the stomach, small and large intestines, the liver, the pancreas, and the spleen. The word "splanchnikos" is Greek and means "inwards." Organs that receive blood flow from the celiac, superior, and inferior mesenteric arteries are classified as having splanchnic circulation. The portal vein receives the blood returning from the three branches of the

abdominal artery that supply the GI tract. The somewhat hypoxic blood is then sent to the liver (Parks et Jacobson., 1985; Ackland *et al*., 2000).

2 Peptic ulcer disease

Peptic ulcer disease (PUD) is one of the most common GI diseases (Venkatesan *et al*., 2017). PUD is a disorder of the continuity of the GI mucosal lining that manifests as sores with a minimum diameter of 0.5 cm in endoscopic examinations (Habeeb *et al*., 2020). In significant part, it is classified as gastric ulcers (GU) and duodenal ulcers (DU) depending on the part of the digestive system affected (GIT) (Eniojukan *et al*., 2017). The commun clinical manifestation is epigastric pain, which may go away after eating or taking an alkali (Habeeb *et al*., 2020).

The prevalence of PUD and its consequences varies globally and has altered over the past few decades as a result of the identification of *H. pylori* as a significant etiological element in the disease pathogenesis and the rising usage of NSAIDs. (Azhari *et al*., 2018)

2.1 Epidemiology and complications

PUD affects approximately 4 million people worldwide, with prevalence of complications in about 10–20% of cases (Lee *et al*., 2011; Habeeb *et al*., 2020). Perforation, bleeding, and obstruction are complications (Malfertheiner *et al*., 2009; Lau *et al*., 2009). Although bleeding occurs more frequently (1:6), perforation is the most frequent cause of emergency surgery for PUD (Wang *et al*., 2010; Güzel *et al*., 2014). Patients with peptic ulcer bleeding had a considerably higher mortality rate, according to Soplepmann *et al (*1997) in a prospective study in Estonia.

2.2 Aetiology

A number of risk factors are thought to contribute to the development of peptic ulcer, including and corticosteroids, a stressful lifestyle, alcohol intake, smoking, family history, the use of NSAIDs and *H. pylori* infection (Sumbul *et al*., 2011; Bandyopadhyay *et al*., 2001). *H. pylori* infection plays a key role in the etiology of peptic ulcers and is responsible for 90–100% and 60–90%, respectively, of individuals with gastric ulcers and deudonel ulcers. This percentage varies by region and socioeconomic condition **(**Miftahussurur, 2015).

2.3 Pathogenesis

PUD is a pathological condition in wich the biological balance between defensive and offensive elements in the GI system is disturbed. Among the principal endogenous aggressive agents are gastric hydrochloric acid, pepsin, reactive free radicals and oxidants, leukotrienes, refluxed bile, and endothelins (Borrelli *et al*., 2000; Repetto *et al*., 2002). Defensive factors include the function of enzymatic and non-enzymatic antioxidants, the gastric mucus barrier,

bicarbonate, mucosal blood flow, surface active phospholipids, PG, and NO (Borrelli F *et al.*, 2000; Cryer B, 2001).

NSAIDs and/or *H. pylori* are the main causes of PUD (NSAIDs). While *H. Pylori* causes an imbalance in acid production and regulation by increasing gastrin secretion and decreasing somatostatin secretion in response to inflammation, NSAIDs have a tendency to affect the Cyclo-Oxygenase (COX) pathways, which leads to the production of prostanoids (prostaglandins, prostacycline, and thromboxane), which changes the mucosal defense mechanism and causes damage to the mucosal surface (Habeeb *et al*., 2020; Rafi *et al*., 2014; Russell, 2001).

2.4 Models of gastric ulcer

For the purpose of inducing gastric ulcers, several experimental models employ a variety of agents, such as ethanol, acetic acid, NSAIDs (indomethacin, aspirin, ibuprofen), histamine, serotonin, reserpine, methylene blue, and diethyldithiocarbamates. Other experimental models involve either a general stressor, such as water immersion stress, or a localized physiological stress, such as ischemia reperfusion (pylorus ligation) (Adinortey *et al*., 2013).

2.5 Ethanol-induced gastric ulcers

Ethanol could be attributed to a disruption in the balance between the stomach protective and aggressive factors of mucosa. While PGs, the production of epidermal growth factor, and the regeneration of epithelial cells work as gastroprotective agents to maintain the integrity of the gastric mucous layer. As well, the vascular endothelial cells of the gastric mucosa are damaged by ethanol, which also causes microcirculatory disruption, hypoxia, and the excessive generation of oxygen radicals (Mezdour *et al.*, 2017). It causes also a decrease in the transmucosal potential difference and the solubilization of mucus components in the stomach. Moreover, it increases the flow of Na^+ and K^+ into the lumen as well as pepsin production, H^+ ion loss, and histamine levels, which are indicators of the exfoliation of the superficial cell layer (Hiruma-Lima *et al*., 1999). Ethanol also inhibits the activity of antioxidant enzymes crucial for the protection of stomach mucosa from injury such SOD, CAT, and GPx while increasing the production of ROS and lipid peroxidation (Pan *et al.*, 2008).

An excessive ethanol consumption leads to acute gastric mucosal lesions through neutrophil infiltration and consequently, production of pro-inflammatory cytokines and expression of nuclear factor-B (NF-B) (Allavena *et al.*, 2008; Gottfried *et al*., 1978; Liu *et al*.,

2012). On the other hand, cytokines such as TNF- and interleukin-6 (IL-6) are important in the pathophysiology of stomach ulcers induced by ethanol (Figure 5) (Li *et al*., 2013).

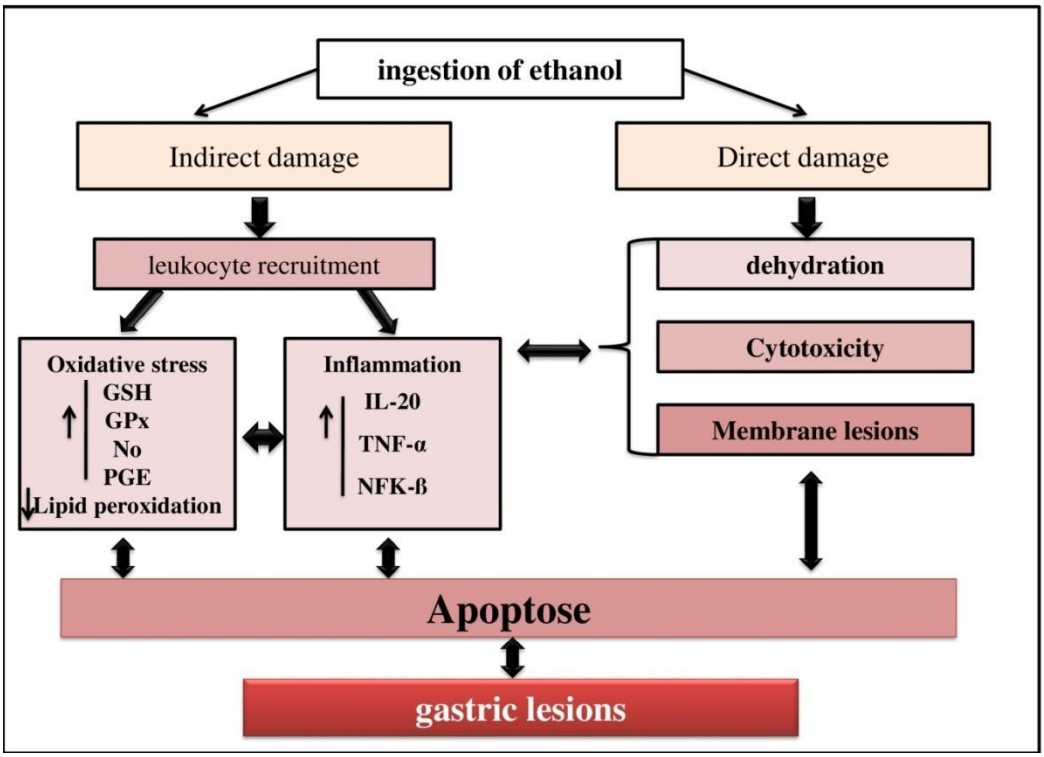

Figure 5. Induction of gastric ulcer by ethanol (Arab *et al*., 2015).

2.6 Therapy

There are already a number of chemical medications available for the prevention and treatment of peptic ulcers, but none are without side effects. H2 receptor antagonists have been linked to headache, an anti-androgenic action, pancreatitis, and disorientation in older persons. Itching, skin rashes, diarrhea, lightheadedness, and the inactivation of some anti-fungal medications have all been associated with proton pump inhibitors. Similar to other medications, sucralfate has a number of common adverse effects, including nausea, constipation, sleepiness, indigestion, and back discomfort. Misoprostol has a variety of side effects, including abdominal pain, diarrhea, vaginal bleeding, constipation, and abortion in pregnant women. It is administered for the prevention and treatment of NSAID-induced gastric ulcers. Both academics and doctors are still interested in finding ways to prevent and treat GI ulcers effectively (Borrelli *et al*., 2000; Repetto *et al*, 2002; Miederer, 1986). Different combination therapy has been used to eradicate this pathogen as well as provide preventive effects or remission of GI disorders due to the clear significance of *H. pylori* in gastritis and GI ulcers. Although combining a proton pump inhibitor with two antibiotics improves the disease's status

and symptoms by increasing eradication rates, this therapeutic strategy is rarely effective, with ulcer recurrence frequently happening. Treatment failure for antibiotic-resistant *H. pylori* infection has been documented in several studies, most notably for antibiotics comprising clarithromycin and metronidazole (Gisbert *et al*., 2011; Toracchio *et al*., 2000). This leads to major issues with peptic ulcer management. Even if a number of pharmacotherapeutic options to decrease one or more pathogenesis-related peptic ulcer pathogenesis processes are being investigated, a cure-all. Polyphenols, secondary plant metabolites that are frequently found in fruits and vegetables, are increasingly seen as natural dietary components that are essential to a balanced diet due to their wide spectrum of biological effects, natural polyphenols showed a benefit for the GI system in a different way, including antispasmodic, anti-colitis, anti-secretory, anti-diarrheal, anti-ulcer, and anti-oxidative stress capabilities (Daglia, 2012; Farzaei *et al*., 2015).

3 Oxidative stress

Oxidative stress results when, the capacities of the endogenous antioxidant defense system are exceeded in tissues and organs by the formation of highly reactive molecules such as ROS, RNS, and RSS, resulting in cellular damage and dysfunction and a variety of diseases. Low amounts of the reactive species are continuously produced by regular metabolic activities inside the cells. They may also be the result of exposure to outside elements such as X-ray and UV radiation, ozone, air pollution, cigarette smoke, bacteria, viruses, and other harmful substances (Pham-Huy *et al*., 2008). Or as the result of a acute or chronic cellular stress. Both free radicals and non-radical oxidants can be reactive species. Due to the existence of unpaired electrons in their outer electron orbit, free radicals are unstable. Free radicals tend to neutralize themselves by interacting with other molecules, which causes their oxidation, because they are extremely unstable and reactive (Cheeseman, 1993). Therefore, they have the ability to cause damage on a broad range of levels by reacting with crucial biological molecules like DNA, lipids, and proteins (Lobo *et al*., 2010). Since proteins are one of the main building blocks of organisms, they serve as prime targets for free radicals (Davies *et al*., 1999). Free radicals can cause some protein alterations, such as protein unfolding or structural changes, but thankfully, the majority of these changes are largely innocuous (Dalle-Donne *et al*., 2005). While reversible oxidative changes play a role in controlling protein activity, irreversible protein modifications can cause proteins to become inactive, which can have long-lasting negative effects on cellular function (Dalle-Donne *et al*., 2005).

The main intracellular sources of chemical reactive species are the plasma membrane, cytosol, lysosomes, and endoplasmic reticulum (Balaban *et al*., 2005). The major types of ROS include free radicals like superoxide anion radicals (O_2^-) and hydroxyl radicals (OH·), as well as non-radical oxidants such hydrogen peroxide (H_2O_2) and hypochlorous acid (HClO). ROS are produced when molecular oxygen is chemically reduced (Schieber *et al*., 2014). The enzymes of the mitochondrial electron transport respiratory chain are the primary sources of ROS generation. Other enzymes catalyze chemical reactions that lead to the generation of ROS, including homologs of NADPH oxidase, PLA2), uncoupled NOS, COX, XO, lipoxygenases (LOXs), glucose oxidase, and myeloperoxidase (MPO) (Swindle *et al*., 2007; Munzel *et al*., 2010; Bhattacharya *et al*., 2014).

3.1 Antioxidants

According to Halliwell and Gutteridge, antioxidants are " molecules that delay, prevent, or remove oxidative damage to a target molecule," (Halliwell, 1995; Halliwell *et al*., 1995).

All living organisms possess endogenous antioxidant defenses that can oppose and eliminate reactive chemical species. According to their mode of action, Gutteridge and Halliwell divided the antioxidants into three categories: primary, secondary, and tertiary antioxidants (Halliwell *et al.*,1995). Secondary antioxidants act as ROS scavengers, while tertiary antioxidants work to repair the damaged molecules. Primary antioxidants prevent the generation of oxidants. Currently, antioxidants are mostly divided into enzymatic and non-enzymatic categories (Figure 6).

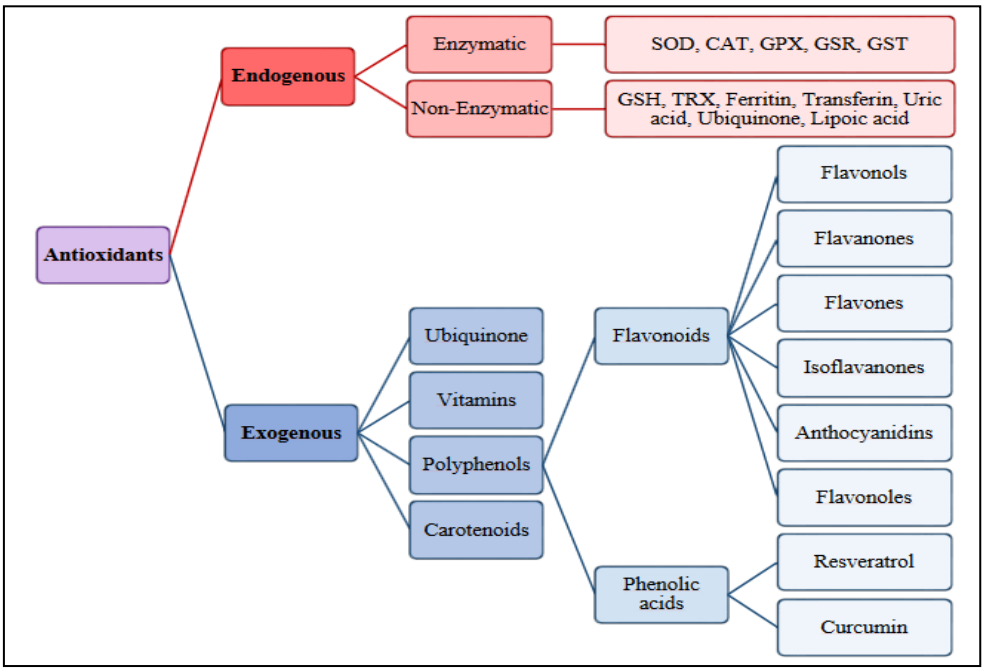

Figure 6. Scheme of endogenous and exogenous antioxidants (Vona R *et al.*, 2021).
SOD, Superoxide dismutase; **CAT**, Catalase; **GPX**, Glutathione peroxidase; **GSR**, Glutathione reductase; **GST**, Glutathione transferase.

3.1.1 Endogenous antioxidants

3.1.1.1 Enzymatic antioxidants

Catalase (CAT), SOD, glutathione peroxidase (GPX), and glutathione reductase are enzymatic antioxidants that contribute in the body's defense against reactive species (GSR). Enzymatic antioxidants are an example of an endogenous antioxidant system and serve both main and secondary defensive purposes. The main defense that limits the creation of reactive species or neutralizes them is comprised of glutathione peroxidase, SOD, and CAT (Rahman, 2007). SOD and CAT in particular offer important antioxidant defenses against ROS.

SOD catalyzes the dismutation of O_2 into O_2 and H_2O. Three SOD isoforms are found in humans (Nozik-Grayck E, 2005): Cytosolic Copper and Zinc-Containing Enzyme, Cu-Zn-SOD, present in the mitochondrial intermembranous space, Manganese-Requiring

Mitochondrial Enzyme, Mn-SOD, and Extracellular Copper and Zinc-Containing SOD) (EC-SOD)(Okado-Matsumoto , 2001)

Catalase, dismutates H_2O_2 to H_2O and O_2, it is largely found in peroxisomes. Although it is primarily produced by the liver, kidney, and erythrocytes, CAT has been discovered in almost all human organs. Lower CATactivity was noted in gastric adenocarcinoma, *H. pylori*-infected stomach, and colorectal cancer (Chang, 2012).

GPX reduces H_2O_2 to H_2O, oxidizes glutathione (GSH) to glutathione sulfate (GSSG), and transforms lipid hydroperoxides (ROOH) into the equivalent stable alcohols. Glutathione reductase (GSR), which keeps levels of reduced glutathione (GSH) stable, is linked with the GPX process. The glutathione system, which includes GSR, GPX, and glutathione S-transferases (GST), serves as an antioxidant barrier in the GIT mucosa. The preservation of cell membranes, red blood cells, and hemoglobin from oxidative stress depends on this enzyme, which produces GSH (Chang, 1978).

The antioxidant GPX protects cells from the negative effects of peroxide breakdown and is present in the mitochondria, cytoplasm, and extracellular space (Toppo *et al.*, 2008). Eight GPX isotypes are present in humans. While GPX2 is unique to the digestive system and guards the gut from absorbing dietary hydroperoxides (Wingler *et al*., 2000).

3.1.1.2 Non-enzymatic antioxidants

Glutathione and thioredoxin (Trx) are both of the endogenous, non-enzymatic antioxidants. The potent antioxidant glutathione is unquestionably one of the most critical antioxidants that the body can generate. Its effects on free radicals as well as compounds like hydrogen peroxide, nitrites, nitrates, benzoates, and others make it relevant. NADPH is a crucial component for its operation. In fact, this molecule, a nicotinic acid derivative, serves as a redox cofactor for the enzyme GSR, which produces reduced glutathione (GSH) from oxidized glutathione (or GSSG) by transferring electrons from NADPH to GSSG. Trx is present in the extracellular space in addition to the cytoplasm, membranes, and mitochondria (Koharyova *et al*., 2008) and it demonstrated cytoprotective effects in a variety of inflammatory situations.

3.1.2 Exogenous antioxidants

Other antioxidants are also used by the body in addition to the endogenous enzymatic and non-enzymatic antioxidant defenses; these antioxidants are classed as exogenous wich must be consumed through diet. Exogenous antioxidants function differently from endogenous ones and in various cellular compartments. They primarily serve as free radical scavengers, neutralizing free radicals, repairing oxidized membranes, and reducing the generation of reactive oxygen

species (Berger, 2005). Exogenous antioxidants include vitamins (A, C, E, and K), enzyme cofactors (Q10), nitrogen molecules (uric acid), minerals (zinc, Zn, and selenium, Se), and polyphenols (Sies, 2007). The catalytic activity of antioxidant enzymes is upregulated by metals such manganese, zinc, copper, iron, and selenium (Vertuani *et al*., 2004). An insufficient dietary intake of these trace elements cn compromise the efficiency of antioxidant defense mechanisms (Gale *et al*., 2004).

3.2 Oxidative Stress and gastric ulcer

Numerous GI conditions, including Barrett's esophagus, peptic ulcer, celiac disease, inflammatory bowel disease, and several adenocarcinomas, are thought to be induced by the breakdown of redox homeostasis (Figure 7) (Perez *et al*., 2017).

Gastritis is characterized as an inflammation of the stomach mucosal lining and can result from a number of factors, including *H. pylori* infection, NSAID use, alcohol intake, and stress. PUD affects the proximal GITand is frequently accompanied with chronic gastritis. The most prevalent and persistent PUDs are ulcers in the stomach and duodenum. PUD can be broadly categorized into the following etiologic groups based on pathophysiology: 1) characterized by high acid secretion (such as Zollinger-Ellison syndrome). 2) related to infections, and 3) caused by NSAIDs (Malfertheiner *et al*., 2001; Sung , 2009).

Decreased levels of the antioxidant enzyme SOD, intake of antioxidant vitamins both contribute to the accumulation of ROS linked to gastroduodenal inflammatory disorders, while ethanol induces stomach inflammation by an increase in O_2 production (Hernandez-Munoz, 2000). The primary source of ROS in chronic inflammation, such as that seen in *H. pylori*-induced gastritis and IBD, is phagocytic leukocytes. During inflammation, significant numbers of neutrophils and/or macrophages enter the stomach mucosa and produce a lot of ROS.

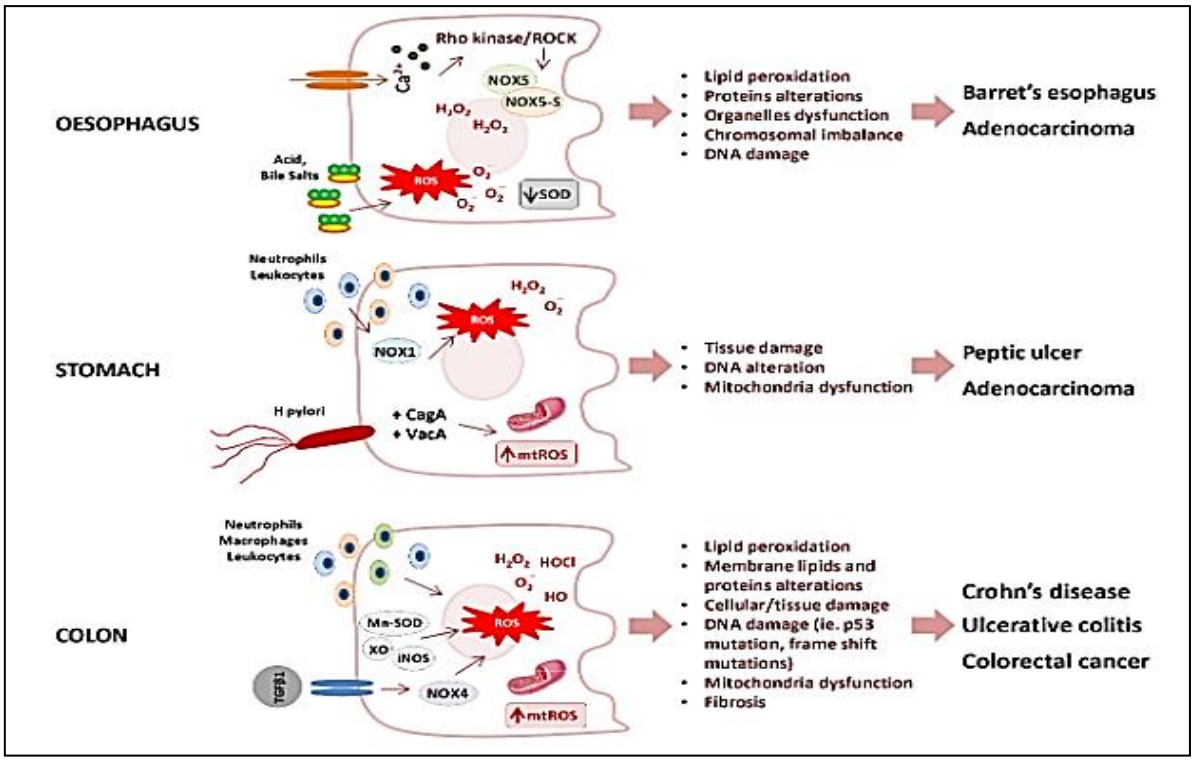

Figure 7. Potential mechanisms of oxidative stress promoting GI diseases. (Vona *et al.*, 2021)

SOD, Superoxide dismutase; **ROS**, reactive oxygen species; **mtROS**, mitochondrial reactive oxygen species; **NOXs** (NOX1, NOX4, NOX5, NOX5-S) NADPH oxidases; **Cag A**, cytotoxin-associated gene A; **Vac A**, vacuolating cytotoxin A; **Mn-SOD**, manganese-dependent superoxide dismutase; **XO**, xanthine oxidase; **iNOS**, inducible nitric oxide synthase.

3.3 Oxidative stress and some metabolic desorders

3.3.1 Oxidative stress and alzheimer

A relation between AD and oxidative stress have been revealed by producing free radicals, the beta-amyloid protein present in the brains of individuals with Alzheimer's disease can cause oxidative stress. Additionally, mitochondrial dysfunction caused by oxidative stress can lead to the accumulation of toxic metabolites and reactive oxygen species (ROS). Additionally, it has been discovered that people with AD have lower levels of antioxidant enzymes such superoxide dismutase, catalase, and glutathione peroxidase, which makes oxidative stress severe (Halliwell, 2019). The brain's neurotransmitter Ach is degraded by the enzymes AchE and BChE, following degeneration of cholinergic neurons that release ach in the brain, ACh levels decrease in AD. ACh inhibitors (AChIs) and BCh inhibitors (BChIs) are frequently used to treat the cognitive symptoms of AD by raising ACh levels in the brain (Perry *et al.*, 2020).

3.3.2 Oxidative stress and diabetes mellitus

Oxidative stress may be a crucial factor in the development of diabetes mellitus, according to recent studies. Reactive oxygen species (ROS) are produced by both endogenous processes, such as energy consumption in mitochondria, and external sources, such as ionizing radiation and chemical carcinogens. In living cells, ROS produced either endogenously or exogenously can simultaneously attack lipids, proteins, and nucleic acids. The excess glucose metabolites moving along these pathways could promote the appearance of DM complications and harm pancreatic b cells through different mechanisms. However, all of these processes result in the production of ROS, which over time and in excess leads to chronic oxidative stress (Yang *et al*., 2011).

4 Gastrointestinal motility

Gastrointestinal (GI) motility is a complex process that includes tone, compliance, transit, and contractile activity. These varieties of motility can be induced and controlled by circulating and local neurohumoral substances (Hansen *et al*., 2003). Multiple mechanisms are involved in controlling GI motility and smooth muscle activity. The main players that react and interact both directly and indirectly with muscle cells are hormones and neurotransmitters (Medhus *et al*., 1999).

4.1 Motility and neurohumoral control of the stomach

The stomach has regionally distinct motility patterns. The distal body and antrum display phasic motor activity, but the fundus and cardia (the pacemaker region) produce tonic contractions. In addition to age and gender, other variables that affect gastric emptying include the volume, caloric density, and osmolarity of the injected substance. stomach nutrient liquid emptying from stomach is properly controlled to deliver 200 kcal/h or less into the duodenum, the impact of carbohydrates and the majority of amino acids on small intestinal mucosal osmoreceptors, which activate neural feedback inhibitory pathways, modulates intestinal nutrient delivery in part (Hansen, 2003).

The neurohumoral control of stomach emptying requires intact vagal innervation. However, vagal nerves are involved in controlling the pattern of contractions during phase III but are not required for the initiation or temporal coordination of global fasting or postprandial gastroduodenal motility patterns. The rate of stomach emptying is influenced by many neurohumoral chemicals. The excitatory regulation of stomach motility is controlled by at least 10 different types of neurons using various transmitter combinations. Numerous neurotransmitters, such as ACh, norepinephrine, 5-HT, substance P, VIP, peptide histidine isoleucin (PHI), and enkephalins, are found in the myenteric neurons of the gastric wall. ACh, SP, or both are present in excitatory neurons, which directly project to the circular muscle layer to regulate contraction. VIP and NO are present in inhibitory motorneurons, which project in the aboral directions to modulate relaxation (Olsson and Holmgren, 2001).

During fasting, vagally mediated cholinergic input maintains proximal stomach tone. After a meal, nitrergic neurons in the gastric wall are likely activated, which relaxes the proximal stomach. Production of cGMP occurs as a result of NO-induced relaxation. As a result, sildenafil (a specific phosphodiesterase-5 inhibitor), modifies esophageal contractions and lowers LES pressure by extending the activity of cGMP (Zhang *et al*., 2001), decreases

liquid emptying rate, raises intragastric volumes after a meal, and inhibits the antrum's and duodenum's interdigestive motor activity (Bortolotti *et al*., 2001).

4.2 Motility and neurohumoral control of the small intestine

The muscularis externa and the muscularis mucosa are two structured muscle regions that makes up the small intestine wall. The primary effector of contractile action is the muscularis externa. It is composed of an inner circular layer and an exterior longitudinal layer that are at a 90-degree angle to one another. Inner and outer layers make up the circular layer. The basic contractile pattern, segmentation, and the circular layer are mediated (mixing and propulsion). Although the longitudinal muscle shortens the gut length and speeds up transit, it probably lacks strong propulsive properties. The muscularis mucosa appears to be crucial for secretory activities. The relationship between the levels is still unclear (Hansen, 2003).

The small intestine is rich on sensory fibers: the mechano, chemo, thermo, and pain receptors transmit information via afferent fibers. The myenteric and submucous plexuses are the projection sites for intrinsic afferent neurons that control regional neuronal reflex activity. Vagal and spinal afferent nerves deliver information from activated sensory receptors to the CNS. The majority of the vagus fibers are afferent and form synapses with no dose ganglion neurons. The splanchnic nerves that carry spinal afferent fibers contain cell bodies in the dorsal root ganglia and synapse in the dorsal horn of the spinal tract, which is where they activate second order neurons that send signals to the gut or to the brain through ascending tracts. In addition, the majority of the splanchnic nerve fibers are efferent. Inhibitory neuronal influences dominate in basal conditions (Hansen, 2003).

A range of neurotransmitters, such as Ach, serotonin, and dopamine, are used by the enteric nervous system to control intestinal motility. Ach induces smooth muscle contractions and increases intestinal motility, whereas serotonin, depending on the receptor subtype activated, can either stimulate or inhibit motility. On the other hand, inhibitory neurotransmitters play a significant role in controlling intestinal motility by reducing smooth muscle contractions and slowing the passage of food through the digestive system. NO and vasoactive intestinal peptide (VIP) are the primary inhibitory neurotransmitters in the enteric nervous system. NO operates on smooth muscle cells to generate relaxation. VIP, which is released in the submucosal plexus by intrinsic and inhibitory motor neurons, can block the contraction of smooth muscles as well as induce the secretion of fluid and electrolytes. Adenosine triphosphate (ATP) and gamma-aminobutyric acid (GABA) are additional

inhibitory neurotransmitters that reduce intestinal motility. Several GI disorders have been linked to the dysregulation of inhibitory neurotransmitter (Furness, 2012).

4.3 Motility of the colon

It has been shown that the colon exhibits both slow waves and spike potentials during myoelectrical activity. Colonic slow wave frequencies are incredibly variable in normal persons. The most prevalent manometric phenomenon are phasic pressure waves. The long spike burst, short spike burst, and GMCs are further forms of activity. There is cyclic contractile activity that has a periodicity of 20 to 30 minutes and may be comparable to the MMC of the small intestine. Following a meal, the motility index rises 20 to 30 minutes later and stays high for up to 3 hours, this gastrocolonic reflex, which involves neuronal and perhaps hormonal pathways, such as CCK, which is also a stimulator of contractile activity in the colon, is still present even after gastrectomy and vagotomy (Philips, 1995). The human myenteric plexus expresses a number of peptide receptors, including GRP, Y2, PACAP type 1, CCK-A, neurotensin type 1, sst2, NK1, and VIP type 2, suggesting that these peptides have a role in motility (Rettenbacher and Reubi, 2001).

5 The plant in study: *Thymelaea hirsuta* L.

T. hirsuta, frequently known as "Methnane" (Badawi, 2019) belonging to Thymelaeaceae family, a flowering plant family with 50 genera, among its well-known genera is Thymelaea, a group of about 31 species of xerophyllous shrubs and herbs (Galicia *et al*., 2006). *T. hirsuta* is from the most prominent species among the thymelaea genus it has been acknowledged throughout history as a significant medicinal herb (Badawi, 2019).

In western Algeria, *T. hirsuta* has been known as " a local herb used by our grandparents" (Deramchia *et al*., 2017).

5.1 Geographical global distribution

T. hirsuta can be found in the Saharo-Arabian deserts, Sinai Peninsula, and coastal plains of the Mediterranean. Regional; Morocco, Algeria, Tunisia, Libya to egypte. and global; The southern part of the Mediterranean, from Morocco to Egypt, stretches from Spain to Greece and Turkey, east Palestine and Lebanon (Badawi, 2019).

5.2 Botanical description

T. hirsuta is an evergreen perennial subdioecious shrub with flowers that are either hermaphrodite or monosexual (either male or female). These flowers are arranged in a compact raceme known as a capitulum and can change from year to year or even within the same flowering season, but floral variation is always present in sexually stable individuals (subgynoecious, subandroecious, protogynous, and prototan- drous) or can change in labile sexual forms, it flowers from September to May (Domme *et al*., 1990; El- Keblawy *et al*., 1996; Hickey & King, 1997). The leaves are sessile, glabrous, obtuse, irregularly arranged on the stem, small (2-6 mm), nerveless, cotonneuses on the underside, and green on the upper surface. They are also olvales-lancéolées. The full length of the plant is approximatelly 100 to 150 cm (Figure 8).

T. hirsuta seed has a firm, impermeable covering that is dark brown, slightly reticulate, and hairy, and is pear-shaped, measuring 2-3 mm in length and 1.5–2 mm in breadth. The embryo is fairly large and is covered by endosperm that is protected by a membrane integument. It has two leafy membranous cotyledons and a radicle that point toward the hilum (endospermic seed). Its germination occurs epigeally (Shaltout *et al*., 1989).

Figure 8. *Thymelaea hirsuta* aerial parts (site 01)

5.3 Systematic classification

Plant taxonomy classifies *T. hirsuta* in a hierarchical manner as described in table 1.

Table 1. The systematic classification of *T.hirsuta* (Badawi, 2019).

Division	Tracheophyta
Class	Magnoliopsida
Superorder	Rosanae
Order	Malvales
Family	Thymelaeaceae
Genus	*Thymelaea*
Species	*Hirsuta*

5.4 Traditional uses

Traditionnaly, *T. hirsuta* has been used as anti-inflammatory, antiseptic and hypertension treatment (Brooks *et al*., 1990; Kumar *et al*., 2010).

Additionally, it is claimed to have leishmanicidal and vermifuge properties (Boudjelal et al., 2013). It is also reported that *T. hirsuta* aerial parts has been used to treat human skin diseases in western Algeria (Deramchia *et al*., 2017).

5.5 Chemical composition and pharmacological properties

Various phytochemical componds have been extracted from *T. hirsuta* areal parts; a high level of total phenolic content was reported by Trigui *et al* in Tunisian *T hirsuta*, thirteen phenolic acids identified by GC–MS analysis in the same study, principally are p-hydroxybenzoic, p-coumaric, ferulic and caffeic acids. Yang identified a mono- and biflavonoids (Genkwanin, genkwanin 5-O-D-glucopyranoside, genkwanin 5-O-D-primeveroside, tiliroside, and neochamaejasmin B), a bi- and tricoumarins (daphnoretin and triumbelletin) and lignans (pinoresinol and syringaresinol). Miyamae isolated from the aerial parts of *T. hirsuta* two novel daphnane diterpenoids, hirsein A and hirsein B, (Miyamae *et al*., 2009).

Since its richeness with bioactive compounds that play a significant role in the prevention and treatment of various ailment, this plant is given great priority to study. For instance, *T. hirsuta* have been reported to have antibacterial, antidiabetic, anti-inflammatory, antioxidant activity, and antihypertensive properties (Bnouham *et al*., 2012; Trigui *et al*., 2013). Additionally, *T. hirsuta* showed anticancer potential against B16 murine melanoma cells, according to Kawano *et al (2010)*.

MATERIALS AND METHODS

1 Materials

1.1 Plant material

The harvest of the plant *T. hirsuta* (figure 09) was carried out on february and march from Batna province, commune of Barika, Municipality of Bitam, at 35° 23′ 50″ north, 5° 21′ 57″ east. After the harvest, the identification and taxonomic classification of the plant were developed by Professor Smain Amira, Department of Animal Biology and Physiology, University Setif 1, Algeria. The voucher number 91 Th12/05/20Bat/OR was deposed at the laboratory of Phytotherapy Applied to Chronic Diseases. The plant material was dried at room temperature for about two weeks. It was then ground by an electric grinder until a fine powder is obtained. Then, stored in clean bags.

Figure 9. *Thymelaea hirsuta* aerial parts (personal photo)

1.2 Animals

Female gender of both rat (Wistar albino strain) and mice (Swiss white) from Pasteur Institute, Algiers, Algeria were used for the experiments, weighing between 180 g and 200 g for rats, and between 25 g and 30 g for mice, animals were maintained under animal facility conditions with free access to water and food.

2 Methods

2.1 Ethnobotanical study of *T. hirsuta* medicinal plant

2.1.1 Description and aim of the study

Traditional medicine indeed plays a crucial role in addressing the health needs of many populations worldwide. This reliance on traditional medicine is due to several factors, including its deep-rooted cultural significance and its affordability compared to conventional

pharmaceutical products. The knowledge of traditional medicinal practices is often passed down through generations via oral communication, which can put this valuable knowledge at risk of being lost over time. Ethnobotany, the scientific study of the relationships between people and plants, is vital for preserving and understanding the use of medicinal plants and their applications in traditional healing practices.

In regions where traditional medicine is still widely practiced, there is a wealth of knowledge about how to use various plants to treat a wide range of diseases. This knowledge is invaluable for maintaining the health and well-being of these communities. Ethnobotanical research can help document and preserve this knowledge, ensuring that it is not lost and can continue to benefit future generations.

The aim of our study was to identify the various medicinal uses of the *T. hirsuta* plant and to document traditional medicinal knowledge associated with its usage in Barika region. This commune belongs to El Hodna region, known for its abundant vegetation cover and the richness of medicinal and aromatic plants. We collaborated closely with traditional therapists and elderly individuals, who are the custodians of this knowledge, as well as representatives from various social groups. The results obtained will provide a comprehensive overview of the plant's healing properties, as described by the local healers in these communities.

2.1.2 Description and choice of survey locations

The ethnobotanical study was carried out in Barika and its different municipalities (Bitam, Medoukel, Ouled Ammar, El Djazar and Ouled Eich) belongs to El Hodna region (Figure 9). El Hodna is located in the central part of Northern Algeria. It covers an area of 18,718 Km2 and is located at an altitude of approximately 500 m, situated between 35°42'07" N 4°32'49" E (Moreau *et al*., 2005). The climate of the investigation area is continental, due in part to Saharan influences. Summer is hot and dry, while winter is very cold, with low and irregular rainfall in the order of 100–250 mm/year (Le Houerou, 1995).

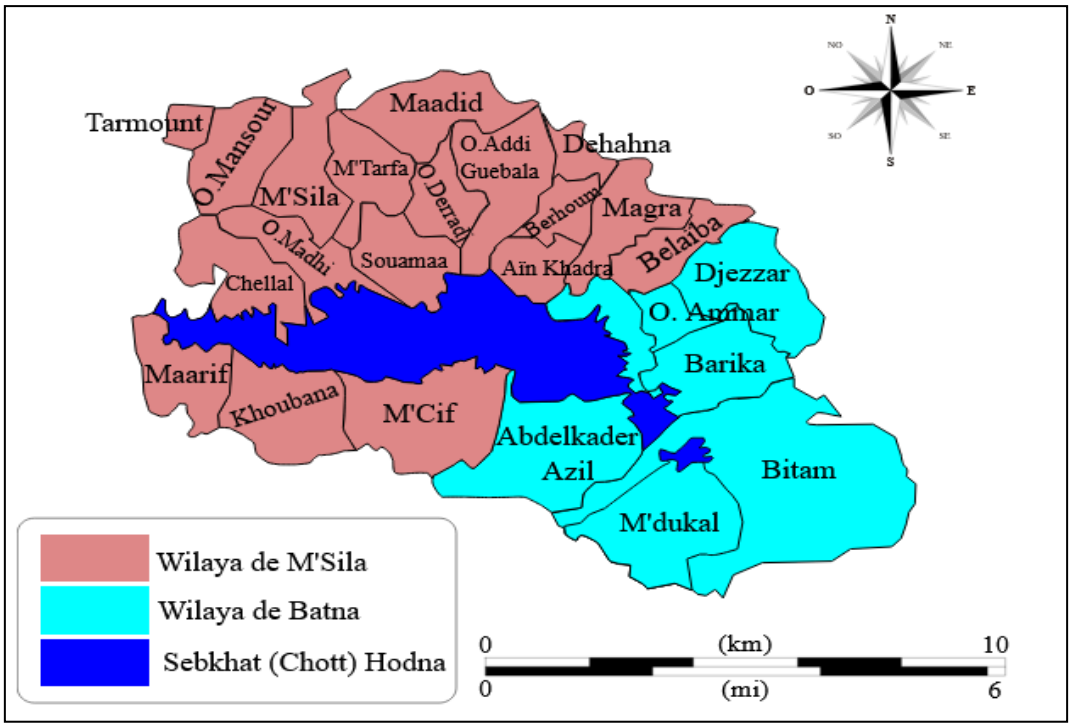

Figure 10. Map of El-Hodna region (Site 02)

2.1.3 Ethnopharmacological survey

All investigations described information regarding the medicinal uses of the plants was obtained through questionnaires (see annex): date, research area (district/village), informants (name/age/sex/educational level), local name of plant, part of the plant used, usage purpose of the plant, dosage, mode of use (decoction, infusion, powder etc.), usage period of the plant and side effects of the plant.

2.2 Experimental studies

2.2.1 Preparation of extracts

2.2.1.1 Methanol and hydromethanol crude extracts

The dried powder of *T. hirsuta* areal parts was extracted by maceration as described by Benchikh *et al.* (2018) (20 g dried powder sample in 400 mL of methanol (95%) for methanol extract and 20 g of dried powder sample in 200 mL distilled water and 200 mL of 95% methanol for the hydromethanol extract). The preparations were left for 72 h at room temperature with continuous stirring. The filtrates were pooled and the solvents were removed under vacuum at 45°C using a rotary evaporator. The obtained crude extracts were stored at 4°C.

2.2.1.2 Decocted extract

The aqueous extraction was conducted according to the method of Ferreira *et al.,* (2005) with slight modification, 20 g of dried powder of *T. hirsuta* areal parts were extracted with 400 mL of distilled water at 100°C for 10 min after boiling. It was filtered with four layers of muslin cloth then a filtration was applied twice on the mixture through a Whatman filterpaper. The water was evaporated to dryness and the residue was storred at 4°C.

2.2.1.3 Extraction yield

The extraction yield is defined as the quantity of extract recovered in mass compared to the initial amount of dry bark and is a measure of the solvent efficiency to extract certain components from the original material.

Extraction yield of the different extracts was calculated using the equation bellow:

$$TEC\ (\%) = \frac{\text{Weight of extract}}{\text{Weight of sample}} \times 100$$

2.2.1.4 *In vitro* studies

2.2.1.4.1 Determination of total phenolic content

FCR was used to determine total phenolic content (TPC), as described by Li *et al.,* (2007). A volume of 100 µL of each extract or various concentrations of the standard (gallic acid) were combined with 500 µL of Folin–Ciocalteu reagent (diluted 10 times). Four minutes later, 400 µL of 7.5% Na_2CO_3 solution was added. The mixture was incubated in the dark for 1 hour at room temperature, and the absorbance was measured at 760 nm. Using a gallic acid calibration plot, TPC was expressed as mg of gallic acid equivalent per gram of dried plant extract weight (mg GAE/g DW).

2.2.1.4.2 Determination of total flavonoid content

Aluminum chloride assay was used to determine total flavonoid content (TFC) (Bahorun *et al*., 1996). To 1 mL of test extract or varied standard (quercetin) concentrations, 1 mL of $AlCl_3$ (2%) was added. After 10 minutes of incubation, the absorbance was measured at 430 nm. Using the quercetin calibration curve, the quantity was reported as quercetin equivalent per gram of dry plant extract weight (mg QE/g DW).

2.2.1.4.3 Determination of total tannins content

The total tannins content (TTC) was estimated using FCR , and tannic acid as reference (NG & Rahate, 2013). For the preparation of reaction mixture, 0.5 mL of extract, 2.5 mL of 10% FCR diluted in water and 2.5 mL of 7.5% Na_2CO_3 were combined. Same procedure was used to prepare a blank without a sample. The samples had been incubated for 45 min at 45°C in a thermostat. Using a spectrophotometer, the absorbance was calculated at λmax = 765 nm. For each analysis, the samples were prepared in triplicate, and the mean absorbance value was calculated. The calibration line was constructed after applying the same procedure to the tannic acid standard solution. The TTC concentration (mg/mL) was derived from the calibration line based on the measured absorbance. Each determination was made three times. In grams of dry extract, the amount of total tannins is represented as mg tannic acid equivalent (TAE).

2.2.1.4.4 HPLC-MS analysis

The separation was accomplished on a thermostated Intertsil ODS-3 reverse phase C 18 column (5 m, 250 mm 4.6 mm i.d). A 20 μL sample volume injection was used. 1.5 mL/min of solvent flowed through the system. The mobile phases used were (A) 0.5% acetic acid in water and (B) 0.5% acetic acid in methanol. With elution gradients: 0–20% B (0–0.01 min); 20–60% B (0.01–2 min); 60–80% B (2–15 min); 100% B (15–30 min); 100–10% B (30–35 min); 10–0% B (35–40 min).

The proposed chromatographic method's linearity, LOD, LOQ, and repeatability were all validated. The retention periods of the phenolic compounds were used to classify them, and UV data were compared to commercial standards. There were three parallel analyses run. Calibration curves were created for the quantitative measurement of phenolic substances by injecting known concentrations.

The proposed chromatographic methods linearity, LOD, LOQ, and repeatability were all validated. The retention periods of the phenolic compounds were used to classify them, and UV data were compared to commercial standards. There were three parallel analyses done. Calibration curves were created for the quantitative measurement of phenolic substances by injecting known concentrations (0.0, 0.00782, 0.01563, 0.03125, 0.0625, 0.125, 0.25, 0.5 and 1.0 ppm) of various standards substances, such as gallic acid, fumaric acid, protocatechuic acid, catechin hydrate, 2,4-dihydroxy benzoic acid, p-coumaric acid, ferulic acid, coumarin, trans-2-hydroxy cinnamic acid, ellagic acid, rosmarinic acid, trans-cinnamic acid, 6,7-dihydroxy coumarin, caffeic acid, vanillin, and trans-cinnamic acid.

Linear concentration range was studied using mixed standard solutions ranging from 0.01 to 1 mg/L. The linearity was examined using coefficient of determination (R 2) values. Determination of signal-to-noise ratio was calculated under the proposed chromatographic condition. LOD was considered as 3:1 and LOQ as 10:1. The analytical parameters and numbers of phenolic compounds are described in table 2.

2.2.1.4.5 Evaluation of antioxidant activity

2.2.1.4.5.1 DPPH scavenging activity

The DPPH assay was based on the measurement of the DPPH radical's transformation from purple to yellow at 517 nm following interaction with an antioxidant agent. Antioxidants' ability to donate hydrogen was assumed to be the reason for their impact on the DPPH radical. A stable free radical called DPPH can take an electron or an hydrogen ion to transform into a stable diamagnetic molecule (Shekhar and Anju, 2014).

According to the procedure prescribed by Ribeiro *et al*., (2007) the DPPH free radical scavenging activity of the extracts was assessed spectrophotometrically by observing the disappearance of DPPH at 515 nm. 180 µL of DPPH reagent (0.004%) was applied to 20 µL of the plant extracts or standard solution (α-tocopherol) on absolute methanol in 96 well plates. The reagent blank was made up entirely of methanol. After being combined, all of the reagents were incubated for 30 minutes at room temperature under darkness. Triplicates of each experiment were performed. Following calculations were made to determine the percentages of the DPPH free radical scavenging activity:

% inhibition = [(Absorbance of control – Absorbance of test sample)/Absorbance of control] x100.

Using a dosage response curve, the IC_{50} values were calculated. The concentration needed to achieve 50% of a maximum scavenging capability is referred to as IC_{50}.

2.2.1.4.5.2 ABTS scavenging activity

The free radical scavenging test toward ABTS was evaluated using the slightly modified method of Re *et al.,* (1999). The solution stock for ABTS radical was prepared by mixing 2.45 mM potassium persulfate and ABTS (7 mM in water) and kept in the dark at room temperature for 16 hours. The solution was then diluted to give an absorbance of 0.7 to 0.75 at 734 nm with methanol. A total of 40 µL of the sample was mixed with 160 µL of ABTS mixture in a 96-well microplate and kept in the dark for 10 min. The reaction mixture absorbance was measured to 734 nm. Butylhydroxytoluene (BHT) has been used as standard antioxidant. Scavenging capacity of the test compounds was estimated from the equation below:

$$\% \text{ inhibition } = (AC - AS) / AS \times 100$$

AS: simple absorbance.

AC: Control absorbance.

2.2.1.4.5.3 Reducing power assay

The extract and BHT reduction power were carried out according to Oyaizu's method (1986). A total of 10 µL of each extract or standard was combined with 40 µL of 0.2 M phosphate buffer (pH 6.6) and 50 µL of 1% potassium ferricyanide in a 96-well microplate. After 20 min of incubation period at 50°C, the mixture was added to 50 µL of 10% TCA. Finally, 10 µL of 0.1 $FeCl_3$ and 40 µL of distilled water were mixed together, and the absorbance at 700 nm was measured. The concentrations at which the absorbance was 0.50 were reported.

2.2.1.4.5.4 Cupric reducing antioxidant capacity (CUPRAC)

The cupric lowering ability of the extracts was tested using the technique described by Apak *et al.* (2004). To 40 µL of sample or standard, 50 µL of 10 mM $CuCl_2$, 50 µL of 7.5 mM neocupronin, and 60 µL of 1 M ammonium acetate solution were added, respectively. The reagent combination was kept at room temperature for 60 min in the dark. At 450 nm, the reaction's absorbance was measured. As a standard antioxidant, BHT was used.

The findings were reported as $A_{0.5}$ (µg/mL), indicating a concentration of 0.5 absorbance.

2.2.1.4.5.5 Phenanthroline reducing antioxidant capacity

The extracts' phenanthroline chelation activities were calculated using a slightly modified version of Szydowska-Czerniak et al., (2008) method. Where, 10 µL of sample or standard, 50 µL of 0.2% $FeCl_3$ solution, 30 µL of 0.5% 1,10-phenanthroline solution, and 110 µL of methanol were all combined for a 20-minute incubation period at room temperature. At 510 nm, the absorbance of an orange-red solution was compared to the blank ($FeCl_3$, phenanthroline, and methanol).

2.2.1.4.5.6 Ferrous ion chelating activity

Ferrous ion chelating effect of extracts were evaluated as describing by Decker and Welch (1990) approach, which is based on the prevention of the formation of Fe^{2+}-ferrozine complex after treatment of materials with Fe^{2+} ions, where, 250 µL of test material or EDTA at different concentration were added to 50 µL of $FeCl_2$ (0.6 mM in distilled water) and 450 µL of methanol. After 5 min of incubation, the reaction was initiated by the addition of 5 mM ferrozine (50 µL), the mixture was stirred and allowed to react at room temperature for 10 min. Except for the

extract and EDTA, the control contained all of the reaction reagents. At 562 nm, the absorbance of the Fe^{2+}-ferrozine complex was determined. The expression of the chelating activity was: Chelating activity (%) = [(Abs of control – Abs of test sample)/Abs of control] x100.

The effective concentration of the test compounds needed to chelate 50% of the iron ions is known as the IC_{50}, it was determined using a dosage response curve.

2.2.1.4.5.7 Antioxidant activity determined by β-carotene bleaching method

According to the methodology of Kartal *et al*., (2007), this assay is based on the ability of antioxidant molecules to prevent the oxidative degradation of β-carotene, which is carried on by oxidative linoleic acid compounds. A mixture of 0.5 mg of beta-carotene, 1 mL of chloroform, 25 µL of linoleic acid, and 200 mg of Tween 40 was used to prepare a ß-carotene/linoleic acid emulsion. A vacuum evaporator was used to completely evaporate chloroform at 40°C. Then, with vigorous shaking, 100 mL of oxygenated distilled water was added. 350 µL of extract or the reference antioxidant (BHT) were added to an aliquot of 2.5 mL of this emulsion and thoroughly mixed.

At 490 nm, the absorbance was measured at 0, 1, 2, 4, 6, and 24 hours. Instead of extract or reference antioxidant, a negative control contained 2.5 mL of distilled water or another solvent. Each sample was analyzed three times.

- The antioxidant activity (AA) was calculated using the following equation in terms of successfully bleaching β -carotene:

$$AA = \left[1 - \frac{A_0 - A_t}{A_0^0 - A_t^0}\right] \times 100$$

Where:

A_0 : the absorbance values for test sample at zero time of the incubation.

A_0^0 : the absorbance values for control at zero time of the incubation.

A_t : the absorbance values for the test sample measred after incubation.

A_t^0: the absorbance values for the control measred after incubation.

2.2.1.4.6 Evaluation of anti-enzymatic activity

2.2.1.4.6.1 α-amylase inhibition assay

The capacity of extracts to inhibit α-amylase was evaluated using the IKI (iodine/potassium iodide) technique created by Quan *et al.,* (2019) with slight modifications. 25 µL of extract was incubated with 50 µL of α-amylase solution (0.1 units/mL) for 10 min at 37°C. Then, 50 µL of soluble starch was added and incubated again for 10 min at 37 °C. At last time, 25 µL o f hcL (0.1 M) and 100 mL Lugol solution were added respectively. A sample without enzyme was included in the blank. The control contained all of the reaction solutions without extract sample. A microplate reader was used to measure the absorbance at 565 nm. Acarbose was used as standard. From the graph of α -amylase inhibitory activity against sample concentrations, the sample concentration providing 50% inhibition activity (IC_{50}) was determined.

2.2.1.4.6.2 α -glucosidase inhibition assay

α-glucosidase inhibitory activity was performed as describing by Kim *et al*., (2010) with slight modifications. 10 µL of extract, 50 µL of Phosphate buffer, 25 µL of Enzyme α-glucosidase (0.1 unit/mL) and 20 µL of PNPG (p-nitrophenyl-α-D-glucopyranoside) were mixed by order and incubated for 20 min at 37°C. After, 90 µL sodium carbonate solution (0.1 M) was added. After 0 and 30 minutes at 37°C, the absorbance was measured against a blank of the sample at 400 nm using a microplate reader. Acarbose was used as standard.

From the graph of α -glucosidase inhibitory activity against sample concentrations, the sample concentration providing 50% inhibition activity (IC_{50}) was determined.

2.2.1.4.6.3 Acetylcholinesterase and butyrylcholinesterase inhibition assay

The modified approach described by Ellman *et al* (1961) was used to assess the inhibitory capabilities of AchE and BchE.

After adding 20 µL of either AchE (5.32 10-3 U) or BchE (6.85 10-3 U) solution to 10 µL of extract dissolved in methanol at various concentrations, 150 µL of 100 mM sodium phosphate buffer (pH 8.0) was added. The mixture was incubated at 25°C for 15 min, and then 10 µL of DTNB (0.5 mM) and 10 µL of acetylthiocholineiodide (0.71 mM) or butyrylthiocholinechloride (0.2 mM) were added. The absorbance measuring was done at 412 nm once for 0 min, twice for 5 min, three times for 10 min, and four times after 15 min. The galantamine is used as a standard.

The following equation was used to calculate the percentage of inhibition of AChE or BChE enzymes in comparison to blank (methanol with phosphate buffer pH 8).

$$\% \text{ inhibition} = (E - S)/E * 100$$

E: enzyme activity without extract.

S: enzyme activity with extract.

2.2.1.4.6.4 Tyrosinase inhibition assay

The spectrophotometric approach reported by Masuda *et al*., (2005) was used to evaluate the inhibitory activity of the tyrosinase enzyme, with slight modification. mushroom tyrosinase was employed and L-DOPA was utilised as the reaction's substrate. 10 µL of the sample solution dissolved in various concentrations of ethanol, 150 µL of 100 mM sodium phosphate buffer (pH 6.8), and 20 µL of tyrosinase enzyme solution in buffer were mixed and incubated for 10 min at 37°C. Then, 20 µL of L-DOPA was added. After 10 minutes at 37°C, the sample and blank absorbances were measured at 475 nm.

2.2.1.5 *In vivo* studies

2.2.1.5.1 Acute oral toxicity

The *in vivo* toxicological properties of *T. hirsuta* extracts was performed using a single-dose 14-days acute oral toxicity study, in accordance with OECD Guideline 423. A limit number of 5 rats of femal sex were used fore each group. They were allowed to acclimatize to laboratory conditions for a week before starting the experiment. The rats were housed in cages and individually marked on the tail for identification. They were maintained on a 12-hour light/dark cycle at room temperature, approximately 23°C, with constant humidity. Drinking water and food were provided throughout the experiment, except for the short fasting period where the drinking water was still in free access but no food supply was provided 12 h prior to treatment. Following the fasting period, body weight of the rats was determined. Then, the extracts were administered at 2 single oral doses (2000 and 5000 mg/kg). The animals were not fed for three hours following administration. The rats were observed in detail for any indications of toxicity effect within the first six hours after the treatment period, and daily further for a period of 14 days. After 14 days, a blood samples were collected for biochemical analyzes. Then, rats were sacrificed, organs were removed for global observation and evaluation of weight changements.

2.2.1.5.2 Gastric emptying and small intestine transit measurements

Gastric emptying was measured according to the method described by Amira *et al*., (2005) with slight modifications. In this investigation, a test meal containing 0.1% phenol red, a non-absorbable and easily measurable marker, was dissolved in 1.5% carboxymethyl cellulose (CMC). A number of 6 mices per groups were given oral pretreatments of DE and HME (250 mg/kg) after 18–20 hours of fasting, as well as atropine (1 mg/kg) as a positive control. Each animal got 0.2 mL of the test meal orally after one hour of treatment, and they were sacrificed 20 min later. After ligating the pylorus and the cardia, a laparatomy was performed to remove the stomach and small intestine. The stomach's contents were homogenized in 25 mL of 0.1 N NaOH. In order to precipitate proteins, 8 mL of the homogenate's supernatant was added to 1 mL of 33% TCA after the mixture had been allowed to sit at room temperature for an hour. The supernatant was then mixed with 2 mL of 2N NaOH following centrifugation (1600 g for 30 min). After homogenization, the absorbance of the mixture was measured at 560 nm. Three animals were sacrificed on the day of each experiment and were used as standards (0% emptying) by being sacrificed immediately after the administration of the test meal. The following formula was used to determine the gastric emptying (GE) rate:

$$GE\ (\%) = (Abs_{control} - Abs_{test}/Abs_{control}) * 100$$

In order to assess intestinal transit, the entire small intestine of the identical animals used for the gastric emptying model was removed immediately after the stomach was excised. The intestine's mesenteric attachments were crudely detached, and the length of the organ was measured. A precise location of the test meal was then determined by dropping 0.1 N NaOH. The ratio of the length of the small intestine to the total distance covered by the test meal was used to calculate the rate of intestinal transit.

2.2.1.5.3 Mechanism of the effect of extract on enhancement of gastric emptying and intestinal transit

The most effective doses of the extract on the rate of gastric emptying and intestinal transit chosen (250 mg/kg) was used in another series of experiments with arginine (1.72 mmol/kg) given intragastrically, L-NNA ($1.36\ 10^{-1}$ mmol/kg) and indomethacin ($1.39\ 10^{-2}$ mmol /kg) and in order to assess a potential interference of the extract with neurotransmitters that regulate GI motility. The pharmacological agents are given just before the administration of the plant extracts.

2.2.1.5.4 Ethanol-induced gastric ulceration in rats

Absolute ethanol was administered by intragastric instillation to female rats weighing between 180 and 200 g to induce gastric ulcers. They were housed in specially designed cages before, during, and after the experiment to prevent coprophagia. The rats were divided into eight groups, each group with six animals, and were given free access to water and starved for 18 hours before the experiment.

The first group received distilled water only, the second group received standard anti-ulcer drug ‘’Ranitidine’’ at dose of 5 mg/Kg 30 min before the oral dose of pur ethanol. The third, fourth and fifth groups were given DE of *T. hirsuta* at a dose of 125, 250 and 500 mg/kg, respectively. The sixth, seventh and eighteeth groups were given HME of *T. hirsuta* at a dose of 125, 250 and 500 mg/kg, respectively.

The animals were killed by cervical dislocation thirty min later. After, each stomach had been incised along its larger curvature, it was photographed and macroscopically examined for linear hemorrhagic lesions in the glandular region. Using the software Image J 1.52 (Wayne Rasband, National Institutes of Health, USA), the total areas of the stomach and lesions were evaluated. Then, ulcer index was calculated.

The formula used to determine the percentage inhibition was as follows:

%inhibition = UI control-UI treated /UI control *100.

2.2.1.5.5 Mechanism of the GI protective effect

The effective dose chosed (250 mg/kg) was used in a second set of studies with arginine (1.72 mmol/kg) given intragastrically, L-NNA (1.36 10^{-1} mmol/kg), indomethacin (1.39 10^{-2} mmol /kg) and yohimbine (1.28 10^{-2} mmol /kg) and injected intraperitoneally, to examine whether the extract might interfere with the neurotransmitters that regulate the GI protective effect. The percentage of ulceration, histological analyses of the glandular gastric mucosa, mucus in the stomach layer and antioxidant activities were measured *in vivo*.

2.2.1.5.6 Histopathological studies

A part of the stomach from each experimental group was fixed in 10% formalin for dehydration, cleared in xylene using a tissue processor (MTP-SLEE, Mainz, Germany), and embedded in paraffin for microscopic investigation. Sections of 5 mm were obtained with a conventional microtome after processing, and they were stained with hematoxylin and eosin (HE). A pathologist examined the tissue sections to check for any undesirable characteristics such edema, erosion, ulceration, and necrosis. Later, photos of the slides were taken.

2.2.1.5.7 Determination of mucus in gastric layer

The alcian blue dye method established by Corne *et al*., (1974) was used to assess adherent mucus in rats. This cationic dye forms insoluble compounds with soluble mucopolysaccharides and glycoproteins without entering mucosal cells. The same grouping of animals as the previous experiment was carried out, immediately, 10 mL of the 0.1% alcian blue solution (0.16 M sucrose/0.05 M sodium acetate, pH 5.8) were poured over each glandular section of the stomach.

After two hours, the unbound dye was removed using two separate washings with 10 mL of 0.25 M sucrose, first for 15 min and subsequently for 45 min. The tissue was then submerged in 0.5 M magnesium chloride and agitated for two hours to elute the mucus-bound dye. Then, four milliliters of the blue extract were briefly shaken with an equal amount of diethyl ether to disperse any potential particles that would have affected the spectrophotometric analysis. The aqueous phase's absorbance was then measured at 605 nm. The results were reported as g of alcian blue/g of tissue. The amount of alcian blue extracted per gram of wet glandular tissue was then determined by linear regression with a calibration curve constructed from standard serial dilutions of different concentrations of the dye.

2.2.1.5.8 Evaluation of *in vivo* antioxidant activity

2.2.1.5.8.1 Preparation of homogenate

The glandular section of each animal's stomach removed and rinced was weighed and homogenized in an ice-cold solution of 50 mM Tris HCl buffer (pH 7.4) to produce 10% (w/v) homogenate. This latter was then centrifuged at 4000 g for 15 minutes at 4°C, and the supernatant was collected and stored at – 20°C for the estimate of the following biochemical parameters: total proteins, GSH level, lipid peroxidation (MDA), and CAT activity.

a) Estimation of catalase (CAT) activity

The method outlined by Clairbone (1985) is used to test CAT activity with some changes. The basis of this test is the ability of CAT to degrade hydrogen peroxide to H_2O and oxygen. In a quartz cuvette, a volume of 19 mM H_2O_2 solution (2.9 mL) and 50 mM phosphate buffer (pH 7.4) were mixed with 50 µL of tissue homogenate. The enzymatic activity is represented in nmol H_2O_2 /min/mg, and the rate of H_2O_2 oxidation in the presence of CAT is measured by spectrophotometry at 240 nm immediately and every 15 seconds for 1 minute.

b) Estimation of lipid peroxidation (LPO)

Malondialdehyde (MDA) formation was assessed to measure the lipid peroxidation of stomach tissue. The basic idea behind this procedure is the formation of a pink MDA-(TBA)2 complex by the interaction of MDA with thiobarbituric acid (TBA) under acidic conditions and at a higher temperature (100°C). A volume of 0.5 mL of TCA (20% w/v) and 1 mL of TBA (0.67% w/v) were added to 0.5 mL of tissue homogenate. The mixture was incubated for 15 min at 100°C, then quickly cooled in ice, mixed with 4 mL of n-butanol, and centrifuged for 15 min at 4000 rpm. At 532 nm, the absorbance of the clear pink supernatant was measured spectrophotometrically against blank. A standard curve of 1,1,3,3 tetraethoxypropane (serial dilutions of the stock 10 mM) was used to determine the concentration of MDA. Results were given in units of nmol TBA per gram of stomach tissue (Ohkawa *et al*., 1979).

c) Assessment of reduced glutathione (GSH)

According to the method of Ellman (1959), reduced glutathione was measured and based on the oxidation of GSH by the 5, 5' -dithio-bis (2-nitrobenzoic acid) (DTNB) (Ellman's reagent), the reaction of DTNB and GSH produce 2-nitro-5-thiobenzoic acid (TNB) with a maximum absorbance at 412 nm (Wendel and Cikryt, 1980). 50 µL of the tissue homogenate were diluted in 10 mL of phosphate buffer for this test (0.1 M, pH 8).

A volume of 20 µL of DTNB (0.01 M) were added to 3 mL of the dilution mixture, and after 5 min of incubation, the yellow color that resulted was measured at 412 nm. The GSH concentration indicated in mmol/g of tissue was approximated from a curve of standard GSH concentrations attained under similar conditions.

d) Estimation of gastric total proteins content

The Biuret kit total protein reagent was used to determine the amount of gastric total proteins according to Gornall *et al*., (1949) method (potassium iodide, potassium sodium tartarate, copper sulphate and sodium hydroxide). In an alkaline medium, proteins produce a blue-violet color with copper sulphate. Briefly described, 25 µL of the tissue homogenate or standard (bovine serum albumen) was combined with 1 mL of the Biuret reagent, and the mix was then incubated at room temperature for 10 min. At 540 nm, the absorbance was then measured.

The following calculation was used to get the total amount of protein:

Total protein (mg/mL) = (Abs of sample / Abs of standard) * n

n: standard concentration.

2.3 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.0.2. Results are evaluated using one-way analysis of variance (ANOVA), followed by the Tukey's test. *In vitro* or *in vivo* assay results are provided as mean ± SD or SEM, respectively.

RESULTS

1 The ethnobotanical study results

The survey encompassed a total of 307 questionnaires, which were diligently filled out by participants encompassed a diverse range of individuals, providing valuable insights into the medicinal plant knowledge within the community. In terms of age distribution, we found that 37.4% fell within the 18 to 29 age group, while an equal percentage, 37.4%, belonged to the 30 to 49 age brackets. The remaining 25.2% of informants were aged over 50, reflecting a broad representation across generations. Family status among the informants exhibited a mix of marital situations. A significant portion, 59.8%, reported being married, while 36% identified as single, and a smaller percentage, 3.7%, had experienced divorce. Gender-wise, the survey had a nearly equal distribution, with 51.4% of informants being male and 48.6% female, highlighting a balanced representation of perspectives.

In terms of educational backgrounds, the informants came from various academic levels. Impressively, 43.9% had university-level education, signifying a strong academic presence in the survey. Secondary education was represented by 19.6% of respondents, while 11.2% had completed a middle-level education, and 13.1% had primary education. Notably, 11.2% of informants had no formal education, underlining the diversity of educational backgrounds within our survey sample. This comprehensive demographic representation has enriched our study, providing a multifaceted understanding of medicinal plant knowledge within the community.

Among the informants surveyed, 50.5% were familiar with the plant referred to as “el methnane,” while the remaining 49.5% had no knowledge of the plant and did not report any utilization of it. This division underscores the varying levels of familiarity with this particular plant within the surveyed population, Furthermore, it’s essential to note that there is a risk of gradually losing traditional medicinal knowledge over time.

The essential results regarding the therapeutic uses of the plant, including the parts utilized, the form of application, preparation methods, dosages, administration modes, recommended usages, and duration of use for treating various illnesses collected through the survey were illustrated in the table (2).

Table 2. Survey results.

	Description	Frequency (%)
Uses of the plant	Therapeutic	91.4
	Cosmetic	8.6
Therapeutic uses	Dermatological problems	27.7
	Respiratory disorders	1.6
	Cardiovascular disorders	5.5
	Neurological disorders	0.8
	Genito-urinary disorders	6.3
	Metabolic disorders	1.6
	Disorders of the digestive tract	13.47
	Gastric ulcer	13.38
	Disorders of glands associated with the digestive system	3.14
	Anti-constipation	1.6
	Against intestinal worms	3.19
	Pregnancy stabilization	1.2
	Influenza	0.8
	Oral surgery treatment and cavity treatment	1.1
	Hair thickening and hydration	1.6
	Blood sugar reduction	4.1
Part used	Whole Plant (Without Roots)	46
	Whole Plant (With Roots)	7.4
	Stem/Branches	1.9
	Leaves alone	44
	Flowers alone	00
	Root	00
	Alone	83.9
	Mixture with other plants	14.3
	Added to food	3.6
Dose used	Sprinkle	13.5
	Spoonful	59.6
	Handful	25
Method of administration	Maceration	1.3
	Infusion	31
	Decoction	16.2
	Powder	16.2
	Poultices	33.8
	Mouthwash	1.4
Duration of use	Once a day / Daily/1 week	79.2
	Once a week	15.3
	Three times a week separately	4.1
Potential of healing effect	Healing	55.9
	Amelioration	44.1
Side effects	Absence	71

	Severe diarrhea in case of overdose	28,9
	Allergic reaction	2,2

The survey findings reveal that *T. hirsuta* known as “Methnan” is widely used in traditional medicine for therapeutic purposes, with a significant majority of respondents (91.4%) reporting its usage for various medical conditions across different biological systems, including the digestive, respiratory, urinary and nervous systems, among others. Notably, it is frequently applied as poultices, particularly in the case of Skin ulcers. Moreover, it is extensively employed in addressing digestive disorders, with a notable emphasis on gastric ulcers (13.38%). The aerial parts of the plant were the most commonly used, often prepared as infusion, decoction or made as poultices. The quantity used was generally conservative, not exceeding a tablespoon for liquid preparations or a handful for poultices. Treatment duration typically spanned about a week, with doses spaced out every day or every two days. The outcomes of Methnan plant use were predominantly satisfactory, ranging from noticeable improvements to complete healing. These results underscore the significant role of Methnan in traditional medicinal practices, particularly for treating a variety of ailments effectively.

Given those important medical uses of this plant, an *in vitro* and *in vivo* studies were conducted with the primary objective of confirming or disproving its efficacy in treating gastric ulcers and exploring its broader biological impact on the GIT and other systems.

2 Comparative study: decocted, hydromethanol and methanol extracts from *T. hirsuta* areal parts

2.1 Extraction yields

Biologically active substances in plants are often found in low amounts. An effective extraction conditions can produce high yields of extracts while requiring minimal modification to the extract's biological properties (Candori *et al*., 2008). The choice of solvent is crucial for obtaining extracts with notable yields. Table 3 presents the yields of extracts using two solvents with different polarities in the following order: Water > water-methanol > methanol

Decocted, hydromethanol and methanol extracts of *T. hirsuta* areal parts showed closer values of yield percentage, 11.6%, 12.05%, 12.80%, respectively (Table 3).

2.2 Quantitative phytochemical analysis

2.2.1 Determination of total phenolic and flavonoid contents

In this study, the total content of polyphenols and flavonoids is suggested to be affected by two factors, the method of extraction and the solvent polarity. Overall, the highest content of total phenols was marked by methanol crude extract with maceration method (200.86 ± 2.20 mg GAE/g DW), followed by HMEwith 180.56 ± 1.91 mg GAE/g DW, while the DEshowed the lowest phenolic content 114.00 ± 1.35 mg GAE/g DW.

The flavonoid content decreased in the same order; methanol, hydromethanol and DE marking 18.26 ± 1.03 mg QE/g DW, 17.01 ± 1.03 mg QE/g DW and 16.80 ± 1.03 mg QE/g DW, respectively (Table 3).

2.2.2 Determination of total tannins content

The quantification of the total tannins present in the different extracts showed that the methanol extract contains 69.90 ± 2.92 mg TAE/g DW of extract as the highest amount, followed by the HME with 51.64 ±2.22 TAE/g DW while the lowest content was obtained by the DEwith 44.30 ± 2.11 TAE/g DW (Table 3)

Table 3. Extraction yield, total polyphenols, flavonoids and tannins in crude extracts from *T. hirsuta* areal parts

	DE	HME	ME
Yield of extracts (%)	11.6 ± 1.1	12.05 ±1.4	12.80 ± 1.2
TPC (mg GAE/g DW**)**	114.0 ± 1.35	180.56 ± 1.91	200.86 ± 2.20
TFC (mg QE/g DW)	16.80 ± 1.03	17.01 ± 1.03	18.26 ± 1.03
TTC (mg ATE/g **)**	44.30 ± 2.11	51.64 ±2.22	69.90 ± 2.92

Abreviations: **DE**: decocted extract; **HME:** hydromethanol extract 50%; **ME**: methanol extract; **TPC:** Total polyphenol content; **TFC:** Total flavonoid content; **TTC**: Total tannins content; DW: dry weight; **GAE**: gallic acid equivalent; **QE**: quercetin equivalent; **TAE:** Tannic acid equivalent**.**

2.3 *In vitro* antioxidant activities

2.3.1 ABTS scavenging activity

The ABTS free radical scavenging test is one of the most widely used methods for measuring the capability of hydrogen donation and oxidation chain breaking to act as antioxidants. ABTS radicals can be oxidized by peroxyl radicals to form stable radicals with absorption between 600 and 750 nm. Absorption is reduced with the presence of antioxidants and ABTS free radical scavenging. The studied extracts showed a scavenging effect on ABTS free radicals, in the following order ME > HME > DE. The ME with an IC_{50}= 20 µg/mL showed a higher activity compared to the DE with IC_{50}=52.93 µg/mL (P<0.0001). In fact, HME marked an IC_{50} value of 22.01 ± 1.20 µg/mL wich expressed a scavenging activity closest to the ME activity. However, no extract showed activity comparable to BHA (1.81 ± 0.10 µg/mL, P< 0.0001) (Figure 11). The ME and HME had a better significant activity than DE.

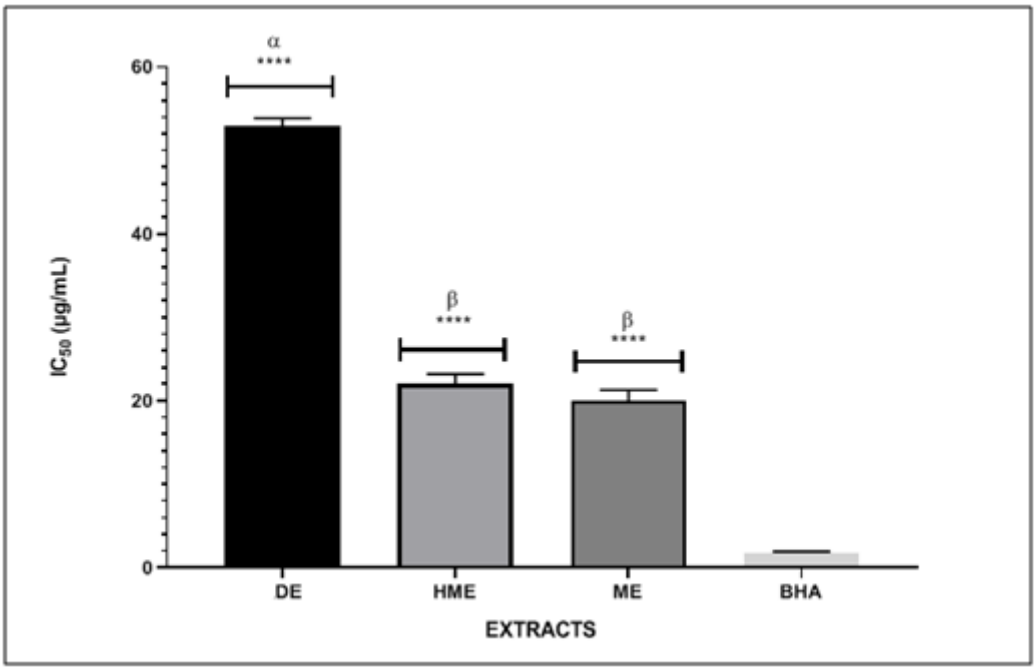

Figure 11. ABTS radical scavenging activity of *T. hirsuta* L. areal parts extracts.

DE: decocted extract; **HME:** hydromethanol extract 50%; **ME**: methanol extract. Data were presented as IC_{50} means±SD (n=3) (****P≤0.0001; ns: not significant) *vs* BHA as standard. Columns carrying different letters are significantly different at P<0.0001.

2.3.2 Reducing Power Assay

The capacity to transform the Fe^{3+} ion into Fe^{2+} by the different extracts studied seems very important. The ME nad HME prepared from *T. hirsuta* showed very similar reducing activities in ascending order with a concentration at $A_{0.5}$ of 81.20 ± 0.64 µg/mL and 85.37 ± 0.38 µg/mL, respectively (Figure 12). On the other hand, DE showed the weakest effect with 124.5 ± 1.79 µg/mL which resulted in a significant difference between DE capacity and both of methanol and hydromethanol one. No one from the tested extracts exhibited an activity comparable to that of BHA (9.30 ± 1.7 µg/mL, P<0,0001).

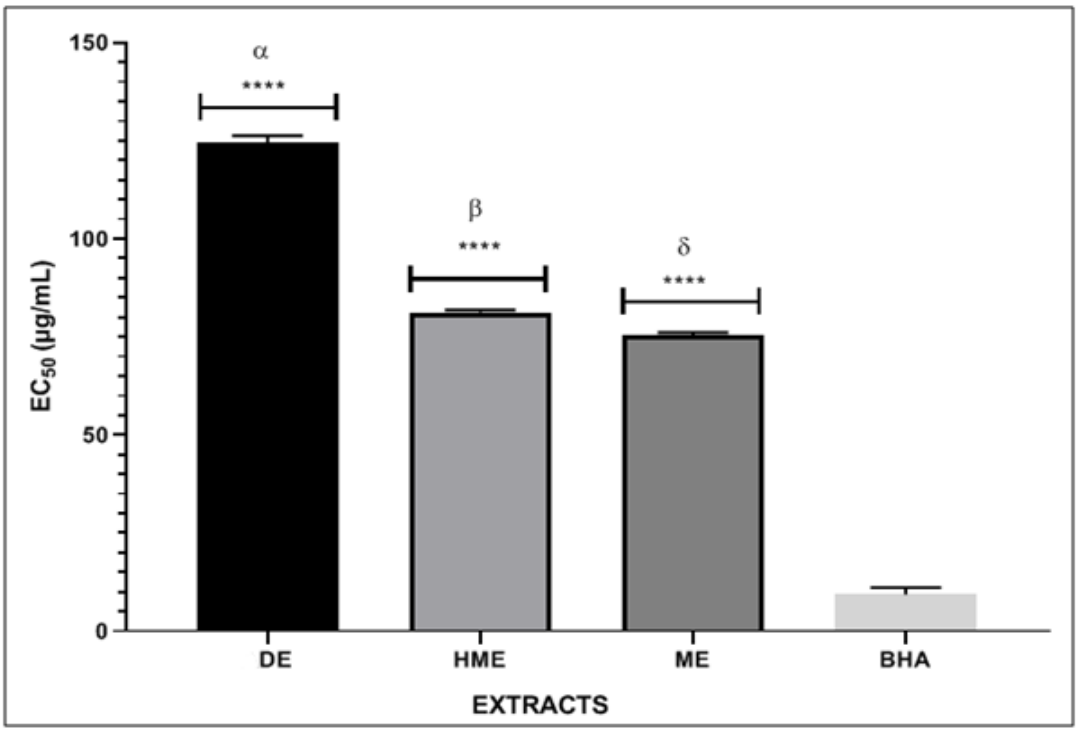

Figure 12. Reducing power activity of *T. hirsuta* L. areal parts extracts.

DE: decocted extract; **HME**: hydromethanol extract at 50%; **ME**: methanol extract. Data were presented as EC50 means ± SD (n=3) (****P≤0.0001; ns: not significant) vs BHA as standard. Columns carrying different letters are significantly different at P <0.0001; β *vs* δ: are significantly different at P<0.001.

2.3.3 Cupric Reducing Antioxidant Capacity (CUPRAC)

One of the most extensively used tests for assessing antioxidant capacity is the CUPRAC test, which involves reducing bis (neocuproine) Cu^{2+} to generate a stable complex that absorbs at 450 nm. Absorption increases with the presence of antioxidants. The increase in the reducing effect of the different extracts is found in the following order ME > HME > DE (Figure 13). The methanol extracts (31.09 ± 1.56 µg/mL) showed a potent activity compared to the hydromethanol ($A_{0.5}$=70.46 ± 0.92 µg/mL) and DE (P≤0.0001). Indeed, the lowest activity is obtained with DE with 134.5 ± 0.44 µg/mL.

The reductive capacity of different extracts against cupper ions was significantly different (P≤0.0001).

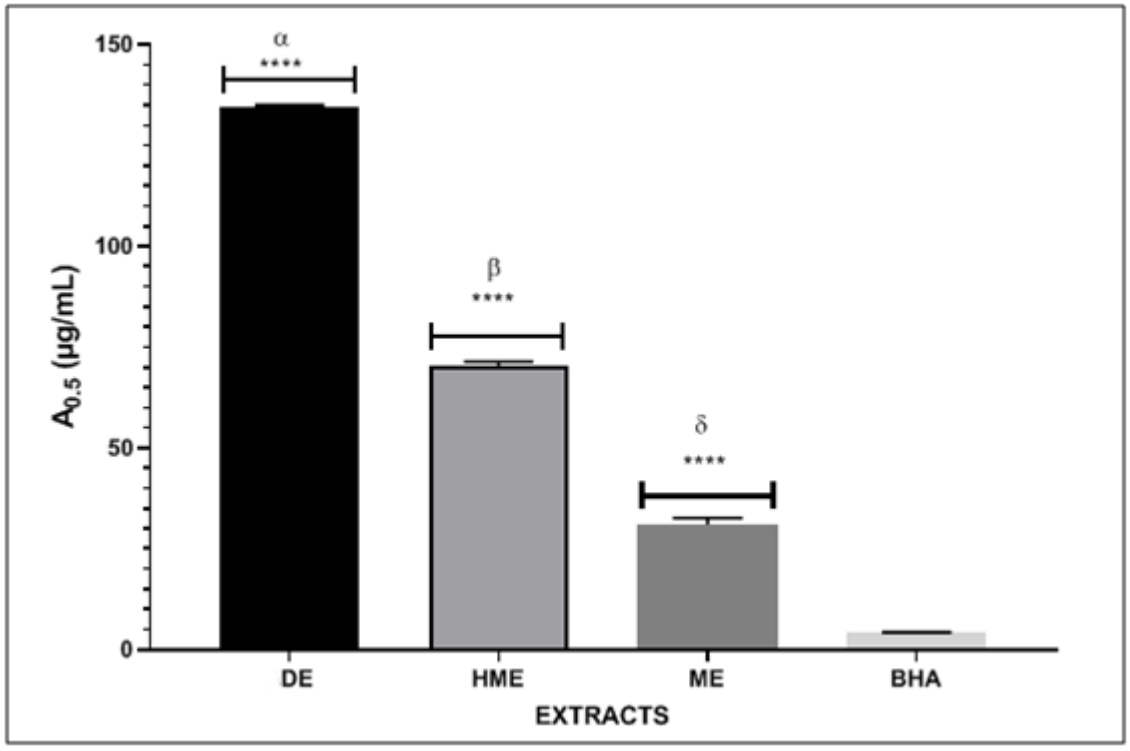

Figure 13. Cupric reducing antioxidant activity of *T. hirsuta* areal parts extracts

DE: decocted extract; **HME:** hydromethanol extract 50%; **ME**: methanol extract. Data were presented as $A_{0,5}$ means ± SD (n=3) (****P≤0.0001; ns: not significant) vs BHA as standard. Columns carrying different letters are significantly different at P< 0.0001.

3 Selected extracts: hydromethanol and DEfrom *T. hirsuta* aerial parts

3.1 HPLC-DAD analysis

The analysis of DE and HME from *T. hirsuta* aerial parts using High-Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD) showed a diverse profile of compounds with distinct UV-Vis absorption spectra (Table 4). The chromatograms revealed several well-defined peaks, each corresponding to specific compounds present in extracts (Figure 14 and 15). The retention times and peak areas were indicative of the presence of various constituents. Notably, HME displayed a dominant peak at a retention time of 31.33 min, with an absorption spectrum indicative of phenolic acid. This compound was identified as trans-cinnamic acid based on its UV-Vis characteristics and retention time, matching standards. Although it is detected in the DE in minimal proportions.

In contrast, DE exhibited a prominent peak at a retention time of 12.35 min, displaying an absorbance profile consistent with Chlorogenic acid, followed by Vanillin.

A variety of polyphenols were identified in both extracts; Catechin, p-hydroxy benzoic acid, Caffeic acid, p-Coumaric acid, Ferulic acid with remarquable abondance in HME.

Rutin, being a flavonoid, exhibited a concentration in HME that was eight times higher compared to its proportion in DE. Coumarin, a type of penzopyrone was present in both extracts with a considerable peak in HME.

Luteolin and apigenin, both recognized as flavonoids, were identified within the composition of HME, while they remained Prominently absent in the DE. This divergence in flavonoid content underscores the distinct chemical profiles of the extracts and suggests potential variations in their biological activities. Some phenolic compounds were conspicuously absent in both examined extracts (Table 4).

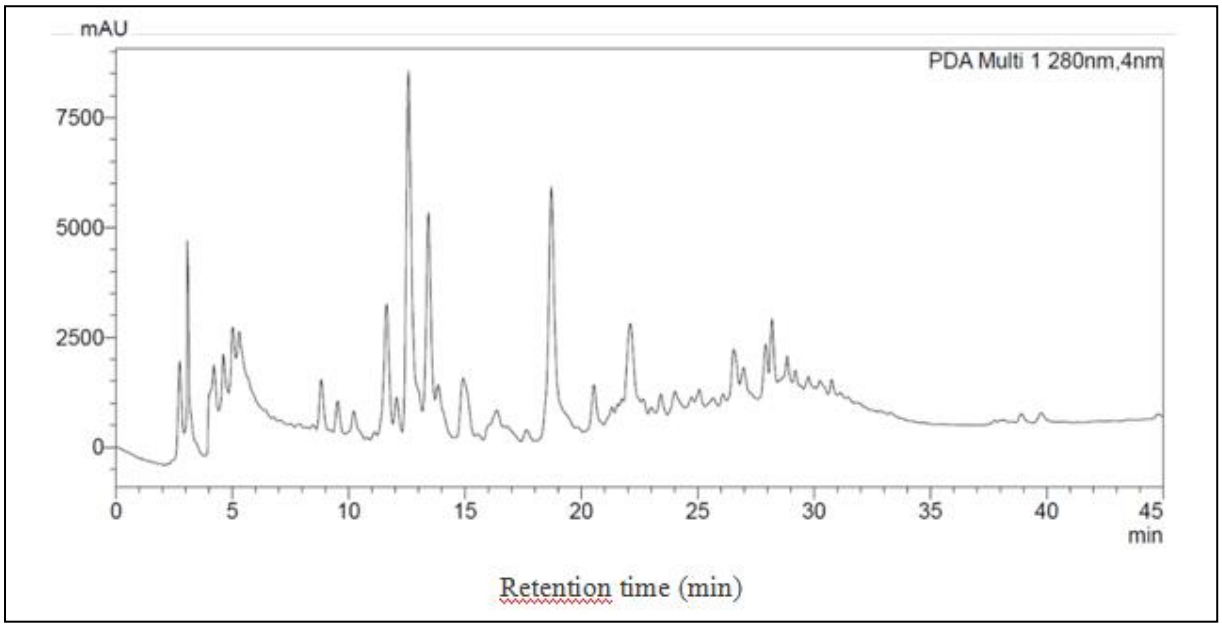

Figure 14. HPLC representative chromatogram of DE.

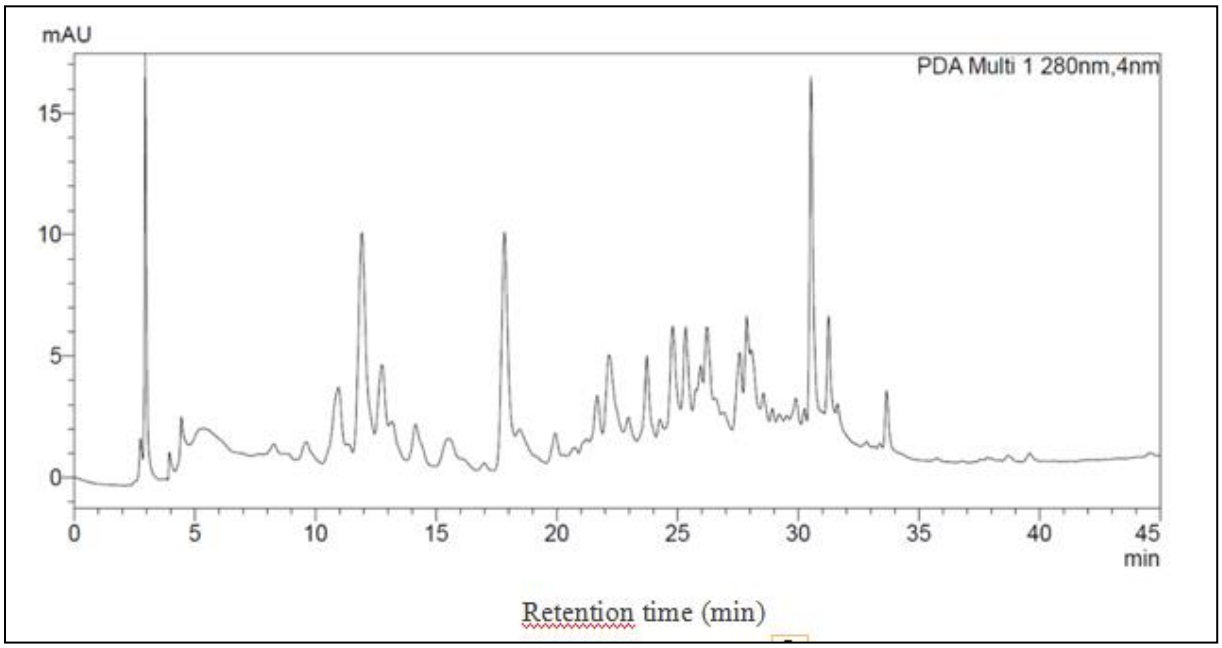

Figure 15. HPLC representative chromatogram of HME.

Table 4. Phenolic composition of DE from *T. hirsuta* areal parts by HPLC-DAD (mg/g).

No	Phenolic compounds	RT (min)	DE	HME
1	Gallic acid	5.70	-	-
2	Protocatechuic acid	8.75	2.56 ± 0.11	0.86 ± 0.02
3	Catechin	10.68	1.68 ± 0.07	5.32 ± 0.13
4	Pyrocatechol	11.04	-	-
5	Chlorogenic acid	12.35	6.20 ± 0.03	11.75 ± 0.05
6	p-hydroxy benzoic acid	12.77	4.27 ± 0.05	7.30 ± 0.10
7	6.7-Dihydroxy coumarin	14.10	-	-
8	Caffeic acid	15.09	3.10 ± 0.08	5.58 ± 0.21
9	3- hydroxy benzoic acid	15.98	-	-
10	Syringic acid	16.56	-	-
11	Vanillin	17.78	4.80 ± 0.05	13.10 ± 0.27
12	p-Coumaric acid	20.56	3.14 ± 0.09	5.20 ± 0.08
13	Taxifolin	21.26	-	-
14	Ferulic acid	22.14	3.92 ± 0.17	7.78 ± 0.23
15	Coumarin	24.49	1.56 ± 0.04	5.20 ± 0.14
16	Rutin	25.30	0.74 ± 0.03	8.15 ± 0.06
17	Ellagic acid	26.11	-	-
18	Rosmarinic acid	26.77		-
19	Myricetin	27.35	-	-
20	Quercetin	30.83	-	-
21	Trans-cinnamic acid	31.33	2.42 ± 0.06	18.78 ± 0.33
22	Luteolin	31.70	-	7.79 ± 0.18
23	Hesperetin	32.14	-	-
24	Kaempferol	33.21	-	-
25	Apigenin	33.77	-	7.50 ± 0.10
26	Chrysin	38.40		-

[a]Values expressed are means ± S.E.M. of three parallel measurements ($p < 0.05$).[b] -: not detected.

3.2 *In vitro* antioxidant activity of selected extracts

In view of the wide range of phytochemical components, the assesement of antioxidant activity and measure the efficiency of these substances in plant extracts require the development of multiple techniques. Most of these procedures are based on the coloring or discoloration of reagents. Some are based on metal reducing power (ferric reducing antioxidant power, FRAP), peroxyl radical scavenging (oxygen radical absorbance capacity, ORAC; total radical trapping

antioxidant parameter, TRAP), hydroxyl radical scavenging, and organic radical scavenging like ABTS and DPPH (Benhammou *et al.*, 2009).

To comprehensively examine the antioxidant potential of the chosen extracts, we conducted an evaluation through various complementary assays. By subjecting the extract to diverse tests, we aimed to elucidate the specific pathways and modes of action through which its antioxidative properties manifeste.

3.2.1 DPPH radical scavenging activity

The anti-radical activity was carried out by the DPPH radical method which is a method frequently used for its simplicity. This method is based on the reduction of a methanol solution of DPPH in the presence of an antioxidant which donates a hydrogen or an electron to give the non-radical form DPPH-H. Antioxidant-reducing compounds decrease the purple chromogen of the DPPH radical to yellow hydrazine. The antioxidant activity of the extracts is expressed in IC_{50}. Figure 16 shows the radical DPPH scavenging capacity of DE and HME. HME exhibited a high antioxidant activity with an IC_{50} of 38.16 ± 0.80 µg/mL closer to α-tocopherol activity (38.15 ± 0.45 µg/mL) as a positive control, the DE displayed a notably lower level of antioxidant activity compared to HME with an IC_{50} of 51.48 ± 1.03 µg/mL. BHA as positive standard exhibited the most potent activity, demonstrating a significant difference compared to both extracts.

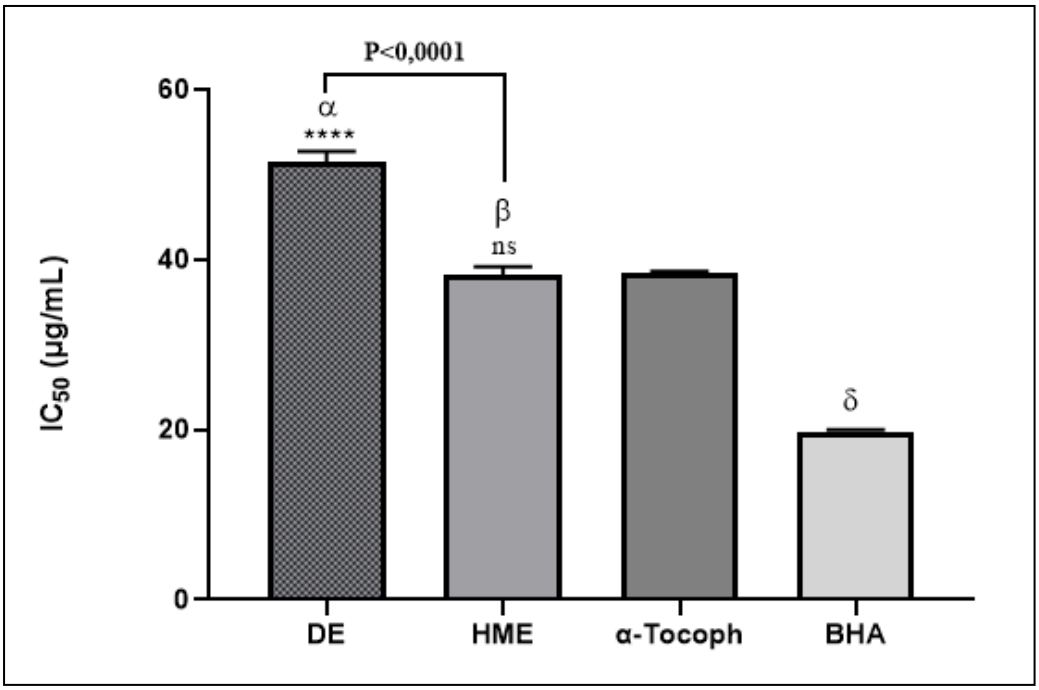

Figure 16. DPPH free radical scavenging activity of *T. hirsuta* crude extracts.

DE: decocted extract; **HME**: hydromethanol extract at 50%. Data were presented as IC_{50} means ± SD (n=3) (****P ≤0.0001; ns: not significant) α-tocopherol as standard. Columns carrying different letters are significantly different at P<0.0001

3.2.2 Ferrous ion chelating activity (FIC)

The FIC assay was used to assess the binding of antioxidant components to metal ions. A better binding ability extract would prevent or block reactions such as Fenton's type reactions, which generate reactive hydroxyl radicals. The assessment of ferrous chelation activity of the two extracts revealed that both of extracts exhibited a low chelator activity to Fe^{2+} ions. HME was more efficient at chelating, with an IC_{50} values of 83.75 ± 0.81 µg/mL followed by DE (93.13 ± 1.13 g/mL) (Figure 17). In this assay, neither of the extracts appeared to be a better Fe2+ ion chelator than the standard EDTA (5.60 ± 0.45 µg /mL). The chelation activity of these extracts, while relatively low, signifies their capacity to interact with ferrous ions, possibly contributing to their antioxidant attributes.

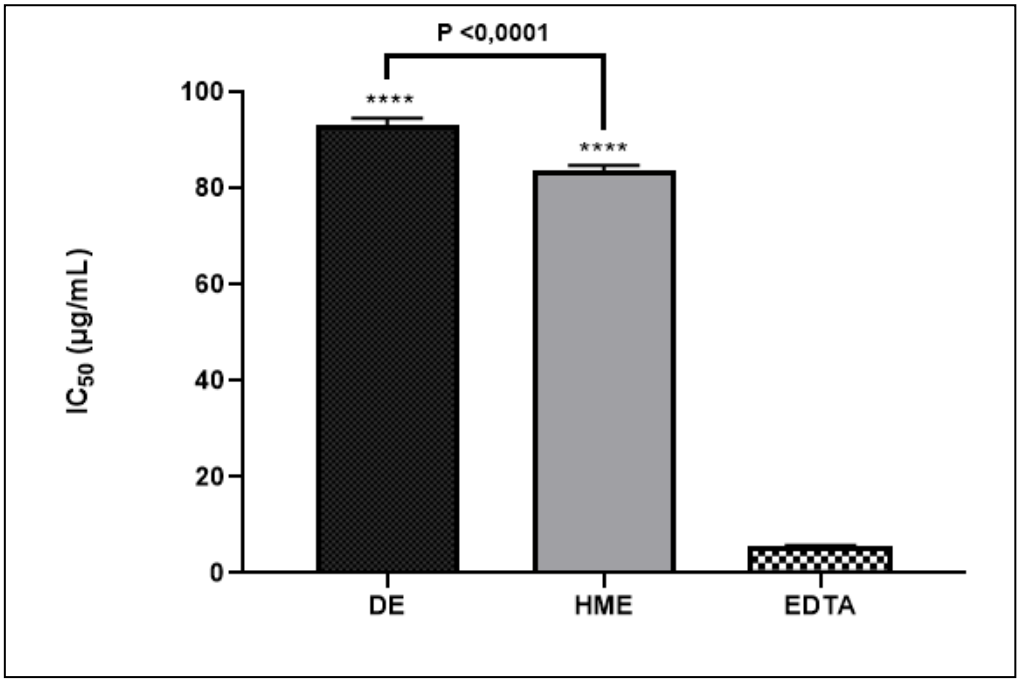

Figure 17. Metal chelating activity of *T. hirsuta* crude extracts.

DE: decocted extract; HME: hydromethanol extract at 50%. Data were presented as IC_{50} means ± SD (n=3) (****P ≤0.0001; ns: not significant) vs EDTA as standard.

3.2.3 Antioxidant activity determined by phenanthroline method

The Phenanthroline method is a commonly employed assay to evaluate the chelating activity of compounds towards metal ions. The potential of the crude extracts from *T. hirsuta* was assessed for the first time by Phenanthroline method. The results demonstrated a strong potential of Fe^{3+} reduction by HME marked at $A_{0,5}$ (the effective concentration at which the absorbance was 0.5) estimated (22.38 ± 2.62 µg/mL). This activity decreased with DE 47.40 ±

0.35 µg/mL. The antioxidant potential of extracts is notably lower compared to both standards utilized, α-tocopherol and BHA (Figure 18).

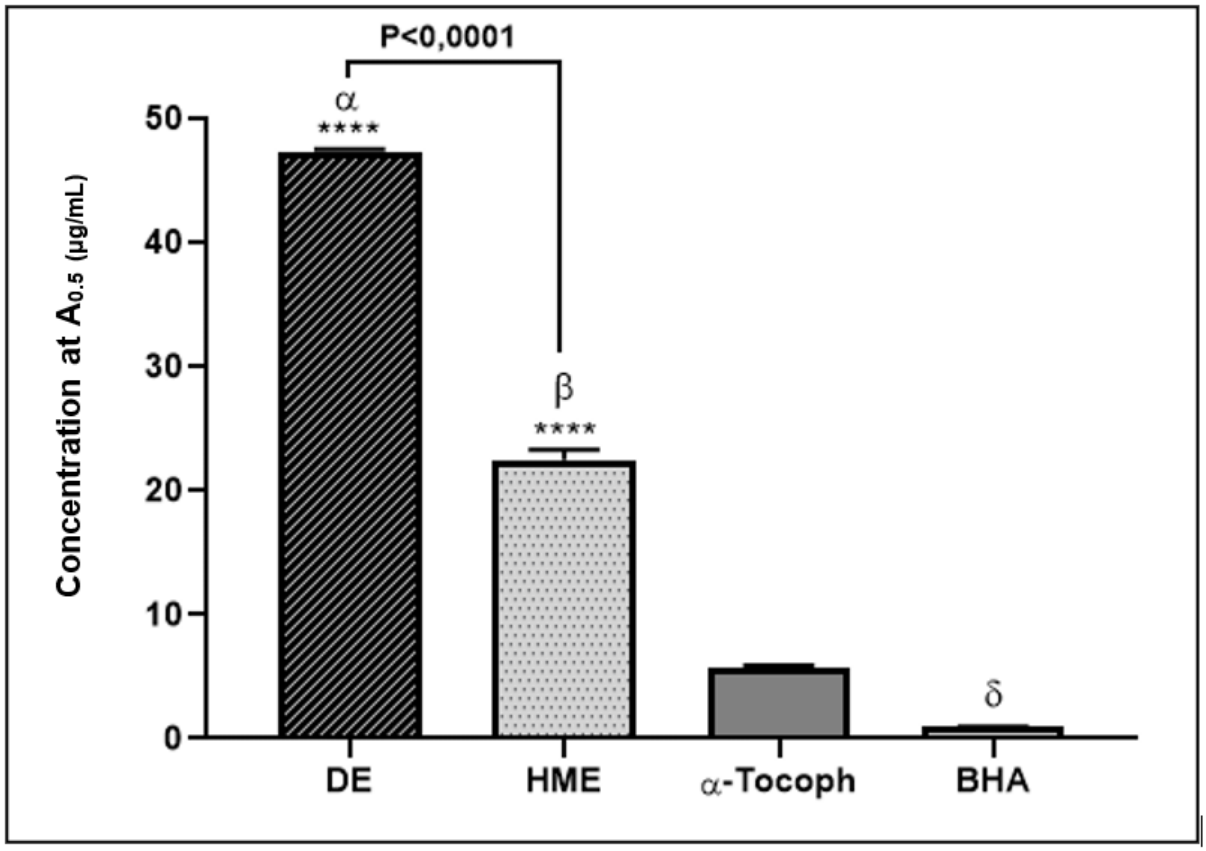

Figure 18. Chelating activity of *T. hirsuta* crude extracts by phenantroline method.

DE: decocted extract; **HME**: hydromethanol extract at 50%. Data were presented as $A_{0,5}$ means ± SD (n=3) (****P ≤0.0001; ns: not significant) vs α-tocopherol as standard. Columns carrying different letters are significantly different at P<0.0001.

3.2.4 Antioxidant activity determined by β-carotene bleaching method

Antioxidants can minimize the extent of ß -carotene degradation by interacting with the linoleate free radical or any other free radical generated in the system. Thus, by simulating the oxidation of membrane lipid components in the presence of antioxidants, this test provides insight into the inhibitory action of extracts on lipid peroxidation. In comparison with the standard, the plant extracts studied and standards at 4 mg/mL inhibited bleaching of ß-carotene and linoleic acid oxidation significantly. HME has the highest Antioxidant activity after 24 hours (23.85 ± 0.94 µg/mL), compared to DE with 40.82 ± 0.66 µg/mL. A significant difference was marked between both standards and extracts (Figure 19).

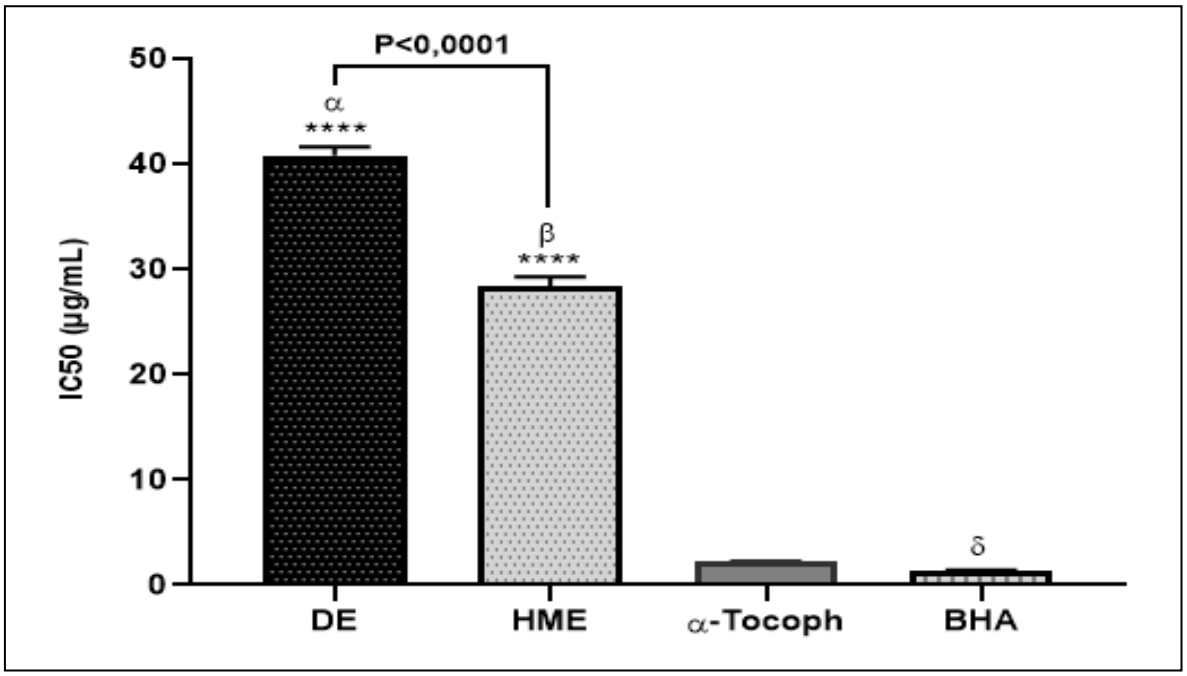

Figure 19. β-carotene bleaching activity of *T. hirsuta* crude extracts.

DE: decocted extract; **HME**: hydromethanol extract at 50%. Data were presented as IC_{50} means ± SD (n=3) (****P ≤0.0001; ns: not significant) *vs* α-tocopherol as standard. Columns carrying different letters are significantly different at P<0.0001.

3.3 *In vitro* enzyme inhibitory activity of *T. hirsuta* crude extracts

In addition to assessing antioxidant activity, the inhibitory effects of extracts against α-amylase, α-glucosidase, AchE, BchE, and tyrosinase enzymes were examined using different standards, including galantamine, Acarbose and Kojic acid as a positive control, the inhibition effect was expressed as pourcentage of Inhibition (%) (at 100 µg/mL).

3.3.1 α-amylase and α-glucosidase inhibition activity

The evaluation of the inhibitory effects of the studied extracts against α-amylase and α-glucosidase diabetes enzymes has yielded interesting results (Figure 20). HME and DE inhibited α-amylase with a pourcentage of Inhibition of 52.80 ± 0.77, 43.71 ± 0.95 at 100 µg/mL, respectively. A more potent inhibitory activity was revealed against α-glucosidase enzyme in the same order, HME exhibited the highest inhibitory effect (69.72 ± 0.95 % at 100 µg/mL), followed by DE with 53.36 ± 0.85 % at 100 µg/mL.

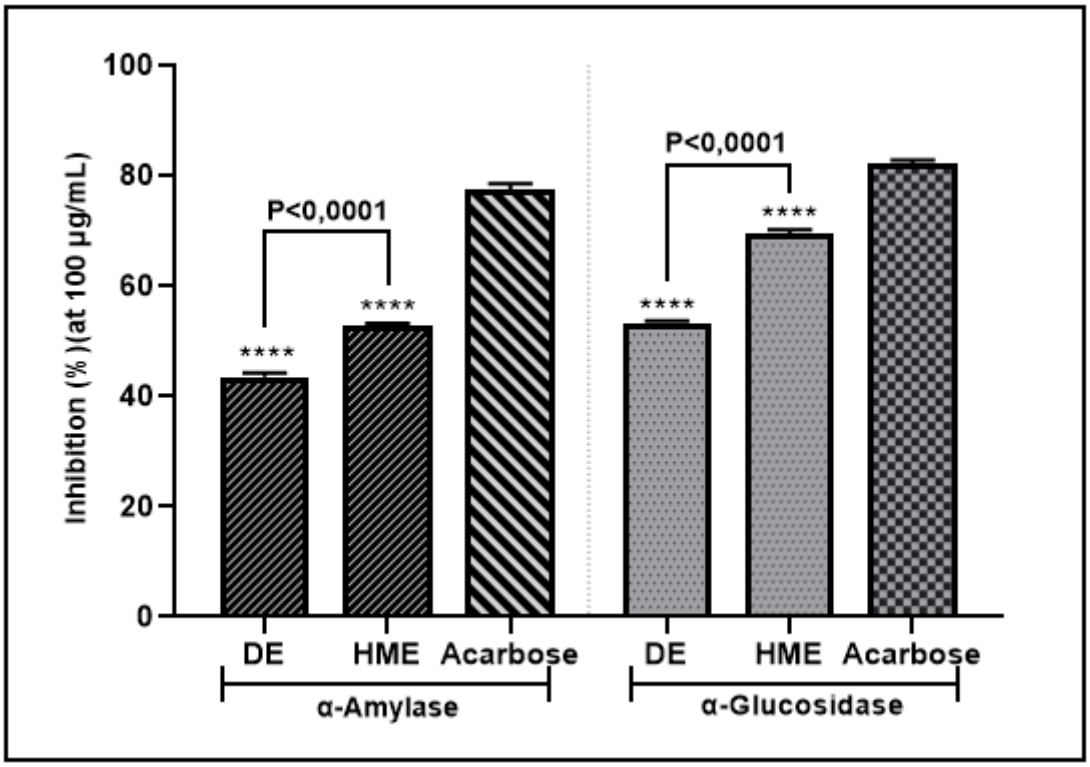

Figure 20. α-amylase and α-glucosidase inhibition activity of *T. hirsuta* crude extracts

DE: decocted extract; **HME**: hydromethanol extract at 50%. Data were presented as pourcentage of inhibition at means ± SD (n=3) (****P ≤0.0001) *vs* Acarbose as standard.

3.3.2 Acetylcholinesterase and butyrylcholinesterase inhibition activity

The cholinesterase enzymes play important roles in neurotransmission, and their inhibition has gained a lot of interest because of their potential in managing cognitive illnesses like Alzheimer (Pessoa, 2008). Low inhibitory activity at 100 µg/mL of extracts was recorded for both HME and DE against AchE enzyme (41.95 ± 0.57 and 31.80 ± 0.58% at 100 µg/mL) respectively. However, the activity of the vegetable extracts studied in inhibiting BchE activity was weaker compared to that against AchE. HME demonstrated a modest inhibition of BchE (39.73 ± 0.68% at 100 µg/mL), suggesting a low interaction between the compounds within the extract and the enzyme. In the same line, DE exhibited relatively the lowest level of inhibition (28.33 ± 0.47% at 100 µg/mL). Neither of the extracts appeared to have an activity close to that of Galantamine as a standard (Figure 21).

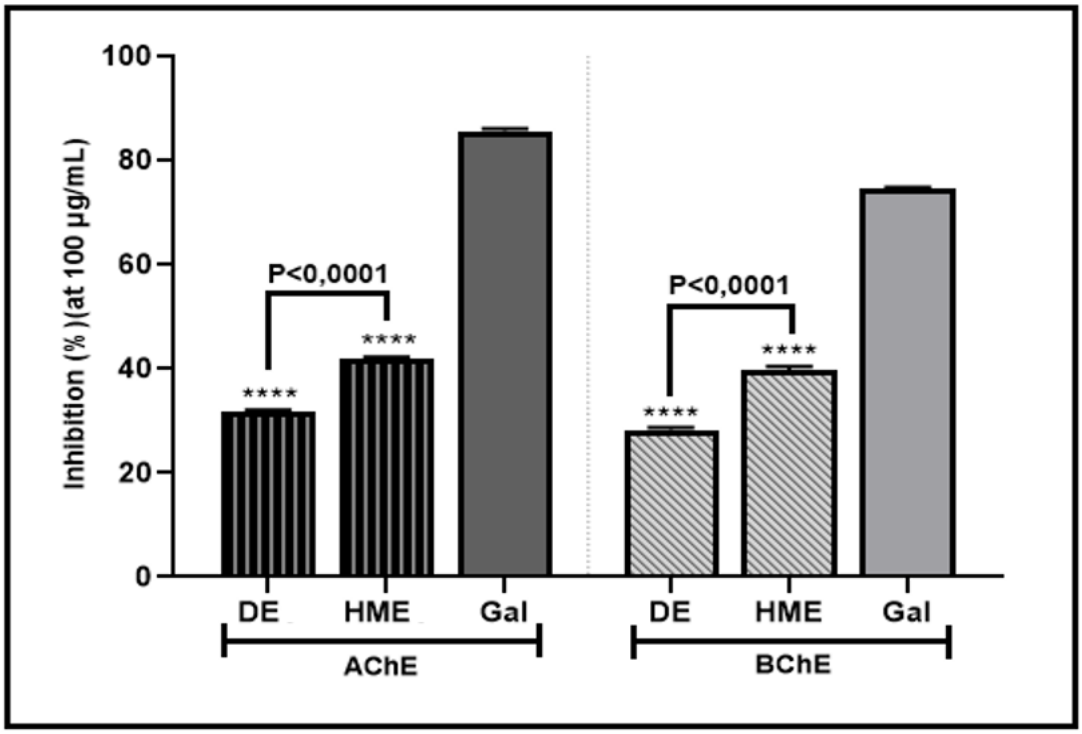

Figure 21. Acetylcholinesterase and butyrylcholinesterase inhibition activity of *T. hirsuta* crude extracts.

DE: decocted extract; **HME**: hydromethanol extract at 50%. **Gal** : Galantamine; **AchE** : Acetylcholinesterase. **BchE**: Butyrylcholinesterase. Data were presented as IC50 means ± SD (n=3) (****P ≤0.0001) *vs* Galantamine as standard.

3.3.3 Tyrosinase inhibition activity

Tyrosinase inhibitory activity, a crucial aspect in evaluating the potential of natural compounds for therapeutic uses, is receiving a lot of attention because of its importance in treating diseases related to tyrosinase activity. In this study, the extracts demonstrated a closer minimal degree of tyroinase inhibition as shown in figure 22. The maximum percent inhibition was noted by HME (27.73 ± 0.93% at 100 µg/mL) followed by DE (16.69 ± 0.55% at 100 µg/mL). The tyrosinase inhibition potential of extracts was significantly lower than positive control drug, kojic acid (79.50%).

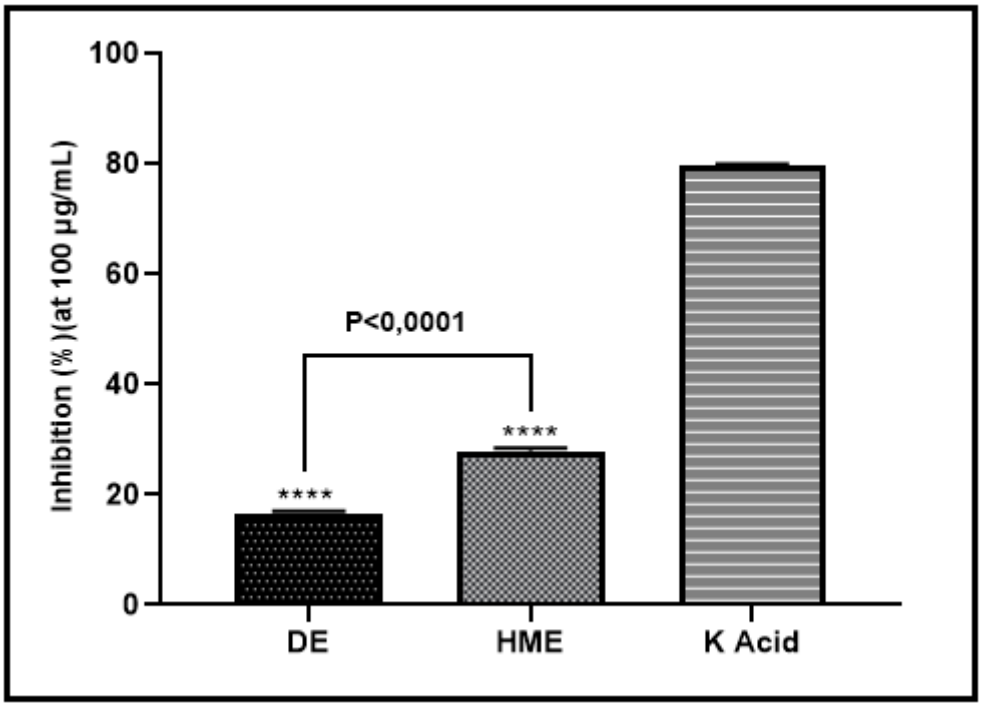

Figure 22. Tyrosinase inhibition activity of *T. hirsuta* crude extracts.

DE: decocted extract; HME: hydromethanol extract at 50%. K acid : Kojic acid. Data were presented as IC50 means ± SD (n=3) (****P ≤0.0001) vs Kojic acid as standard.

4 *In vivo* pharmacological effects

4.1 Acute oral toxicity of DE and HME

4.1.1 Physiological and Behavioral Responses

Rats were exposed to increasing doses of both HME and DE (2000 mg/kg and 5000 mg/kg body weight), and their physiological and behavioral responses were closely monitored. Remarkably, throughout the 14 days of the observation period, no significant alterations in clinical signs, behavior, skin effects, breathing, impairment in food intake or water consumption or mortality rates were observed in the treated rats compared to the control group. These findings suggest a relatively low acute oral toxicity of the extract in rats and indicate a favorable safety profile at the tested doses.

4.1.2 Biochemical parameters changes

The results of table 5 illustrates that following the oral administration of both extracts, there were no remarkable and statistically significant alterations in the serum levels of key biomarkers, including urea, creatinine, alanine aminotransferase (ALAT), and aspartate aminotransferase (ASAT), when compared to the control group (P<0.05). This overall lack of significant change suggests that the administration of these extracts did not exert substantial

adverse effects on renal function or liver enzymes. However, noteworthy exceptions were observed. Specifically, the DE, when administered at both doses, exhibited a slightly a significant increase in ALAT levels, suggesting a minimal impact on liver function. Additionally, a subtle statistically significant change in ASAT levels was detected in the case of the HME at both administered doses.

Table 5. Biochemical parameters.

	UREA	CREAT	ASAT	ALAT
Control	0.38±0,04	4.5±0.2	20.66±3.11	70.01±1,9
DE 2 g/kg	0.37±0,02 ^{ns}	3,54±0.43 ^{ns}	26.2±2.64 ^{ns}	54.5±2.7*
DE 5 g/kg	0.42±0.02 ^{ns}	3.78±0.33 ^{ns}	38,8±1.76*	90,2±2.4*
HME 2 g/kg	0.34±0,03 ^{ns}	3.7±0.16 ^{ns}	42.2±3.44**	81.4±2.3 ^{ns}
HME 5 g/kg	0.37±0.01 ^{ns}	3.48±0.06 ^{ns}	39.66±0,88*	76.5±1,7 ^{ns}

Values are expressed as mean ± SEM (n=5). (ns: not significant, *P<0.05, **P<0.01,), ALAT: alaline aminotransferase, ASAT: aspartate aminotransferase, CREAT:creatinine.

4.1.3 Body and organ weights changes

Acute toxicity results showed that body weight in femal rats was not highly affected after treatment with both DE or HME at the used doses, a slight increase in body weight was recorded in administration of 5 mg/kg of decocted extract. In the other hand, a significant loss of body weight was remarqued in group of rats received a dose of 2 mg/kg from HME (Figure 23).

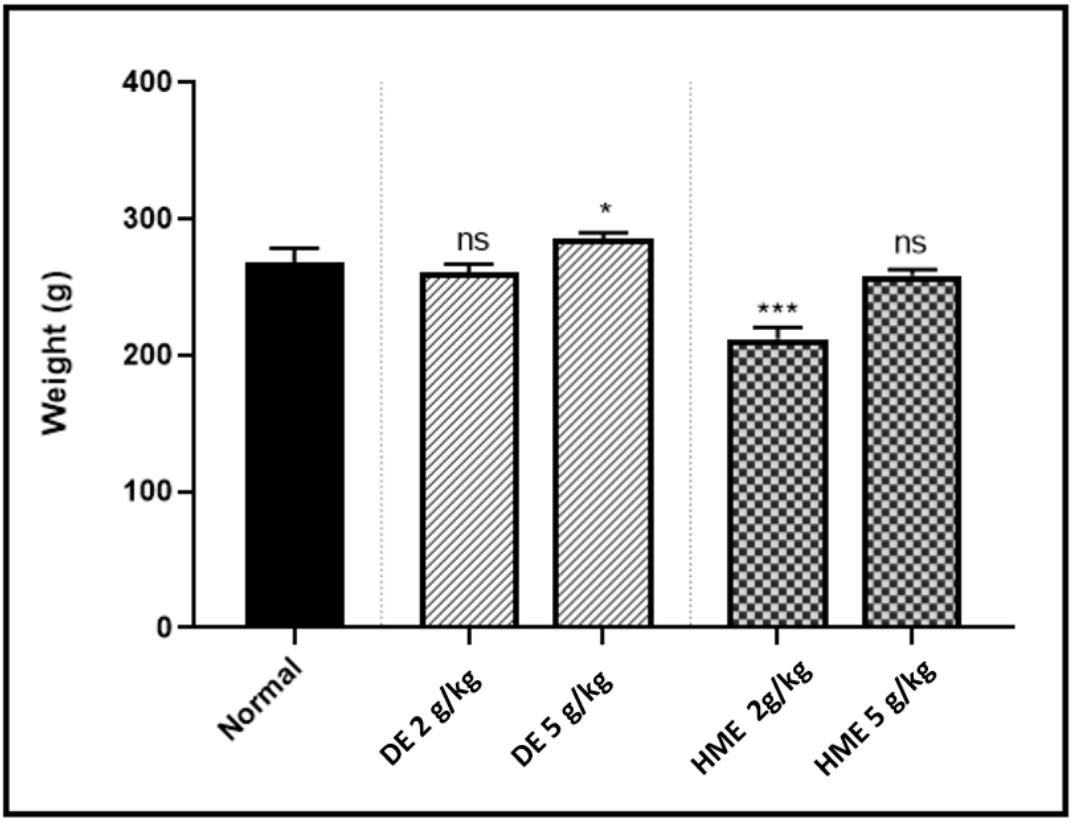

Figure 23. Changes in body weight of female rats treated with the extracts.

DE : Decocted Extract; **HME** : Hydromethanol extract. Values are expressed as mean ± SEM (n=5). (ns: not significant P>0,05 ; *P<0.01 ; ***P<0.0005)

The results of the relative organ weights are presented in figure 24. The relative organ weights (liver, kidneys) of the traited groups at the two different doses of both extracts (2000 and 5000 mg/kg), showed no significant difference (P > 0.05) compared to the normal group with the exception of the HME administered at a dose of 2000 mg/kg, which demonstrated a significant increase in kidney weight, the other experimental groups exhibited relatively consistent results in terms of organ weight changes.

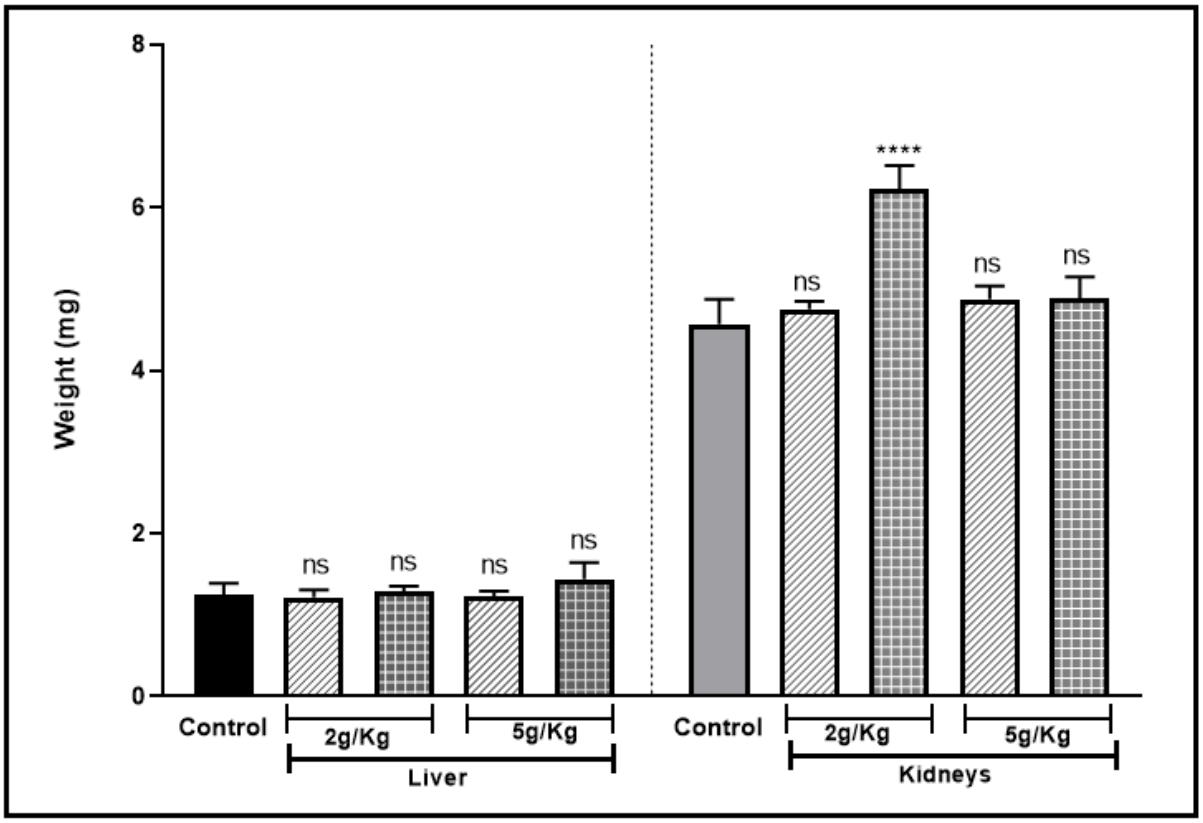

Figure 24. Comparison of relative organ weights in female rats after 14 days from treatment with DE and HME.

DE: decocted extract; HME: hydromethanol extract. The values of the bars chart are expressed as means ± SEM (n=6). (****$P \leq 0.0001$ *vs* control (vehicle, Nacl 0,9%).

4.2 Evaluation of *in vivo* pharmacological effect of *T. hirsuta* selected extracts

4.2.1 Decocted and hydromethanol extracts effects on gastric emptying and intestinal transit in mice and possible mechanism (s) underlying

4.2.1.1 Gastric emptying

Gastric emptying, a pivotal process in digestion, plays a crucial role in the efficient transfer of nutrients from the stomach to the small intestine. The administration of DE and HME from *T. hirsuta* areal parts led to a decrease in the activity of the emptied quantity of the test meal compared to the negative control (CMC) as shown in figure 25. Importantly, this effect did not follow a dose-dependent pattern. DE at the lower doses (125, 250 mg/kg) decreased the gastric emptying significantly to 48.68 ± 2.95 % and 38.60 ± 1.67 %, respectively in comparison to negative control, with a rate close to that of atropine (51.98 ± 2.32 %) suggesting its potential to slow down this digestive process. While, at the highest dose of this experimental range (500 mg/kg) the extract continued to exert a modest inhibitory effect on gastric emptying (55.98 ± 2.19 %), this diminution was noticeably less pronounced compared to the lower doses. In the same line, HME, while demonstrating an activity level lower than that of the first extract,

exhibited a remarkable efficacy. In particular, at highest concentrations tested (250 and 500 mg/kg), the second extract showcased a performance notably superior to the negative control and approached to that of the positive control, highlighting its promising attributes in specific concentration ranges. This intriguing result prompts further investigation into the underlying mechanisms of the extracts on gastric emptying.

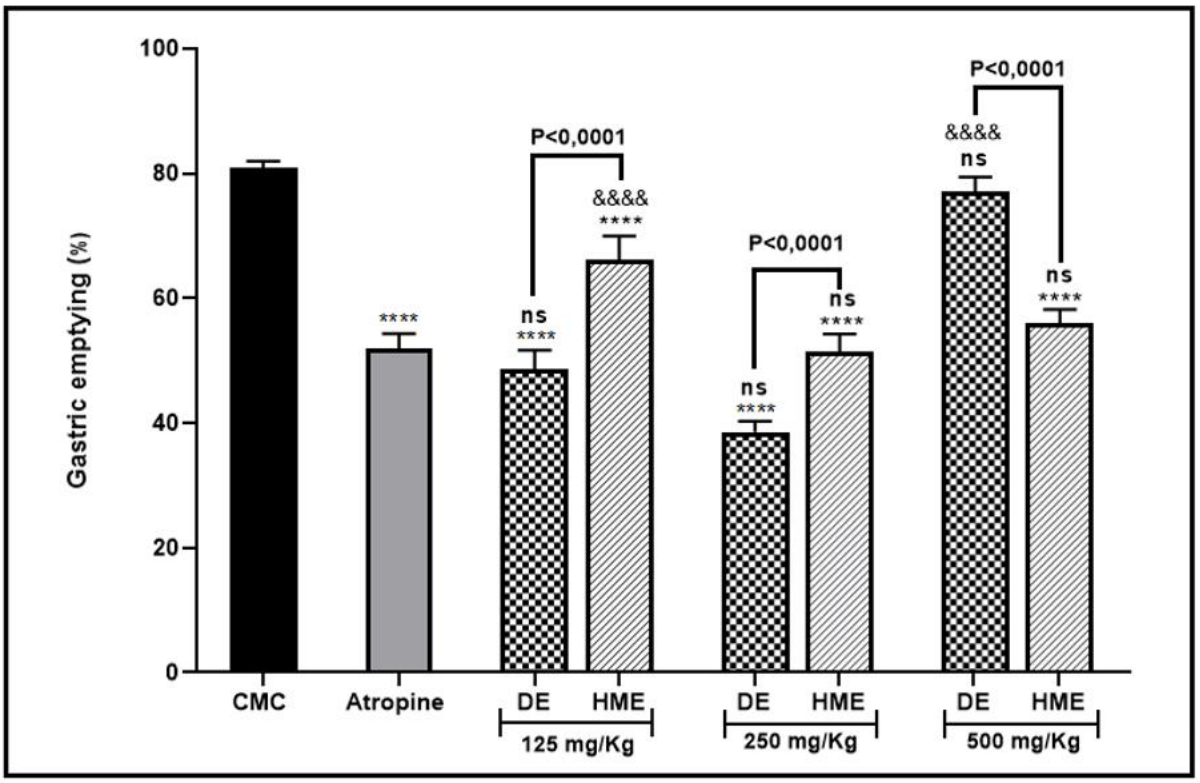

Figure 25. Effects of DE and HME on gastric emptying in mice.

DE: decocted extract; **CMC**: Carboxymethyl Cellulose. The values of the bars chart are expressed as means ± SEM (n=6-8). (****$P \leq 0.0001$; ***$P \leq 0.001$) *vs* vehicle (CMC 1.5% *p.o.*). (&&&&$P \leq 0.0001$, *&&&P* ≤0.001) *vs* positive control group (Atropine sulphate, 1 mg/kg *i.p.*).

4.2.1.1.1 Mechanisms of the DE effect on gastric emptying

As previously described, the extracts have demonstrated a significant slowing effect on gastric emptying. To gain a more comprehensive understanding of the mechanisms behind these effects, a standardized approach was chosen using different pharmacological susbstances L-NNA, L-Arg, indomethacin and the selected dose of 250 mg/kg from both extracts to explore potential synergistic effects that might arise from the interaction of these substances. As shown in figure 26, treatment of mice with indomethacin and DE 250 mg/kg simultaneously reduced gastric emptying rates significantly to 52.89% (P≤0.0001) in comparison with negative control where only the vehicle CMC was administered. However, when indomethacin was administered in isolation, the decrease in gastric emptying was even more pronounced 45.52% (P<0,005).

Oral administration of L-Arg with the extract induces a decrease in gastric emptying (67.86 ± 3.8%), this reduction was not significantly different from the activity observed when the extract was administered in isolation (62.60 ± 3.24). However, the rate was highly elevated compared to L-Arg alone (35.99 ± 2.99%; P≤0.0001). On the other hand, the use of L-NNA with DE significantly reduces emptying to the lowest rate 11.5% in compared to each of negative control, extract and L-NNA administred alone (P≤0.0001).

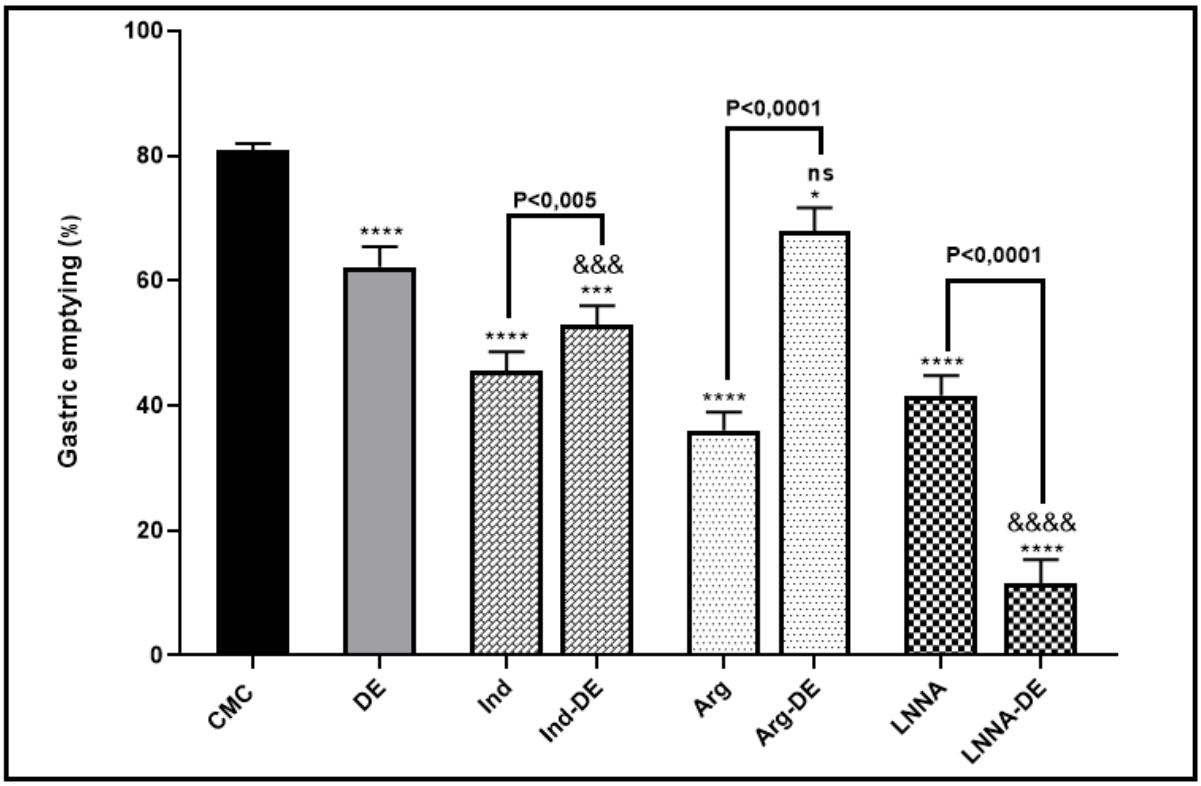

Figure 26. Effect of DE in absence and in presence of indomethacin, L-Arg or L-NNA on gastric emptying.

DE: decocted extract; **CMC**: carboxymethyl cellulose. The values of the bars chart are expressed as means ± SEM (n=6-8). (*P < 0,05; **** P≤0.0001 ***vs*** vehicle (CMC 1.5% *p.o.*). ns; no significant difference (P>0.05). (&&&&*P* ≤ 0.0001, &&&*P* ≤0.001) *vs* DE; P≤0.0001in comparison to both effects of DE in absence and in presence of indomethacin, L-arginine or L-LNNA.

4.2.1.1.2 Mechanisms of the HME effect on gastric emptying

The HME administered to mice pretreated with indomethacin, had a significant effect on decreasing gastric emptying (61.16%) compared to both effects observed with HME alone and negative control (70.53 ± 2.27 % and 81.0 ± 1.17 %, respectively; P<0,0001) (Figure 27). However, the significant increase of gastric emptying rate noted when using L-Arg in the presence of HME at a dose of 250 mg/kg was the highest achieved rate (91.41 ± 2.52 %) wich reflects the loses of the extract effect on slowing gastric emptying in the presence of L-Arg,

while a significant decrease in emptying was recorded in the presence of L-Arg alone in comparison with both cases, negative control and L-Arg-HME association (81.00 ±1.17 and 34.43 ± 1.85, respectively; P<0,0001).

On the other hand, on presence of L-NNA such an inhibitor of NOs, a reduction even more pronounced of gastric emptying than that observed with the negative control (40.17 ± 1.11 and 81.00 ±1.17, respectively; P<0,0001). This potent effect of the extract, when combined with L-NNA, surpassed the impact marked when the extract was administered alone.

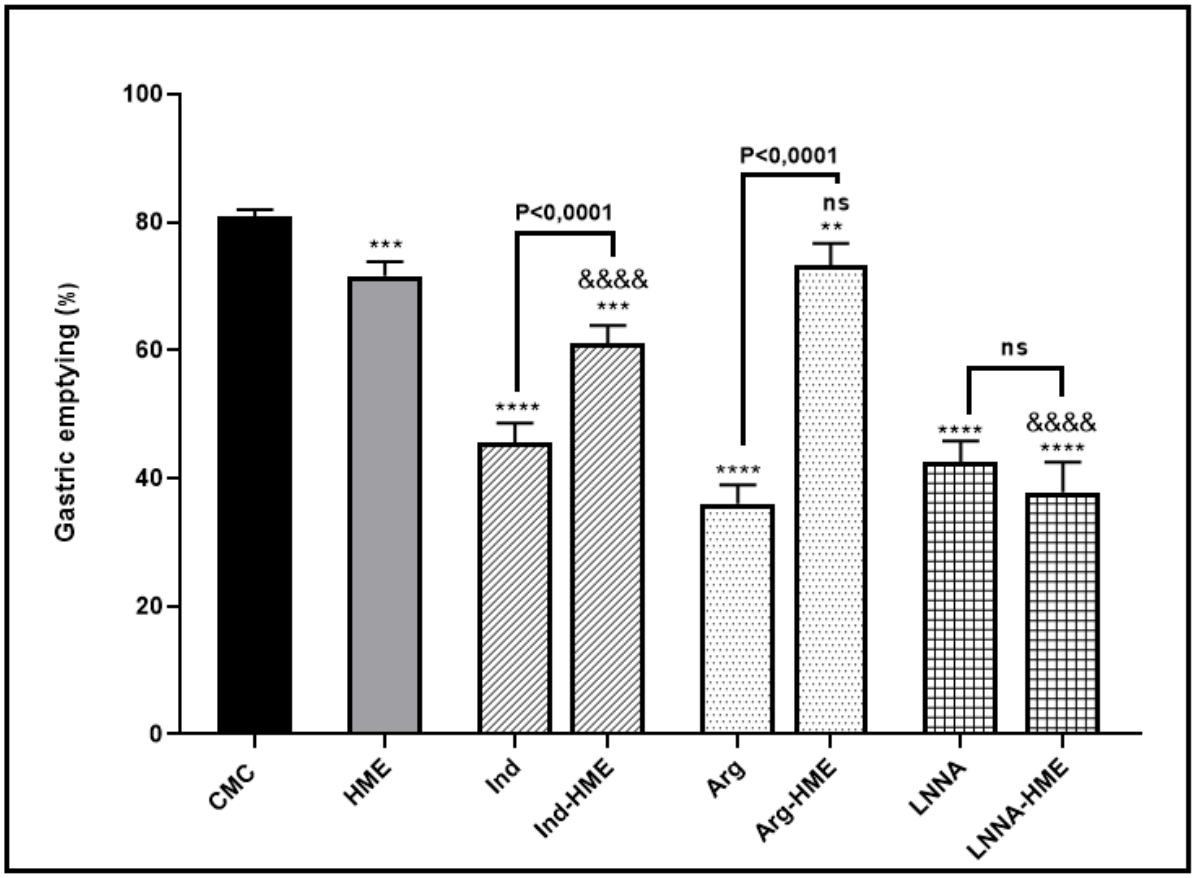

Figure 27. Effect of HME in absence and in presence of indomethacine, L-Arg or L-NNA on gastric emptying.

HME: hydromethanol extract; **CMC**: carboxymethyl cellulose. The values of the bars chart are expressed as means ± SEM (n=6-8). ***; P≤0.001, ****; P≤0.0001 *vs* vehicle as negative control. ns; no significant difference (P>0.05), (****$P \leq 0.0001$; ***$P \leq 0.001$; **$P \leq 0.01$) *vs* vehicle (CMC 1.5% *p.o.*). (&&&&$P \leq 0.0001$, &&&$P \leq 0.001$, &&$P \geq 0.05$) *vs* effects of HME in absence and in presence of indomethacine, L-arginine or L-LNNA.

4.2.1.2 Intestinal transit

In the present study, the animals are treated by oral administration of 0.1 ml/20g of test solution of the extract before the administration of the test meal. Intestinal transit and gastric emptying are measured after the gavage of phenol red solution in the stomach for 20 min. The rate of intestinal transit in control mice (treated with 1.5% CMC only) one hour before gavage

with phenol red are 61.40 ± 2.29 % (n = 8). The administration of both DE and HME at different doses induces an important increase in intestinal transit in dose dependant manner compared to the vehicle (CMC) as negative control (44.57 ± 2.75; P≤0.0001). As shown in figure 28; the tested doses (125, 250 and 500 mg/kg) from DE showed an increasement from 50.69%, 54.59% to 68.78%, respectively; (P≤0.0001) and more highly for HME, from 65.98%, 67.24% to 70.05% (P≤0.0001). These results provide preliminary evidence for the potential of both extracts as a natural agent for promoting intestinal transit.

Additional research, including mechanistic analyses, will be conducted to uncover the underlying mechanisms accountable for these observed effects.

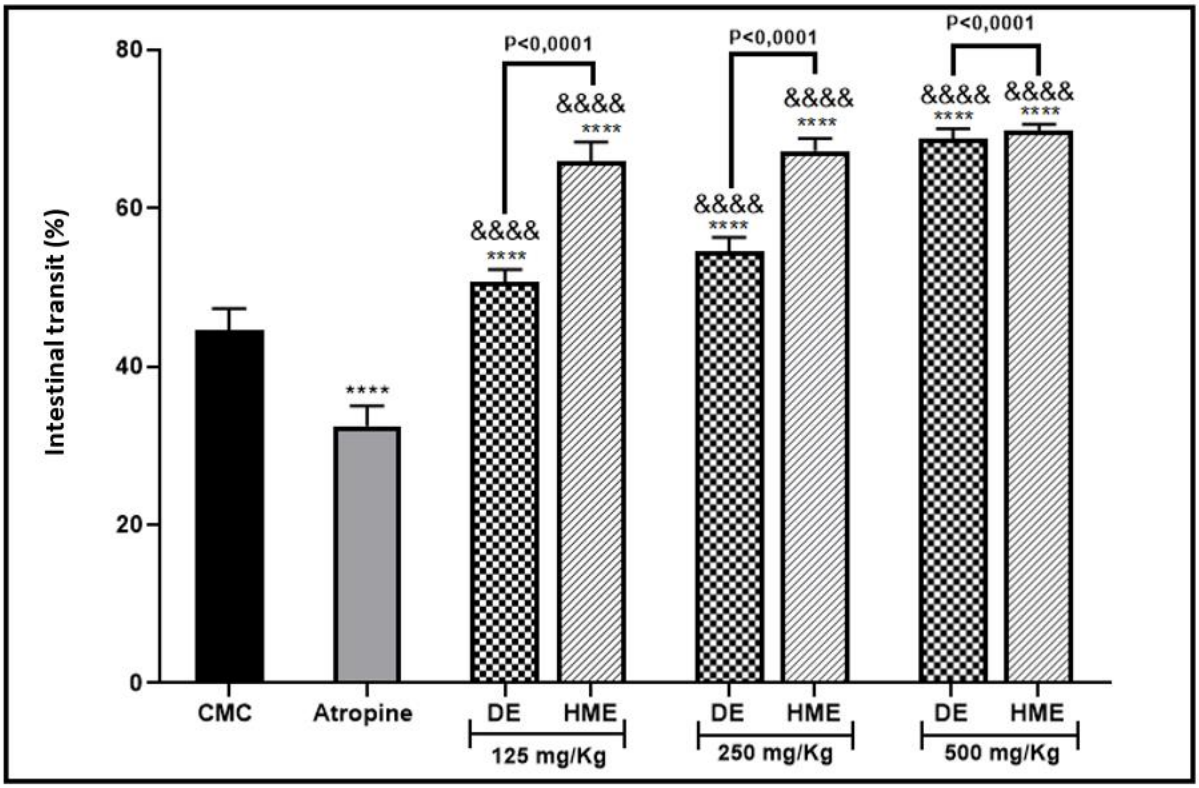

Figure 28. Effect of DE and HME on gastric intestinal transit in mice.

DE: decocted extract; **HME**: hydromethanol extract; **CMC** stands for Carboxymethyl Cellulose. The values of the bars chart are expressed as means ± SEM (n=6). (****$P \leq 0.0001$) *vs* vehicle (CMC 1.5% *p.o.*). (&&&&$P \leq 0.0001$) *vs* Atropine (1 mg/kg).

4.2.1.2.1 Mechanisms of the DE effect on intestinal transit

The DE at doses of 250 mg/kg showed an improvement in intestinal transit. While, this activity indicated a significant decline of intestinal transit rate when DE was administered in rats pretreated with indomethacin compared to both negative control and DE administred alone. Contrary, the administration of L-Arg concurrently with the extract showed an important

increase in the rate of intestinal transit (71.84 ± 3.44%; P≤000.1) (Figure 29). The oral administration of DE under the effect of L-NNA showed a rate of 40,58% significantly lower than that observed in effect of DE alone.

These findings suggest that DE from *T. hirsuta* at 250 mg/kg exhibited an active effect in accelerating intestinal transit. However, a synergistic effect between the extract and arginine results in a significant acceleration of intestinal transit. This observation underscores the potential for enhanced outcomes when combining the extract with Arg in influencing this physiological process.

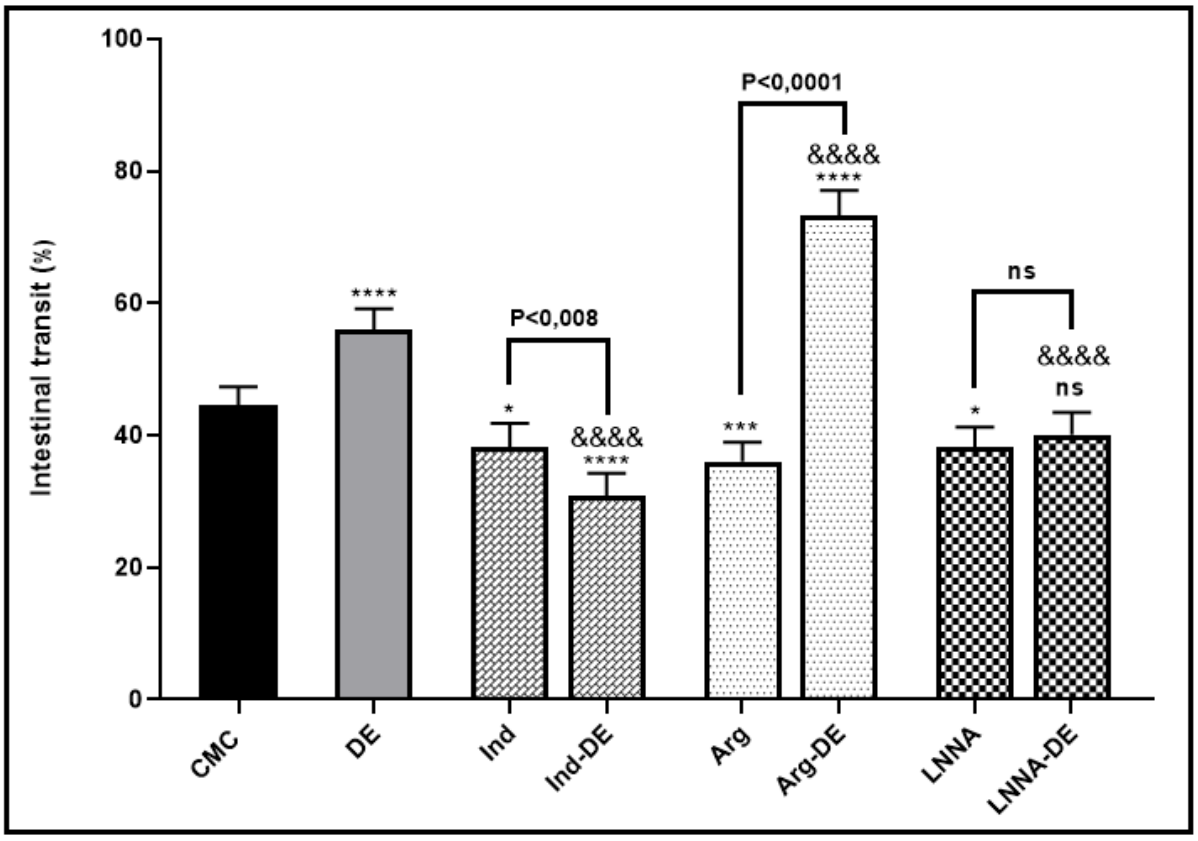

Figure 29. Effect of DE in absence and in presence of indomethacin, L-Arg or L-NNA on intestinal transit.

DE: decocted extract; **CMC** stands for carboxymethyl cellulose. The values of the bars chart are expressed as means ± SEM (n=6). (****$P \leq 0.0001$; ***$P \leq 0.001$; *$P > 0.05$) *vs* vehicle (CMC 1.5% *p.o.*). (&&&&$P \leq 0.0001$) *vs* DE.

4.2.1.2.2 Mechanisms of the HME effect on intestinal transit

In a compelling experimental study, the administration of HME following the treatment of mice with indomethacin demonstrated remarkable results; HME induced a considerable increase in intestinal transit (48.65 ± 3.6%), surpassing the outcomes observed in both the

negative control group and the indomethacin-only treated group (44.57±2.7, 38.14±3.6, respectively; P<0,0001).

In the presence of Arg, DE enhances significantly the intestinal transit compared to both its corresponding control and the negative control (P<0.0001) with a rate of 61.31 ± 3.1% closer to that showed by the administration of the tested dose of extract in isolation. While, this acceleration is reduced in the presence of L-NNA; The administration of HME in mice pre-treated with L-NNA exhibited a comparable level of activity to that observed in mice pre-treated with CMC alone and L-NNA control, indicating no significant difference in their observed activities (P≤0.0001) as shown in figure 30.

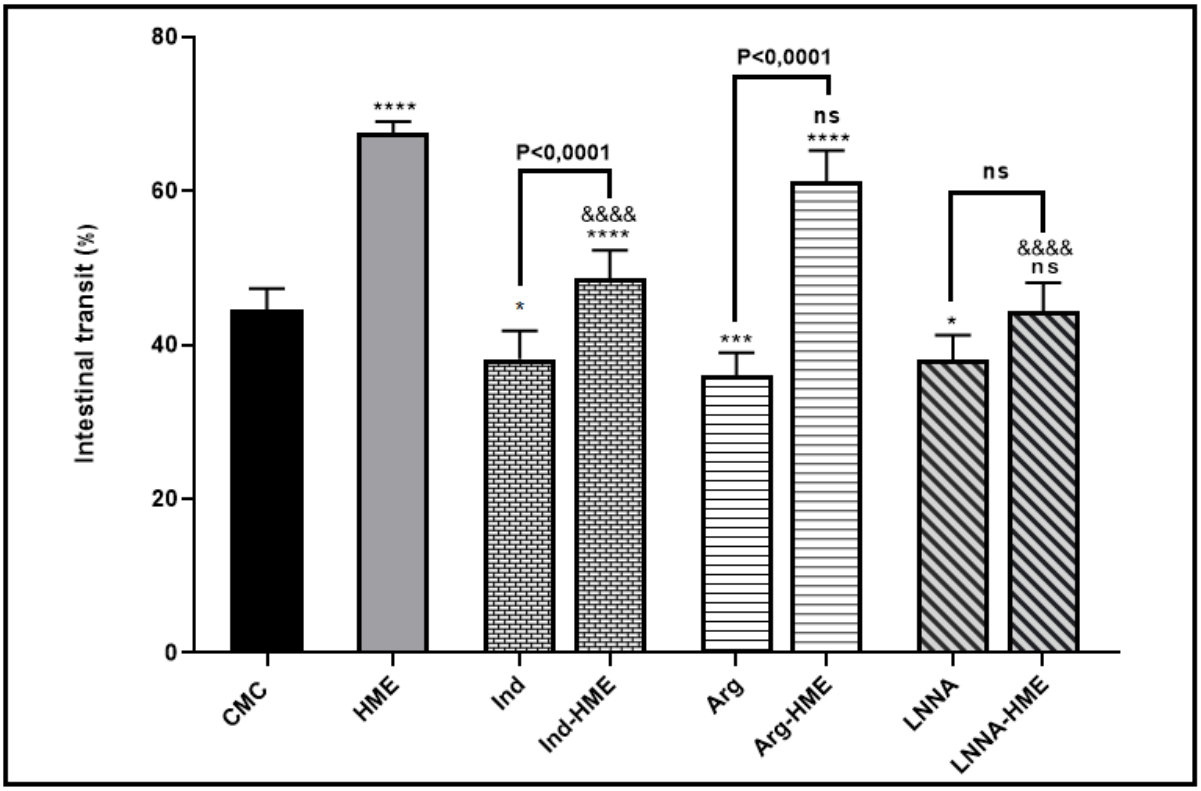

Figure 30. Effect of HME in absence and in presence of indomethacin, L-Arg or L-NNA on intestinal transit.

HME: hydromethanol extract; **CMC** stands for carboxymethyl cellulose. The values of the bars chart are expressed as means ± SEM (n=6). (*****P* ≤ 0.0001; ****P* ≤ 0.001; ***P* ≤ 0.01, **P*> 0.05) vs vehicle (CMC 1.5% *p.o.*). (&&&&*P* ≤ 0.0001) vs HME.

4.2.2 Effect of *T. hirsuta* studied extracts against ethanol-induced gastric ulceration in rats

4.2.2.1 Effect of DE and HME on macroscopic and histopathological appearance

a) Macroscopic examination

An excessive alcohol consumption stands out as the primary contributor to damage in the gastric mucosa (Mousa *et al*., 2019). According to the experimental results, animals administered 100% ethanol consistently displayed severe macroscopic damages apparent in the examination. These damages manifested as a distinct loss of the normal color and mucus lining of the stomach. Furthermore, the presence of petechiae, indicating small, pinpoint hemorrhages, was observed, along with more extensive hemorrhages and the development of edema, or tissue swelling. However, this damage was notably reduced by the administration of DE or HME at varying doses. Pre-treatment of rats with DE or HME at the specified doses (125, 250, and 500 mg/kg) effectively reduced the damage induced by ethanol (Figure 31). These outcomes were further confirmed through histopathological analysis, which revealed fewer signs of hemorrhagic lesions, reduced infiltration, and less edema in the gastric mucosa of rats treated with DE and HME at 125 and 250 mg/kg doses. Notably, the group treated with HME showed an even more substantial amelioration compared to the DE-treated group, suggesting a higher effectiveness of HME at these doses. Animals receiving the highest doses (500 mg/kg) were entirely protected from the harmful effects of ethanol, preserving all histological aspects compared to the control group of animals. These results underscore the potential protective effects of both DE and HME against ethanol-induced gastric damage.

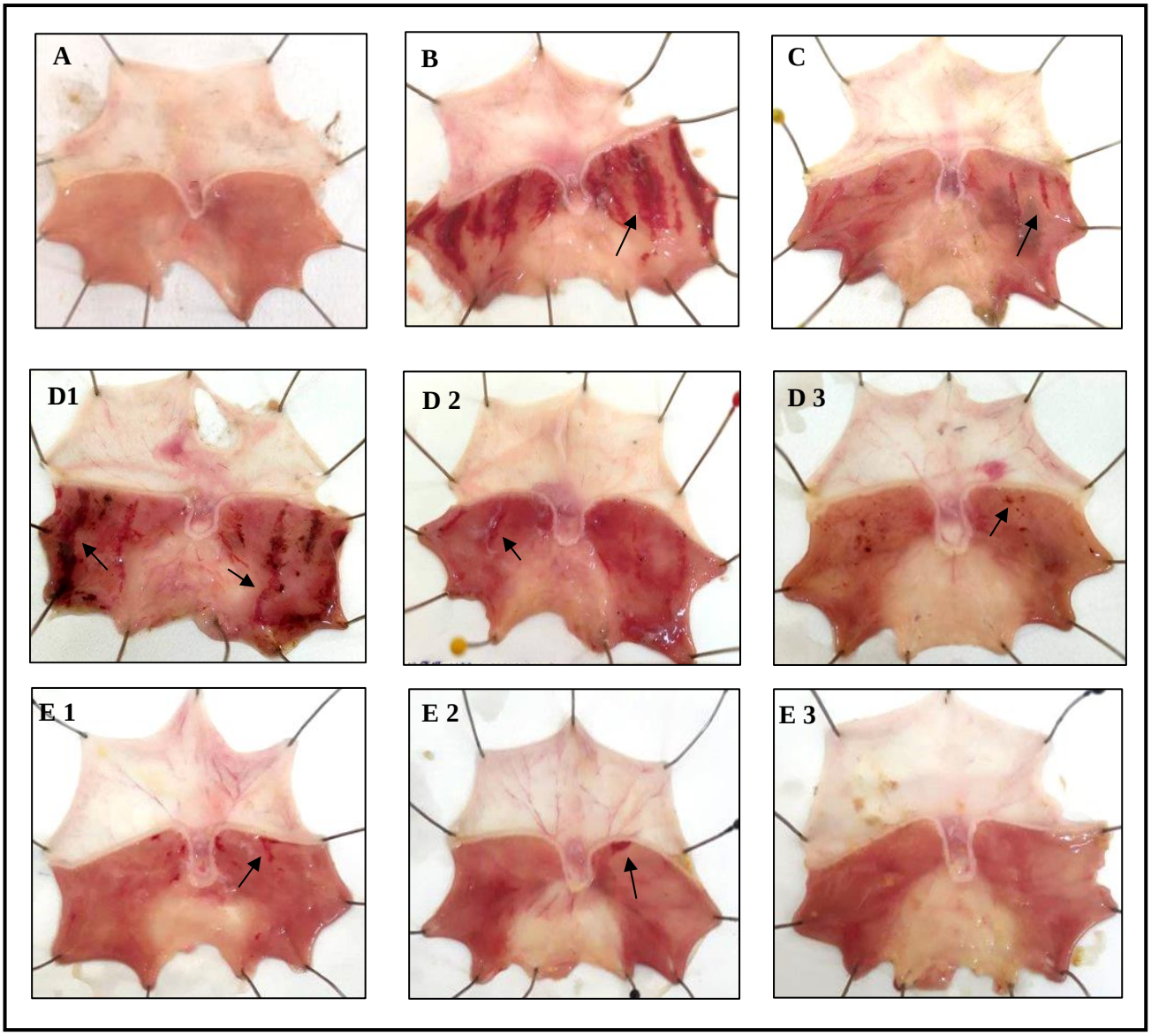

Figure 31. Effects of DE and HME on the macroscopic appearance of the gastric mucosa.

(A)Normal control group, (B) The group pre-treated with Nacl 0,9% vehicle as negative control. (C) The group pretreated with ranitidine (5 mg/kg) as positive control. (D1, D2 and D3): The group pre-treated with DE (125, 250 and 500 mg/kg, respectively). DE: Decocted Extract. (E1, E2 and E3): The group pre-treated with HME (125, 250 and 500 mg/kg, respectively). HME: Hydromethanol extract. Black arrow: elongated bands of hemorrhagic lesions.

b) Histopathological examination

The histopathological examination of stomach tissue samples from the control negative group, received ethanol and NaCl, and both other groups receiving DE and HME at increasing doses (125 mg/kg, 250 mg/kg, and 500 mg/kg), revealed significant differences in the amelioration of stomach ulcerated tissue, with increasing doses (Figure 32). Notably, HME demonstrated superior efficacy compared to DE. This observation can be attributed to several factors, including the analysis of necrosis in the superficial mucosal epithelium, oedema, inflammatory, cell infiltration, and congestion of blood vessels in the submucosa.

In the negative control group, histopathological examination revealed a pronounced necrosis in the superficial mucosal epithelium, indicated by an orange arrow (Figure 32 A). This necrosis was a result of the damaging effects of ethanol. As the dosage of both DE and HME increased, there was a clear trend of decreased necrosis in the superficial mucosal epithelium. However, HME consistently outperformed DE in reducing this necrosis. This improvement was more prominent with the highest dose of 500 mg/kg of HME. Oedema was evident in the negative control group due to the inflammatory response triggered by ethanol administration. With increasing doses of DE and HME, there was a notable reduction in oedema. Once again, HME exhibited a more significant reduction in oedema compared to DE, particularly at the highest dose of 500 mg/kg. The inflammatory cell infiltration, indicated by a black arrow, was observed in the negative control group, indicative of the inflammatory response to ethanol. The histological examination demonstrated that both DE and HME were effective in reducing inflammatory cell infiltration with increasing doses. HME consistently showed a superior anti-inflammatory effect compared to DE, with the greatest reduction observed at 500 mg/kg. The congestion of blood vessels in the submucosa, marked by a blue arrow, was a common feature in the negative control group.

These findings suggest that both DE and HME were effective in ameliorating ulceration induced by ethanol. However, HME consistently outperformed DE in reducing necrosis, oedema, inflammatory cell infiltration, and congestion of blood vessels. This superior efficacy of HME became more apparent with increasing doses, with the highest dose of 500 mg/kg yielding the most remarkable results. HME is a more effective therapeutic agent for mitigating the histopathological changes associated with gastric ulceration compared to DE.

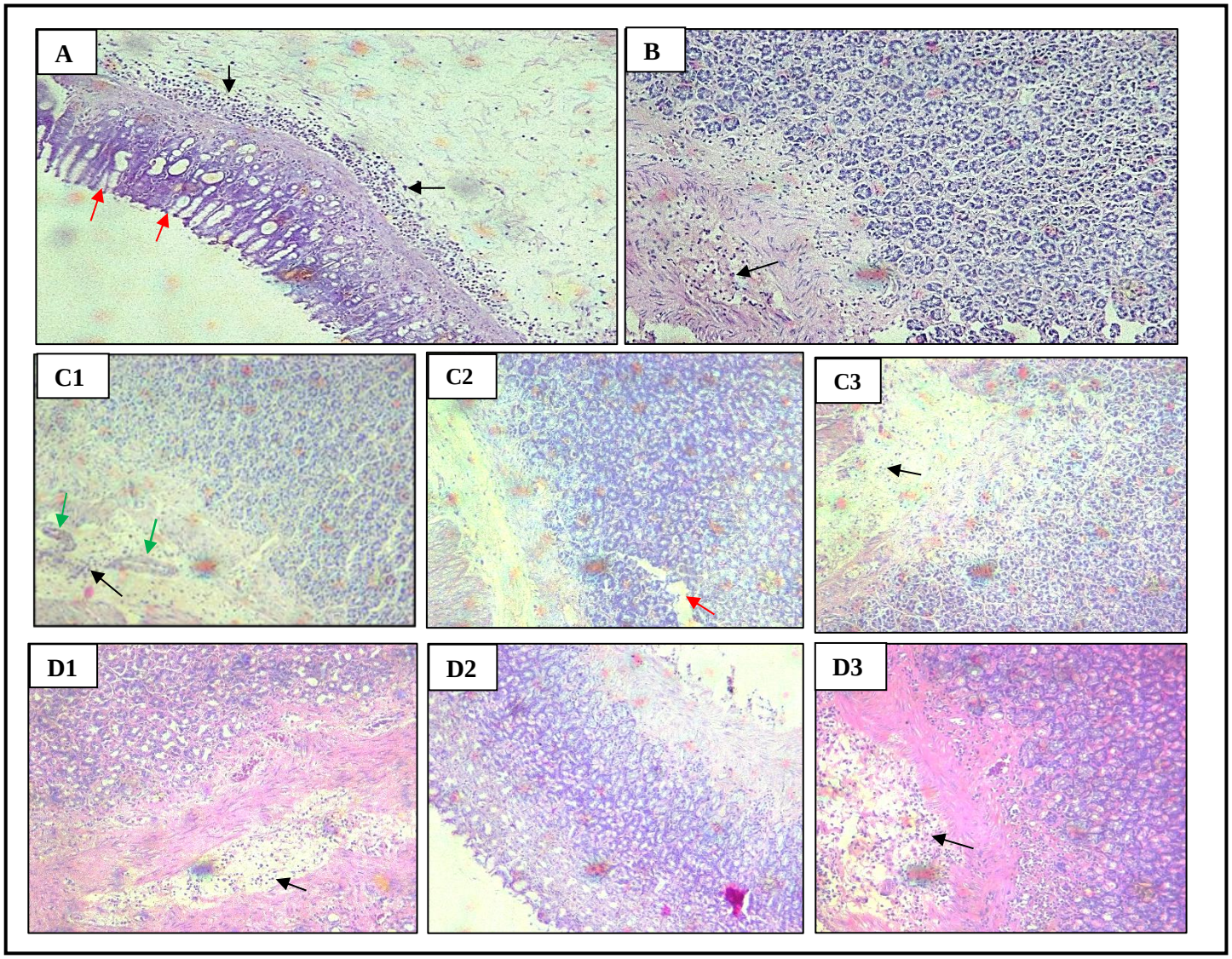

Figure 32. Effects of DE and HME on the histopathological appearance of the gastric mucosa.

(A) The group pre-treated with NaCl 0,9% vehicle as negative control. (B) The group pretreated with ranitidine (5 mg/kg) as positive control. (C1, C2 and C3): The group pre-treated with DE (125, 250 and 500 mg/kg, respectively). DE: Decocted Extract. (D1, D2 and D3): The group pre-treated with HME (125, 250 and 500 mg/kg, respectively). HME: Hydromethanol extract. Red arrow: indicate necrosis. Bleue arrow: indicate the congestion of blood vessels. Black arrow: indicate the inflammatory cell infiltration.

4.2.2.2 Macroscopic and histopathological examination of DE mechanisms effect on ethanol-induced gastric mucosa damage in rats

a) Macroscopic examination

As shown in figure 33, after the administration of different pharmacological substances to assess the mechanism of action of DE on gastric ulcer healing**.** The results of experiments demonstrated that rats pretreated with the Arg in the absence of DE slightly developed injuries, while in presence of extract, these damages were significantly attenuated. In the presence of

LNNA (Nos inhibitor) and in absence of the extract, the stomach tissue exhibited more severe injuries. However, following pre-treatment with the extract, these injuries were partially reduced. For indomethacin, a notable ulceration is observed in the case of its administration alone. However, in the presence of the DE, tissue damage was partially moderated. In a similar case, the observed damage to the rat tissue were severe in the presence of yohimbine (an alpha-2 adrenergic receptors antagonist) in absence of DE. However, after pre-treatment of the rats with the extract (DE), the severity of these lesions was slightly reduced. Globaly, in comparison with negative control (rats pretreated with CMC), the DE(DE) had significantly reduced the stomach tissue damages in rats in presence of Arg and slightly ameliorated in presence of LNNA, indomethacin and yohimbine at different degrees.

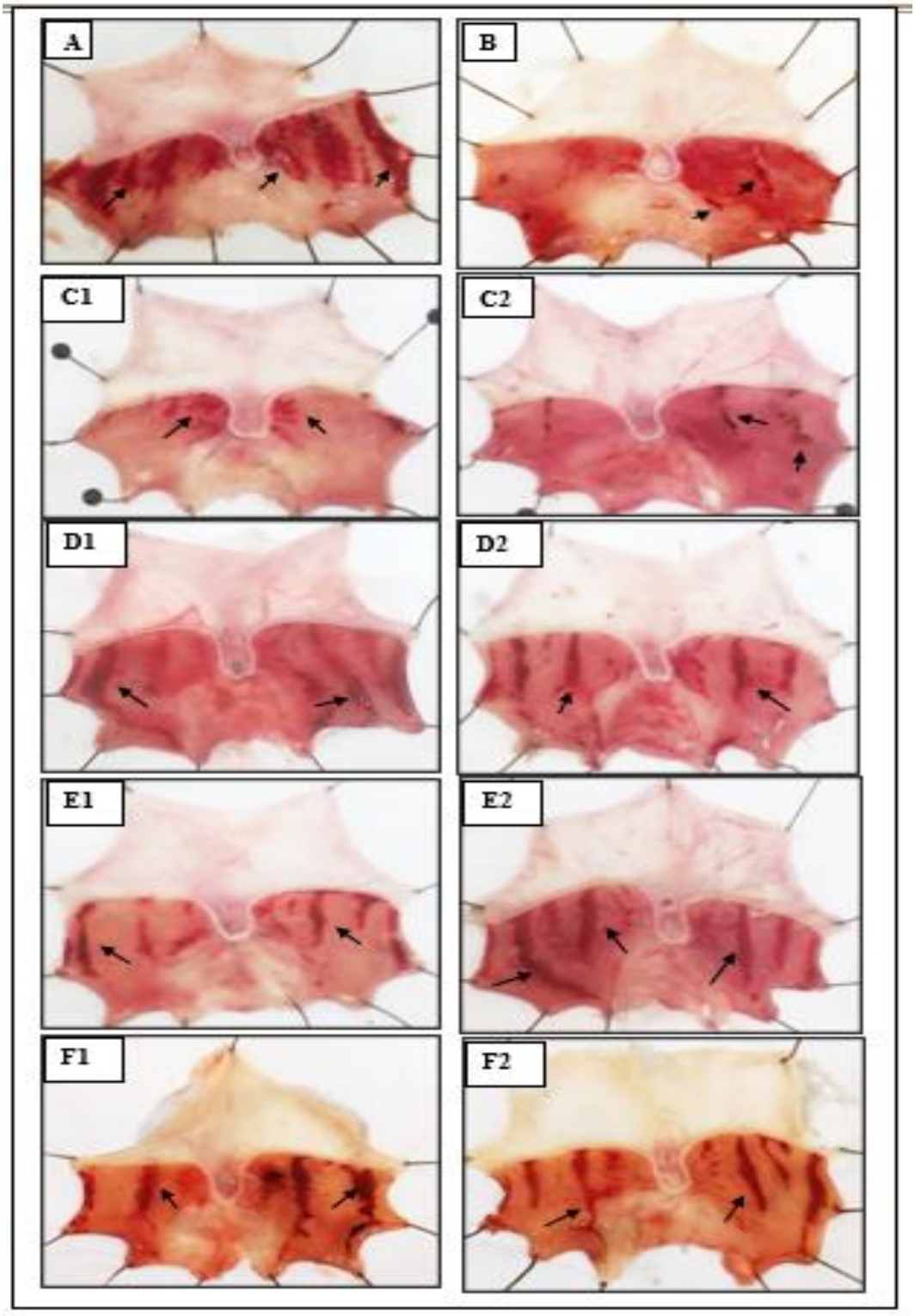

Figure 33. Effect of DE in absence or presence of different pharmacological substances on the macroscopic appearance of the gastric mucosa in ethanol-induced gastric mucosal lesions in rats.

(A) The group pre-treated with Nacl 0,9% vehicle as negative control. (B) The group pre-treated with DE (250 mg/kg). (C1, D1, E1 and F1): The group pre-treated with arginine, L-NNA, indomethacin or yohimbine in absence of DE). (C2, D2, E2 and F2): The group pre-treated with L-arginine, L-NNA, indomethacin or yohimbine in presence of DE. **DE**: Decocted extact. Black arrow: elongated bands of hemorrhagic lesions.

b) Histopathological examination

The histopathological findings of ethanol-induced rat's stomach tissue revealed diverse findings across several experimental circumstances including the administration of different pharmacological agents in presence and in absence of extract. Figure 35 C1, shows that rats pretreated with Arg in the absence of DE exhibited minor modifications such as edema, inflammatory cell infiltration, and blood vessel congestion in the submucosa. These alterations, however, were significantly attenuated in the presence of the DE extract (Figure 34 C2).

Furthermore, when L-NNA, a NO_S inhibitor, was introduced, the stomach tissue displayed more severe manifestations (Figure 34 D1). Nevertheless, following pre-treatment with the DE, these histopathologial factors exhibited partial amelioration showing a moderate inflammation (Figure 34 D2).

A major ulceration component was seen in a second experiment involving the injection of indomethacin alone, demonstrating obvious swelling and distortion of the tissue structure, inflammatory cell infiltration, and congestion of blood vessels in the submucosa (Figure 34 E1). Tissue changes were partially reduced when the DE was present (Figure 34 E2). A similar pattern appeared in the case of yohimbine, in presence and in absence of extract (Figure 34 F1 and F2, respectively).

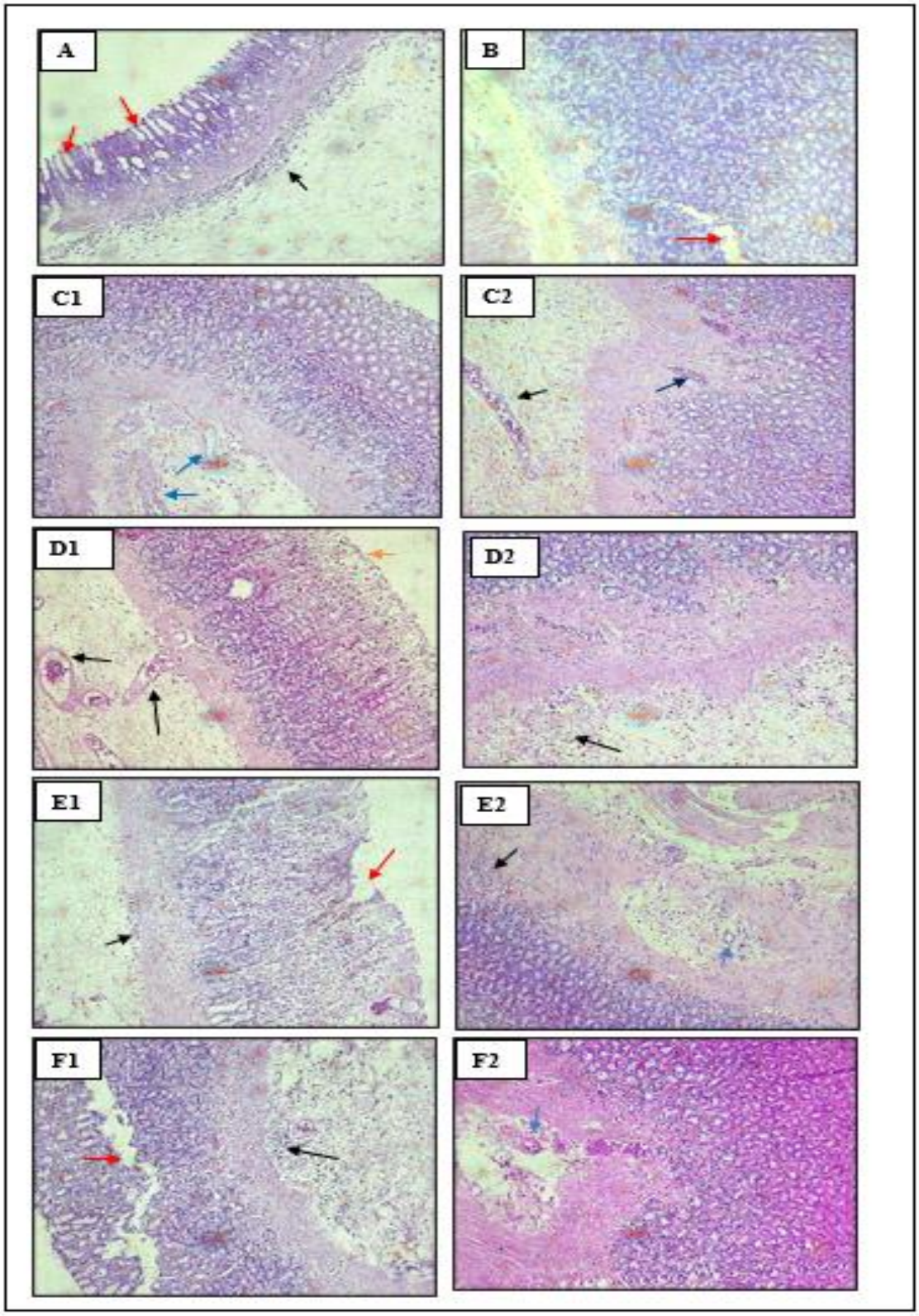

Figure 34. Effect of DE in absence or presence of different pharmacological substances on the histopathological appearance of the gastric mucosa in ethanol-induced gastric mucosal lesions in rats.

(A)The group pre-treated with Nacl 0.9% vehicle as negative control. (B) The group pre-treated with DE (250 mg/kg). (C1, D1, E1 and F1): The group pre-treated with arginine, L-NNA, indomethacin or yohimbine, respectively in absence of DE. (C2, D2, E2 and F2): The group pre-treated with arginine, L-NNA, indomethacin or yohimbine, respectively in presence of DE. Red arrow: indicate necrosis. Bleue arrow: indicate the congestion of blood vessels. Black arrow: indicate the inflammatory cell infiltration.

4.2.2.3 Macroscopic and histopathological examination of HME mechanisms effect on ethanol-induced gastric mucosa damage in rats

a) Macroscopic examination

As demonstrated, the animals that received 100% ethanol developed a consistent macroscopic damage which was evidenced by a loss of normal colour and mucus along with the presence of petechiae, haemorrhage and oedema. In an extremely unique and specialized case, the administration of HME resulted in a significant reduction of all observable tissue damages, approaching complete healing. The effect was detected in the presence of Arg, L-NNA, indomethacin or yohimbine, four different experimental circumstances caused severe tissue damages in absence of HME at different degrees (Figure 35). The ability of HME to reduce and potentially facilitate tissue recovery against consistent macroscopic damage induced by ethanol indicates its extraordinary therapeutic potential.

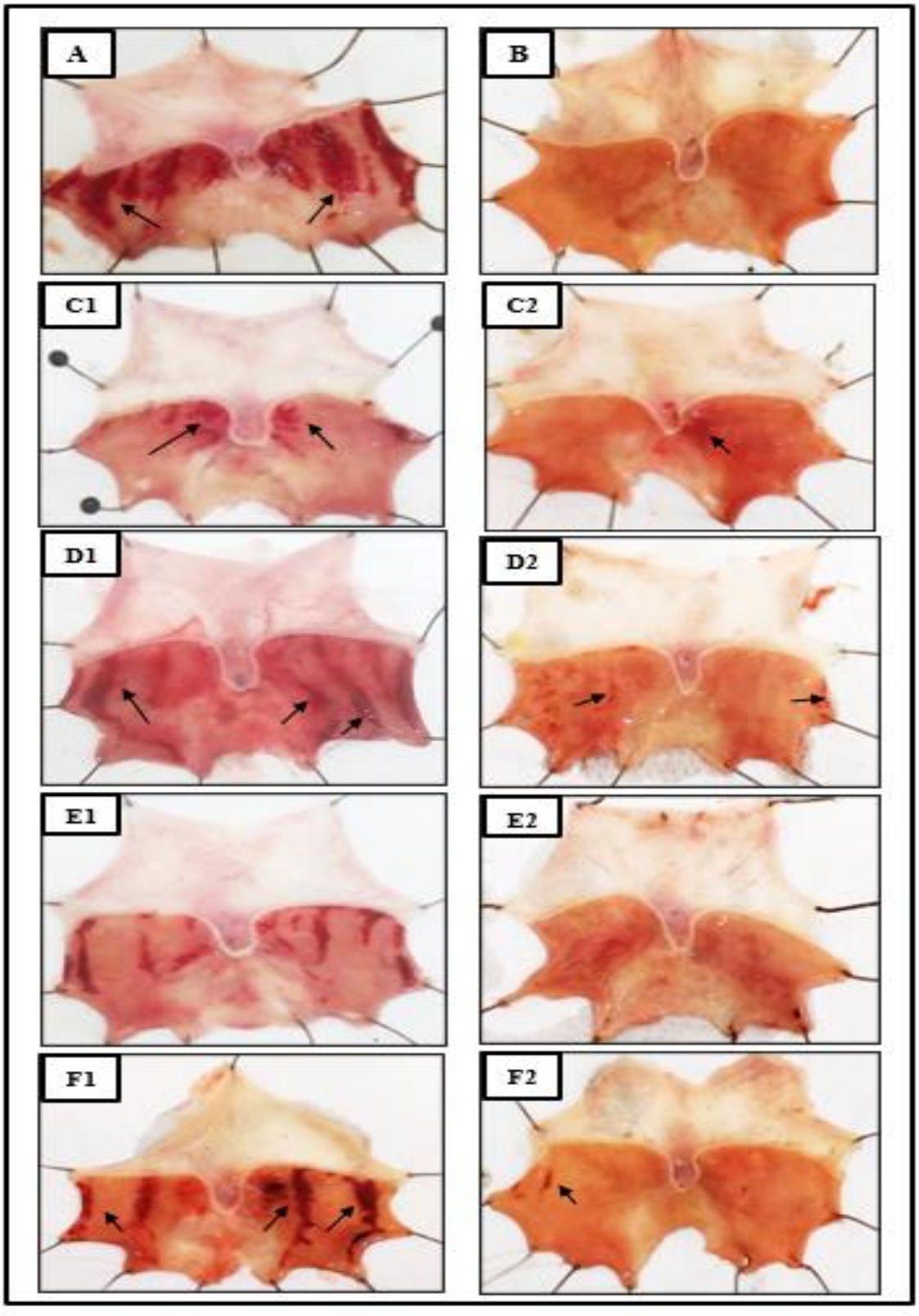

Figure 35. Effect of HME in absence or presence of different pharmacological substances on the macroscopic appearance of the gastric mucosa in ethanol-induced gastric mucosal lesions in rats.

(A)The group pre-treated with Nacl 0,9% vehicle as negative control. (B) The group pre-treated with DE (250 mg/kg). (C1, D1, E1 and F1): The group pre-treated with arginine, L-NNA, indomethacin or yohimbine in absence of HME). (C2, D2, E2 and F2): The group pre-treated with arginine, L-NNA, indomethacin or yohimbine in presence of HME. **HME**: hydromethanol extact. Black arrow: elongated bands of hemorrhagic lesions.

b) Histopathological examination

The assay revealed that rats pretreated with arginine in the absence of DE exhibited minor modifications such as edema, inflammatory cell infiltration, and blood vessel congestion in the submucosa (Figure 36 C1). Those alterations, were significantly attenuated in the presence of the DE extract (Figure 36 C2). Furthermore, when LNNA was introduced, the stomach tissue displayed more severe manifestations showing a severe inflammation and a remarquably congestion of blood vessels (Figure 36 D1). Nevertheless, following pre-treatment with the DE extract, these histopathologial factors exhibited partial amelioration (Figure 36 D2).

A major ulceration component was seen in a second experiment involving the injection of indomethacin alone (Figure 36 E1), demonstrating obvious swelling, necrosis, distortion of the tissue structure and inflammatory cell infiltration. Tissue changes were partially reduced when the DE extract was present (Figure 36 E2). A similar manifestation appeared in the case of yohimbine, showing a moderate inflammation after the tratement of rats with HME (Figure 36 F2).

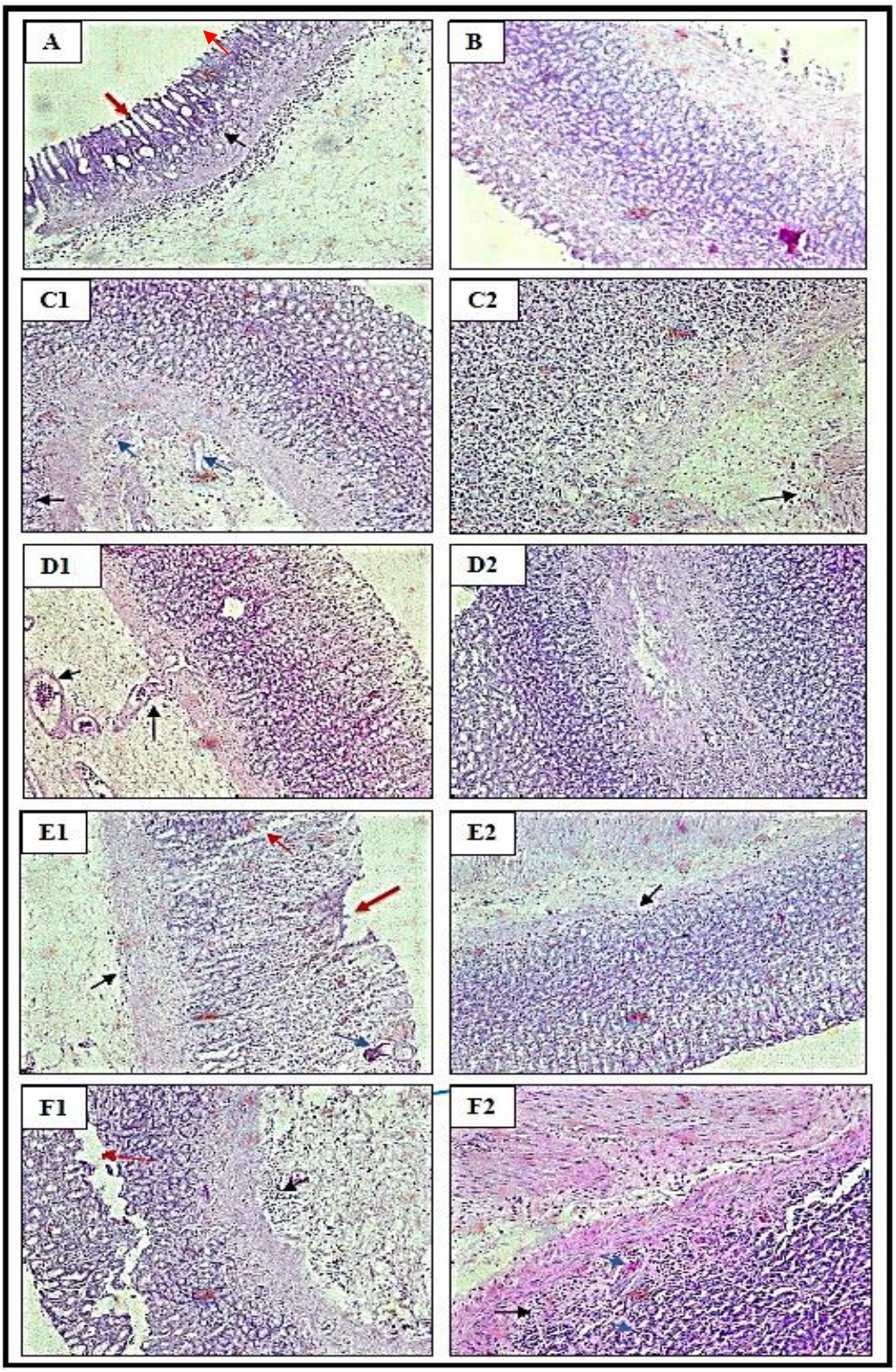

Figure 36. Effect of HME in absence or presence of different pharmacological substances on the histopathological appearance of the gastric mucosa in ethanol-induced gastric mucosal lesions in rats.

HME; Hydromethanol extract, (**A**) The group pre-treated with Nacl 0,9% vehicle as negative control. (**B**) The group pre-treated with HME (250 mg/kg). (C1, D1, E1 and F1): The group pre-treated with arginine, L-NNA, indomethacine or yohimbine in absence of HME. (C2, D2, E2 and F2): The group pre-treated with arginine, L-NNA, indomethacin or yohimbine in presence of HME. Red arrow: indicate necrosis. Bleue arrow: indicate the congestion of blood vessels. Black arrow: indicate the inflammatory cell infiltration.

4.2.2.4 Gastroprotective effect of DE and HME

Intragastric administration of 100% ethanol to untreated rats served as a negative control resulted in widespread bandlike hemorrhagic erosions in the glandular stomach and the extent of protection was remarkably limited expressed a high ulcer index of (34.54 ± 2.26%). While, pretreatment with DE at the assessed doses (125, 250, and 500 mg/kg) provided increasing degrees of protection to the mucosa from all such ethanol-induced damages 10.89 ± 3.25%, 3.96 ± 1.85%, and 2.20 ± 1.57%, respectively.

Oral administration of 125, 250 and 500 mg/kg of HME induced a maximale protection marking an uncountable minimal ulcer index of 0.61 ± 0.55 %, 0.37 ± 0.47% and 0.17 ± 0.25 %, respectively. These values were significantly lower than that of ranitidine 5 mg/kg (10.05 ± 0.57%. P≤0.0001) (Figure 37). No significant difference is recorded by comparing these three highly protective doses (P>0,1).

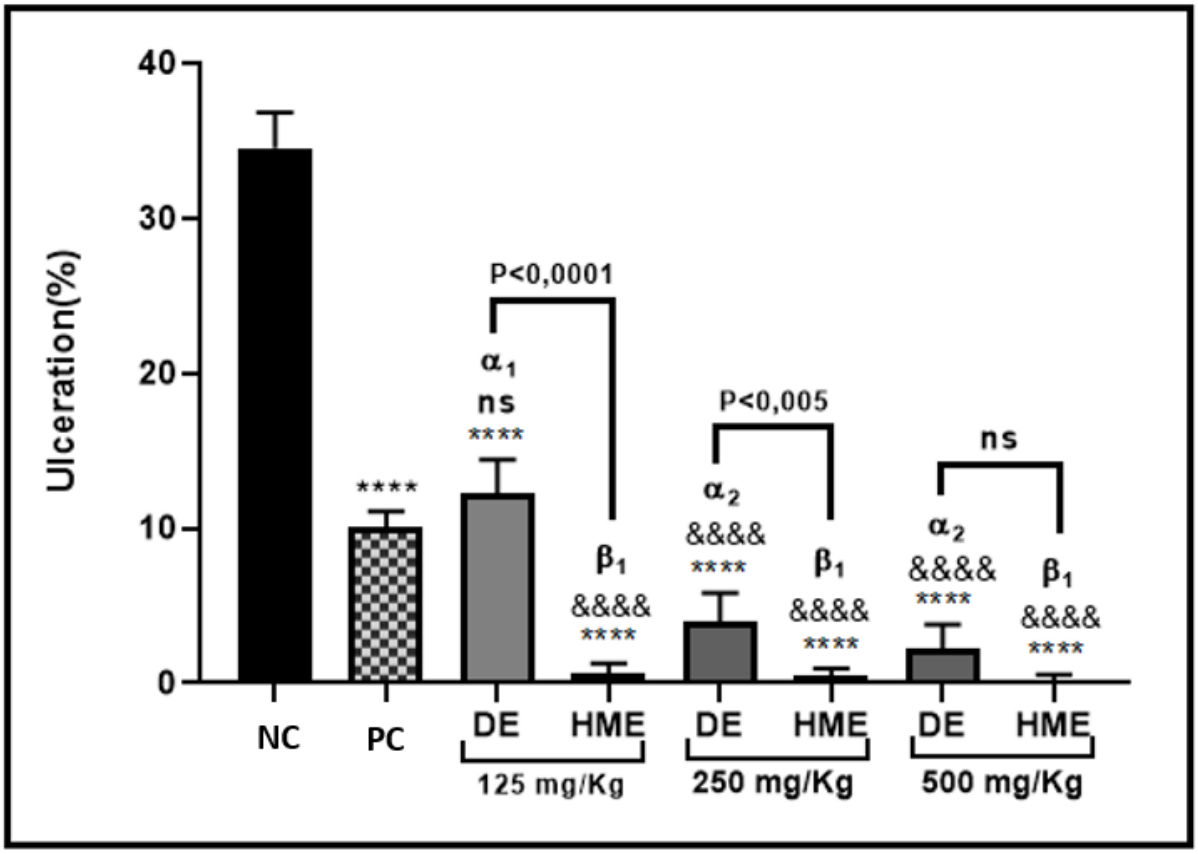

Figure 37. Effects of DE and HME on gastric ulcer in rats.
DE; Decocted extract. **NC**: Negative Control, **PC**: Positive Control. Bars represent means±SEM (n=8). **; P≤0.01, ***; P≤0.001, ****; P≤0.0001 vs vehicle as negative control. Ns: no significant difference (P>0.05), &&&&; P≤0.0001 vs Positive control. Columns carrying different letters are significantly different at P<0.0001.

4.2.2.5 Mechanisms of the DE on gastroprotective effect

Figue 38 showed the results of the series of experiments carried out in order to identify the potential mechanism (s) inderlying in the gastroprotective effect of the DEfrom *T. hirsuta*

areal parts showed that L-Arg, L-NNA, indomethacin or yohimbine administered in presence and in absence of extract at a dose of 250 mg/kg.

The percentage of ulceration marked when administering the hydromethanol extract alone or in presence of each one of pharmacological agents used was significantly reduced expressing a high protection against damages induced by ethanol compared to negative control in acceptance of the case of L-NNA alone. However, in presence of Arg, DE showed no significant difference effect (4.59 ± 1.66%) compared to DE alone (3.96 ± 1.85%, P>0,1). Contrary, rats pretreated with L-NNA followed by extract had a very highly significantly increased gastric ulcer than to those treated only with extract (13.38 ± 2.66%; P≤0.0001). On the other hand, DE in the presence of indomethacin, had significant increase on gastric ulcer compared to DE alone (20.90 ± 3.94%; P≤0.0001). In the same line, yohimbine had provided a significant protection to the mucosa against damages caused by ethanol (16.76 ± 2.11%; P≤0.0001) compared to NaCl vehicle treated animals, and no significant difference was reported in the presence of DE (17.75 ± 2.56%; P≤0.0001).

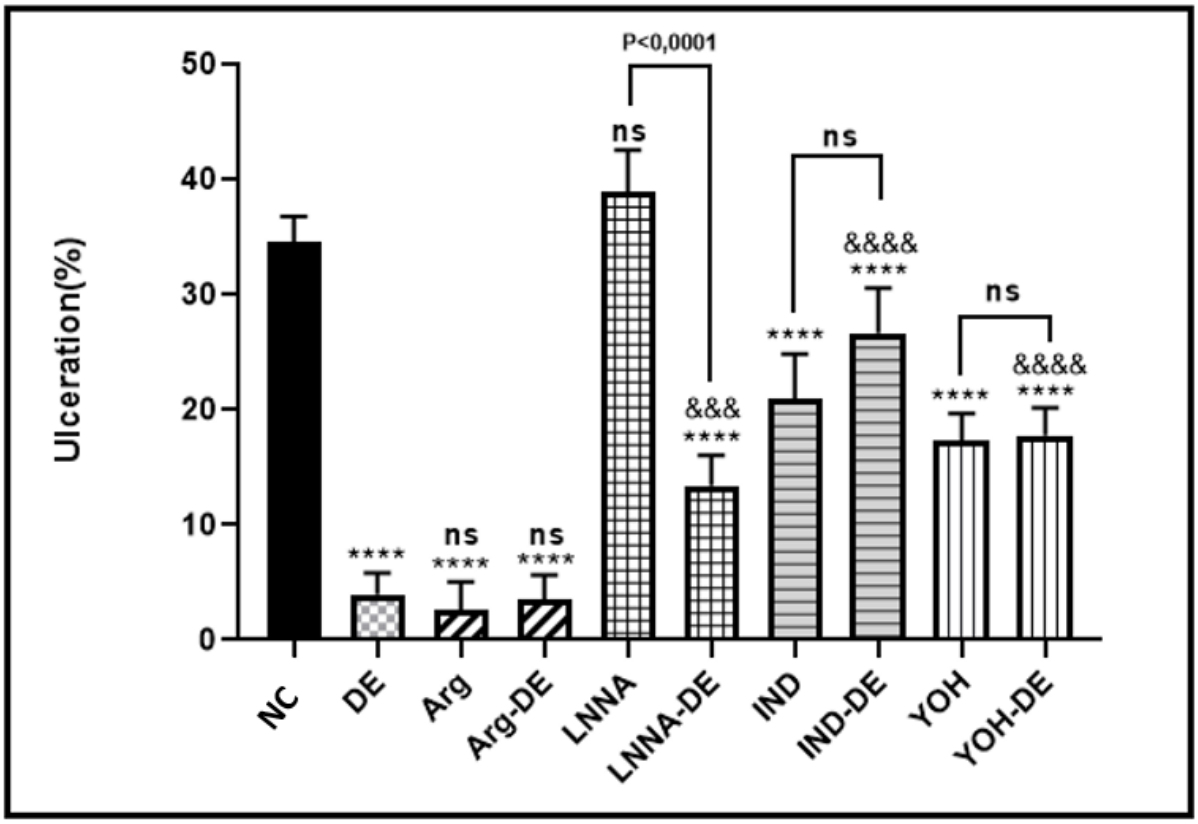

Figure 38. Effect of DE in absence and in presence of L-Arg, L-NNA, indomethacin or yohimbine on gastric ulcer in ethanol-induced gastric mucosal lesions in rats

DE: DE(at dose of 250 mg/Kg); NC: negative control. The values of the bars chart are expressed as means ± SEM (n=6-8). (****; P≤0.0001) *vs* vehicle as negative control. ns; no significant difference (&&&&*P* ≤ 0.0001, &&&*P* ≤0.001) *vs* DE; P≤0.0001in comparison to both effects of DE in absence and in presence of L-Arg, L-NNA, indomethacin or yohimbine, respectively.

4.2.2.6 Mechanisms of the HME on gastroprotective effect

The results of the administration of different pharmacological agents and the evaluation of HME effect and its mechanism of action have been presented in the figure 39 of which, the HME, in the presence of each of the pharmacological agents showed very high protection against gastric ulcer induced by ethanol, expressed by a very low percentages of ulceration with significantly high difference in comparison to negative control. Those activities of the HME in presence of L-Arg, L-NNA, indomethacin or yohimbine (0.085 ± 0.04%; 1.91 ± 1.18% and 0.13 ± 0.22%; 1.55 ± 2.14 %, respectively) were comparable to that provided by HME alone (0.47 ± 0.37%; P> 0,1).

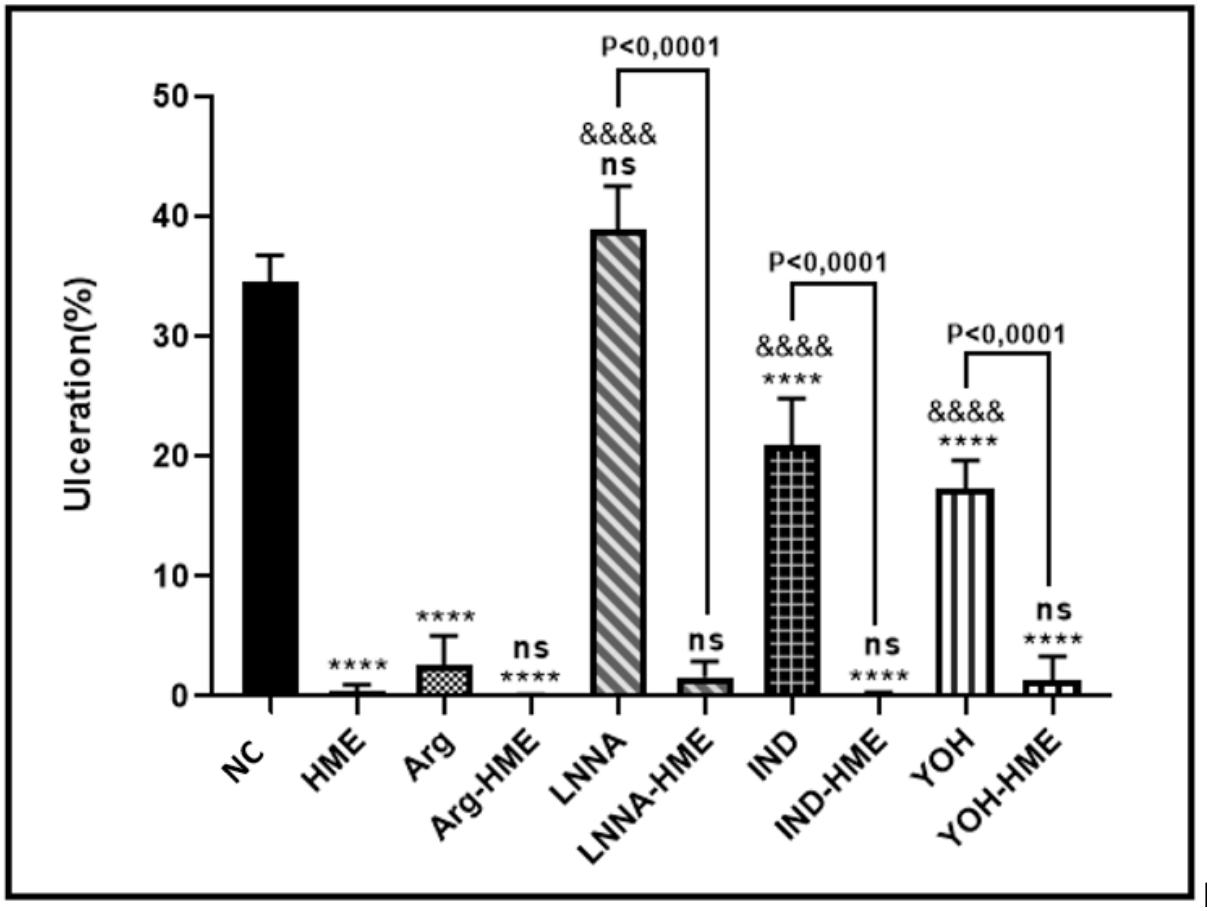

Figure 39. Effect of HME in absence and in presence of L-Arg, L-NNA, indomethacin or yohimbine on gastric ulcer in ethanol-induced gastric mucosal lesions in rats.

HME: Hydromethanol extract (at dose of 250 mg/Kg); NC: negative Control. The values of the bars chart are expressed as means ± SEM (n=6-8). (****; P≤0.0001) vs vehicle as negative control. Ns; no significant difference (&&&&*P* ≤ 0.0001) *vs* DE; in comparison to both effects of HME in absence and in presence of L-Arg, L-NNA, indomethacin or yohimbine, respectively.

4.2.2.7 Determination of mucus in gastric layer

The effects of DE and HME on gastric mucus content are shown in figure 40. Both of extracts had a negligible effect on preserving the gastric mucus content, showing comparable amounts to that exhibited by the negative control (62.85 ± 3.4 µg of alcian blue/ g of wet tissue; P>0.05). The DE, at doses of 125, 250, and 500 mg/kg, demonstrated mucus contents of 46.81 ± 4.1; 66.60 ± 3.5; 76.46 ± 4.5 µg of alcian blue/ g of wet tissue; respectively). Similarly, HME, administered at the same doses, showed mucus contents of 53.09 ± 2.20 ;56.00 ± 1.64; 64.34 ± 2.54 µg of alcian blue/g of wet tissue; respectively. Notably, all of these rates observed with both extracts were lower than those observed with the positive control (62.85 ± 3.4 µg of alcian blue/ g of wet tissue; P>0.05).

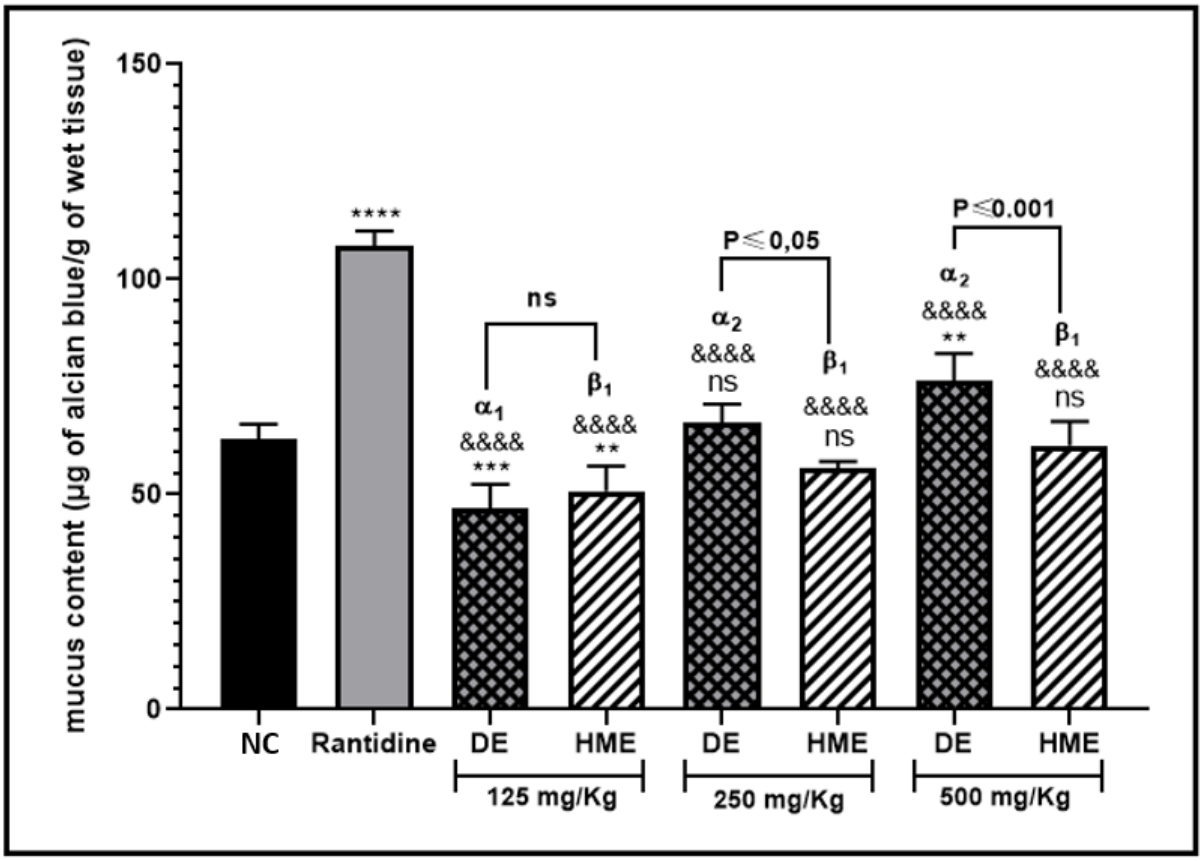

Figure 40. Effects of DE and HME on gastric mucus content in ethanol-induced gastric mucosal lesions in rats.

DE; Decocted extract, **HME**; Hydromethanol extract, NC: Negative Control. Bars represent means±SEM (n=8). *; P≤0.05, ***; P≤0.001 and ****; P≤0.0001 vs vehicle as negative control. Ns; no significant difference (P>0.05), &&&&; P≤0.0001 vs ranitidine as positive control. Ns; no significant difference (P>0.05) in comparison between the effects of DE and HME at different doses (125, 250, 500 mg/kg). Columns carrying different letters are significantly differentat P<0.0001.

4.2.2.8 Evaluation of *in vivo* antioxidant activity in gastric homogenate

The evaluation of the antioxidant effect of extracts on the stomach wall was conducted by assessing several key parameters within gastric homogenate, this assessment aimed to understand the potential protective properties of the extracts against oxidative stress and damage, this evaluation was conducted by the determination of CAT activity, examination of GSH level, the concentration of MDA and measurement of the total protein content within the stomach tissue. Results are represented in table 6.

4.2.2.8.1 Estimation of catalase activity

Catalase, a crucial enzyme responsible for neutralizing harmful hydrogen peroxide in the body. At a dose of 250 mg/kg of DE, a remarkable enhancement in CAT activity was observed at the minimal dose of 125 mg/kg, as well as the maximal dose of 500 mg/kg. However, the CAT activity was significantly lower at the maximal dose of 500 mg/kg compared to the dose of 250 mg/kg, where it demonstrated the highest activity. In comparison with negative control all doses had significantly improving CAT activity ($P \leq 0.0001$). In the same line, HME at ascendant doses (125, 250 and 500 mg/kg) marked a significant enhancement in CAT activity compared to both negative and positive control ($P \leq 0.0001$).

4.2.2.8.2 Estimation of glutathione level

The *in vivo* assessment of antioxidant activity conducted on extracts, specifically by evaluating glutathione (GSH) levels revealed that both DE and HME exhibit an increase in GSH levels across all doses compared to negative control ($P > 0,0001$). In fact, GSH levels were found to be highly stronger than those observed in positive control groups ($P > 0,0001$). These findings suggest that neither DE nor HME had a significant impact on elevating GSH levels, indicating a limited antioxidant effect compared to the controls.

4.2.2.8.3 Estimation of gastric total proteins content

The assessment of gastric homogenate's total protein content revealed a significant increase at the highest dosage of 500 mg/kg ($P > 0,0001$). At the lower doses of 125 mg/kg and 250 mg/kg, the protein content remained comparable to that of the negative control group. Conversely, HME demonstrated a substantial and statistically significant rise in total protein content, surpassing the negative control ($P \leq 0.01$) and approaching levels akin to the positive control ($P > 0.05$).

4.2.2.8.4 Estimation of lipid peroxidation

As shown in table 06, DE exhibited a substantial effect by significantly reducing MDA levels in a dose-dependent manner (P>0,0001). Strikingly, as the dosage increased, MDA levels decreased progressively, suggesting a potential protective effect against lipid peroxidation significantly greater than ranitidine, serving as the positive control. Conversely, HME failed to demonstrate any significant potential in decreasing MDA levels, as they remained comparable to the negative control group that received NaCl pretreatment (P>0.05).

Table 6. Effects of DE and HME on CAT activity, GSH level, total protein level, MDA level of stomach tissue in ethanol induced gastric mucosal lesions in rats.

		Extracts doses			Vehicle	Ranitidine
		125mg/kg	250mg/kg	500mg/kg		
Catalase activity (nmol/min/mg)	DE	13.46±1.5$^{***ans\ \alpha}$	25.83±2.9$^{****A\beta}$	14.74±1.8$^{***ans\ \alpha}$	2.99±0,05	8.65±0.93****
	HME	31.39±1.2$^{****A\ \alpha}$	33.13±3.6$^{****A\ \alpha}$	39.03±4.25$^{****A\ \beta}$		
GSH level (nmol/g tissue)	DE	10.66±1.71$^{***A\ \alpha}$	26.34±3.7$^{****A\beta}$	34.31±1.30$^{****A\ \delta}$	2.93±0.19	5.82±0.49****
	HME	17.15±2.6$^{****A\ \alpha}$	20.83±1.8$^{****A\ \alpha}$	18.62±3.2$^{****A\ \alpha}$		
Gastric MDA level (nmol/g tissue)	DE	6.65±2.9$^{****ans\ \alpha}$	4.72±0.6$^{****ans\ \alpha}$	2.14±0.32$^{****D\ \alpha}$	13.16±1,04	5.22±1.12****
	HME	13.32±4.3$^{ns\ A\ \alpha}$	10.72±1.8$^{ns\ D\ \alpha}$	9.67±0.80$^{ns\ ans\ \alpha}$		
Total proteins (mg/mL)	DE	2.06±0.01$^{*A\ \alpha}$	2.54±0.37$^{ns\ A\ \alpha}$	3.84±0.06$^{****\ ans\ \alpha}$	2.99±0,55	4.42±0.93****
	HME	3.5±0.06$^{**ans\ \alpha}$	3.54±0.26$^{**ans\ \alpha}$	3.54±0.09$^{**ans\ \beta}$		

DE; Decocted extract, **HME**; Hydromethanol extract. Bars represent means±SEM (n=6-8). Ns; no significant difference (P>0.05), *; P≤0.05, **; P≤0.01, ***; P≤0.001, ****; P≤0.0001 vs vehicle as negative control. Ans; no significant difference (P>0.05), D; P≤0.05, C; P≤0,01 B; P≤0.001, A; P≤0.0001 vs Ranitidine as positive control. Columns carrying different letters are significantly differentat P<0.0001 in comparison between the effects of different doses of the same extract (125, 250, 500 mg/kg).

DISCUSSION

Ethnobotanical study

A significant majority of respondents reporting the usage of *T. hirsuta* for various medical conditions across different biological systems, including a notable emphasis on the application of *T. hirsuta* in addressing disorders of the digestive tract, gastric ulcer, disorders of glands associated with the digestive system, anti-constipation, against intestinal worms also its uses in addressing respiratory, urinary, and nervous system-related issues, aligns with findings highlighted by previous studies such as those conducted by Borris *et al.* (1988) and Lev (2002) reporting that the plant was recommended as a remedy for hemorrhoids, stomach issues, intestinal problms, asthma, coughing and sexual diseases. In Moroccan traditional medicine, *T. hirsuta* found extensive use, with the aerial parts specifically employed as both a purgative and laxative, as documented by Bellakhdar (1997) and Bellakhdar *et al*. (1991).

According to the results, the prevalent method in traditional medicine, and the specific focus on treating skin ulcers were ‘’Poultices’’ underscores the perceived therapeutic value of *T. hirsuta* for dermatological concerns. As reported by Lev (2002), *T. hirsuta* was used to treat skin diseases in the middle east.

The wide use of *T. hirsuta* on reduction of blood sugar included in our study aligns with traditional medicinal knowledge documented by Alami *et al*. (2015) and Yabrir *et al*. (2018), where both leaves and seeds have been historically employed as remedies for diabetes.

The use of *T. hirsuta* on hair loss were predominantly cited by formants, and previously reported by other ethnobotanical studies. The highlighting of these results was conducted to demonstrate the traditional uses of *T. hirsuta* and the importance of reffering to the folk medicine.

Extraction yields and determination of total phenolic, flavonoid and tannins contents

Several techniques and different solvents have been applied to extract the bioactive components from *T. hirsuta,* few of them focuced on the effect of solvent polarity and extraction techniques used. In the present study, the chosen solvents were 100% methanol, 50% methanol and 100% water with polarity index values of 6.6, 7.8 and 9, respectively (Kumoro *et al*., 2006) in order to highlight the effect of solvent polarity for the extraction of bioactive components from *T. hirsuta,* and its antioxidant activity.

A slow variability of extraction yields was detected. Where, the aqueous DE of *T. hirsuta* showed the lowest yield with 11.6% in comparison with the HME and ME obtained with maceration method. This difference can be explained by the effect of temperature in the

breakdown of cellular constituents during the decoction process (Hamrouni *et al*., 2012), since some bioactive compounds are extremely sensitive to oxygen and heat (Ishida *et al*., 2005).

The highest extraction yield was recovered by absolute methanol (12.8%), followed by HME at 50% methanol (12.05%). A similar remarque was marked by Yahyaoui *et al.*, (2018) in different conditions of temperature and duration of extraction with Tunisian genus of *T. hirsuta*. Such variation can be explained by difference of polarity's degree, where water the most polar solvent showed the minutest yield then the subsolvant of water/methanol mixture, arriving to absolute methanol with low polarity. These results showed that the increasement of solvent polarity with water addition induce a decrease in extraction yield. This is consistent with what has been found in previous studies (Yahyaoui *et al*, 2018; Djermane *et al.*, 2020).

Flavonoids and phenolic compounds are reported to be natural antioxidants (Shahidi *et al*., 2015). The antioxidant activity of polyphenols is mostly owing to their redox characteristics, which allow them to function as reducing agents, hydrogen donors, singlet oxygen quenchers, and metal chelators (Javanmardi *et al*., 2003). The mechanisms of action of flavonoids are exerted through scavenging or chelating process (Schmitt-Schillig *et al*., 2005). *T. hirsuta* was shown to contain flavonoids and polyphenols in several studies (Trigui *et al.*, 2014; Kristanti *et al*., 2018; Djermane *et al*., 2020).

The present study revealed that all extracts were rich in polyphenols, values were higher than those cited by Yahiaoui *et al*. (2020) regarding ME and HME. This difference may be due to extraction techniques conditions. Our results were slightly less than those reported by Djermane *et al*. (2020), with aqueous and HME, this variance can be explained by temperature effect on chemical compound and /or methanol proportion. A considerable total flavonoid content has been found in *T. hirsuta* areal part in all extracts. These findings are in accordance with those reported by Amari *et al*. (2014) showing an acceptable flavonoid content in the diffrent areal parts of *T. hirsuta.* Additionally, Trigui *et al.* (2014) demonstrated a higher flavonoid content by treatement of the areal parts of *T. hirsuta* with solvents with increasing polarity.

Overall, the absolute methanolic extract had the highest total content of both phenolic and flavonoid compounds, followed by HME and DE. Such variation can be explained by the simple fact that the chemical composition varied considerably according to the solvent polarities. Such differences have been reported in the littérature by Javaprakasha *et al*. (2001). Lesjak *et al*. (2011) indicated that TPC is affected by the solvent type and polarity. In the same line, Do *et al*. (2014) declared that the TFC level in extracts is influenced by solvent type and polarity index, plant species, and plant parts used.

Tannins are high molecular weight polyphenolic compounds which can be divided into condensed tannins and hydrolysable tannins (Mohanty *et al*., 2013). The quantification of total tannins in the different extracts revealed variations in content. ME demonstrated the highest tannin levels, followed by HME, while DE exhibited the lowest content. These results suggest that the choice of extraction solvent, and extraction methods significantly influences tannin extraction efficiency. Yahiaoui *et al., (2018)* found the highest amount of tannins in medium to polar extracts while the lowest content was obtained by the non-polar extract from *T. hirsuta*.

Arapitsas (2012) found that polar organic solvents demonstrated higher efficacy in extracting tannins compared to non-polar solvents. This can be attributed to the increased propensity of large molecular weight polyphenols to conjugate with proteins and form water-soluble tannin-protein complexes. Aditionnaly, Zhang (2015) had previously recommended polar solvent, specially, methanol to extract condensed tannin from walnuts green husk.

In vitro antioxidant activities of decocted, hydromethanolic and methanolic extracts

From the previous results, ME with the highest amount of total phenolics and flavonoids was the stronger radical scavenger of ABTS radical, followed by HME and DEwith decreasing in total phenols and flavonoids content, respectively. These findings, were comparable to those reported by Amari *et al*. (2014) with different areal parts of *T. hirsuta* and also to those described by Yahiaoui *et al.* (2018). Harvesting season and geographic region can also affect the phytochemical content of extracts, declared Ben Farhat *et al*. (2015)

From our results of method based on free radical scavenging, a highly significant difference was marked between DE and both HME and ME, whereas these lasts were nearly similar and not significantly different (P>0,5) marking a more powerful scavenging effect against the radical ABTS. Overall, these findings are in accordance with findings reported by Djermane *et al*. (2020). A similar conclusion was reached by Ioannou *et al.* (2015). A number of studies have found a link between free radical scavenging activity and total phenolic components (Zheng and Wang, 2001; Wangensteen *et al*., 2004; Kanatt *et al*., 2007; Sarikurkcu *et al*., 2008). One of the primary mechanisms of polyphenol action is electron donation, these compounds contain phenolic rings with hydroxyl groups, making them electron-rich compounds. They readily donate electrons to ABTS radicals, neutralizing them and converting them into stable molecules (Rice-Evans *et al*., 1997). Polyphenols can also act via hydrogen atom transfer, where they donate an hydrogen atom to neutralize ABTS radicals (Amorati *et al*., 2018). Supporting both ways hydrogen donation (HAT) or electron transfer (SET).

The reductive capacity of the extracts assessed by two different assays:

CUPRAC, an electron-transfer (ET)-based method, where, a redox reaction of the CUPRAC reagent with chain-breaking antioxidants form the neocoprine (Nc) chelate. The redox potential of the extracts in study were significantly different, DE had the lowest capacity to reduct the copper ions with a very low value in comparison with previous study by Djermane *et al.*, (2020) on aqueous maceration extract of *T. hirsuta*. This difference may be justified by the fact of temperature in the dielectric constant of water **(**Owe *et al.*, 1961; Dyer *et al.*, 2006; Carr *et al*, 2011). Where, increasing temperatures cause an overall decrease in the water interactions known as "polarity" (Zahra *et al.*, 2016). Wherease, ME and HME showed a considerable reduction ability of copper ions from CU(II) to CU(I). This result ties well with previous study, wherein HME at 70% of *T. hirsuta* showed remarquable reduction ability (Djermane *et al.*, 2016). This potential may be conducted by its richness on polyphenols, wich, particularly those with catechol (ortho-dihydroxy) groups in their structure, can act as reducing agents by donating an electron to Cu (II) ions, converting them into Cu(I) ions (Veskoukis *et al.*, 2012).

In the reducing power assay, the reduction of the Fe^{3+}/ferricyanide complex to its ferrous form is due to the presence of antioxidant reductants in the extracts and is monitored by measuring the formation of Perl's Prussian blue of ferrous form at a wavelength of 700 nm (Gülçin, 2006). The results of the present study clearly indicated that ME with the highest amount of phenolic compound was the strongest reductant one, which proved its richeness with compound with electron transfer ability, followed by HME and DE with a considerable potential. These studies also indicated sub solvent water-methanol in maceration to be a better solvent than water in extraction of these compounds. Contrary, Djermane *et al.* (2020), declared a higher reducing potential of aqueous macerated extract than the hydromethanolic extract at 70%. These results suggest that *T. hirsuta* areal parts are rich in antioxydants with high reductant activity.

Selected extracts: decocted and hydromethanolic crude extracts from *Thymelaea hirsuta* areal parts

HPLC-DAD analysis

The chemical profiles of DE and HME from *T hirsuta* aerial parts using HPLC-DAD revealed a distinct diversity wich can be explained by the fact of the difference in method and conditions of extraction and/or the solvent used; Rafińska *et al.*, (2019) found that the choice of solvent used in the extraction process significantly influences the chemical composition of the extract. where, water was found to be especially efficient in extracting polyphenolic compounds from *Lepidium sativum* (*L. sativum*) seeds. In the other hand, supercritical fluid extraction (SFE) with the addition of a moderately polar solvent, specifically 96% ethanol, was

effective in extracting flavonoids from the plant material. However, Our findings have led to a conclusion contrary to this outcome, it reported the presence of trans-cinamic and chlorogenic acid in both extracts with high levels in hydromethanolic one, it is a naturally occurring phenolic acid with a wide range of biological activity and minimal toxicity found in plants. This diversity can be explained by the effect of the biggest impacts come from climatic conditions, cultivation, fertilization and time of harvest as outlined by Arceusz *et al.*, (2013). Cinnamic acid derivatives are significant and promising molecules with a high potential for development into medications in the search for novel pharmacologically active chemicals. Numerous derivatives of cinnamic acid, particularly those containing the phenolic hydroxyl group, are well-known antioxidants with a host of health benefits attributed to their potent ability to scavenge free radicals (Sova, 2012). Yahiaoui *et al*. (2018) also reported the discovery of two derivatives of cinnamic acid; 3,4-dihydroxy-5-methoxycinnamic acid and dihydromyricetin 2,4-dihydroxycinnamic acid by HPLC-UV-Vis analysis in *T. hirsuta* areal parts; as well as the chlorogenic acid have been detected in hexane extracts in the same study.

Catechin, p-hydroxy benzoic acid, Caffeic acid, p-Coumaric acid, Ferulic acid were major phenolics reported predominantly in HME on our study. In their meticulous investigation of *T. hirsuta,* Trigui *et al*. (2013) corroborated the presence of the aforementioned phenolic compounds and also unveiled additional constituents, notably alphalinolenic acid, octadecanoic acid, ohydroxybenzoic acid and phydroxyphenylacetic acid.

Our investigations have revealed the presence of flavonoids ; rutin, luteolin and apigenin in both of extracts, several studies confirmed the presence of aforementioned flavonoids in thymelaea genus; *Soltani et al (2023)* noticed a smaller amount of rutin and a considerable quantity of luteolin and apigenin in *Thymelaea tartonraira* (L.), These flavonoids are known for their antioxidant qualities and potential cardiovascular benefits and may play a key role in reducing chronic disease risk (Boyer and Liu, 2004).

Coumarin and vanillin were identified in both extracts, DE and HME obtained from the aerial parts of *T. hirsuta*. This observation aligns with the findings of several other studies investigating the chemical composition of extracts from *T. hirsuta*. The presence of coumarin and vanillin in multiple solvent extracts underscores the robustness and consistency of these compounds within the plant material.

In the investigation of the studied extracts, the absence of pyrocatechol, 6,7-dihydroxy coumarin, syringic acid, taxifolin, ellagic acid, rosmarinic acid, myricetin, quercetin, hesperetin, kaempferol, and chrysin suggests a unique chemical profile for these extracts. The absence of these particular compounds could be attributed to various factors, including the

specific plant species, geographic location, extraction methods employed, or the maturity stage of the plant material. Those findings align with the results reported by Yahiaoui *et al.*, (2018) in their study on HPLC-UV-Vis analysis of different extracts obtained from the aerial parts of *T. hirsuta*, confirming the absence of the aforementioned compounds. When, Trigui *et al.*, (2014) detected the galic acid during the identification of phenolic acids in the ethyl acetate extract of *T. hirsuta* using a derivatization method followed by GC–MS analysis.

In vitro antioxidant activity of selected extracts

Antioxidant activity determined by DPPH scavenging assay

In the investigation of the antioxidant potential of *T. hirsuta* extracts, the study revealed compelling results, with HME demonstrating significant scavenging activity against the DPPH radical. DE also exhibited noteworthy antioxidant properties. These findings are consistent with previous research, such as a study conducted in the Kasserine region of Tunisia, where *T. hirsuta* from this area exhibited substantial antioxidant activity in both DPPH and ABTS assays (Yahyaoui *et al*., 2018). The exploration of various extraction methods unveiled that the methanol extract consistently displayed the highest antioxidant activity, as evidenced by its IC_{50} values ranging from 14.8 ± 0.35 to 16.55 ± 0.44 mg/l in the DPPH assay. This was followed by the ethanol extract (14.45 ± 0.957 to 37.9 § 0.62 mg/l), while acetate and hexane extracts showed IC_{50} values equal to or greater than 123 ± 8.4 mg/l. In the ABTS assay, ME again exhibited the strongest activity (6.4 ± 0.43 to 22.23 ± 1.62 mg/l), with the ethanolic extract following closely behind (18.43 ± 0.75 to 25.42 ± 0.40 mg/l) (Yahyaoui *et al*., 2018).

Moreover, Amari *et al*. (2014) explored the antioxidant activities of different aerial parts of *T. hirsuta* from the west of Algeria, finding that the extracts displayed concentration-dependent scavenging activity, with the flower showing the highest activity followed by the leaf and stem. The antioxidative properties were attributed to the phenolic hydroxyl groups present in the aerial parts of *T. hirsuta* (Amari *et al*., 2014). Additionally, Dahamna *et al*. (2015) reported that the aqueous extract of dried aerial parts of *T. microphylla* exhibited robust scavenging activity with an IC_{50} of 0.1 mg/ml, while the ethanolic extract had an IC_{50} of 0.2 mg/ml. Kerbab *et al*. (2015) investigated the free radical-scavenging activity of the hydroalcoholic extract and its fraction from *T. microphylla*, with the extract demonstrating an EC50 of 180.80 mg/ml and fraction VI showing an EC_{50} of 11.2 mg/mL, comparable to the well-known antioxidant α-tocopherol (EC_{50}= 10.1 mg/ml) (Dahamna *et al*., 2015; Amari *et al*., 2014). This potent capability on scavenging DPPH radical excerted by both DE and HME may be conffered by the diverse array of compounds identified in, including trans-cinnamic acid, chlorogenic acid, vanillin, catechin, p-hydroxy benzoic acid, caffeic acid, p-coumaric acid,

ferulic acid, rutin, coumarin, luteolin, and apigenin, as hydrogen donors. The hydrogen-donating properties of these compounds, facilitated by the presence of hydroxyl groups in their chemical structures, contribute to their ability to neutralize free radicals effectively (Halliwell and Gutteridge, 2007). Specifically, catechin, p-hydroxybenzoic acid, caffeic acid, p-coumaric acid, ferulic acid, rutin, luteolin, and apigenin, with their inherent hydrogen-donating capabilities, play a pivotal role in the antioxidant defense mechanisms of the extracts. This assertion aligns with established literature highlighting the antioxidant prowess of polyphenols such as catechin, emphasizing their significance as hydrogen donors (Scalbert *et al*., 2005). Furthermore, the hydrogen-donating role of p-hydroxybenzoic acid and other polyphenols in human nutrition underscores their contribution to antioxidant effects (Jaganath and Crozier, 2009). The studies on caffeic acid also underscore their potential as hydrogen donors, reinforcing their antioxidant capabilities and their role in scavenging free radicals (Clifford, 2000). Additionally, the presence of ferulic acid, rutin, luteolin, and apigenin in the extracts further supports their antioxidant effects by acting as hydrogen donors, as highlighted in the literature on bioavailability and bioefficacy (Manach *et al*., 2005; Cushnie & Lamb, 2005; D'Archivio *et al*., 2007)

This substantial difference in component concentration with nearly twice the quantity of crucial antioxidant components as revealed in HPLC-DAD results of our study can also highlights the enhanced antioxidant potential of HME, making it more effective in neutralizing free radicals in comparison with DE.

Antioxidant activity determined by ferrous ion chelating activity

The ferrous chelation activity assay provides valuable insights into the extract's ability to chelate or bind with ferrous ions, A crucial mechanism in neutralizing free radicals and preventing oxidative damage in the view that ferrous ions are the most powerful pro-oxidants among various species of transition metals present in the food system (Yomauchi *et al*., 1988; Hultin, 1994). To the best of our knowledge, it has not been previously explored on *T. hirsuta* plant extracts. The chelation ability of both extracts was observed to be relatively low wich reflects its composition lacks compounds that are highly conducive to binding with metal ions that are essential in inhibiting metal-catalyzed reactions that can contribute to the generation of harmful ROS. Despite the abundance of polyphenolic components in both extracts, a notable low ferrous activity was evident. This observation aligns with the findings of Wang *et al*., (2009) who reached a similar conclusion in their correlation study. Wang *et al*. (2009) conducted a quantitative correlation study, evaluating the relationship between TPC and antioxidant properties in both water and 70% acetone extracts. Their results supported our

observation, indicating that no correlation was found between chelation ability and TPC, suggesting that other components such as polysaccharides, proteins or peptides in the extracts were more effective chelators of ferrous ions than phenolic compounds (Wang *et al*., 2009). In contrast, other authors have claimed that Polyphenols obtained from brown seaweeds exhibit strong ferrous ion chelation properties (Senevirathne *et al*., 2006; Chew et al., 2008). The effectiveness of phenolic compounds in chelating metals depends on their distinctive phenolic structure, as well as the quantity and positioning of hydroxyl groups (Santoso, Yoshie-Stark, & Suzuki, 2004).

Antioxidant activity determined by Phenanthroline method

In the case of direct electron donation, another approach conducted for the first time in the genus of *T. hirsuta* is the use of the o-phenanthroline method to assess the ability of extracts to decrease the Fe^{3+} ion revealed also that HME exhibited the highest chelating activity, followed by DE. This difference can be attributed to the addition of methanol as a solvent wich is known to yield higher concentrations of bioactive compounds due to its ability to extract a broader spectrum of phytochemicals as seen in the phytochemical composition obtained by HPLC-DAD analysis, this higher extraction efficiency likely resulted in a more concentrated and potent mixture of chelating agents, what imparts electron donor properties and the ability to reduce Fe^{3+} ions to both extracts, HME and DE.

Antioxidant activity determined by β-carotene bleaching method

The β-carotene bleaching assay was applied in this investigation to assess the impact of extracts on lipid peroxidation, offering valuable understanding into their antioxidant properties. The DE had a moderate effect on inhibiting β-carotene bleaching, whereas HME showed a remarkably high activity. These results are consistent with those of Akrout *et al*., (2011) who found that the alcohol/water extract had more antioxidant activity than the infusion and that the *T. hirsuta* infusion had minimal or negligible activity. Merghem *et al*. (2020) reported a noteworthy percentage of β-carotene bleaching, which is in line with our findings and highlights the superiority of the methanolic extract over its aqueous equivalent. On the other hand, Djermane *et al*., (2020) found that HME with 70% methanol showed a lower IC_{50} than the aqueous extract, indicating the former's greater ability to inhibit β-carotene bleaching.

In a similar line, further research on *Thymelaea microphylla*, a different species in the same genus, provided insightful results. According to Allam *et al*., (2020) the methanol extract showed remarkable β-carotene bleaching inhibition after 48 hours, with more efficacy in preventing the oxidative degradation of β-carotene than the ethyl acetate and water extracts. Moreover, Dahamna *et al*., (2015) affirmed the same, stating that the alcoholic extract,

specifically the ethanolic extract, exhibited a superior percentage of inhibition compared to the aqueous extract from *Thymelaea microphylla* leaves and flowers.

In a similar vein, further research on *Thymelaea microphylla*, a different type within the same plant genus, some intriguing outcomes emerged. According to Allam *et al*., (2020), ME stood out for its impressive ability to prevent β-carotene bleaching over 48 hours, proving more effective than the ethyl acetate and water extracts. Confirming this, Dahamna *et al*., (2015) noted a similar trend, highlighting that the alcoholic extract, specifically the ethanolic extract displayed higher inhibitory percentages compared to the aqueous extract. Their focus on *Thymelaea microphylla* leaves and flowers adds to our understanding of how diverse extracts from the same plant species may exhibit similar or closely aligned antioxidant properties.

Globally, plant phenols serve a crucial role as antioxidants, exhibiting multifunctional properties such as acting against ROS, scavenging singlet oxygen, functioning as reducing agents, and donating hydrogen atoms (Gulcin *et al*., 2019).

Evaluation of anti-enzymatic activity *in vitro*

α-amylase and α-glucosidase inhibition assay

In our study and in the context of diabetes, the evaluation of the inhibitory effects of the examined extracts on α-amylase and α-glucosidase, pivotal enzymes in this disease, showed compelling results, both HME and DE displayed significant inhibitory potential specifically, HME. These results imply that both extracts possess a high potential as inhibitors of both α-Amylase and α-glucosidase key enzymes associated with diabetes. This high inhibitory effect, particularly in the case of HME, indicate promising anti-diabetic effect. The difference in inhibitory profiles of extracts against α-amylase and α-glucosidase suggest potential variations in the mechanisms of action, reflecting the potential of the extracts in managing hyperglycemia. In similar studies, Djermane *et al*. (2020), observed a remarkable inhibitory effect on the α-glucosidase enzyme with the HME from *T. hirsuta* with a considerable effect from aqueous extract. Additionally, in an *in vivo* study conducted by Abid *et al*. (2020), the evaluation of α-glucosidase inhibition activity focused on the aqueous infused extract and the ethyl acetate fraction from Moroccan *T. hirsuta*, the results indicated that the ethyl acetate fraction extract demonstrated inhibitory activity against intestinal α-glucosidase. When, the aqueous extract did not exhibit this inhibitory effect. It has previously been documented that *T. hirsuta* methanol extract reduces blood glucose levels in diabetic–hypertensive (DH) rats, demonstrating an antidiabetic action (Bnouham *et al*., 2012).

The inhibition of α-glucosidase and α-amylase in the small intestine is one of the ways that plants exercise their anti-hyperglycemic action; this inhibits the breakdown of complex

carbohydrates and interferes with their absorption (Shim *et al*., 2003; Oboh and Ademiluyi, 2011). This mechanism to prevent postprandial hyperglycemia have been reported to be the capabilities of several phytoconstituents, such as flavonoids, phenylpropanoids, terpenes, and alkaloids (Yin *et al*., 2014). These compounds have been identified in both HME and DE from *T. hirsuta*, worth mentioning phenylpropanoids such as trans-cinnamic acid, coumarin, and Chlorogenic acid. Also flavonoids such as Catechin and 6,7-Dihydroxy coumarin. The enhanced inhibitory capacity of HME over the DE against both α-amylase and α-glucosidase activities can be attributed to the higher concentration of the aforermentioned compounds likely contributes to its increased inhibitory potential. Furthermore, it was observed that additional bioactive compounds, specifically luteolin and apigenin, were exclusively identified in the HME. These compounds have been previously recognized for their inhibitory effects on key enzymes such as α-amylase and α-glucosidase (Shobana *et al*., 2009).

Acetylcholinesterase and butyrylcholinesterase inhibition activity

In the context of neurodegenerative disorders, we evaluated the inhibition of cholinesterase enzymes (AchE and BchE). These enzymes are thought to be promising in the treatment of neurological and neurodegenerative disorders, including AD. By hydrolyzing Ach to choline and acetate, AchE reverses the effects of this neurotransmitter at cholinergic synapses (Hajlaoui *et al*., 2021). Our findings reported a moderate inhibitory activity at 100 µg/mL of both extracts against AchE enzyme. While, their activity in inhibiting BchE activity was weaker compared to that against AchE. Those results are in contradictions with those reported by Djermane *et al*., (2020) in an investigation of the pharmacological effect of crude extrats (Dichloromethane, Acetone, aqueous methanol (70%, v: v) and water) of *T. hirsuta* from M'sila region in southern-east of Algeria over the period of March, declaring that the extracts showed no inhibitory effect on AchE, but they excercing interesting inhibition against BChE in particular the Dichloromethane extract of *T. hirsuta*. This disparity could be explained by the difference in the phytochemical profiles of the extracts wich can likely influenced the type of solvents used (polarity), extraction techniques, geographic location and harvest time (Hayouni *et al*., 2007; Ben Farhat *et al*., 2015).

Several phytochemicals revealed in the analysis of both HME and DE have been previously identified as inhibitors of cholinesterase enzymes. Krzysztoforska *et al*., (2019) declared that protocatechuic acid has extremely promising properties that may be helpful in preventing the development of neurodegenerative diseases. Where, Wang *et al*., (2017) discovered three hydroxycinnamoylated catechins (HCCs) (1-3) in Zijuan tea and investigated their bioactivity through molecular modeling simulation and biochemical experiments showing that they bind AchE (AChE) tightly and have strong AChE inhibitory activity. Caffeic acid and

chlorogenic acid, both phenolic acids, were observed to inhibit the activities of AChE and BChE in a dose-dependent manner. Notably, caffeic acid exhibited a more pronounced inhibitory effect on both AChE and BChE activities compared to chlorogenic acid. Interestingly, when were combined, they demonstrated an antagonistic inhibition of AChE and BChE activities (Oboh *et al*,. 2013).

The poverty of extracts in quercetin, kaempferol and myricetin may be the cause of the modest activity observed in the inhibition of cholinesterase enzymes, given that their high effectiveness in inhibiting these enzymes is well-established in previous studies (Katalinić *et al*., 2010; Ademosun *et al*., 2016)

Tyrosinase inhibition activity

Melanin, a natural pigment offers color to skin, hair, and eyes and protects them from the damaging effects of UV light (Masum *et al*., 2019). Tyrosine, a copper-dependent enzyme, starts the process of melanogenesis, or the production of melanin, by catalyzing the conversion of L-tyrosine to L-3,4,-dihydroxyphenylalanine (L-DOPA), which is then further processed by tyrosinase. The rate-limiting stage in the synthesis of melanin is represented by this enzyme reaction, which eventually results in the pigment's production (Wu *et al*., 2009; Videira *et al*., 2013). In this study, the extracts demonstrated a considerable level of tyrosinase inhibition potential with slightly greater potency from hydromethanolic one, This finding aligns with the confirmation provided by Kawano *et al*., (2007) who reported that *T. hirsuta* extract might have potent anti-melanogenesis effect through decreasing the synthesized melanin content in the murine melanoma B16 cells without cytotoxicity after 48 hours of incubation (Kawano *et al*., 2007). The presence of some phytochemicals with a inhibitory effect aginst tyrosinase must be the source of this considerable inhibitory effect displayed by extracts. Song *et al*., (2021) conducted research on catechins as tyrosinase inhibitors and revealed a potent potential for the inhibition of tyrosinase revealing a synergetic effect of two type of catechin. Nevertheless, it is suggested that the absence of other phytochemicals known for their antityrosinase activity renders the activity weak, such as quercetin, which has previously exhibited strong antityrosinase activity with a percent inhibition of 95%, equivalent to the positive control (kojic acid), in tyrosinase inhibition assays in previous studies conducted by Sirat *et al*., (2010).

***In vivo* pharmacological effects**

Acute oral toxicity of DE and HME

The results from our study indicate a favorable safety profile for the tested doses of HME and DE, with no significant alterations in physiological and behavioral parameters, including

clinical signs, behavior, skin effects, breathing, food intake, water consumption, and mortality rates. The lack of significant impact on key serum biomarkers (urea, creatinine, ALAT, ASAT) suggests minimal adverse effects on renal function or liver enzymes. Notably, exceptions were observed with DE, showing a slight but significant increase in ALAT levels, indicating a subtle impact on liver function. Additionally, a statistically significant change in ASAT levels was noted with HME at both administered doses. Comparing our findings with those of Azza *et al*. (2012), their study reported no signs of toxicity after oral administration of a single dose of the extract (5 g/kg body weight). In their sub-chronic dose study, where the extract was administered daily for four weeks, they observed no variation in biochemical parameters such as serum glucose, creatinine, and urea levels compared to the control group. However, they noted a marked decrease in serum levels of ALAT and ASAT compared to controls, suggesting a potential hepatoprotective effect. Similarly, the study by Merghem *et al*., (2020) on the acute toxicity of *T. hirsuta* in rabbits found no mortality during the observation period. They observed an increase in ASAT and ALAT levels in treated groups at different doses, along with a decrease in both cholesterol and glucose levels.

The historical use of *T. hirsuta* in folk medicine is now supported by the confirmed safety observed. However, further research is imperative to reinforce and deepen our understanding of the underlying mechanisms and long-term effects of *T. hirsuta* extracts.

Evaluation of *in vivo* pharmacological effect of *T. hirsuta* selected extracts

Decocted and hydromethanolic extracts effects on gastric emptying and intestinal transit in mice and possible mechanism (s) underlying

Gastric emptying

The exploration into the influence of *T. hirsuta* plant extracts on gastric emptying yields intriguing insights into digestive processes. Administering DE and HME from *T. hirsuta* aerial parts resulted in a reduction in the activity of the emptied quantity of the test meal compared to the negative control (CMC). Notably, this effect didn't follow a straightforward dose-dependent pattern.

At lower doses (125, 250 mg/Kg), DE significantly decelerated gastric emptying in comparison with the negative control. These reductions approached the inhibitory effect of atropine, suggesting the potential of DE to influence the digestive process. It's interesting to note that DE continued to decelate gastric emptying at the highest dose (500 mg/Kg), but a little less than at lower doses.

Although having lower activity levels than DE, HME showed considerable efficiency, especially at the highest tested doses (250 and 500 mg/Kg). Within the lowest concentration

level, the second extract showed promising characteristics; its performance was higher than that of the negative control and nearly reached that of the positive control. This emphasizes how HME can control the emptying of the stomach.

The observed effects of both DE and HME on gastric emptying may be explained by the distinct bioactive compounds present in each extract. These compounds probably affect the GI tract's smooth muscle contractions or potentially impacting neurotransmitter release, which modifies the rate of gastric emptying. Catechins, which are found in both extracts, have been associated with the relaxation of smooth muscle in the GIT in a study on green tea catechins (Maeda *et al*., 2003), suggests a potential contribution to a smoother digestive process. Another dietary polyphenolic compound, chlorogenic acid found in both HME and DE, has been previously demonstrated in experimental studies to activate the nitric oxide-sGC-PKG pathway, leading to the relaxation of vascular smooth muscles. Given that chlorogenic acid is implicated in increasing the basal tone of ileal smooth muscles (Berman *et al*., 2019). This suggests a potential mechanism for the observed effect of both extracts in slowing the gastric emptying process. The relaxation of vascular smooth muscles, mediated by chlorogenic acid, may contribute to this impact on gastric emptying. Baggio *et al.,* (2009) discovered that intraperitoneal administration of a flavonoid-rich extract significantly inhibited gastric emptying in mice. This finding reinforces our results, given that both extracts are considerably rich in flavonoids. Additionnaly, the antioxidant properties of some polyphenols may protect neurons from oxidative stress, preserving neurotransmitter release and preventing neuronal damage (Jang *et al*., 2003).

In this study, it is interesting to note that HME showed less of a delaying impact on stomach emptying than DE, despite the fact that it included more polyphenols, flavonoids and tannins. This unexpected finding suggests that the effect on stomach motility is not exclusively influenced by the amount of phytochemicals. The reported results could also be explained by other factors, such as the particular type of compounds, interactions with other substances, or even the concentration gradient inside the GIT. Additionally, an elevated viscosity was observed in the aqueous extract, provides a compelling link to its potential as a contributing factor to the more pronounced slowing effect on gastric emptying. This noteworthy viscosity may have influenced the aqueous extract's ability to form a gel-like mass in the digestive tract, thereby impeding the smooth movement of chym. This suggests that the viscosity, as a specific characteristic of the aqueous extract, may play a crucial role in shaping the observed physiological outcomes, highlighting the intricate interplay between the physical properties of the extracts and their effects on digestive processes. This notion has been substantiated in studies exploring the dynamic effects of starch on GI processes reporting that

alterations in the characteristics of starch, such as modifications in viscosity and osmolality, have the potential to contribute to the delay in gastric emptying. Changes in starch, whether through cooking, cooling, or other chemical modifications, can impact its glycaemic and/or insulinaemic index (Peracchi *et al*., 2000).

Beyond the apparent differences in the action of the two extracts and in polyphenol content, the dynamic interplay of factors contributing to the delay invites a deeper exploration into the mechanism of action of the plant. Insights into how the plant extracts modulate neural signals or perhaps influence the contractility of smooth muscles within the digestive tract could unravel the nuanced ways in which these extracts interact with the GI system.

Mechanisms of the DE and HME effect on gastric emptying

Due to their significant role in the intricate regulation of GI motility, neurohumoral chemicals and their receptors have been the main focus of therapeutic research. The numerous receptors involved in motility are mostly found on smooth muscle cells and neuronal structures in the intrinsic and extrinsic regions of the enteric nervous system. Where, receptor agonists and antagonists work together directly in this system to alter excitatory or inhibitory signals (Hansen, 2003). Given this principle, different pharmacological agents were used and an observable change in the activity of extract was observed. As previously described, DE showed a significantly deley in gastric emptying. The initial administration was indomethacin, a known strong prostaglandin production inhibitor. Noting that, some prostaglandin series have been shown to have varying effects on gastric motility. The circular layer of muscle is relaxed and the longitudinal smooth muscle layer is contracted, both by E-series prostaglandins. The F series prostaglandins, on the other hand, contract both layers of smooth muscle. Indomethacin inhibits the ability of longitudinal muscle to contract and stimulates the circular muscle found in the stomach and small intestine in several species, typically resulted in a delayed gastric emptying (Stein *et al*., 1994). Indomethacin, a NSAID, inhibits gastric emptying primarily by reducing prostaglandin synthesis. Prostaglandins, particularly PGE2, regulate smooth muscle activity in the GIT, promoting coordinated contractions and relaxations for efficient food movement. Inhibition of COX enzymes by indomethacin decreases prostaglandin levels, leading to increased smooth muscle tone and diminished contractions in the stomach. This altered motility results in delayed gastric emptying. The mechanism is supported by various studies, including those exploring the impact of indomethacin on GI function (Wallace *et al*., 2000). The intervention of indomethacin has been observed to enhance the delay in gastric emptying, suggesting its role in modulating this physiological process. This improvement in delayed gastric emptying is attributed to the action of both DE and HME on the prostaglandin

pathway. It appears to exert their effect by influencing prostaglandins, particularly prostaglandin E2 (PGE2). Notably, the effect of DE and HME on gastric emptying delay was sustained even in the presence of L-arginine wich precludes the involvement of the nitrenergic pathway. This observation highlights the complexity of both DE and HME mechanisms in influencing gastric emptying, implicating the prostaglandin with the DEdemonstrating more potency. Basic studies regarding the effect of *T. hirsuta* on gastric emptying have not been reported.. Grzanna *et al*., (2005) found that Ginger exerts influence on both the nitric oxide (NO) pathway and prostaglandin synthesis. The bioactive compound gingerol, present in ginger, is suggested to play a role in the prostaglandin pathway and affect NO production. This dual action is believed to contribute to smooth muscle relaxation within the GIT. Amira *et al* (2008) it has been reported that the relaxation of mouse stomach muscles induced by various types of flavonoids such as apigenin, quercetin, rutin, naringenin, and catechin primarily depends on the structure of these flavonoids and the position of hydroxyl groups, rather than on neural transmission, NO/prostaglandin production, or the activation/inhibition of potassium channels.

Effect of DE and HME on intestinal transit

The control of intestinal transit is a complex process that involves both neuronal and myogenic mechanisms mediated by numerous neurotransmitters and mediators. Both DE and HME administered at varying doses, significantly enhance intestinal transit in a dose-dependent manner. Notably, the effects are more pronounced by HME. This observed effect is likely an outcome of the intrinsic phytochemical composition of the extracts. Rutin, an abundant flavonoid in HME, has attracted attention for its potential impact on GI function, particularly in contributing to improved bowel regularity. Studies, including the work by Ganeshpurkar *et al.*, (2017), have explored the pharmacological potential of rutin, shedding light on its beneficial effect on improving intestinal transit. Catechins, including epicatechin, found in both extracts with high content in HME, have been subjects of study due to their potential to modulate gut function and accelerate intestinal transit (Chacko *et al*., 2010). In the same line, chlorogenic acid, abundant in both extract and highly in HME, has been examined for its effects on GI motility and transit time (Naveed *et al*., 2018).

Mechanism of DE and HME on improving intestinal transit

The studied extracts DE and HME demonstrated an improvement in intestinal transit. However, with intervention of indomethacin, a significant decline in the intestinal transit rate was marked. This outcome aligns with the well-known inhibitory effect of indomethacin on intestinal motility, attributed to its ability to block COX enzymes and consequently reduce

prostaglandin synthesis. Prostaglandins, particularly PGE2, play a pivotal role in regulating smooth muscle activity in the GIT (Dey *et al*., 2007). The concurrent administration of L-Arg with the extracts led to a substantial increase in the rate of intestinal transit. On the other hand, the oral administration of extracts under the influence of L-NNA resulted in a significantly lower transit rate than that observed with extracts alone. These results strongly suggest the exceptional involvement of the nitrinergic pathway and the prostaglandin pathway in the mechanism of enhancement of intestinal transit approved by both extracts. The intricate interplay between these pathways underscores the complexity of the extract's effects on intestinal motility and the nature of their pharmacological actions. In the same line, Liang *et al* (2005) examined the impact of lipopolysaccharide on diarrheogenic activity, GI transit, and intestinal fluid content, exploring the potential roles of NO and PGE2 in GI functions of endotoxin-treated mice, concluding that Pretreatment with L-NAME, a non-selective NO_S inhibitor, or indomethacin, an inhibitor of prostaglandin synthesis, significantly attenuated the effects of lipopolysaccharide on diarrheogenic activity and intestinal content and reversed the GI transit induced by lipopolysaccharide highlighting the involvement of these agents on the GI effects initiated by lipopolysaccharide in mice. In a study conducted by Fraser *et al* (2005), aimed to assess the impact of the NO_S inhibitor NG-monomethyl-l-arginine (l-NMMA) on the regulation of small intestinal nutrient transit resulted in a significant delay of duodenocaecal transit compared to control. Their findings suggested a potential role for endogenous NO in the modulation of small intestinal nutrient transit through the regulation of small intestinal motility. Polyphenols from tea, grapes, cacao, berries, and plants have been shown to increase the formation of potent vasoprotective factors including nitric oxide (NO) and to delay endothelial ageing (Oak *et al*., 2018). Contrary, the dichloromethane fraction of *Origanum majorana* induces relaxation in the smooth muscles of the rabbit jejunum with an effect independent of several pathways, including cholinergic, nitrergic, adrenergic, or guanylyl cyclase (Makrane *et al*., 2018). The relaxation observed in the stomach muscles of mice, induced by various flavonoids such as apigenin, genistein, quercetin, rutin, naringenin, and catechin, is predominantly relies on the structural attributes of these flavonoids and the specific positioning of hydroxyl groups (Amira *et al*., 2008). Ultimately, it has also been demonstrated that the pathological condition of the digestive tract plays a key role in the impact of medicinal plant extracts on intestinal transit (Capasso *et al*., 2008).

Effect of *T. hirsuta* studied extracts against ethanol-induced gastric ulceration in rats

Effect of DE and HME on macroscopic and histopathological appearance

A substantial consequence of alcohol use on the stomach mucosa was observed in the rats of the negative control group given ethanol, leading to severe macroscopic damage in the gastric mucosa. This damage was characterized by a loss of normal color and mucus lining, as well as the presence of petechiae, extensive hemorrhages, and tissue swelling (edema). These macroscopic changes indicate significant harm caused by ethanol to the stomach. According to Tarnawaski *et al.,* (2014), ethanol consumption may damage the lining of the stomach and increase the risk of ulcer development, causing an irritation and inflammation of the gastric mucosa, which might compromise the stomach's barrier and increase the risk of ulcer development. It causes ulcers by a number of different ways, such as increased gastric acid output, changes in mucosal blood flow, and impairment of the mucosal defensive mechanisms. Ethanol can also solubilize the protective mucus, exposing the mucosa to the proteolytic and hydrolytic effects of hydrochloric acid and pepsin. It easily passes through the gastric mucosa, damaging the membrane (Sener *et al*., 2004). Additionally, alcohol potentially causes microvascular damage by disrupting the vascular endothelium and increasing vascular permeability (Adinortey *et al*., 2013).

The administration of DE and HME at different dosages showed a significant decrease in the damage caused by ethanol with a dose-dependent pattern. Regaining normal color and mucus lining, reducing petechiae, and lessening hemorrhages and edema were all signs of the protective benefits. To date, no studies have explored the potential gastroprotective properties of *T. hirsuta*. Several studies reported the bennefical effect of plant extracts against macroscopic damages caused by ethanol in stomach tissue (Kim *et al*., 2017; Sattar *et al*., 2019; Liu *et al*., 2021).

Ethanol-induced ulceration was effectively mitigated by both DE and HME. In contrast, HME continuously exceeded DE in the reduction of important histopathological indications such as blood vessel congestion, inflammatory cell infiltration, necrosis, and oedema. Higher dosages of HME demonstrated this improved efficacy increasingly. Consequently, HME finds out to be a more potent therapeutic agent than DE at reducing the histological alterations linked to stomach ulceration. The distinct composition of HME may be the cause of the observed variations in efficacy. In a comparative study investigating the effects of *Rumex nepalensis* crude HME and solvent fractions at different doses, Sisay *et al*. (2020) reported that gastroprotection provided by the *R. nepalensis* hydromethanolic crude extract at 400 mg/kg was comparable to the standard treatment and even more potent than the solvent fractions. A gross and histo-morphological study of anti-ulcerogenic effects of *Cissampelos owariensis*

methanolic extract in Wistar rats was conducted by Dayo *et al* (2019) found that the methanolic extract of *Cissampelos owariensis* enhances both the microscopic and macroscopic appearances of stomach ulcerated tissue suggests a potential therapeutic efficacy of the extract in alleviating gastric ulceration.

Macroscopic and histopathological examination of DE and HME mechanisms effect on ethanol-induced gastric mucosa damage in rats

In examining the gastric ulcer healing potential of the DE macroscopically and microscopically aligning a cohesively results. Rats pre-treated with arginine and DE exhibited significantly attenuated injuries and demonstrated amelioration of corresponding histopathological factors, emphasizing the extract's efficacy. Notably, the intensified stomach injuries observed with L-NNA, a NOS inhibitor, were significantly alleviated with DE pre-treatment, indicating the potential involvement of NO pathway. Given that NOs established role in promoting angiogenesis and collagen production, essential for mucosal healing, the observed effects align with existing literature where NO donors have been shown to promote gastric ulcer healing while NO synthase inhibitors delayed the process (Wallace *et al*., 2000). Furthermore, the macroscopic moderation of ulceration induced by DE after indomethacin pretreatement suggests its potential anti-inflammatory properties, consistent with the partially reduced tissue damage observed histopathologically. Similarly, yohimbine-induced severe tissue damage was slightly reduced with DE pre-treatment. These compelling macroscopic and histopathological results collectively indicate a multifaceted mechanism of action for DE, possibly involving interactions with arginine, modulation of NO pathway, and anti-inflammatory processes.

The comprehensive evaluation of the impact of HME on both macroscopic and histopathological aspects of stomach ulceration revealed a strong therapeutic potential. The observed decrease in tissue damage points to a complex mechanism that may include NO pathway regulation, antioxidant, cytoprotective, and anti-inflammatory properties. The process of ulcer healing is a multifaceted one, wherein the injured tissue repairs in an attempt to restore its original integrity. It has been suggested that these processes can be divided into the following sequential phases: remodeling, proliferation, inflammation, and hemostasis (Fornai *et al*., 2011).

Gastroprotective effect of ADE and HME

The ulcer index significantly decreased as the dosage was increased, suggesting a stronger protective effect at higher doses. This directly evidenced dose-responsive gastroprotective impact of both extracts which provides increasing levels of protection notablly HME. This activity is likely attributable to the substantial content of phytochemicals present in the extract.

Sumbul *et al.,* (2011) supported the contribution of polyphenols in several biological activities in the gastroprotective area, including anti-secretory, cytoprotective, and antioxidant activities. The potential benefits and treatments of dietary polyphenols for peptic ulcers include: enhancing cytoprotection, re-epithelialization, neovascularization, and angiogenesis; up-regulating prostaglandins and tissue growth factors; down-regulating anti-angiogenic factors; enhancing endothelial NOS-derived NO; suppressing oxidative mucosal damage; amplifying antioxidant performance, antacid, and antisecretory activity; increasing endogenous mucosal defensive agents; and preventing *H. pylori* colonization-related changes in the stomach and the development of gastric ulcers in the stomach (Farzaei *et al*., 2015). Many compounds identified in both DE and HME were been reported to have gastroprotective activity such as caffeic acid, ferulic acid, p-coumaric acid and catechin (Wang *et al*., 2012; Li *et al.*, 2018; Beber *et al*., 2018; Boeing *et al*., 2021; Costa *et al*., 2021). However, apigenin is exclusively present in HME with a high concentration, indicating a potential role in the observed differences between the two extracts. Costa *et al.,* (2021) confirmed the gastroprotective effect of apigenin in a study on the role of isolated compounds from red propolis extract. Luteolin, a promising flavone belonging to flavonoids is also present only in HME. Luteolin is known to inhibit inflammation in gastric cells via inhibition of neutrophil infiltration, inhibition of MPO, inhibition of COX-1 and COX-2 activity and inhibition of the production of pro-inflammatory cytokines such as IL1, IL6, IL10 and TNFα (Osakabe *et al*., 2004; Antonisamy *et al*., 2016; Kangwan *et al*., 2019; do Nascimento *et al*., 2020).

The majority of phytochemicals detected in both extracts were notably more abundant in HME, ranging from three to five times higher. This is in line with litterature, Jakobek (2015) found that phenolic compounds represented by the flavonoids, tannins, phenolic acids and other plant metabolites are present mainly in polar fractions of extracts of several plants. The potent gastroprotective impact of HME is suggested to be attributed also to its high concentration of tannins, which exhibit beneficial effects on gastric protection. Tannins are powerful scavengers of peroxyl radicals and can additionally interact with mucus proteins. This interaction enhances their cytoprotective efficacy by creating a protective protein layer on the GI mucosa (da Silva *et al*., 2010).

Mechanisms of DE and HME on gastroprotective effect

The evaluation results indicates that both DE and HME exhibited a strong antiulcerogenic effect specially, the HME. Indeed, there are several mechanisms for protecting gastric mucosa, such as production of prostaglandins, nitric oxide, antioxidants enzymes and non-enzymatic antioxidant systems, mucus-bicarbonate barrier and others (Wallace, 2008). Thus, to evaluate

possible mechanisms related with the antiulcerogenic properties of DE and HME from *T. hirsuta*.

The COX involvement was evaluated using indomethacin (5 mg/kg, s.c.) as an inhibitor of biosynthesis of cytoprotective prostaglandins, and administration of ethanol as the necrotizing agent. The indomethacin dose used at this experiment is not able of inducing ulcers by itself, at least within the evaluated time (Reyes-Trejo *et al*., 2008).

Treatment with DE yielded significant decrease in ulceration index when compared with negative control. On the other hand, when indomethacin was administrated (aiming to block COX), a significant increasing ulceration index was observed, demonstrating the potential involvement of prostaglandins in the gastroprotective mechanism of DE by maintenance of the gastric mucosal integrity. In fact, prostaglandins (mainly PGE2) have antiulcerogenic effect against lesions caused by ethanol, acting as an important mediator for mucosal integrity (Júnior *et al*., 2014).

In a comparable manner, increasing NO levels could play a role in protecting the stomach mucosa (Caldas *et al*., 2015). Thus, NOS inhibitors were used to assess the role of NO in the formation of ulcers generated by ethanol. Where, DE showed a high gastroprotection when compared to the CMC group. Interestingly, following the administration of L-NAME (a non-selective NO_S inhibitor), a slight influence was indicated on the protective effect. Altogether the results suggest that NO pathway is moderatly involved in the gastroprotective effect DE *T. hirsuta* areal parts. In addition, it was demonstrated that NO and COXs interact mutually, with NO synthase inhibitors and indomethacin, blocking the production of PGE2 and cGMP, respectively, while maintaining bioavailable NO may increase the activity of these enzymes (Khattab *et al*., 2001). Gastric acid secretion is influenced by the adrenergic system. It has been demonstrated to be inhibited in the GIT by activating presynaptic α_2-adrenoceptors on the vagus nerve. In a number of gastric lesion models, antagonists prevent stomach damage (Sasmini *et al*., 2000). The administration of yohimbine, an α2-adrenoreceptors antagonist significantly blocked the anti-ulcer effect of DE and increased the ulcer index suggesting a pivotal role for the adrenergic pathway in the gastroprotective mechanism of DE. Wich reinforces the notion that DE's gastroprotective effect operates through modulation of the adrenergic system, specifically via α2-adrenoreceptors involvement. Similar studies, reported that subcutaneous yohimbine (5 mg/kg) attenuated the protective effect of intragastric nicotine against ethanol-induced gastric mucosal injury. Koc *et al* (2012) demonstrated that presynaptic α2-adrenoreceptors play a crucial role in inhibiting ulcers induced by factors such as indomethacin, aspirin, ethanol, stress, and pyloric ligation.

In elucidating the mechanism of action associated with HME, the extract preserves its efficacy in both instances of intervention with indomethacin and yohimbine. Remarkably, the potent effect of the extract remains unaffected even when both prostaglandin inhibition and a2-adrenergic receptor inhibition are concurrently employed. HME highlighting the fact that HME operates independently of both the prostaglandin pathway and the adrenergic pathway. One of the multiple gastro protective mechanisms of α2-adrenoreceptors may be the stimulation of COX-1, previous experimental studies have shown a direct relationship between those processes reporting that the stimulation of α2-adrenoreceptors produced an increase in the activity of COX-1 (Suleyman *et al*., 2009). In the other hand, the potent protective activity of HME from *T. hirsuta* was partially decreased in the presence of NOS inhibitors (L-NAME); suggesting a modest involvement of NO pathway in its mechanism of action. The potent protective mechanisms linked to NO donors harnesses many mechanisms: reduction in polymorphonuclear leukocyte infiltration into the mucosa, inhibition of neutrophil adherence to the vascular endothelium, antithrombotic action, antiaggregatory property, attenuation of lipid peroxidation, and gastric mucosal vasodilatation (Leung *et al*., 2009). The presence of NO in the mucosa can contribute for maintain blood flow protecting from damage caused by ethanol, which have a cytotoxic action on parietal cells, as well as by NSAIDs. Furthermore, antioxidants like polyphenols can increase the bioavailability of NO, preventing the formation of peroxynitrites by a non-enzymatic reaction between NO formed and superoxide (Khattab *et al*., 2001; Farzaei *et al*., 2015). Similar findings have appeared in the literature, such as the outcomes obtained by Júnior *et al*. (2014), also showing that the protective effect of hydroalcoholic extract from the leaves of *Croton campestris* A. St.-Hill on the gastric mucosa is NO-dependent due to the potentiation of NO/cGMP pathway. This mechanism is involved by several phytochemical compounds marking their presence in both extracts Significantly higher in HME . Kolgazi *et al* (2021) found that caffeic acid attenuates gastric mucosal damage induced by ethanol in rats via NO modulation. Catechin, present with high concentration in HME has been studied for its potential to enhance NO bioavailability and contribute to gastroprotection (Zhang *et al*., 2020). Additionally, Min *et al* (2005) reported a strong gastroprotective effect of apigenin-7-O-β-d-glucuronopyranoside (AGC) and apigenin in gastritis induced by indomethacin. In a study conducted by Shimoyama *et al*., (2013), revealed that treatment with chlorogenic acid exhibited a gastroprotective effect on a gastric ulcer model induced by ethanol, leading to a reduction in the percentage of the lesioned area.

Determination of DE and HME effect on mucus in gastric layer

At the tested doses, both DE and HME showed negligible effects on preserving gastric mucus content, comparable to the negative control. This suggests that, under the conditions of a single administration, these extracts do not significantly impact gastric mucus production or preservation. However, it is important to mention that with prolonged administration, there may be a potential for a protective effect. On the contrary a rapid reflex to involve their protective activity, these extracts, at the tested doses, do not seem to utilize the property of increasing gastric mucus making other mechanisms as previously reported. Khattab *et al* (2001) confirmed that other possible mechanisms of gastric protection may include the COX pathway, the NO pathway, inhibition of gastric secretion, and enhancement of blood flow to the mucosa. Noting that, DE preserved the mucus content slightly more than HME, especially at the highest dose (500 mg/kg). This effect may be attributed to the activation of the COX-1 pathway for gastric mucosal protection by DE, which plays a crucial role in the synthesis of prostaglandins. Through its action on the prostaglandin pathway, DE slightly enhanced the protection of mucus. Giving that, prostaglandin is crucial in the regulation of gastric mucus secretion reduced by ethanol (Zhao *et al*., 2009). Prostaglandins are recognized for their gastroprotective effects, contributing to the maintenance of the integrity and defense mechanisms of the gastric mucosa and increasing the secretions of mucus, bicarbonate and sulfhydryl compounds to strengthen the resistance of gastric mucosal cells to the necrotizing effect of strong irritants (Takeuchi *et al*., 2002; Brzozowski *et al*., 2005; Laine *et al*., 2008).

Evaluation of *in vivo* antioxidant activity in gastric homogenate

ROS play an important role in the pathogenesis of acute gastric mucosal injuries induced by ethanol and ischemia-reperfusion in rats, creating an imbalance between oxidant and antioxidant cellular processes (Hamaishi *et al*., 2006; Nassini *et al*., 2010). Plants have evolved an antioxidant defence mechanism that includes non-enzymatic substances such ascorbate, glutathione, and others to avoid or lessen damage caused by ROS like tocopherol, carotenoids, flavonoids, and enzymes such polyphenol oxidase (PPO), glutathione reducase (GR), ascorbate peroxidase (APX), peroxidase (POX), CAT, and SOD (Núñez *et al*. 2003, Sergi and Alegre 2003; Agarwal and Pandey 2004). As expected, the ethanol-induced ulcer markedly reduced the CAT and GSH activity and increased lipid peroxidation (an important biomarker of oxidative damage) in gastric tissues. Contrarily, traitement with DE and HME resulted in the restoration and enhancement of GSH levels, CAT activity in both extracts with dose-dependent response, and a reduction in lipid peroxidation (MDA) in the case of DE when HME failed to reduce MDA levels suggesting that DE exhibits a mechanism of action involving catalase,

GSH, and lipid peroxidation reduction, providing a comprehensive defense against oxidative stress. In contrast, HME primarily acts through CAT and GSH, with limited efficacy in protecting against lipid peroxidation wich is in line with outcomes declared by Gawlik-Dziki et al., (2014) that crude spice extracts had a relatively low capacity to inhibit lipid peroxidation giving that digestion in the simulated *in vivo* conditions released high level of active compounds that are able to permeate across a dialysis membrane .The diverse mechanisms exhibited by these extracts in reducing oxidative stress *in vivo* can be elucidated by variations in their phytochemical content and its specific interactions. The anti-ulcer action of dietary polyphenols is largely different, due to their anti-inflammatory activity, which is caused by down-regulating proinflammatory cytokines and cellular and intercellular adhesion agents, suppressing leukocyte-endothelium interaction, inhibiting nuclear signaling pathways of the inflammatory process, and modulating intracellular transduction and transcription pathways (Farzaei *et al*., 2015). The impact on protein content is more pronounced at higher doses of DE. When, HME have a potent effect on protein content. This renforce our findings concerning the high gastroprotective activity of the HME, as previously concluded, can be attributed to the significant interaction between high levels of tannins and proteins giving that tannins are powerful scavengers of peroxyl radicals and can additionally interact with mucus proteins. This interaction enhances their cytoprotective efficacy by creating a protective protein layer on the GI mucosa (da Silva *et al*., 2010).

CONCLUSION AND PERSPECTIVES

The ethnobotanical study revealed that *T. hirsuta*, or "Methnan," is extensively utilized in traditional medicine. The majority of applications encompass various medical conditions across different biological systems, such as the digestive, respiratory, urinary, and nervous systems. Among these, Methnan finds notable usage as poultices for skin ulcers and is particularly emphasized in addressing digestive disorders, including gastric ulcers. Expanding on this traditional knowledge, we conducted an *in vitro* and *in vivo* studies to elucidate the biological activities of *T. hirsuta* revealing its richness on total phenolic, flavonoids, and tannins content. Additionnaly, the extracts, including HME, ME and DE demonstrated notably high antioxidant activity, respectively. including different mechanisms for scavenging free radicals (such as DPPH and ABTS assays), reducing power, and metal chelation, highlighted the diverse antioxidant mechanisms of *T. hirsuta*.

T. hirsuta, suggested an antidiabetic activity by inhibiting the enzymes α-amylase and α-glucosidase. They also exhibited a moderate anti-Alzheimer's activity, with a more pronounced effect on the inhibition of AChE compared to BChE. The origin of these activities could be attributed to the abundantly detected phenolic compounds (Chlorogenic acid, Caffeic acid, Ferulic acid, trans-cinnamic acid, etc.) and may be linked to the radical-scavenging properties of these phenolic compounds and their derivatives. Additionally, *T hirsuta* showed a considerable potential of tyrosinase inhibition, wich lead to a more even skin tone and makes it a promising natural ingredient in skincare formulations aimed at addressing skin discoloration and promoting a healthier complexion.

The absence of toxic effects in rats provides compelling evidence for the safe use of *T hirsuta* in both traditional and modern medicinal contexts. This underscores its potential as a secure and effective choice for healthcare practices.

T. hirsuta has been shown to guarantee beneficial activities for the GIT, including a delay in gastric emptying regulated by both the nitrergic and prostaglandin pathways. Additionally, there is a significant improvement in intestinal transit through the involvement of the aforementioned pathways. Additionally, *T. hirsuta* has demonstrated a pronounced a high protective activity against gastric uler, affirming its traditional medicinal uses, particularly with the HME, as compared to the decocted one. The two extracts exhibited distinct pathways in their mechanisms of action. This protection was manifested by the improvement of macroscopic and microscopic aspects of stomach tissues and *in vitro* through the increase in GSH content, enhancement of antioxidant enzyme activity such as CAT, preservation of protein levels and reduction in lipid peroxidation.

Despite attempting to assess as many biological activities as possible in *T. hirsuta* there are still several aspects that can be explored as future prospects. For instance, numerous unexplored avenues remain for further investigation and development, presenting opportunities for future research and advancements in our understanding of this plant species potential benefits. including among them:

- Isolate the molecules implicated in the various studied activities.
- Conduct an *ex-vivo* study on the anti-Alzheimer's activity to substantiate and evaluate its potential effect.
- Explore the anti-ulcerative effect of *T. hirsuta* in various ulcer induction models.
- Conduct studies on other protective mechanism pathways of HME due to its potent effects.
- Examine the potential involvement of other pathways in the inhibitory effect on gastric emptying and/ or intestinal transit such as neural transmission and adrenergic receptors.

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ANNEX

Questionnaire card of survey

Medicinal plants and phytotherapy

This study is carried out as part of the preparation of a doctoral thesis, we ask you to respond to the following questionnaire:

Informant:

- Age: ....

– Address (municipality): ……..……..

– Marital status: Single □ Married □ Divorced □

– Gender: Male □ Female □

– Academic level: None □ Primary □ Secondary □ University □

Plant material:

– Vernacular name:**نبات المثنان**

– Scientific name: Thymelaea hirsuta ENDL L.

– Use of the plant: Therapeutic □ Cosmetic □ Others □

– Single plant □ Possible combination (of plants) □: ....

– Part used: Stem □ Flowers □ Leaves □ Whole plant □

Other combinations □: ....

– Form of use: Herbal tea □ Powder □ Essential oils □ Fatty oils □ Extract (tincture, solution, capsule) □:.Others: ....

– Method of preparation: Infusion □ Decoction □ Poultice □ Raw □ Cooked □ Others □:

.... …

– Dose used:

- Pinch □ Handle □ Spoon □

- Precise dose: Quantity in g / glass:

Others: ....

– Method of administration: Oral □ Massage □ Rinsing □ Brushing □

Others □: ....

– Dosage: number of doses per day.

- For children: 1 time/day □ 2 times/day □ 3 times/day □ Others □:.....

- For the elderly: 1 time/day □ 2 times/day □ 3 times/day □ Others □:.....

- For Adults: 1 time/day □ 2 times/day □ 3 times/day □ Others □:.....

– Duration of use (TRT duration): One day □ One week □ One month □ Until healed□.

Use:

– Type of illness:

- Dermatological conditions □
- Respiratory disorders □
- Cardiovascular disorders □
- Genitourinary disorders □
- Osteo-articular disorders □
- Metabolic disorders □
- Disorders of the digestive tract □ - case of gastric or duodenal ulcer □
- Affects of the accessory glands of the digestive tract □
- Neurological disorders □

OTHER USES:..... ....

– Diagnosis by: Himself □ The doctor □ The herbalist □ Others □:..... .....

– Results: Healing □ Improvement □ Ineffective □

- Side effects: ..... . Toxicity: ....

Site 01:

https://www.teline.fr/fr/photos/thymelaeaceae/thymelaea-hirsuta

Site 02:
https://fr.m.wikipedia.org/wiki/Fichier:Les_communes_de_la_r%C3%A9gion_de_la_Hodna.svg