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In vitro evaluation of protiens anti-denaturation and antioxidant activities of commercially available *Matricaria chamomilla L.*

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Dedication

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DAJMILA

Dedicated

First

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Abstracts

Abstract

This study extensively investigated the in vitro therapeutic potential of *Matricaria chamomilla* (chamomile) extracts prepared using ethanol, acetone, and water, bay evaluating their anti-denaturation and antioxidant activities. The anti-denaturation activity, employing bovine serum albumin, determined the aqueous extract to be highly active, exhibiting 75.93% inhibition of protein denaturation at its maximum concentration, significantly more than diclofenac sodium (21.81%) and Vitamin C (41.15%). At low dilutions , chamomile extracts still showed positive inhibitory activities, demonstrating their continuing effectiveness. The DPPH assay indicated the aqueous extract as a powerful scavenger (67.62% inhibition) and the ethanolic extract next at a close second (61.38%), with pro-oxidant activity in acetonic and aqueous extracts at dilutions that were very low. The FRAP assay indicated the greatest ferric reducing antioxidant power for the ethanolic extract (92.66% inhibition).

These findings collectively emphasize the tremendous potential of chamomile as a natural source of anti-inflammatory and antioxidant agents worthy of further work on characterization of compounds, in-vivo studies, finding of molecular mechanisms, and comprehensive toxicity and pharmacokinetic studies.

Key words: *Matricaria chamomilla*, protein anti-denaturation, antioxidant, DPPH, FRAP, anti-inflammatory.

Résumé

Cette étude a examiné de manière approfondie le potentiel thérapeutique in vitro des extraits de *Matricaria chamomilla* (camomille), préparés avec de l'éthanol, de l'acétone et de l'eau, en évaluant leurs activités anti-dénaturation et antioxydantes. L'essai d'activité anti-dénaturation, utilisant la sérumalbumine bovine, a déterminé que l'extrait aqueux était très actif, présentant une inhibition de 75,93% de la dénaturation des protéines à sa concentration maximale, ce qui est significativement plus élevé que le diclofénac sodique (21,81%) et la vitamine C (41,15%). Même à de faibles dilutions, les extraits de camomille ont montré des activités inhibitrices positives, démontrant leur efficacité continue. L'essai DPPH a indiqué que l'extrait aqueux était un puissant piégeur (67,62% d'inhibition) et l'extrait éthanolique un second proche (61,38%), avec une activité pro-oxydante dans les extraits acétoniques et aqueux à de très faibles dilutions. L'essai FRAP a indiqué la plus grande puissance antioxydante réductrice de fer pour l'extrait éthanolique (92,66% d'inhibition). Ces résultats soulignent collectivement le formidable potentiel de la camomille en tant que source naturelle d'agents anti-inflammatoires et antioxydants, méritant des travaux supplémentaires sur la caractérisation des composés, des études in vivo, la découverte des mécanismes moléculaires, et des études complètes de toxicité et de pharmacocinétique.

Mots-clés : *Matricaria chamomilla*, anti-dénaturation des protéines, antioxydant, DPPH, FRAP.

ملخص

هذه دراسة مكثفة تناولت الإمكانات العلاجية لمستخلصات البابونج (*Matricaria chamomilla*). تم تقييم فعاليتها في المختبر بعد تحضير المستخلصات باستخدام الإيثانول والأسيتون والماء، مع التركيز على خصائصها المضادة للتمسخ والمضادة للأكسدة. أظهرت النتائج المتعلقة بالنشاط المضاد للتمسخ، باستخدام ألبومين المصل البقري كنموذج، أن المستخلص المائي للبابونج كان ذا فعالية عالية جدًا, حيث حقق تثبيطًا لتمسخ البروتين بنسبة 75.93% عند أعلى تركيز له، متفوقًا بذلك بشكل ملحوظ على ديكلوفيناك الصوديوم (21.81%) وفيتامين ج (41.15%). اللافت للنظر أن مستخلصات البابونج أبدت نشاطًا تثبيطيًا إيجابيًا حتى عند التخفيفات المنخفضة، مما يؤكد فعاليتها المستمرة. وفيما يخص النشاط المضاد للأكسدة، أثبتت تجربة DPPH أن المستخلص المائي كان ماسحًا قويًا للجذور الحرة بنسبة تثبيط بلغت 67.62%، يليه المستخلص الإيثانولي بفارق بسيط بنسبة 61.38%. كما لوحظ أن المستخلصات الأسيتونية والمائية أظهرت نشاطًا مؤكسدًا حتى عند التخفيفات المنخفضة جدًا. ومن خلال تجربة FRAP، تبين أن المستخلص الإيثانولي يمتلك أكبر قوة مضادة للأكسدة مختزلة للحديد، بنسبة تثبيط مذهلة بلغت 92.66%. هذه النتائج مجتمعة تسلط الضوء بقوة على الإمكانات الهائلة للبابونج كمصدر طبيعي للمواد المضادة للالتهابات ومضادات الأكسدة. ولهذا، فإن هناك حاجة ملحة لمزيد من العمل لتوصيف المركبات النشطة، وإجراء دراسات في الجسم الحي، وفهم الآليات الجزيئية لهذه التأثيرات، بالإضافة إلى دراسات شاملة حول السمية حركة الدواء .

الكلمات المفتاحية : البابونج الألماني (*Matricaria chamomilla*) ، مضاد للأكسدة، ، مضاد للتمسخ، DPPH ، FRAP.

Abbreviations

DPPH: 2,2-diphenyl-1-picrylhydrazyl

FRAP: Ferric Reducing Antioxidant Power

SFE: Supercritical Fluid Extraction

EO: Essential Oil

ZRT: Temporary Restricted Zone

HCl: Hydrochloric acid

ROS: Reactive Oxygen Species

SET: Single Electron Transfer

Fe^{3+}: Ferric iron

Fe^{2+}: Ferrous iron

$K_3Fe(CN)_6$: Potassium hexacyanoferrate

$FeCl_3$: Ferric chloride

BSA: Bovine Serum Albumin

EPR: Electron Paramagnetic Resonance

H: Hydrogen (often refers to a hydrogen atom in chemical contexts like H donors)

UV-vis : Ultraviolet-visible (spectrophotometer)

NSAID: Non-Steroidal Anti-Inflammatory Drug

NSAIDs: Non-Steroidal Anti-Inflammatory Drugs (plural)

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Introduction

Introduction

Humanity has consistently confronted a myriad of diseases, discomforts, and health challenges throughout history, prompting the development of diverse strategies to counteract these adversities **(Picard et *al*., 2018; Ferroni et *al*., 2018)**. Among the time-honored approaches employed in the battle against illness is the utilization of medicinal plants for treating a wide spectrum of ailments **(Lawal et *al*., 2016; Kruk et *al*., 2019)**. Despite the remarkable advancements in mainstream medical therapies, the inclination towards herbal medicine is experiencing a resurgence. This renewed interest is partly fueled by the escalating concerns surrounding the toxicities and adverse effects associated with many conventional treatments **(Akram et *al*., 2014; Khalid et *al*., 202l)**. Consequently, in recent years, the use of medicinal plants is increasingly being recognized as a valuable source of complementary and alternative treatments, often integrated alongside conventional medical interventions to enhance therapeutic outcomes and mitigate side effects **(Kawamura et *al*., 2018)**.

Within this rich tapestry of plant-based remedies, chamomile (*Matricaria chamomilla L.*), belonging to the Asteraceae family, stands out for its diverse array of reported therapeutic properties. This unassuming plant has been traditionally used for centuries, and modern scientific investigations are increasingly validating its ethnomedicinal applications. *Matricaria chamomilla L.* has demonstrated protective effects against a range of health challenges, including oxidative stress and inflammation, bacterial and fungal infections, cancers, ulcers, and neuropathologies. These beneficial effects are largely attributed to its inherent therapeutic antibacterial, antioxidant, and anti-inflammatory activities. Notably, Matricaria chamomilla is implicated in providing protection against skin photo-damage, aging processes, neuropathologies, and even cancer development, highlighting its significant potential in promoting overall health and well-being **(Akram et *al*., 2024)**.

The multifaceted medicinal properties of chamomile are attributed to its complex phytochemical profile **(Smith et *al*., 2010)**. Key bioactive compounds identified in chamomile include various flavonoids (such as apigenin, quercetin, and luteolin), terpenoids (including bisabolol and chamazulene, which is formed during steam distillation), and phenolic acids **(Jones et Brown, 2015)**. Apigenin, in particular, has been extensively studied for its potent anti-inflammatory, antioxidant, and anti-cancer activities **(Chen et *al*., 2018)**. Bisabolol is known for its anti-irritant, anti-inflammatory, and antimicrobial properties **(Garcia et Lee, 2012)**, while chamazulene contributes to the characteristic blue color of chamomile oil and

exhibits anti-inflammatory and antiallergic effects **(Weber et *al*., 2019)**. The synergistic action of these and other constituents likely contributes to the broad therapeutic spectrum of chamomile **(Miller et Wilson, 2020)**.

To harness the therapeutic potential of chamomile, various extraction methods can be employed to isolate its bioactive compounds **(Robards, 2003)**. Traditional methods often involve the preparation of aqueous extracts (teas) by infusing the dried flower heads in hot water. This method is simple and widely accessible, yielding a range of water-soluble compounds **(Lu et Foo, 2000)**. However, to obtain a more concentrated extract rich in specific lipophilic or volatile compounds, other techniques such as solvent extraction using ethanol, methanol, or ethyl acetate can be utilized **(Smith, 2005)**. Supercritical fluid extraction (SFE) using carbon dioxide is another advanced technique that offers the advantage of being solvent-free and producing high-purity extracts **(Turner et *al*., 2010)**. Steam distillation is specifically used for the extraction of the volatile essential oil of chamomile, which is rich in bisabolol and chamazulene and is often used in aromatherapy and topical applications **(Salehi et *al*., 2018)**. The choice of extraction method significantly influences the chemical composition and, consequently, the biological activity of the resulting extract **(Dai et Mumper, 2010)**.

Therefore, this in vitro study seeks to address the following key question**: Can chamomile extracts effectively inhibit protein denaturation under stressed conditions and exhibit significant antioxidant activity in vitro, thereby supporting its potential as a natural therapeutic agent in mitigating inflammation-induced complications?**

Objective

This research aims to investigate whether chamomile extracts can effectively inhibit protein denaturation under stressed conditions and exhibit significant in vitro antioxidant activity. By examining these properties, the study seeks to support the potential of chamomile extracts as a natural therapeutic agent in mitigating inflammation-induced complications. The ability of the extracts to maintain protein stability and function under conditions mimicking the inflammatory environment, such as elevated temperatures, oxidative stress, pH changes, and the presence of inflammatory mediators, will be examined. Furthermore, the efficacy of the extracts in neutralizing free radicals will be assessed using various antioxidant assays. By correlating these findings, the research aims to provide a scientific rationale for understanding the mechanism by which chamomile extracts may contribute to combating inflammation and reducing its associated damage, thereby supporting their potential use as a natural alternative or adjunct to conventional treatments.

PART ONE:

Generalities

I-Definition of Ethnobotany

Ethnobotany is the scientific study of how people in different cultures use plants. This includes looking at all the ways plants are used, such as for food, building homes, making medicine, tools, fuel, dyes, and even poisons. It also involves understanding the cultural and ritual importance of plants in different societies. Today, scientists use knowledge from ethnobotany, along with chemistry (phytochemistry), genetics, and the study of how drugs work (ethnopharmacology), to discover new ways plants can be helpful **(Sarova, 2017) (Figure 01)**.

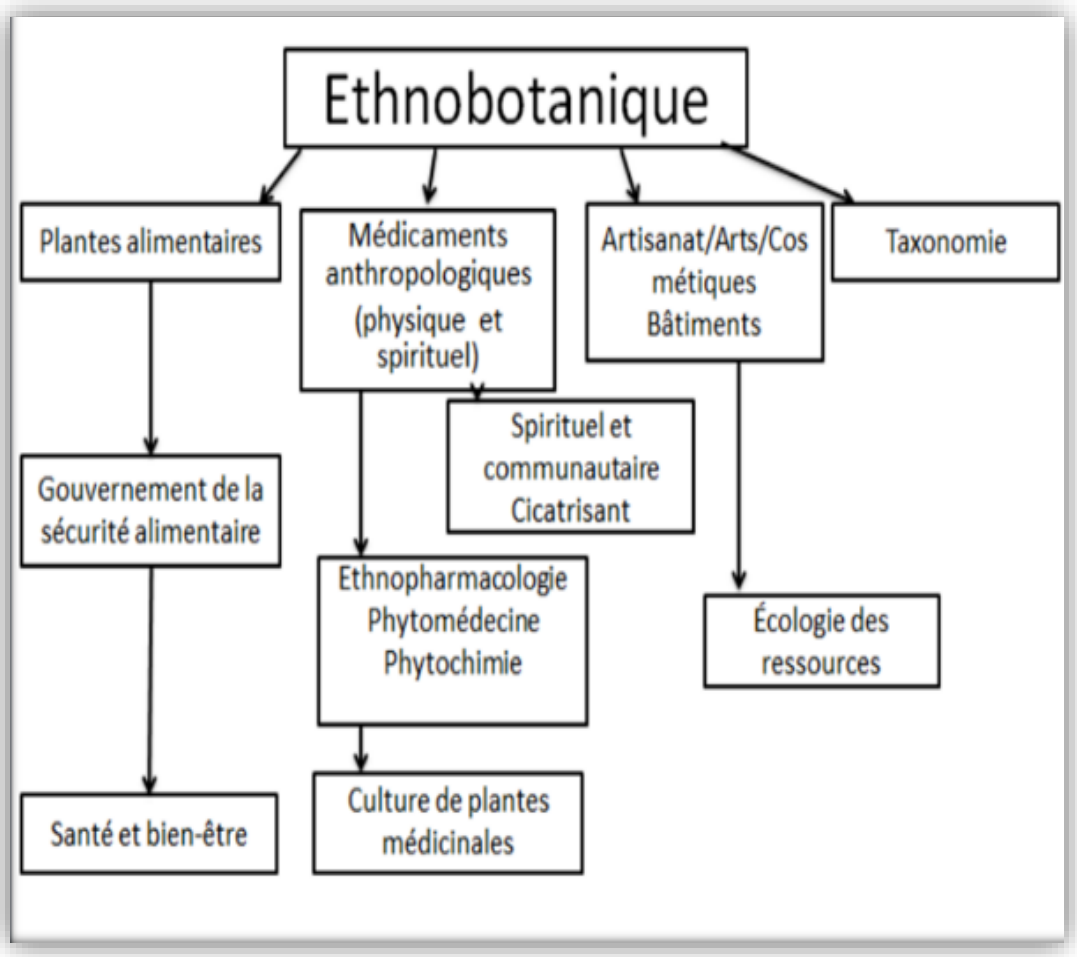

Figure 1. Integrative and multidisciplinary nature of ethnobotany **(Sarova, 2017)**.

I-1.Botany

I-1-1. *Matricaria chamomilla L.*

Matricaria chamomilla L. is a well-known medicinal plant from the Asteraceae family that has been called the "star among medicinal species **(Ali et *al*., 2020)**.

*M. chamomilla i*s an old-time drug famously known as chamomile, German chamomile, Roman chamomile, Hungarian chamomile, and English chamomile **(Franke et *al*., 2005)**.

The issue of misidentification between true chamomile (*Matricaria chamomilla* or sometimes Chamaemelum nobile) and plants like Anthemis cotula, commonly known as stinking chamomile or mayweed, is significant due to the potential for adverse effects. While real chamomile is widely used for its soothing and medicinal properties, Anthemis cotula is considered toxic and has a distinctly disagreeable smell **(Franz et *al*., 2007).**

Figure 2 : *Matricaria chamomilla L*. (Source : NLS Faculty, 2025) .

I-1.2. Botanical and Ecology Description

Matricaria chamomilla is an annual herbaceous plant characterized by slender, tapering roots resembling spindles. Its upright, branching stem typically reaches heights ranging from 10 to 80 centimeters (approximately 4 to 31 inches). The leaves are finely divided, described as bi- to tripinnate, meaning they are compound leaves where the leaflets are further subdivided, resulting in a delicate, feathery appearance. These leaves are typically narrow and elongated **(Singh et *al*., 2011; Lim et *al*., 2014)**.

The flower heads of *M. chamomilla* are borne on individual stalks (pedunculate) and exhibit a heterogamous structure, meaning they contain two types of florets. These flower heads are distinctly separate and measure between 10 to 30 millimeters (roughly 0.4 to 1.2 inches) in diameter. The central disc florets are golden yellow, tubular in shape, and possess five small teeth at their apex. These tubular florets are quite small, ranging from 1.5 to 2.5 millimeters in length, and consistently terminate in a glandular tube, a characteristic feature aiding in identification **(Singh et *al*., 2011; Lim et *al*., 2014)**.

Surrounding the central disc are the ray florets, which are what most people recognize as the "petals" of the chamomile flower. These are typically 11 to 27 in number and are a pure white color. Each ray floret is oblong in shape, measuring 6 to 11 millimeters in length and 3.5 millimeters in width, and they are arranged in a concentric manner around the yellow disc **(Singh et *al*., 2011; Lim et *al*., 2014)**.

The receptacle, the part of the flower head to which the individual florets are attached, is initially flat but becomes conical as the flower matures. It measures 6 to 8 millimeters in width and is notably hollow, a key distinguishing feature from similar-looking species like Anthemis cotula. The fruit of M. chamomilla is a small, dry, one-seeded fruit called a cypsela. These cypselae are yellowish-brown in color and are characterized by the presence of 3 to 5 subtle longitudinal ribs along their surface. This detailed botanical description allows for accurate identification of true chamomile and helps differentiate it from other plants with similar appearances **(Singh et *al*., 2011; Lim et *al*., 2014)**.

Although Matricaria chamomilla exhibits adaptability to various soil types, cultivation in fertile, dense, and waterlogged soils is not recommended. This species thrives in temperatures between 7 °C and 26 °C and requires an annual precipitation of 400 to 1400 mm. While it

demonstrates tolerance to chilly conditions, maximum essential oil (EO) production is achieved when the plant receives ample sunlight, long summer days, and warm temperatures **(Alberts et *al*., 2009)**

I-1-3. Ecological Aspects of Chamomile

Chamomile (*Matricaria chamomilla L.*) is a Mediterranean plant species exhibiting moderate thermophilic requirements. Its cardinal temperatures for germination include a minimum of 6-7 °C and an optimum temperature range of 20-25 °C. During its vegetative growth phase, the plant thrives under an average ambient temperature of 19-20 °C, which represents its optimal growth temperature **(Rafieian-Kopaei et *al*., 2015)**.

Temperature is a critical abiotic ecological factor influencing the physiological and biochemical attributes of chamomile. Empirical studies indicate an inverse relationship between increasing temperatures and biomass accumulation (manifested as decreased wet weight), individual flower weight, and the phenological development rate (prolonged duration from germination to anthesis). Conversely, elevated temperatures correlate with an increased accumulation of the secondary metabolites apigenin and essential oil **(Rafieian-Kopaei et *al*., 2015)**.

Chamomile demonstrates tolerance to transient low temperatures during the Temporary Restricted Zone (ZRT) presumably referring to a specific developmental research trial). However, exposure to cold temperatures during the stem thickening growth stage in spring can induce cryogenic stress, leading to developmental arrest and a subsequent reduction in floral yield **(Rafieian-Kopaei et *al*., 2015)**.

Chamomile exhibits salt tolerance as a glycophyte, accumulating salt in roots for drought resistance in saline conditions, although high salinity reduces flower yield

It adapts to various soils but prefers light, calcareous, sandy soils with a pH between 4.8 and 8.0, showing potential for alkaline soil growth **(Zargari et Amirbeygi, 1995-1996)**.

I-1-4. Geographic Distribution

Matricaria chamomilla L. originates from the southern and eastern regions of Europe, as well as the northern and western parts of Asia. It has since been brought to numerous other countries and has successfully established itself in the wild in places like Great Britain, Australia, and

North America **(Singh et *al*., 2011)**. Currently, it enjoys broad distribution and is commercially farmed in nations such as Germany, Hungary, France, the former Yugoslavia, Russia, Brazil, New Zealand, and North Africa **(Singh et *al*., 2011)**.

I-1-5. Scientific classification

Table 01 : Scientific classification of *Matricaria chamomille L.*

Taxonomic Rank	Classification
Kingdom	Plantae
Phylum	Angiosperms
Sub-Phylum	Eudicots
Class	Asterids
Order	Asterales
Family	Asteraceae
Tribe	Anthemideae
Genus	Matricaria
Species	*M. chamomilla*

II.Therapeutic effects

Chamomile, a versatile herb, exhibits numerous therapeutic effects. It strengthens nerves and sexual ability, acts as a brain tonic and diuretic, and regulates menstruation and lactation. It

alleviates headaches, migraines, bladder stones, and urinary dribbling **(Carl et *al*., 1991; Sarris et *al*., 2011)**.

Furthermore, chamomile relieves teething pain, insomnia, anorexia, enteritis, and anemia. It eliminates intestinal worms, menstrual cramps, and jaundice. Chamomile baths and topical applications with almond oil relieve pain, skin problems (eczema, hives, itching), and ear pain. It also acts as an emetic in food poisoning and eases backache, joint pain, and gout **(Carl et *al*., 1991; Sarris et *al*., 2011)**.

Chamomile brightens blonde hair, alleviates menopausal disorders, and combats allergies. It aids digestion, relieves gastrointestinal spasms, and treats various gastrointestinal issues. It enhances memory, strengthens nerves, and exhibits sedative, anti-migraine, hypnotic, anticonvulsant, anticancer, anti-anxiety, anti-epileptic, and anti-seizure activities. It also lowers blood pressure and has anti-inflammatory, eczema treatment, acne treatment, skin disinfectant, wound healing, and skin strengthening properties **(Carl et *al*., 1991; Sarris et *al*., 2011)**.

Additionally, chamomile boosts the immune system, relieves coughs and congestion, treats colds, lung and laryngeal inflammation, joint inflammation, and rheumatic symptoms. It addresses kidney and bladder swelling, urinary issues, premature ejaculation, and menopausal disorders. It also manages ulcerative colitis, reduces fatigue, and exhibits wound-healing, bacteriostatic, bactericidal, antifungal, and anticancer properties **(Carl et *al*., 1991; Sarris et *al*., 2011)**.

Chamomile's therapeutic effects are largely attributed to its polyphenolic compounds and antioxidant activity, which neutralize harmful free radicals and mitigate disease complications **(Rahnama et *al*., 2014; Jafarpoor et *al*., 2014)**.

II-1. Denaturation

In scientific literature, denaturation is defined as the process in which proteins or nucleic acids lose their native, functional three-dimensional structure. This disruption of the molecule's higher-order structure (secondary, tertiary, and quaternary for proteins; double helix for DNA and RNA) occurs due to the breaking of weak chemical bonds like hydrogen bonds, hydrophobic interactions, and salt bridges. Factors that can cause denaturation include changes in temperature, pH extremes, chemical agents (such as detergents, urea, and organic solvents),

and even physical agitation. While denaturation alters the molecule's conformation and often its biological activity, the primary structure (the sequence of amino acids or nucleotides) typically remains intact. The process can sometimes be reversible (renaturation) if the denaturing conditions are removed, but in many cases, it is irreversible **(Khan et *al*., 2025)**.

Denaturation, in biology, proces modifying the molecular structure of a protéine or nucleic acid. Denaturation invioles the Brea King of many of the weak linkages, or noncovalent bonds, such as hydrogen bonds and hydrophobic interactions, within a protein or nucleic acid molecule **(Khan et *al*., 2025)**.

II-2. Antidenaturation

Anti-denaturation, in a biochemical context, refers to the ability of a compound or agent to prevent or reverse the structural changes in a protein molecule that lead to the loss of its native conformation and function. Protein denaturation can be induced by various stressors, including heat, extreme pH, organic solvents, and certain chemicals. At a molecular level, anti-denaturation involves stabilizing the non-covalent interactions (such as hydrogen bonds, hydrophobic interactions, and electrostatic forces) that maintain the protein's intricate three-dimensional structure. By preserving this native conformation, anti-denaturing agents help to maintain the protein's biological activity and prevent its aggregation or precipitation (**Ramalingam et *al*., 2010)**.

The in vitro anti-denaturation effects induced by natural products and non-steroidal compounds in heat treated (immunogenic) bovine serum albumin is proposed as a screening assay for the detection of anti-inflammatory compounds, without the use of animals, in the early stages of the drug discovery process The effects of anti-desnaturalization in vitro induced by natural products and non-steroidal compounds in the bovine serum album (immunogenic) tratada with heat, it is recommended to try to test for the detection of inflammatory compounds without the use of animals in the early stages of the drug discovery process **(Williams et *al.*, 2008)**.

II-2.1. Proteins denaturation in human body

Various environmental factors can induce protein denaturation within the human body:

2.1.1. Fever (Hyperthermia)

When the body temperature rises significantly, the increased kinetic energy disrupts the weak bonds (like hydrogen bonds) that maintain the intricate three-dimensional structure of proteins, causing them to unfold and lose their function. This can impair various cellular processes **(Hopkins et *al*., 2022)**.

2.1.2. Extreme pH Changes

The body tightly regulates pH levels. However, in certain pathological conditions, localized or systemic pH can deviate significantly. Extreme acidity or alkalinity can disrupt the ionic and hydrogen bonds within proteins, leading to denaturation. For instance, the stomach acid (HCl) denatures proteins in food to aid digestion, but drastic pH changes within other bodily fluids can be harmful to the body's own proteins **(Hopkins et *al*., 2022)**.

2.1.3. Heavy Metal Poisoning

Heavy metals like mercury, lead, and cadmium can bind to proteins, disrupting their structure and function. They might interfere with disulfide bonds or bind to amino acid side chains, causing denaturation. The search results mention that heavy metals can change the structure of proteins by binding to functional groups **(Hopkins et *al*., 2022)**.

2.1.4. Cellular Stress

Various cellular stresses, including high concentrations of certain chemicals or the presence of misfolded proteins, can trigger denaturation of other proteins within the cell. The body has mechanisms to refold or degrade these denatured proteins to maintain cellular health **(Hopkins et *al*., 2022)**.

2.1.5. Drugs that contribute in proteins denaturation

drugs that cause protein denaturation, it's important to know that various chemical and physical agents can lead to this process. Some examples of such agents include:

A. Organic solvents

Alcohols (like ethanol) can disrupt hydrophobic interactions in proteins **(Walker et *al*., 2009; Kharaz et *al*., 2017; Mehak et *al*., 2024)**.

B. Detergents

These can interfere with hydrophobic interactions and disrupt the protein structure **(Walker et *al*., 2009; Kharaz et *al*., 2017; Mehak et *al*., 2024)**.

C. Urea and guanidinium chloride

These chaotropic agents disrupt hydrogen bonds and other non-covalent interactions **(Walker et *al*., 2009; Kharaz et *al*., 2017; Mehak et *al*., 2024)**.

D. Acids and bases

Extreme pH levels can alter the charges on amino acid side chains, leading to denaturation **(Walker et *al*., 2009; Kharaz et *al*., 2017; Mehak et *al*., 2024)**.

E. Heavy metals

Some heavy metals can bind to proteins and disrupt their structure and function **(Walker et *al*., 2009; Kharaz et *al*., 2017; Mehak et *al*., 2024)**.

II-2-2. Example of antidenaturation

A. Flavonoids from various plants: These polyphenolic compounds, found in fruits, vegetables, and tea, have shown the ability to inhibit protein denaturation in in vitro studies **(Anandavadivelan et Girija, 2015)**.

B. Specific plant extracts: Extracts from plants like *Ficus racemosa* bark have demonstrated significant in vitro anti-denaturation activity **(Dharmadeva et *al*., 2019)**.

C. Fatty acids (e.g., from fish oil): Polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found in fish oil, have exhibited anti-denaturing effects (**Mizushima et *al*,.1999)**.

Certain traditional medicinal plants: Extracts from plants like *Acacia nilotica* have four shown notable protein anti-denaturation properties (**Eswaran et *al*,. 2013)**.

II-3- Oxydation

The fundamental chemical process involving the interchange of electrons between two distinct chemical entities is termed an oxidation (electron relinquishment)–reduction (electron acquisition) or redox reaction. The chemical species that accepts electrons functions as the oxidant, undergoing a reduction in its oxidation state. Conversely, the chemical species that donates electrons acts as the reductant, experiencing oxidation characterized by an elevation in its oxidation state **(Franco et *al*., 2018)**.

II-4-Antioxydant

Antioxidants are groups of compounds that neutralize free radicals and reactive oxygen species (ROS) in the cell **(Abuajah et *al*., 2015)**.

These antioxidants provide protection against damage caused by free radicals played important roles in the devolopment of many chronic diseases including cardiovascular diseases, aging, heart disease, anaemia, cancer, inflammation **(Vaibhav et *al*., 2015)**.

An antioxidant is characterized as a molecule capable of significantly impeding or entirely inhibiting the oxidative degradation of substrate molecules, even when present in minute concentrations **(Gulcin et *al*., 2010)**, these compounds act as electron donors to free radicals, thereby neutralizing them and mitigating oxidative damage within biological systems **(Gulcin et *al*., 2010; Shantabi et *al*., 2014)**, antioxidants mitigate the formation of free radicals by modulating the free radical-mediated oxidative process across its principal phases: initiation, propagation, and termination **(Gocer et *al*., 2011; Gülçin et *al*., 2012)**. Reaction kinetics constitute a significant parameter influencing the protective efficacy of an antioxidant over both short and long timescales. This encompasses the thermodynamics governing the interaction between the antioxidant and various oxidants, the reaction rate constant, and the inherent reactivity of the antioxidant molecule. A comprehensive evaluation of antioxidant effectiveness necessitates the consideration of all these kinetic and thermodynamic parameters **(Aksu et *al*., 2015**), this mechanism allows them to maintain redox homeostasis within their metabolic processes **(Halliwell et *al*., 1996)**.

Antioxidants can be classified into two groups according to their nature and origin:

A-Natural antioxidants

A-1 Enzyme antioxidants: superoxide dismutase, catalase, glutathione peroxidase

A-2 Non-enzymic antioxidants: vitamin C, vitamin E, beta-carotene, glutathione, trace elements (copper, zinc, selenium, manganese, and iron), polyphenols (flavonoids, phenolic acids, tannins, coumarin, etc.)

B-Synthetic antioxidants: butylhydroxyanysole, butylhydroxytoluene

II-5- Oxidative Stress

Cellular survival hinges on oxidation processes, notably aerobic respiration, which catabolizes organic molecules like glucose to yield energy. However, this metabolic activity concurrently generates reactive oxygen species (ROS), also known as free radicals, that can induce oxidative damage to cellular components (**Gulcin et *al*., 2020)**. A free radical is characterized by the presence of an unpaired electron, which possesses an intrinsic quantum mechanical property known as spin. This open-shell electronic configuration typically confers high chemical reactivity upon the species **(Buyukokuroglu et *al*., 2001)**. However, currently, numerous stable free radical species exist under standard laboratory conditions, specifically in ambient air and at room temperature **(Ionita et *al*., 2021)**. Oxidative stress is predominantly mediated by free radicals **(Gulcin et *al*., 2006; Altay et *al*., 2019)**.

The concept of oxidative stress is a relatively recent paradigm that has gained significant traction within the medical sciences **(Balaydın et *al*., 2010; Munteanu et *al*., 2021)**. This phenomenon arises from the overproduction of reactive oxygen species (ROS) within the cellular mitochondrion. The inherent generation of free radicals in biological systems is a well-established process, and their accumulation is implicated in the pathogenesis of numerous degenerative conditions, including carcinogenesis, acute inflammation, hypertension, diabetes mellitus, preeclampsia, acute kidney injury, atherosclerosis, Alzheimer's disease, Parkinson's disease, mutagenesis, senescence, and cardiovascular disorders **(Kedare et *al*., 2011; Cetinkaya et *al*., 2012)**.

Environmental factors, including ultraviolet (UV) radiation and pollutants, contribute to oxidative stress, exerting a daily impact on human health **(Koksal et *al*., 2011)**.

Excessive generation of free radicals within the human body leads to significant deleterious effects across various tissues **(Hamad et *al*., 2017)**.

A critical complication arising in this context involves the generation of lipid peroxidation within the plasma membrane. This process subsequently enhances the production of reactive nitrogen species (RNS) and reactive oxygen species (ROS). Concurrently, the presence of transition metals, such as iron and copper, facilitates Fenton and Haber-Weiss **(Gulcin *al*., 2008); Cakmakcı et *al*., 2015; Topal *al*., 2016)**.

Furthermore, the Haber-Weiss reaction generates hydroxyl radicals (OH·) from superoxide anions ($O2^{-}$) and hydrogen peroxide (H_2O_2) through catalysis by iron ions (Fen+). This mechanistic implication was initially proposed by Fritz Haber **(Haber et *al*., 1934)**.

II-5-1. Natural products and their role to combact oxidative stress

Natural substances, especially antioxidants such as flavonoids, are presented as promising in fighting the oxidative stress and potentially lessening the risk and impact of diseases **(Muscolo et *al*., 2024)**. The importance of further research into the molecular mechanisms of these natural compounds is emphasized to strengthen their use in prevention and treatment **(Chen et *al*., 2016)**.

Figure 3: Free radical/antioxidant balance **(Shimizu, 2004)**

II-5-2. consequences of oxidative stress

If not strictly controlled, oxidative stress can be responsible for the induction of several diseases, both chronic and degenerative, as well as speeding up body aging process and cause acute pathologies (i.e., trauma and stroke). Depending on its intensity, oxidative stress induces a variety of cellular responses. Mild stresses can stimulate cell growth, while more severe stresses lead to irreversible damage, ranging from apoptosis to necrosis. The long-term consequences of oxidative stress are numerous and varied, including genetic mutations, cellular dysfunction, chronic diseases, and premature aging **(Favier,2003)**.

The importance of further research into the molecular mechanisms of these natural compounds is emphasized to strengthen their use in prevention and treatment. The primary goal of the discussed research collection is to demonstrate the role of natural products in reducing oxidative stress and related health issues **(Wei Chen et *al*., 2016)**.

PART TWO :

Materials and methods

I-Material and methods

I-1. Acquisition and laboratory preparation of plant material

Various extraction methodologies were employed to obtain different fractions from the *Matricaria chamomilla* plant material. Initially, a specific quantity of dried chamomile was purchased from the local market in the city of Tiaret and botanically authenticated at the university. Thereafter, the plant material was mechanically processed to yield a fine powder, thereby increasing the surface area for subsequent solvent interaction. Three distinct extraction procedures were then performed.

I-1-1.Preparation of Chamomile flowers

Whole, dried *Matricaria chamomilla* flowers were utilized as the starting plant material. These were subjected to gentle comminution, a process involving a slight crushing action, to increase the specific surface area of the plant matrix. This physical pretreatment enhances the accessibility of intracellular components to the extraction solvent, thereby improving the overall efficiency of the subsequent extraction process by facilitating mass transfer kinetics.

Figure 4 : Preparation of Chamomile flowers s (Original

I-1-2.Preparation of ethanolic extract

We initiated the ethanolic extraction by carefully weighing 7.5 g of the powdered Matricaria chamomilla and transferring it into a beaker. Subsequently, we added 100 mL of a 60% (v/v) ethanol-water solution (prepared by mixing 60 mL of absolute ethanol with 40 mL of distilled water) to the beaker, ensuring complete immersion of the chamomile powder. The resulting mixture was then subjected to maceration, with continuous agitation provided by a mechanical stirrer, maintained at a temperature of 10°C for a duration of 2 hours. This process was implemented to facilitate the efficient dissolution of ethanol-soluble phytochemicals from the plant matrix into the solvent.

In the context of plant material extraction, including that of *Matricaria chamomilla*, methanol is often selected as a solvent due to several key physicochemical properties. Firstly, its effectiveness as a solvent stems from its ability to solubilize a wide array of phytochemicals, encompassing both polar and moderately polar compounds. This broad solvent capacity allows for the extraction of diverse classes of plant metabolites, including various active compounds with potential medicinal or industrial applications. Secondly, methanol exhibits a high degree of penetrability into plant tissues. This characteristic facilitates the efficient mass transfer of intracellular constituents into the extraction solvent by effectively permeating the cell walls and membranes, thereby maximizing the recovery of target analytes from the plant matrix.

I-1-3.Preparation of acetonic extract

For the acetonic extraction, we prepared a 35% (v/v) acetone-water solution by carefully mixing 52.5 mL of acetone with 97.5 mL of distilled water to achieve a final volume of 150 mL. Subsequently, we introduced 10 g of the powdered *Matricaria chamomilla* into this solution, ensuring complete submersion of the plant material to maximize solvent-solid interaction. The resulting mixture was then subjected to infusion with continuous agitation using a mechanical stirrer, maintained at a temperature of 1 °C for a duration of 2 hours. This process was implemented to facilitate the extraction of acetone-soluble phytochemicals from the chamomile powder into the solvent phase.

I-1-4. Preparation of aqueous extract

For the aqueous extraction, we initiated the process by carefully weighing 10 g of the powdered *Matricaria chamomilla* and transferring it into a suitable vessel. Subsequently, we added 150 mL of distilled water, ensuring that the powdered plant material was completely submerged in the aqueous solvent. The resulting mixture was then subjected to infusion, facilitated by continuous agitation using a mechanical stirrer, and maintained at a temperature of 10°C for a duration of 2 hours. This procedure was implemented to promote the dissolution and extraction of water-soluble phytochemicals present within the chamomile powder into the aqueous medium.

Following the respective extraction procedures, each obtained extract was subjected to a filtration process to remove any residual solid particulate matter, ensuring a clarified liquid extract. Subsequently, the filtered extracts were transferred into separate Petri dishes, serving as evaporation vessels with a large surface area to volume ratio. These Petri dishes containing the extracts were then placed in a laboratory incubator maintained at elevated temperatures. This incubation step facilitated the evaporation of the extraction solvent, driving the transition of the dissolved solutes into a solid, powdered form. The resulting dried extracts, representing concentrated fractions of the chamomile's phytochemical constituents based on the initial solvent used, were then collected for further analysis or experimental applications.

II-Methods for Evaluating the Antioxidant Activity of Chamomile

The current surge in research interest prominently features the exploration of molecular antioxidant compounds derived from natural sources. In line with this trend, our study was designed to comprehensively evaluate the antioxidant activities inherent in different extracts obtained from various parts of *Matricaria chamomilla* (chamomile). The antioxidant activity of the chamomile extracts was evaluated through two distinct assays that provide complementary perspectives on their radical scavenging and reducing abilities: the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) free radical scavenging assay and the Ferric Reducing Antioxidant Power (FRAP) assay 1 **(Georgieva et *al*., 2010)**.

It is a common and insightful practice within antioxidant research to categorize assays based on the specific radical-generating substrate utilized as an indicator molecule. In our investigation, we adhered to this principle, recognizing the importance of employing assays that

capture different facets of antioxidant activity. As such, we selected and utilized both the FRAP assay, which measures the ability of antioxidants to reduce ferric ions (Fe 3+ to Fe 2), and the DPPH• radical scavenging assay, which quantifies the capacity of antioxidants to neutralize stable free radicals. These assays, along with others commonly employed in the field, offer valuable and often complementary insights into the multifaceted mechanisms through which antioxidant compounds exert their protective effects **(Georgieva et *al*., 2010)**. By employing both the DPPH and FRAP assays, we aimed to gain a more holistic understanding of the antioxidant potential of the different parts of *Matricaria chamomilla.*

II-1.FRAPP test

In our laboratory, we employed the Ferric Reducing Antioxidant Power (FRAP) assay, a widely recognized technique based on the single electron transfer (SET) mechanism, to assess the total reducing power of our samples. This assay specifically measures the ability of antioxidants present in an acidic solution to reduce a ferric (Fe^{3+})-ligand complex to its ferrous (Fe^{2+}) form. This reduction is accompanied by the development of an intensely colored ferrous complex, the formation of which is directly proportional to the antioxidant capacity of the sample. By quantifying the absorbance of this colored complex, we can determine the reducing power of the antioxidants present in our extracts **(Antolovich et *al*., 2002).**

Initially, the ferric iron complex exhibits a yellow coloration. Upon reduction in the presence of electron-donating antioxidants, ferrous iron is formed, resulting in a color change to blue or green. The magnitude of this color change is directly proportional to the extract's antioxidant activity **(Hamadou Habibou et *al*., 2019)**.

II-1-1.Protocol

To determine the ferric reducing property of our extracts, we followed the assay procedure outlined by Yen and Chen **(Yen et *al*., 1994)**.

To assess the reducing power of our sample, which indicates its antioxidant potential, we followed a specific protocol.

We started by taking a precise volume of 0.5 mL of our sample solution, which had a concentration of 1 milligram of the extract per milliliter of solvent, and transferred it into a clean test tube. To this sample solution, we then added 1.25 mL of a phosphate buffer solution.

This buffer had a concentration of 0.2 Molar and maintained the pH of the reaction at 6.6. Following the buffer, we added 1.25 mL of a 1% solution of potassium hexacyanoferrate [$K_3Fe(CN)_6$]. This chemical compound contains iron in its ferric (Fe^{3+}) state, which we aim to reduce. We then placed the test tube containing this mixture into a water bath that was kept at a constant temperature of 50°C. The mixture was allowed to incubate under these conditions for a duration of 30 minutes to allow the reaction to proceed. After the incubation period, we stopped the reaction by adding 1.25 mL of a 10% trichloroacetic acid solution to the test tube. This step helps to precipitate any large molecules, like proteins, and ensures the reaction does not continue further. From this reaction mixture, we carefully took three equal portions, each with a volume of 0.625 mL, and transferred them into separate, clean test tubes. To each of these portions, we added 0.625 mL of distilled water and 0.125 mL of a freshly prepared 1% ferric chloride ($FeCl_3$) solution in water. The ferric chloride will react with any ferrous iron (Fe^{2+}) that was formed during the incubation, resulting in a colored complex **(Hamadou Habibou et *al*., 2019)**.

To ensure our measurements were accurate, we also prepared a blank sample. This blank contained all the same reagents – the phosphate buffer, potassium hexacyanoferrate, and trichloroacetic acid – but instead of our sample extract solution, we used the same volume of the solvent that was used to dissolve our extract. This blank was treated exactly the same way as our actual samples.

II-2.DPPH test

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was discovered 100 years ago by Goldschmidt and Renn in 1922 **(Goldschmidt et *al*., 2022). Then,** Blois has developed this method (Blois, 1958).

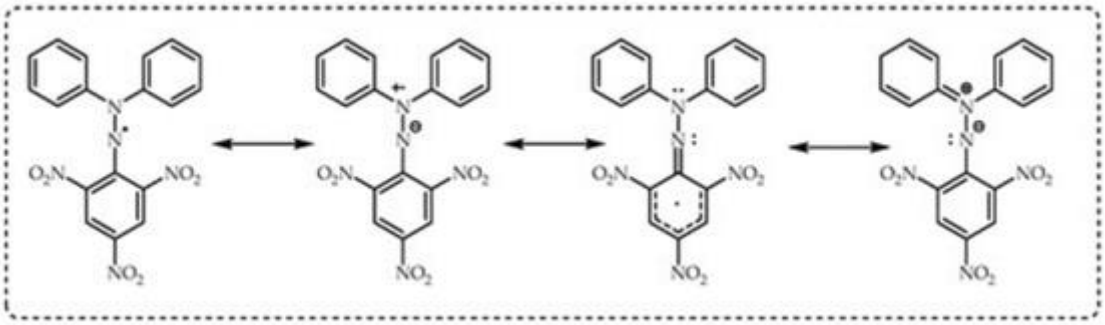

Figure 5 : The chemical structures of a 1,1-diphenyl-2-picrylhydrazyl radical (DPPH·)

This spectrophotometric assay assesses antioxidant capacity by measuring DPPH radical scavenging. Developed further by Brand-Williams et *al*. in 1995, it became a widely used method. Gulcin's group effectively applied this antioxidant technique with a minor modification **(Bondet et *al*., 1997)**.

The nitrogen atom's single electron in the DPPH radical is reduced to its corresponding hydrazine form by accepting a hydrogen atom from an antioxidant. The DPPH radical is notable for its stable nature and intense coloration. These two characteristics have led to its widespread use in solution. This radical is frequently employed in polymer chemistry, particularly in Electron Paramagnetic Resonance (EPR) spectroscopy, and for assessing the antioxidant capacities of various chemical compounds **(Bondet et *al*., 1997)**.

The telltale violet blue of a DPPH solution disappears when it encounters a compound capable of donating a hydrogen. This loss of color signifies the reduction of the DPPH radical to its DPPH-H form **(Yapıcı et *al*., 2021)**. The deep violet color we see in the DPPH· solution comes from that wider band in its spectrum. The moment hydrazine (DPPH-H) forms, that visible band disappears, and the solution's color shifts from violet to a pale yellow. This color change is a direct result of the radical being reduced by a hydrogen atom donated by the antioxidants (the H donors). This reaction, which the literature calls the "DPPH test," is something we can easily measure by looking at it with a UV-vis spectrophotometer. It's a common technique for assessing how strong the antioxidant capacity is in pure antioxidant molecules, particularly things like herbal extracts or phenolic compounds **(Xie et *al*., 2014)**.

As a widely used spectrophotometric technique, DPPH radical scavenging is a go-to method for assessing the antioxidant power of various samples, including beverages, pure compounds, foods, and plant extracts. Its simplicity, sensitivity, speed, and reproducibility make it the most convenient and popular approach for evaluating the radical-scavenging capacity of both individual substances and complex herbal mixtures **(Gulcin et *al*., 2023)**.

II-2-1.Protocol

In the careful preparation of the DPPH stock solution utilized for the experimental procedure, there was a specific protocol that had been adhered to in order to maintain accuracy and consistency. First, 0.025 g of 2,2-diphenyl-1-picrylhydrazyl (DPPH) powder was weighed accurately with the aid of a calibrated analytical balance, a crucial step towards attaining the

required concentration. The aforementioned accurately weighed DPPH was then transferred quantitatively into a 1-liter Class A volumetric flask. The utilization of a volumetric flask, whose volume measurement is very precise, was at the focal point in this case.

Dissolution and Homogenization Then, methanol, a suitable solvent for DPPH, was carefully added to the flask. Addition of the solvent was gradual, with initial swirling to promote dissolution of the DPPH powder. Methanol was added in such a way that the meniscus of the solution precisely reached the 1-liter calibration mark on the neck of the volumetric flask. This provides for the final volume to be precisely 1 liter.

In order to attain full dissolution and to give a homogenous solution, the flask was then closed and moderately agitated using gentle inversion and stirring. This agitation is required to guarantee that molecules of DPPH are evenly dispersed within the solvent's volume so concentration gradients will not exist.

Aliquoting for Experimental Use In the present experimental arrangement, with the use of varied reaction vessels, a known volume of the prepared DPPH stock solution was needed. From calculations using the concentration of the stock solution (0.025 g DPPH in 1 liter methanol), a total volume of 24 mL of the DPPH stock solution was found to be required. The 24 mL aliquot volume was accurately measured, typically using a burette or calibrated pipette to ensure accuracy, and then transferred into the respective single laboratory tubes. The accurate aliquoting guaranteed that each reaction vessel received an equal and accurate volume of DPPH, which represented 0.0006 g of the original mass of DPPH. Attention to detail in the preparation phase is required to achieve dependable and reproducible results in quantitative experimental techniques.

II-3.Antidenaturation test

The anti-denaturation assay utilizing Bovine Serum Albumin (BSA) presents a valuable in vitro approach to streamline the early stages of drug development. This method is predicated on the principle that the application of heat to BSA induces denaturation, leading to the exposure of antigens associated with type III hypersensitivity reactions, which are implicated in the pathogenesis of conditions such as serum sickness, glomerulonephritis, rheumatoid arthritis, and systemic lupus erythematosus. Consequently, the BSA assay serves as an efficient tool for

identifying compounds capable of stabilizing proteins and preventing this denaturation process **(Williams et *al*., 2008)**.

To specifically investigate the capacity of our *Matricaria chamomilla* extracts to prevent protein denaturation, we conducted a heat-induced denaturation assay, utilizing BSA as our model protein. This particular assay allows us to effectively evaluate the protective effects of our extract against the unfolding and subsequent aggregation of proteins under thermal stress, thereby providing crucial insights into its potential as a potent inhibitor of protein denaturation.

II-3-1.Protocol

In this experiment, we meticulously prepared test samples to investigate the potential of different extracts to protect bovine serum albumin (BSA) against heat-induced denaturation. Our procedure in olved several key steps, each designed to provide specific insights into this protective effect

II-3-2.Preparation of Test Samples:

We initiated the process by creating a series of test tubes, each containing a specific combination of solutions. Into each tube, we introduced 1 mL of the extract solution. Recognizing that the solvent might influence the extract's activity, we employed three different extraction solvents: ethanol, distilled water, and acetone. This allowed us to assess the impact of solvent polarity on the potential protective properties of the extracts. Following the addition of the extract, we introduced 4 mL of a phosphate buffer solution (0.1 M, pH 7.4). The phosphate buffer was crucial in maintaining a stable physiological pH throughout the experiment, ensuring that any observed effects were due to the extract components rather than pH fluctuations. Finally, to each tube, we added 1 mL of a 1 mM albumin solution, prepared in the same phosphate buffer. This ensured a consistent concentration of the target protein across all test samples.

Once the mixtures were prepared, we subjected the test tubes to an initial incubation period of 15 minutes at 37°C. This temperature is physiologically relevant and this step was included to allow ample time for any potential interactions between the diverse components present in the extracts and the albumin molecules to occur before the introduction of the denaturation stress.

We hypothesized that any protective molecules within the extracts would need this initial contact period to bind to or interact with the albumin, potentially stabilizing its structure

II-3-3. Heat-Induced Denaturation

To induce protein denaturation, we transferred the test tubes to a precisely controlled water bath maintained at 60°C. This elevated temperature was applied for a duration of 15 minutes, a condition known to disrupt the delicate non-covalent interactions that maintain the native tertiary structure of albumin, leading to its unfolding and subsequent.

By applying a consistent heat stress across all samples, we aimed to isolate the effect of the different extracts on the protein's stability.

Following the heat treatment, we allowed the test tubes to cool down to room temperature. This cooling step was crucial to effectively halt the denaturation process at a specific endpoint. By rapidly reducing the temperature, we prevented further uncontrolled aggregation of the denatured albumin, allowing for a more accurate and reproducible measurement in the subsequent spectrophotometric analysis.

The extent of albumin denaturation was quantified using a spectrophotometer. Specifically, we measured the absorbance of each solution at a wavelength of 660 nm. This wavelength is sensitive to the turbidity of the solution, which directly correlates with the degree of protein aggregation. As albumin denatures and aggregates, the solution becomes more turbid, leading to a higher absorbance reading. Conversely, if an extract possesses protective properties, it would reduce the extent of denaturation and aggregation, resulting in a lower absorbance reading at 660 nm. This quantitative measurement allowed us to directly compare the effectiveness of the different extracts in preventing albumin denaturation.

To ensure the validity and proper functioning of our assay, we included a positive control. This control consisted of 1 mL of phosphate-buffered saline (PBS), 4 mL of PBS, and 1 mL of the albumin solution, subjected to the exact same incubation and reading steps as our test samples. The positive control represented the albumin under the heat stress in the absence of any potentially protective extract. We expected this sample to exhibit a high degree of denaturation and thus a high absorbance reading, confirming that our heat treatment was indeed effective in

inducing protein denaturation. This control served as a benchmark against which to evaluate the protective effects of our extracts.

To eliminate any background interference from the buffer solution itself, we included a blank. Our blank consisted of 6 mL of the phosphate buffer (0.1 M, pH 7.4), treated identically to the test and positive control samples before measuring the absorbance By using this buffer blank to zero the spectrophotometer, we ensured that our absorbance readings for the test samples and the positive control specifically reflected the turbidity caused by protein aggregation, providing a clear and accurate baseline for our measurements.

II-4.PBS

The preparation of 11 Phosphate Buffered Saline (PBS) at pH 7.4 is a fundamental procedure in numerous biological applications. This isotonic buffer meticulously mimics the physiological environment in terms of pH, osmolarity, and ion concentrations, closely resembling those found within the human body. This characteristic makes PBS an excellent choice for applications such as washing delicate cells, transporting vital tissues, and diluting various substances without causing any cellular damage or stress. Furthermore, owing to its non-toxic nature to cells, PBS is extensively utilized in rinsing cell culture vessels and other preparations where residual substances could interfere with subsequent experiments. While its preparation is straightforward and it boasts a good long-term shelf life, it's crucial to note that PBS can precipitate in the presence of zinc ions, necessitating caution when used in applications that might involve these ions. **(Sambrook, J ., Russell, D ., 2001) , (Greenfield , E, A ., 2014).**

II-4-1.Protocol

To prepare a liter of PBS (pH 7.4), we start by taking 800 milliliters of good quality distilled water in a suitable container. We then carefully add 8 grams of sodium chloride (NaCl) with a molecular weight of 58.44 g/mol. Sodium chloride is an important constituent in bringing the osmotic pressure of the solution to a value such that the cell neither shrinks nor swells

when it comes in contact with it. Additionally, we include 0.2 grams of potassium chloride (KCl), which has a molecular weight of 74.55 g/mol and also helps to provide ionic balance as well as osmotic stability.

In order to make the phosphate buffer system that is responsible for sustaining a stable pH of 7.4, we combine 1.44 grams of disodium phosphate (Na_2HPO_4) with a molecular weight of 141.96 g/mol and 0.245 grams of monopotassium phosphate (KH_2PO_4) with a molecular weight of 136.09 g/mol. The two components act together to create an effective buffering system, which resists pH change with the addition of small amounts of acids or bases and thereby keeps the environment constant for biochemical reactions. After addition of all salts, pH of the resulting solution is subsequently adjusted with care to achieve the target value of 7.4 using dilute hydrochloric acid (HCl) or sodium hydroxide (NaOH), which is regarded as ideal for most applications in cellular and molecular biology. Distilled water is subsequently added incrementally in small amounts until the volume of solution achieves the final volume of 1 liter, thereby achieving proper concentrations of all reagents.

II-4-2.Diclofenac Sodium

For the experimental determination of diclofenac sodium, a dilution series was carefully prepared in order to plot a calibration curve. A 75 mg/ 3 ml stock solution of diclofenac sodium was used. An exact volume of 1 mL from the stock solution was diluted with 9 mL of distilled water. The initial dilution is required for the sake of bringing the concentration to a level at which it would be suitable for further spectrophotometric measurement.

Preparation of Dilution Series and Spectrophotometric Analysis Following the initial dilution, there was a scheduled sequence of additional dilutions. In this sequence, the concentrations actually utilized were based on the anticipated concentrations of diclofenac sodium in the samples to be determined, thereby enabling the preparation of an extensive calibration curve. The absorbance of the resulting diclofenac sodium solutions was ascertained after each dilution procedure, using a spectrophotometer.

The precise wavelength used to make these early measurements was not defined; however, it would typically be that at which the absorption maximum of solution diclofenac sodium occurs, determined through an initial spectral scan. This method allows for accurate quantitation in accordance with the Beer-Lambert Law.

II-5.Protein Binding Assay

For the interaction of diclofenac sodium with proteins, in the second phase of the study, a reaction mixture was specifically prepared. It included 1 mL of Bovine Serum Albumin (BSA), a widely used model protein for drug-protein interaction studies, 1 mL of Phosphate-Buffered Saline (PBS) to provide a fixed pH and osmotic environment, and 1 mL of the previously prepared diclofenac sodium solution. This combined solution was then measured at 660 nm wavelength. This wavelength suggests a possible turbidimetric or colorimetric assay, maybe the precipitation of protein or a reaction to form a colored product as a result of diclofenac binding to BSA. It differs from the typical UV absorbance of diclofenac itself, suggesting a different analytical target.

Diclofenac Sodium as a Benchmark Standard As noted by **Adeyeye et *al*. 1990**, diclofenac sodium was used as a reference standard within the framework of this experiment. This practice is not new in quantitative analysis, where a pure and known material is used as a benchmark for comparison. Diclofenac sodium is specifically characterized as an odorless white to off-white crystalline powder with known analgesic and anti-inflammatory activity. Its borderline hygroscopic tendency to attract moisture from the air requires careful storage and handling to preserve its integrity and purity. Utilization of a well-characterized standard, such as diclofenac sodium, is crucial in developing a reliable calibration curve, which in turn enables accurate determination of diclofenac concentrations in samples of unknown origin.For the experimental determination of diclofenac sodium, a dilution series was carefully prepared in order to plot a calibration curve. A 75% stock solution of diclofenac sodium was used. An exact volume of 1 mL from the stock solution was diluted with 9 mL of distilled water. The initial dilution is required for the sake of bringing the concentration to a level at which it would be suitable for further spectrophotometric measurement.

Preparation of Dilution Series and Spectrophotometric Analysis Following the initial dilution, there was a scheduled sequence of additional dilutions. In this sequence, the concentrations actually utilized were based on the anticipated concentrations of diclofenac sodium in the samples to be determined, thereby enabling the preparation of an extensive calibration curve. The absorbance of the resulting diclofenac sodium solutions was ascertained after each dilution procedure, using a spectrophotometer.

The precise wavelength used to make these early measurements was not defined; however, it would typically be that at which the absorption maximum of solution diclofenac sodium occurs, determined through an initial spectral scan. This method allows for accurate quantitation in accordance with the Beer-Lambert Law.

III- Statistical analysis

Readings were performed in triplicate for each parameter. The mean percentage values were calculated using Excel 2010.

Results and discussion

I-Extraction yield by weight

The extracts yield is the ratio of the quantity of vegetal mass collected after drying to the quantity of biomass, expressed as a percentage.

➢Yield is calculated using the following formula :

$$R\ (\%) = M1 / M0 * 100$$

Où :

R (%) : Extracts yield as a percentage.

M1: Mass of extracted extract, expressed in grams.

M0: Mass of the plant used for extraction, expressed in grams.

Table 2 : Extraction weight yields

Extract	Species name	Extraction rate (%)
Ethanolic extract	*Matricaria chamomilla*	11
Acetonic extract	*Matricaria chamomilla*	97.6
Aqueous extract	*Matricaria chamomilla*	41.8

Based on our findings, the acetone extract showed the highest yield at 97%, followed by the aqueous extract at 41.8%, and the ethanol extract with the lowest yield at 11% **(Table 2)**.

II-FRAP antioxidant activity

Table 3: FRAP antioxidant activity (Percentage inhibition %)

Concentrations	Ethanolic extract	Acetonic extract	Aqueous extract	Vitamin C
Stock solution (10 mg/10 ml) (1000 µg/ml)	49.43	92.66	48.62	96.71
0.5 mg/ml (500 µg/ml)	43.97	72.80	36.60	96.52
0.25 mg/ml (250 µg/ml)	33.47	61.08	31.12	96.25
0,125 mg/mL (125 µg/ml)	28.11	58.27	26.12	95.71
0,0625mg/mL (62,5 µg/ml)	20.87	53.32	22.09	95.57
0,03125 mg/mL (31 µg/ml)	16.58	33.96	15.33	94.80
0,015625 mg/mL(15,62 µg/ml)	12.69	21.77	11.24	92
0,0078125 mg/ml (7.81 µg/ml)	10.88	7.59	7.59	/
0,00390625 mg/ml(3.90 µg/ml)	4.29	4.29	3.98	/

The FRAP (Ferric Reducing Antioxidant Power) assay determines plant extract ferric ion ,reduction capability, which is directly equivalent to their antioxidant potential. In this study the results showed concentration-dependent increases in antioxidant activity for each of the .three extracts (acetonic, ethanolic, and aqueous). This assay relies on the reduction of a ferric tripyridyltriazine (Fe(III)-TPTZ) complex to its ferrous (Fe(II)-TPTZ) form at low pH, which produces a characteristic blue color measurable at 593 nm **(Gulcin et *al*., 2003; Al-Farsi et *al*., 2005)**.

The highest FRAP activity was exhibited by the acetonic extract at all concentrations with a maximum inhibition percentage of 92.66%. This is an indicator that acetone extracted antioxidant compounds that have high electron-donating ability such as flavonoids and polyphenols ***(*Siddhuraju et *al.,* 2002*)***.

The ethanolic extract exhibited moderate activity with a maximum value of 49.43% while that of aqueous had the lowest antioxidant capacity and had a maximum value of 48.62%, declining faster at lowconcentration.

These results indicate that the type of solvent contributes significantly to the extraction of antioxidant compounds. Acetone, which is more non-polar than water and ethanol, may be able to extract a broad selection of lipophilic antioxidants and thus contribute to greater reducing .power

In a study involving 13 cultivated *Matricaria chamomilla* varieties, methanol extracts demonstrated notable antioxidant activity as measured by FRAP assay. The level of this activity differed based on environmental conditions and the extracts' chemical composition **(Formisano et *al*., 2015)**.

In a Colombian study, the authors have evaluated the antioxidant power of many fruits like banana passion fruits, Macadamia, Peach tomato, Umarí… this antioxidant capacity is especially related to vitamin C. But the synergetic effect of Ascorbic acid (AA) with polyphenols is more important in the antioxidant power of fruits more than the vitamin C alone (Scalbert et al., 2005; Blomhoff, Carlsen, Andersen, et al., 2006). In this same work the Banana passion fruits (P. tarminiana, P. mollisima) demonstrated the highest antioxidant capacity **(Contreras-Calderón et *al*., 2011)**.

III-DPPH antioxidant activity

Table 4: DPPH antioxidant activity (Pourcentage inhibition %)

Concentrations	Ethanolic extract	Acetonic extract	Aqueous extract	Vitamin C
Stock solution (10 mg/10 ml) (1000 µg/ml)	61.38	41.70	67.62	97.05
0.5 mg/ml (500 µg/ml)	60.15	37.92	48.07	96.58
0.25 mg/ml (250 µg/ml)	58.42	26.33	47.21	96.50
0,125 mg/mL (125 µg/ml)	58.12	24.14	40.39	95.81
0,0625mg/mL (62,5 µg/ml)	56.08	20.88	37.02	95.52
0,03125 mg/mL (31 µg/ml)	44.79	19.21	33.29	93.90
0,015625 mg/mL (15,62 µg/ml)	43.44	11.34	19.25	89.17
0,0078125 mg/ml (7.81 µg/ml)	41.16	8.91	16.92	/
0,00390625 mg/ml(3.90 µg/ml)	28.85	-8.32	-19.39	/

The DPPH free radical test is widely utilized by researchers to assess the ability of extracts to scavenge free radicals. It is regarded as a straightforward and convenient method compared to other techniques. This test relies on the reduction of DPPH during a reaction. Initially, DPPH has a purple color, which changes to light yellow as it gets reduced **(Sridhar et Charles, 2018)**.

Aqueous extract possessed the strongest inhibition activity of DPPH at the highest concentration (10 mg/10 ml), showing 67.62% reading at the level of stock solution. Its activity decreases significantly with decreasing concentration, showing negative values at the lowest level of concentration (3.90 µg/ml), possibly indicating the lack of antioxidant activity or even a pro-oxidant effect at such very low levels of concentration. This strong inhibition of aqueous extract could be explained by the important contents of polyphenols and, flavonoids and tannins **(Hadj Mohamed et *al*., 2021)**.

Ethanolic extract has uniformly high and relatively consistent antioxidant activity across the greater part of the concentrations from 61.38% for the stock solution concentration and decreasing steadily. It possesses a percentage of inhibition of over 40% to the 7.81 µg/ml, demonstrating its good effectiveness across a wide range of concentrations. It has been demonstrated that all chamomile flower extract samples obtained through either warm or cold extraction with ethanol-water solutions exhibited significant antioxidant activity. In contrast, the samples analyzed from the stem, root, or leaves showed very low antioxidant activity **(Costescua et *al*., 2008)**.

The activity of acetonic extract is low to moderate compared to the other two extracts, starting at 41.70% at the stock soolution. Its activity decreases faster, and it even shows negative values at the lowest concentration (3.90 µg/ml) such as aqueous extract, showing loss of antioxidant activity or pro-oxidant effect at very low concentrations

The scavenging effectiveness of vitamin C has been found to be independent of its concentration. Significant antioxidant activity is observed even at minimal doses, such as 0.02 mg/ml. Similarly, β-carotene demonstrated a comparable reaction with DPPH as vitamin C did. **(Liu et *al*., 2008)**.

Our current study's findings (as shown in the table) are consistent with this observation**,** demonstrating that scavenging capacity was elevated regardless of concentration **(Liu et *al*., 2008)**.

IV-Proteins antidenaturation inhibition pourcentages (%)

Table5: Proteins antidenaturation inhibition pourcentages (%)

Concentrations	Ethanolic extract	Acetonic extract	Aqueous extract	Concentrations	Sodium diclofenac	Vitamin C
Stock solution (10 mg/10 ml) (1000 µg/ml)	50.00	46.18	75.93	12.5 mg/mL (12500µg/ml)	21.81	41.15
0.5 mg/ml (500 µg/ml)	43.32	37.34	53.47	6.5 mg/ml (6500 µg/ml)	15.25	34.58
0.25 mg/ml (250 µg/ml)	38.77	23.40	36.64	3.25 mg/ml (3250 µg/ml)	13.48	33.60
0,125 mg/mL (125 µg/ml)	30.04	17.04	25.90	1.62 mg/ml (1620 µg/ml)	10.71	26.81
0,0625mg/mL (62,5 µg/ml)	23.46	13.55	18.76	0.81 mg/ml (810 µg/ml)	9.05	24.52
0,03125 mg/mL (31 µg/ml)	21.34	9.50	14.34	0.4 mg/ml (400 µg/ml)	8.33	15.20
0,015625 mg/mL (15,62 µg/ml)	12.70	6.07	12.56	0.2 mg/ml (200 µg/ml)	7.46	11.89
0,0078125 mg/ml (7.81 µg/ml)	10.33	4.88	8.93	0.1 mg/ml (100 µg/ml)	/	1.17
0,00390625 mg/ml (3.90 µg/ml)	8.19	2.24	5.54	0.05 mg/ml (50 µg/ml)	/	-3.00

Chamomile extracts Show superior anti-denaturation activity compared to standard anti-Inflammatory agents. The antidenaturation assay highlighted significant variations in the ability of different chamomile extracts, Vitamin C, and diclofenac sodium to inhibit protein denaturation under thermal stress. This assay serves as a valuable indicator of potential anti-inflammatory properties.

Among the tested samples, the aqueous extract of chamomile exhibited the highest inhibition rate of 75.93% at its highest concentration. Such a high rate implies that the extract may contain active water-soluble compounds that are accountable for its effectiveness in preventing protein denaturation.

The ethanolic extract followed, with an inhibition rate of 50.00%, and the acetonic extract was least effective, with 46.18%. These differences can most likely be explained due to each solvent's varying ability to extract bioactive constituents for anti-denaturation action.

Compared to the control tested, diclofenac sodium inhibition was much lower, at a maximum of 21.81%. This suggests that the chamomile extracts especially the aqueous extract could be a more effective natural alternative for reducing inflammation-induced protein damage in this in vitro setting.

Vitamin C was 41.15% inhibited at highest concentration tested. However, when it was tested at lowest dilution **(3.90 µg/ml)**, it had a negative inhibition reading of -3.00%, which could be suggestive of response variability in the assay at extremely low levels.

Across various dilutions, a clear dose-dependent effect was observed, with higher concentrations yielding stronger inhibition of protein denaturation for all extracts and controls. The aqueous chamomile extracts consistently maintained the strongest antidenaturation activity across most concentrations. Notably, even at their lowest tested concentrations, chamomile extracts maintained a positive (though weaker) inhibitory effect, unlike diclofenac sodium, for which no inhibition was reported at the lowest concentrations **(3.90 µg/ml** and **7.81 µg/ml)**, and Vitamin C, which showed a negative inhibition rate at **3.90 µg/ml**.

The findings of the current research, as indicated in the anti-denaturation test, are consistent with the overall scientific knowledge outlined by **Williams et *al*. 2008** who noted the

importance of the bovine serum albumin (BSA) anti-denaturation assay as a valid screening in vitro test for the identification of potential anti-inflammatory compounds.

The two studies indicate the significant anti-denaturation activity of plant extracts of natural origin. In the present work, aqueous chamomile extract proved to have superior activity, inhibiting by 75.93% at the highest concentration tested. This was considerably superior to that of the ethanolic extract (50.00%) and that of the acetonic extract (46.18%). These results were in agreement with Williams et al.,2008 wherein the ethanolic extracts of *Boehmeria jamaicensis* and *Gliricidia sepium* and *Artocarpus altilis* fractions displayed good protection against BSA, even at very low levels of 0.012 to 1.0 µg/mL.

A substantial difference was then evident when natural extracts were compared to traditional pharmaceutical chemicals. In the same study by Williams et al. 2008, diclofenac sodium, a widely used non-steroidal anti-inflammatory drug (NSAID), had a highest inhibition rate of just 21.81%. Although **Williams et *al*. 2008** recorded the protein-stabilizing potential of other NSAIDs such as indomethacin and salicylic acid.

Chamomile (*Matricaria recutita L*., also known as *M. chamomilla* or *Chamomilla recutita*) is one of the most widely used herbs for treating indigestion, cramps, inflammation, and various minor ailments **(Ganzera et *al*., 2006)**. The health-promoting properties of chamomile are primarily attributed to its rich content of flavonoids, which include compounds with a flavone core (such as apigenin and luteolin) and flavonol derivatives (like quercetin and patuletin). These flavonoids are present in various forms, including aglycones, mono- and di-glycosides, as well as acylated derivatives. In addition to flavonoids, chamomile contains essential oils as key active components most notably terpenoids such as α-bisabolol and its oxides, azulenes like chamazulene, and acetylene derivatives **(Ganzera et *al*., 2006)**.

In a study conducted by **Ballo et *al*. 2023**, the aqueous extract of *Cota tinctorium* (Yellow *Chamomile*) ($42.07 \pm 1.16\%$) and the hydroethanolic extract of *Ximenia americana* ($41.33 \pm 0.96\%$) demonstrated the highest effectiveness in inhibiting protein denaturation among the tested extracts. Their effects were statistically comparable to that of diclofenac ($p > 0.05$).

Our present data suggest that natural extracts like aqueous chamomile can exert higher direct protein-stabilizing activity in this test than certain synthetic analogues.

Significantly, chamomile extracts maintained their positive inhibition even in their lowest concentrations used compared to diclofenac sodium and vitamin C, which gave no inhibition or even reverse inhibition values at very extreme dilutions. These findings support the premise that compounds with activity at low doses are the best bets for new drug creation, as highlighted by **Williams et *al*. 2008**.

Conclusion

Conclusion

This study meticulously investigated the therapeutic potential of *Matricaria chamomilla* (chamomile) extracts, a natural plant widely recognized for its medicinal applications. Our research systematically evaluated the anti-denaturation and antioxidant activities of chamomile extracts prepared using distinct solvents: ethanol, acetone, and water.

The anti-denaturation assay yielded significant results, with the aqueous extract of chamomile demonstrating superior efficacy. At its highest concentration, it achieved a remarkable 75.93% inhibition of protein denaturation, profoundly outperforming the standard anti-inflammatory drugs diclofenac sodium (21.81%) and Vitamin C (41.15%) at their respective highest concentrations. Crucially, even at their lowest tested concentrations (e.g., 3.90 µg/ml), chamomile extracts maintained positive inhibitory effects (e.g., 5.54% for aqueous extract), contrasting sharply with diclofenac sodium and Vitamin C which showed diminished or even negative inhibition (-3.00% for Vitamin C). This highlights the sustained efficacy of chamomile compounds even at very low doses.

In the antioxidant assessments, the DPPH assay revealed the aqueous extract as a potent scavenger, exhibiting 67.62% inhibition at its highest concentration, followed closely by the ethanolic extract at 61.38%. However, a critical observation was the pro-oxidant effect displayed by both acetonic (-8.32%) and aqueous (-19.39%) extracts at their very low 1/256 dilutions, underscoring the concentration-dependent nature of their antioxidant activity. Conversely, the FRAP assay identified the acetonic extract as having the highest ferric reducing antioxidant power, with an impressive 92.66% inhibition at its highest concentration, suggesting its efficiency in extracting electron-donating compounds like flavonoids and polyphenols. The ethanolic and aqueous extracts showed moderate FRAP activity at their highest concentrations (49.43% and 48.62% respectively).

These findings collectively underscore the profound importance of natural products like chamomile as invaluable reservoirs for novel therapeutic agents. The demonstrated superior anti-denaturation activity, particularly of the aqueous extract, when compared to established anti-inflammatory drugs, positions chamomile as a promising natural anti-inflammatory candidate. The significant antioxidant properties observed further highlight their potential in mitigating oxidative stress-related conditions.

Conclusion

To ensure a comprehensive and robust understanding, future work should critically focus on:

Isolation and chemical characterization of the specific bioactive compounds responsible for the observed activities.

In-vivo efficacy and safety studies using appropriate animal models to validate the therapeutic potential in living systems.

Elucidation of molecular mechanisms to precisely understand how these extracts exert their anti-denaturation and antioxidant effects at a cellular and molecular level.

Comprehensive toxicity and pharmacokinetic assessments to ensure the safety and optimal absorption, distribution, metabolism, and excretion of the extracts.

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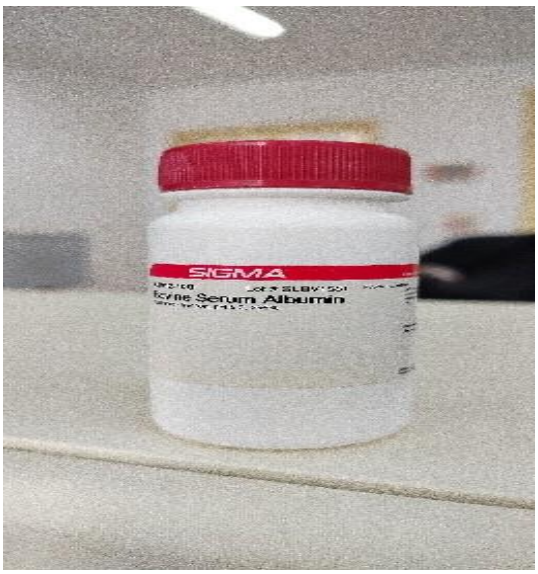
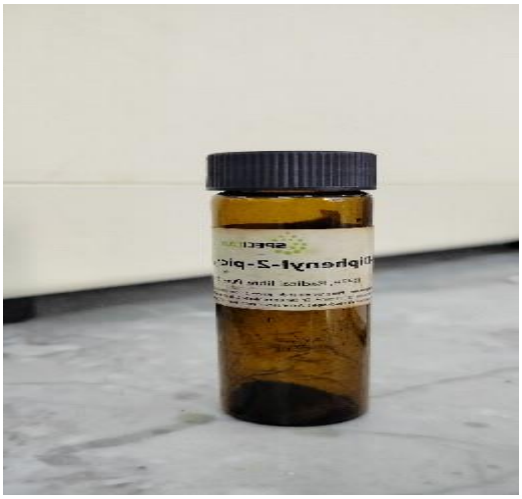
Annexes



BSA

Commercial chamomile



DPPH