PEOPLE'S DEMOCRATIC REPUBLIC OF ALGERIA
MINISTRY OF HIGHER EDUCATION AND SCIENTIFIC RESEARCH
University Frères Mentouri Constantine 1
Faculty of Nature and Life Sciences
Department of Animal Biology

Order N° : 56/D3C/2023
Serial N°: 03/BA/2023

Thesis
Presented for the fulfillment of the Doctorate degree, 3rd cycle
Domain : **Nature and Life Sciences**
Field : **Biological Sciences**
Option : **Cellular and Molecular Immunology**

Defended by :

KHATTABI Latifa

Entitled:

Impact of Pirimicarb on Neuro-Immune-Endocrine System Sensitivity and the Preventive Effect of *Ephedra alata* monjauzeana

Jury members:

Chairman:	**BAAZIZ Nasser**	**Prof.**	**University Frères Mentouri Constantine 1**
Supervisor:	**CHETTOUM Aziez**	**Prof.**	**University Frères Mentouri Constantine 1**
Examiner :	**EL OUAR Ibtissem**	**Prof.**	**University Frères Mentouri Constantine 1**
Examiner :	**FRIH Hacene**	**Prof.**	**Badji Mokhtar University – Annaba**
Examiner :	**DJERROU Zouhir**	**Prof.**	**20 August 1955 University – Skikda**

Academic year
2022-2023

Dedication

I dedicate my thesis to you, my beloved

FATHER.

It was dark to achieve this goal

without your light.

My eternal love to you pushed me to

fulfil your wish, and making you happy

where you are now.

May GOD bless you!

"Papito"

Acknowledgments

"Mamita", the most worthy and honest recognition should be conveyed to you. Without a doubt, the achievement of this project is a result of your sacrifices, patience, support, confidence, motivation, and love.

My brother and sisters, I want to express my gratitude for your warm presence, empathy, complicity, and humor which have lifted my spirits and provided me with inspiration and courage to persue my thesis project.

Acknowledgements and gratefulness are cordially addressed to my supervisor, Prof. Aziez CHETTOUM for his guidance and valuable pieces of advice.

I would like to express my great honor and appreciation for the interest and willingness of Prof. Nasser BAAZIZ for being the president of the thesis committee and of Prof. lbtissem EL OUAR, Prof. Hacene FRIH and Prof. Zouhir DJERROU for being the thesis examiners.

I wish to show my distinguished recognition to my line managers at Biotechnology Research Center (CRBt) Constantine, notably the Director, Prof. Ammar AZIOUNE, and Dr. Chawki BENSOUCI for authorizing me to perform a part of the work at CRBt and for offering me a day off by a week for the accomplishment of the experimental study.

I would acknowledge the Algiers Pasteur Institute managers for allowing me to use their materials in order to execute the H&E preparation of tissue sections. Particularly, the staff of the Veterinary Pathological Anatomy and Cytology Laboratory for their guidance and kindness.

I am deeply thankful to my colleagues of the Scientific and Technical Research Center in Physico-Chemical Analysis (CRAPC), who generously provided me with their service and expertise, notably: Dr. Mustapha Mounir BOUHENNA, Dr. Moussa MOKHTARI, Dr. Farid CHEBROUK, Dr. Youcef RAHMANI and Dr. Walid BOUSSEBAA.

Many thanks to Dr Laid BAHRI, who taught me a lot about how to deal with laboratory animals, he is a real Leader.

I wish to extend my special thanks to my friends and colleagues of CRBt, for their help and encouragements.

LIST OF PUBLICATIONS AND COMMUNICATIONS

International publications

1. **Khattabi, L., Boudiar, T., Bouhenna, M. M., Chettoum, A., Chebrouk, F., Chader, H., ... & Akkal, S.** (2022). RP-HPLC-ESI-QTOF-MS qualitative profiling, antioxidant, anti-enzymatic, anti-inflammatory, and non-cytotoxic properties of Ephedra alata monjauzeana. *Foods*, 11(2), 145.
2. **Khattabi, L., Chettoum, A., Hemida, H., Boussebaa, W., Atanassova, M., & Messaoudi, M.** (2023). Pirimicarb Induction of Behavioral Disorders and of Neurological and Reproductive Toxicities in Male Rats: Euphoric and Preventive Effects of Ephedra alata Monjauzeana. *Pharmaceuticals*, 16(3), 402.

Communications

- **National**

1. **Khattabi, L., Boudiar, T., Chettoum, A.** (2020).Anti-oxidant activities of *Ephedra* hydro-alcoholic extract: nine in-vitro methods. *1 st Scientific Day on the Biology of Medicinal Plants BMP, 22 Janvier, Tebessa*.
2. **Khattabi, L., Chettoum, A., Bouhenna, M. M.** (2021). Total phenolic and flavonoid content, antioxidant and non cytotoxic effects of *Pulicaria Odora*. *Séminaire National : Ressources végétales, Produits Naturels et Santé (RVPNS), 09-10-11 Juin, Blida*.
3. **Khattabi, L., & Chettoum, A.** (2021). Anti-inflammatory and anti-diabetic effect of *Pulicaria odora* crude extract. *Séminaire national: Bio-ressources:Nutition, Santé et Environnement , 17 et 18 Mai, M'sila*.

- **International**

1. **Khattabi, L., & Chettoum, A.** (2022). Euphoric effects of *Ephedra alata* monjauzeana against neuroendorcine perturbations induced by pirimicarb in wistar rat. *Séminaire International sur la Valorisation des Ressources Agronomiques, Ecologiques et Alimentaires, 18-19-20 Octobre, Skikda.*
2. **Khattabi, L., Bahri, L., Derghine, R., Chettoum, A.** (2023). Anti-paw edema of *Ephedra alata* Decne and its protective effect against Pirimicarb neutrophil induced cytotoxicity. *Third international congress on biological and health sciences, 14-15-16 April, Turkey*.

LIST OF ABBREVIATIONS

A 0.5	Concentration at 0.5 absorbance
ACTH	Adrenocorticotropic hormone
ANS	Autonomic nervous system
AVP	Arginine vasopressin
BBB	Blood–brain barrier
BPC	Base peak chromatogram
CCL CC	Chemokine ligand
CNS	Central nervous system
CRH	Corticotrophin releasing hormone
CRHR1	Type 1 CRH receptors
CUPRAC	Cupric reducing antioxidant capacity
CXCL	Chemokine (C-X-C motif) ligand
DAD	Diode array detector
DHEA	Dehydroepiandrosterone
DPPH	2,2-diphenyl-1-picrylhydrazyl
EamCE	*Ephedra alata* monjauzeana crude extract
EPM	Elevated plus maze
EOS	Entries into the open section
ES	Endocrine system
ESI	Electro spray ionization interface
EZM	Elevated Zero maze
FRAP	Ferric reducing ability of plasma
FSH	Follicule -stimulating hormone
FST	Force swim test
GOR	Galvinoxyl radical
GPx	Glutathione peroxidase
GR	Glutathione reductase
GST	Glutathione-S-transferase
GSH	Glutathione
H&E	Hematoxylin and eosin
Hep2	Human epithelial type 2
HD	Head dips
HPA	Hypothalamic pituitary adrenal
IC50	Concentration of 50% inhibition
IFN-γ	Interferon gamma
IL-1	Interleukine-1
IL-6	Interleukine-6
IS	Immune system
LC	Locus ceruleus
LCMS/MS	Liquid chromatography coupled to tandemmass spectrometry
LH	Luteinizing hormone
LTB	Leukotriene B
LTD	Leukotriene D
MCP	Monocyte chemo-attractant protein
MDA	Malondialdehyde
MDA-Lys	Malondialdehyde-lysine
OC	Organochlorines
OFT	Open field test

LIST OF ABBREVIATIONS

OP	Organophosphorus
OS	Oxidative stress
PAF	Platelet activating factor
PGE	Prostaglandin E
PMNs	Polymorphonuclear neutrophils
PNS	Peripheral nervous system
PVN	Periventricular nucleus
QTOF	Quadrupole-time-of-flight mass
Rd	Rhabdomyosarcoma
ROS	Reactive oxygen species
SNS	Sympathetic nervous system
SNPs	Spherical silver nanoparticles
SOD	Superoxide dismutase
T3	Tri-iodothyronine
T4	Thyroxine
TGF	Transforming growth factor
TFC	Total flavonoid content
TNF-α	Tumor necrosis factor
TPC	Total phenolic content
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone
VIP	Vasoactive intestinal polypeptide
VP	Vasopressin
WHO	World Health Organization

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

TABLE OF CONTENTS

1 Introduction

The maintenance of the living organism homeostasis is ensured, in mammals, by three major communication and integration systems: nervous system (central nervous system (CNS) and autonomic nervous system (ANS)), endocrine system (ES) and immune system (IS).The function of each three systems has long been thought to be independent, but a significant amount of recent data showed that they communicate with each other in a way that is both multidirectional and accurate. This communication takes place due to the production of common mediators (hormones, cytokines, neuromediators, neuropeptides) and the presence of their specific receptors **(Blalock, 1984)**. Stresses from different natures (physical, psychological, infectious or toxic) can have convergent effects owing to the common pathways they undergo, mediators and receptors they use. Each system is subject to a set of activation factors and delicate regulation, any deficit at this level can have serious consequences. The term of "psychoneuroimmunology" was introduced in 1960s in order to define the bidirectional relationship between brain and IS. The IS plays systemic surveillance and an intervention role in brain. An excessive response of glial cells (immune cells of the CNS involved in immune reactions) can have deleterious effects on neurons. Similarly, in the ES, the feedback loops if they no longer fulfill their role (in particular that involve corticosteroids), they could promote the establishment of several psychiatric disorders, including anxiety, depression and other mood issues **(Verhoeven et al., 2005)**. Moreover, changes in the IS can lead to profound alterations in psychological state. The most possible link interacting behavior, nervous, endocrine and immune systems is established by cytokines which act as signaling molecules of the IS. Several studies indicated a close association between activation of pro-inflammatory cytokines including interleukin-1 (IL- 1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and psychiatric symptoms **(Rhie et al., 2020)**. Pro-inflammatory cytokines contribute to the development of depressive disorder and induce major depressive disorders in physically ill patients with no history of mental disorders. The activation of the inflammatory response leads to release inflammatory cytokines and mobilization of immune cells both of which have been shown to access the brain and alter behavior. Evidently, inflammation is an important biological event that can increase the risk of major depressive episodes, much like the more traditional psychosocial factors **(Bayramgürler et al., 2013).**

Exposure to pesticide is expected for workers in production and application, although, public population can also be affected from contaminated water and food. Pesticides have been extensively exploited, and mostly uncontrolled in several developing countries **(Blair et al., 2015)**. Through food chain contamination and low chronic exposure, adverse effects reach to human life. These ingestions have resulted serious carcinogenic, endocrine, neurological, reproductive and other ailments. Likewise, many casualties have been reported due to poisonous pesticide exposure. Bundle of research indicated that cancer risk and mental health problems are enlarged by 25–30% after exposure to pesticides. In a similar vein, there is a significant association between paternal exposure to pesticides and a 50% increased risk of leukemia, lymphoma, and brain cancer in children **(Corsini et al., 2008; Ali et al., 2021)**. In further, pesticide exposure is associated with mental disorders, including depression, especially in occupationally exposed populations, such as farmers. Findings from experimental studies have attributed the negative effects of pesticides on mental health to their neurotoxic and endocrine disrupting activities **(Koh et al., 2017)**.

Due to the limited availability of research studies focusing on toxicological risk assessments specifically regarding the effects of pirimicarb, we have appointed to study its impact on, hypothalamic pituitary adrenal (HPA) axis, neurobehavior, brain ,immune and reproductive function along with eventual induction of oxidative stress (OS) (these checkpoints are relevant for the previously mentioned reasons). On the other hand, *Ephedra alata* monjauzeana was selected and included in the same survey, in order to engender probable preventive effects towards pirimicarb nuisance. Indeed, this plant has aroused our interest since it is usually consumed from Algerian desert people as herbal tea to reduce the stress of the day and to enhance the quality of slumber. Therefore, the objective of the current thesis is to:

1) Identify and evaluate the disturbances of the HPA axis in wistar rats exposed to pirimicarb.

2) Survey the behavioral disturbances induced by pirimicarb by measuring the status of anxiety and depression through behavioral tests.

3) Evaluate specific biomarkers, such us pro-inflammatory cytokines, hormones and hematological parameters.

4) Conduct histopathological examination and assess OS parameters.

5) Investigate the eventual preventive effect of *Ephedra alata* monjauzeana.

II Literature Review

II.1. Pesticides

II.1.1. History and etiology

Human activities are a part of struggle for meeting basic needs of life. In order to sustain life; humans must discover better means for addressing the development challenges, including those relating food security, for a peaceful and secure life. Since ancient times, specifically before 2000 BC, humans have employed pesticides as a means of safeguarding crops. In Mesopotamia, about 4500 years ago, they used elemental sulfur dusting as pesticide for their crops. In other places, they used poisonous plants for pest control. In the 1500s, the initial usage of "para-pesticides," specifically mercury and arsenic, began to emerge. These substances were used until the start of synthetic pesticide era (1940 and beyond), initially for the destruction of food reserves during the World War II and later on as precious tools for cultivating processes of daily consumed foods. The serious use of pesticides in agriculture started in the nineteenth century and expanded in the twentieth century, pesticides were used to control various pests and disease carriers, like mosquitoes, fleas, ticks, mice and rats **(Abubakar et al., 2019; Larramendy & Soloneski, 2019)**. The term pesticide (which includes phytosanitary products) originates from a double etymology: English for pest (harmful animal, insect or plant) and Latin for the suffix-cide (to kill). It refers to inorganic substances (iron sulphate, copper, etc.) and synthetic organic substances, used to control organisms considered harmful **(Joly, 2014).** According to the food and agriculture organization (1989) a pesticide is any substance or mixture of substances intended for preventing, destroying, or controlling any pest including vectors of human or animal diseases, unwanted species of plants or animals causing harm during, or otherwise interfering with the production, processing, storage, or marketing of food, agricultural commodities, wood and wood products, or animal feedstuffs, or which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies. The term includes chemicals used as growth regulators, defoliants, desiccants, fruit thinning agents, or agents for preventing the premature fall of fruits, and substances applied to crops either before or after harvest to prevent deterioration during storage or transport. The term, however excludes such chemicals used as fertilizers, plant and animal nutrients, food additives and animal drugs **(Stoytcheva, 2012)**.

II. 1.2. Classification:

Pesticides could be categorized according to their application, target organism and chemical nature. On the basis of application, pesticides can be grouped as agriculture (used to protect the crop pest, insects and weeds), public health (used to kill vector which causes diseases) and domestic pesticides (used to kill insects like cockroach, bacteria, protozoa, mice etc) **(Rani et al., 2021)**. Based on the target organism, pesticides are classified based on the target pest object and are given special names to reflect their activities. The category names for these pesticides come from the Latin word cide (means killer), which is used after the name of the target pest. The pesticide categories based on target pests are summarized in table 1 **(Hassaan & El Nemr, 2020)**.

Table 1: Type of pesticide according to their specific target.

Based on chemical nature, pesticides can be designated as:

Type of pests	Pesticides example	Target pests/Function
Avicides	Avitrol (aminopyridine)	Kill birds
Acaricides	Bifenazate	Kill mites that feed on plants and animals
Attractant	Pheromones	Attracts wide range of pests
Algaecides	Copper sulfate	Control or kill growth of algae
Bactericides	Copper complexes	Kill bacteria or acts against bacteria
Biopesticide	Bacillus thuringiensis	Wide range of organisms
Bait	Anticoagulants	Wide range of organisms
Desiccants	Boric acid	Act on plants by drying their tissues
Defoliants	Tribufos	Removes plant foliage
Fungicides	Azoxystrobin, Chlorothalonil	Kill fungi (including blights, mildews, molds, and rusts)
Fumigants	Aluminum phosphide	Wide range of organisms
Herbicides	Atrazine, glyphosate, 2,4-D	Kill weeds and other plants that grow where they are not wanted
Insecticides	Aldicarb, Carbaryl, imidacloprid	Kill insects and other arthropods

Insect growth regulator	Diflubenzuron	Insects
Lampricides	Trifluromethyl	Target larvae of lampreys which are jawless fish latching on vertebrate fish in rivers
Larvicides	Methoprene	Inhibits growth of larvae
Molluscicides	Metaldehyde	Inhibit or kill molluscs i.e. snails usually disturbing growth of plants

II.1.2.1. Organochlorines (OC)

OC are organic compounds (also called chlorinated hydrocarbons) of low molecular weight, with a cyclic structure and five or more than five chlorine atoms in their structure. They belong to one of the earliest categories of pesticides ever synthesized and extensively used in agriculture. Most of them are usually used as insecticides for the control of a broad range of insects, and have a long-term residual effect in the environment **(Abubakar et al., 2019; Hernández, 2021)**. These synthetic compounds are chemically stable and hydrophobic, they are present in aquatic systems worldwide as a consequence of their widespread usage, long-range transport, and persistence **(Niaounakis, 2017)**. OC are the most successful, profitably utilized and commercialized group of pesticides, they have gained huge popularity and prominence in a short span of time by virtue of their ability to control almost all kinds of pests including insect, fungi and rodent **(Kaushik & Kaushik, 2007)**.

II.1.2.2. Organophosphorus (OP)

They are basically esters of phosphoric acid with varying combinations of oxygen, nitrogen, carbon and sulphur attached to them. All the OP compounds shared a common structural pattern, a phosphorus atom present in the center, which is double bonded with either oxygen or sulfur atom and single bonded with alkoxy/aryloxy/ thioalkoxy groups (R and R') and X the any leaving group **(Pundir et al., 2019)**. OP compounds are a class of substances frequently encountered in surface water and groundwater coming from agro-industrial processes. OP are toxic and can be bio-accumulated. Due to their hydrophobic and nonvolatile nature, they are mainly degraded in the interfacial region of cavitation bubbles **(Torres-Palma & Serna-Galvis, 2018)**.

II.1.2.3. Carbamates

Carbamate compounds are esters of carbamic acid that are commonly used as insecticides. These compounds are referred to as N-methylcarbamates. Generally have the formula: RHNCOOR', and are relatively polar, highly soluble in water, and chemically reactive. Carbamates tend to hydrolyze easily, resulting in a low level of persistence in the environment, both in soil and water. Derivatives of carbamic acid, thiocarbamic acid, and dithiocarbamic acid are used as herbicides. When used properly, carbamate pesticides offer significant benefits to society, as they protect and increase agricultural production, as well as protect human and animal health from insect-vector-mediated diseases **(Horsak et al., 1964; Gupta, 2014)**.

Most commonly used organophosphate and carbamate pesticides are nerve poisons and frequently involved in poisoning and suicide. These OP and carbamates irreversibly inhibit acetylcholine esterase and accumulate acetylcholine at muscarinic and nicotinic synapses in the central nervous system **(Kaphalia, 2011)**.

II.1.2.4. Pyrethroids

Pyrethroids are a class of synthetic organic insecticides derived from pyrethrins. They have been used worldwide since the 1980s because of their high level of effectiveness and low toxicity compared to other insecticides, such as OP and carbamic ester compounds **(Tang et al., 2018)**. Resmethrin, the first generation synthetic pyrethroid was developed from naturally occurring pyrethrins by altering their structure to increase stability in sunlight and insecticidal activity in 1962. The World Health Organization (WHO), recommended the use of pyrethroids including deltamethrin and permethrin owing to the less environmental persistency and low toxicity to humans and other mammals **(Ravula & Yenugu, 2021)**.

According to chemical composition, pesticides are mainly grouped into four categories, namely organochlorine, carbamates, pyrethroids, and organophosphate **(Dar et al., 2019)**. However, other chemical categories of pesticides can also be numbered, phenyl amides (carbanilates, acylanalides, toluidines and acetamides), phenoxyalkonates, trazines, benzoic acid derivatives, benzonitriles, phtalimide derivatives, dipyrids and miscellaneous **(Rani et al., 2021)**.

II.1.3. Impact of pesticides and their toxicity to human health

Expanding utilization of pesticides can provoke serious issues due to their bio-magnification and persistent nature **(Sharma et al., 2019)**. Although, eco-toxicological studies of the natural environment have become very important in recent decades since pesticides regularly enter the environment. They may disturb the natural balance of the ecosystem and cause substantial ecological changes even if used according to good agricultural practices. Whereas problems arising from pesticide use are most often linked with agriculture or forestry practices, they are also present as a common component of urban wastewater accumulating as the result of weed treatment along roads or rail lines, as well as from gardens, parks, and urban woodland areas **(Lushchak et al., 2018)**. Human exposure to pesticides can occur directly from occupational, agricultural, and household use, while they can also be transferred indirectly through diet. Moreover, the general population may be exposed to pesticides due to their application on golf courses, around major roads, etc. The main routes of human exposure to pesticides are through the food chain, air, water, soil, flora, and fauna. Pesticides are distributed throughout the human body via the bloodstream but can be excreted through urine, skin, and exhaled air. There are four common ways pesticides can enter the human body: dermal, oral, eye, and respiratory pathways **(Kim et al., 2017),** figure 1.

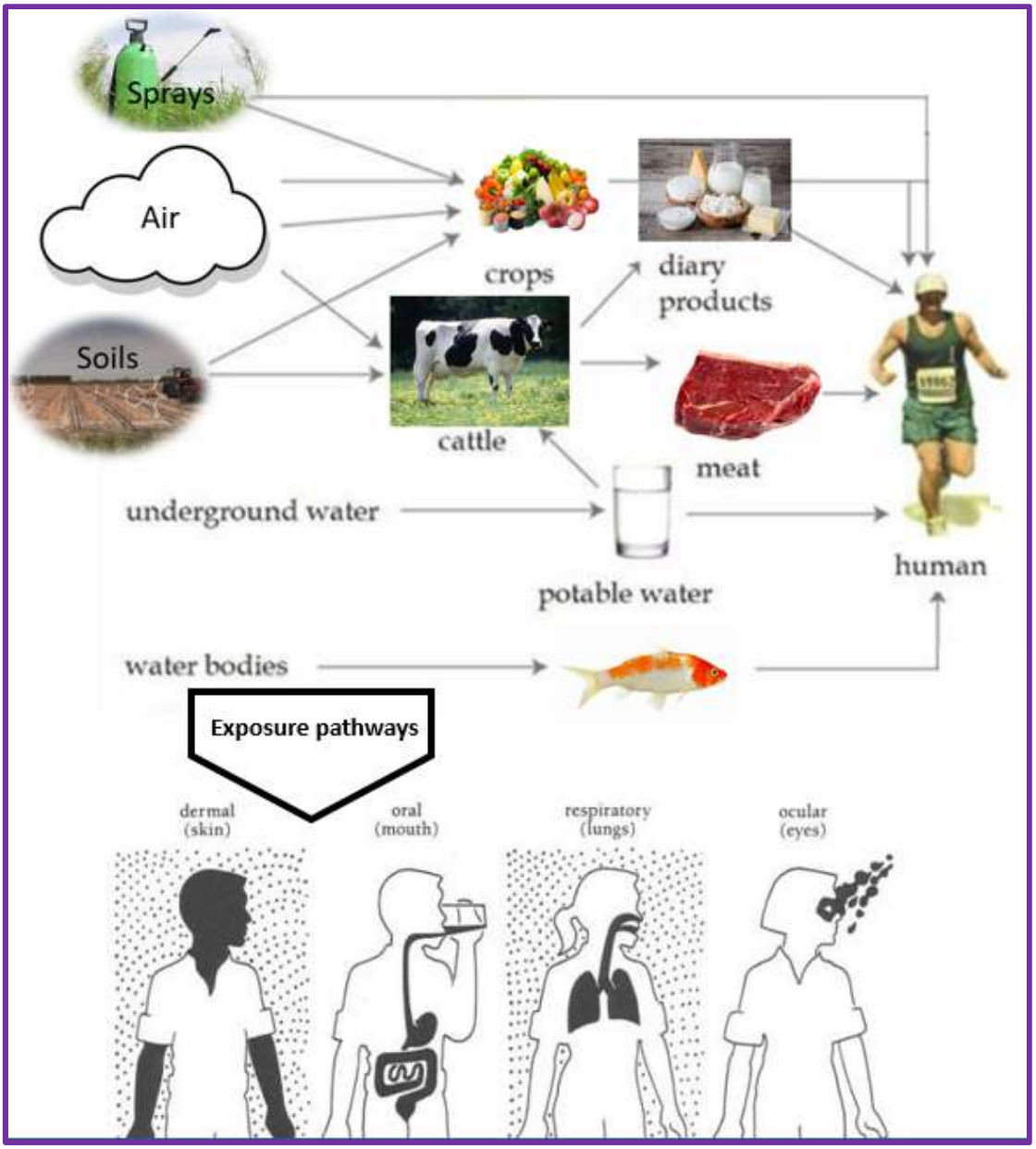


Figure 1: Routes of exposure to pesticides **(Terziev & Petkova-Georgieva, 2020)**.

The interaction between several types of pesticides may induce multiple responses, depending on differences in the chemical properties and modes of toxic action of each compound. When pesticides have the same mechanism of action, their combined effect is the sum of the potency-corrected doses of each individual. Some mixtures having the potential of producing greater toxicity than it would be predicted, based on the potencies of the individual compounds (synergism; synergistic effects could also occur when exposures are above dose thresholds) **(Hernández et al., 2013)**. Pesticide residues in food and water are gradually

accumulated in body tissues and have adverse effect on internal organs like liver, stomach, large intestine, and pancreas, inducing malignant tumor in them. These chemical contaminants are responsible for DNA adduct formation, which, in turn, can lead to chronic diseases, including cancer **(Barzegar et al., 2021)**. WHO classified pesticide toxicity, into different classes as shown in table 2 **(Hashimi et al., 2020)**.

Table 2: WHO classification of pesticides toxicity.

WHO class	**LD50* for the rat (mg/kg body weight)**		**Example in terms of active ingredients**
	Oral	**Dermal**	
Extremely hazardous	< 5	< 50	Aldicarb, Parathion, Mercuric Chloride
Highly hazardous	5–50	50–200	Acrolein, Cadusafos, Ca-arsenate
Moderately hazardous	50–2000	200–2000	Alachlor, Bentazone, Copper f sulfate
Slightly hazardous	Over 2000		Hexaconazole, Atrazine, Butachlor
Unlikely to present acute hazard	5000 or higher		Mancozeb, Captan, Bifenox

*LD50: the lethal dose refers to the quantity of a substance required to cause the death of half the animals in a tested population.

Pesticides have shown to be involved in the pathogenesis of Parkinson's and Alzheimer's diseases as well as various disorders of the respiratory and reproductive tracts. OS caused by pesticides is an important mechanism through which many of the pesticides exert their harmful effects. OS is known to cause DNA damage that in turn may cause malignancies and other disorders. Many pesticides have shown to modulate the gene expression at the level of non-coding RNAs, histone deacetylases, DNA methylation patterns suggesting their role in epigenetics. Poisoning from pesticides is a global public health problem and accounts for nearly 300,000 deaths worldwide every year **(Sabarwal et al., 2018)**. Common health problems

caused by pesticides are summarized in figure 2.

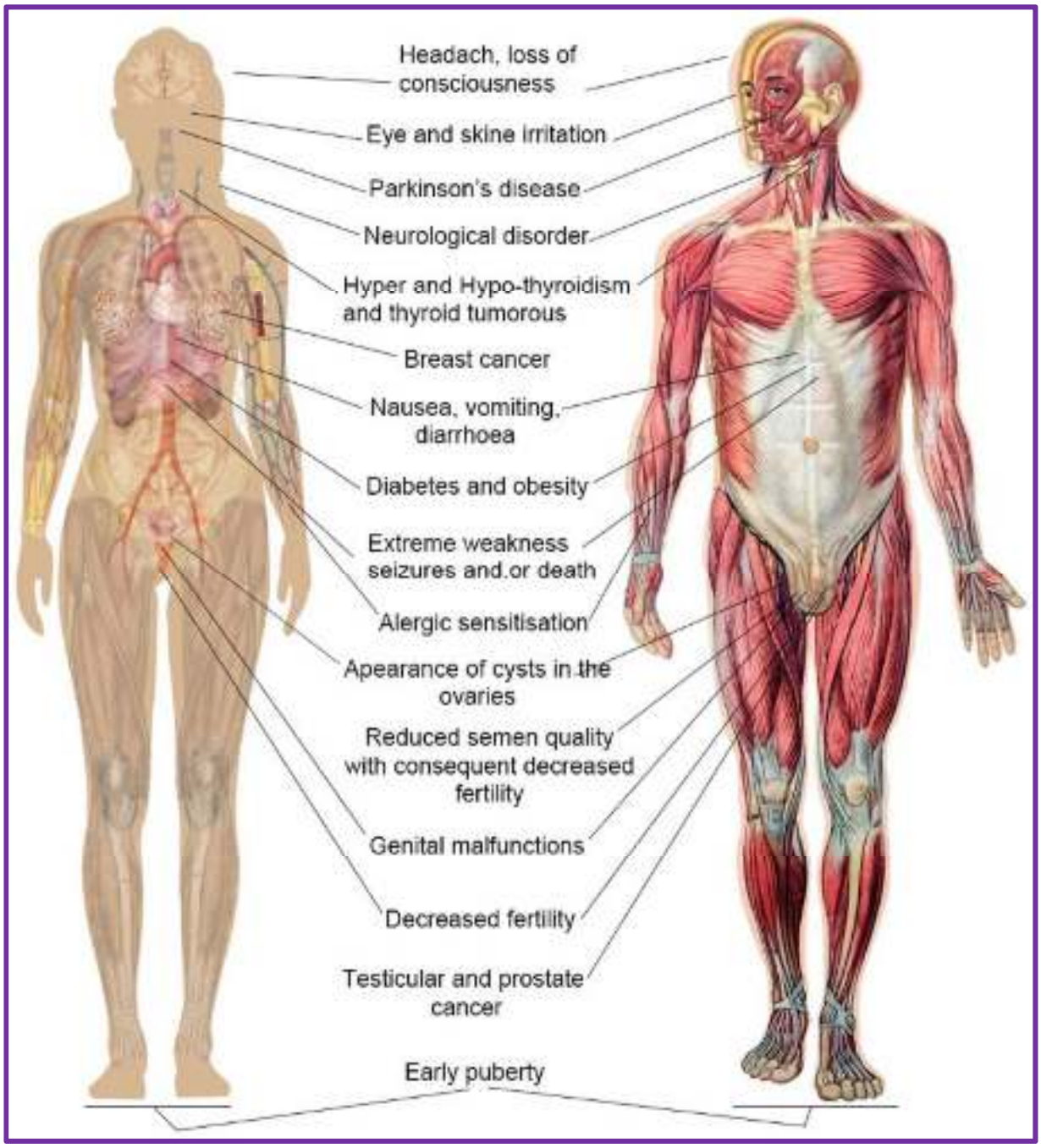


Figure 2: Most frequent health issues associated to pesticide exposure **(Tudi et al., 2022)**.

II.1.4. Pirimicarb

Pirimicarb is an aminopyrimidine that is N,N,4,5-tetramethylpyrimidin-2-amine substituted by a (dimethylcarbamoyl)oxy group at position 4. It is a carbamate ester, an aminopyrimidine and a tertiary amino compound (figure 1). It derives from a dimethylcarbamic acid. It is also an acetylcholinesterase inhibitor that is used as a pesticide. Besides, it is characterized as a colorless, an agrochemical, an environmental contaminant, a xenobiotic and an insecticide **(PubChem, 2023)**. As a potent selective systemic insecticide, it is effective against aphids in fruit and vegetable cultures **(Schwack & Kopf, 1993)**.

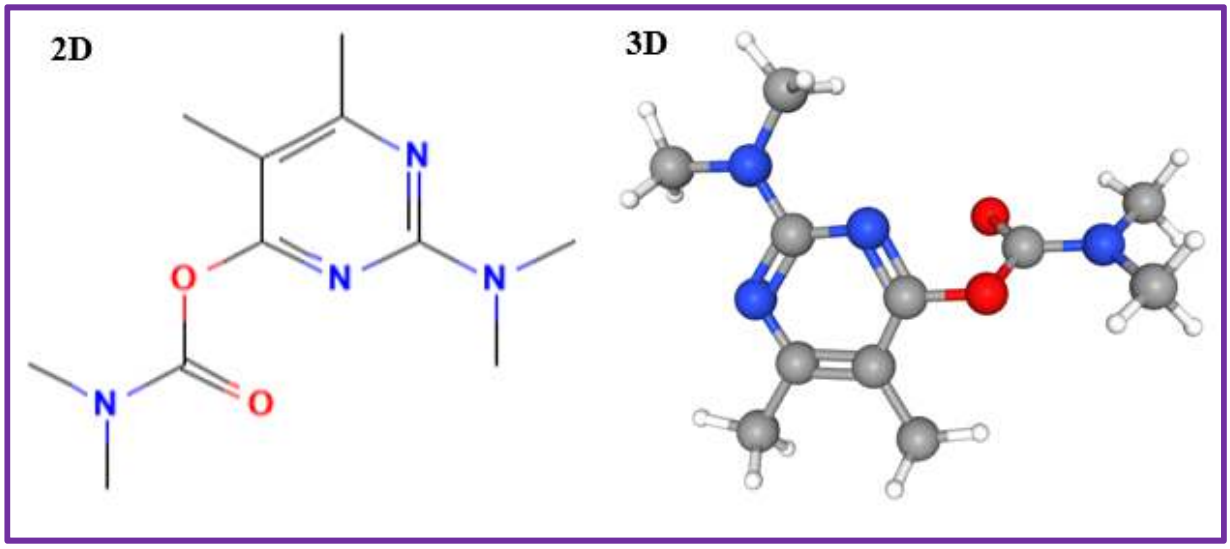


Figure 3: Chemical structure of pirimicarb with 2D and 3D conformations.

II.2. The neuro-immune endocrine interactions and their different stressors

II.2.1. Overview of the neuro-immune endocrine axis

The CNS controls the IS over two pathways, the autonomic nervous system and neuro-endocrine efflux. Numerous conditions have to be satisfied by a particular neurotransmitter of the autonomic nervous system to ensure neural-immune interactions. Namely, chemical association of specific nerve fibers with central and peripheral lymphoid tissues, release of the neural derived substance and its availability for immune cells, the presence of appropriate receptors on target immune cells and the identification of immune-regulatory effects of the neurotransmitter. These conditions have been fulfilled for noradrenaline and substance P and largely also for somatostatin and vasoactive intestinal peptide **(Mössner & Lesch, 1998)**. The brain has long been considered a privileged organ, since the blood brain barrier (BBB) and its tight junctions prevent the transmigration of systemic immune cells **(Laflamme & Rivest, 2001)**. The efficacy of this barrier is based on the innate immune properties of resident cells (neurons and glia), that interact with pathogens, endogenous or exogenous toxins, to boost the adaptive response and to minimize the collateral damage **(Lampron et al., 2013)**. The coordination of neural, behavioral and endocrine responses to inflammation provide an important first-line defense and help restore homeostasis in the body. Activated immune cells synthesize pro-inflammatory substances named cytokines that are IL-1β, IL-6 and TNF α; these cytokines bind to receptors on endothelial and perivascular cells of BBB and stimulate production of a variety of other inflammatory molecules, including prostaglandins and additional cytokines. As a part of neuronal responses to immune activation, these molecules alter the activity of neuroendocrine cells to influence peripheral hormone levels and endocrine dependent behaviors. The hypothalamus is the principal structure mediator of these effects, through its ability to modulate both the pituitary hormone secretion and the sympathetic nervous system (SNS) **(Leonard, 2018)**.

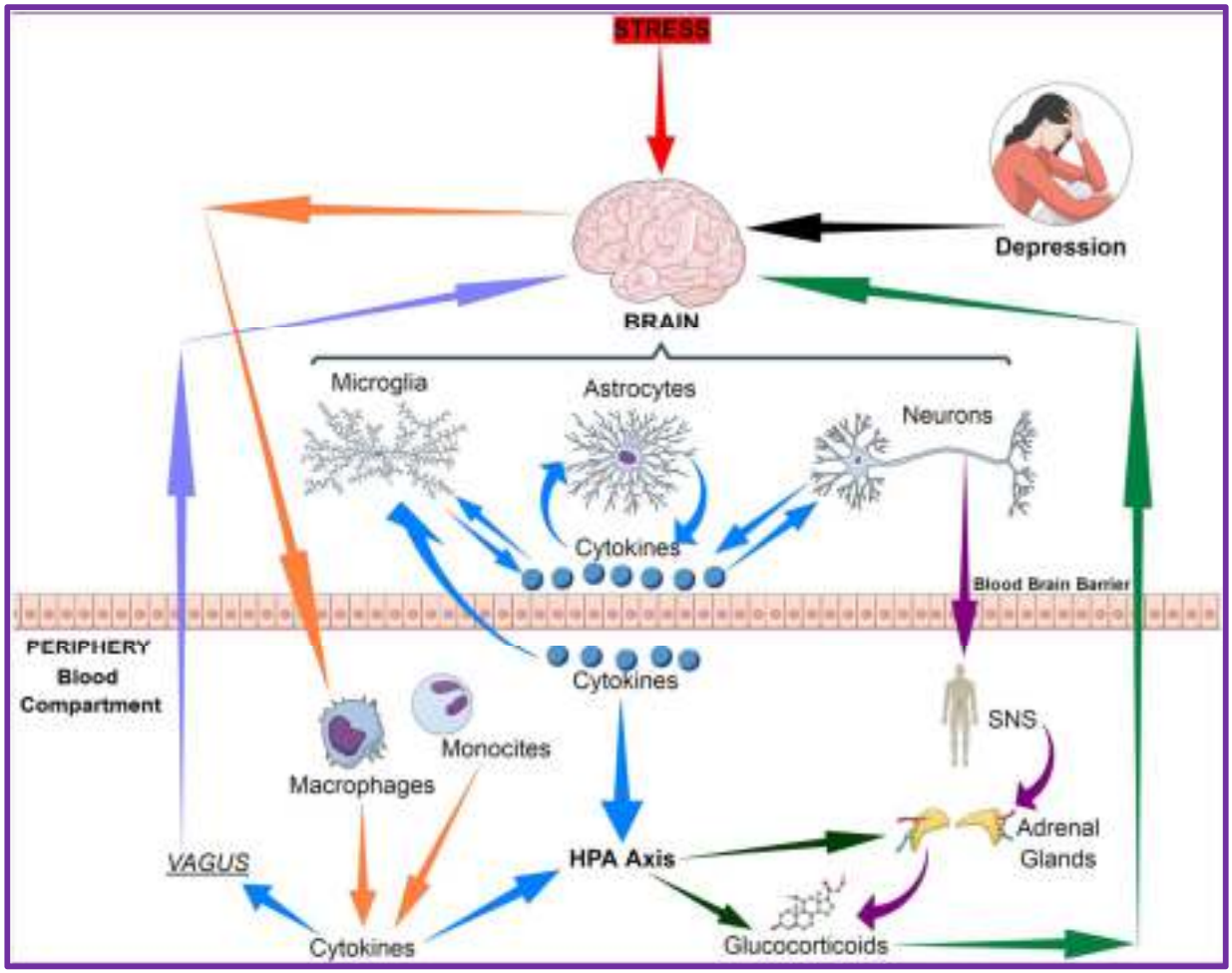


Figure 4: Bio-directional connections between the stress, the brain and inflammatory cytokines. SNS, sympathetic nervous system **(Leonard, 2018)**.

Neurons in the PVN of the hypothalamus that synthesize corticotrophin releasing hormone (CRH) are activated initially by prostaglandins and, subsequently, by circulating cytokines to prolong the HPA response. There are additional stimulatory effects of cytokines at both the pituitary and the adrenal to further enhance the HPA response **(Pittman, 2011)**. The primary hormonal pathway by which the CNS regulates the IS is the HPA axis, through the hormones of the neuroendocrine stress response. Neuroendocrine regulation of immune function is essential for survival during stress or infection and to modulate immune responses in inflammatory disease. Glucocorticoids are the main effector end point of this neuroendocrine system and, through the glucocorticoid receptor, have multiple effects on immune cells and molecules **(Webster et al., 2002)**. The HPA axis is the key player in stress responses; it is well

established that both external and internal stressors activate the HPA axis. Cytokines are chemical messengers that stimulate the HPA axis when the body is under stress **(Haddad et al., 2002)**. Activation of the HPA axis, initiated by the release of CRH and vasopressin (VP) to the pituitary portal circulation, is essential to maintain homeostasis and ultimately for survival during severe stress. Its regulation depends on adrenal steroids and afferent neural inputs to CRH neurons in the hypothalamic periventricular nucleus (PVN). CRH is the main regulator but VP, co-expressed in about 50% of CRH neurons, potentiates the stimulatory effect of CRH on adrenocorticotropic hormone (ACTH) release. CRH and VP stimulate ACTH secretion through activation of type 1 CRH receptors (CRHR1) and type V1b VP receptors in the pituitary corticotrophin **(Aguilera, 2011)**. Hormones released by adrenals and gonads, like glucocorticoids, estrogen, progesterone, and testosterone, work in parallel with neurotransmitters and neuropeptides to regulate the IS (Figure 5). In turn, cytokine signaling provides feedback to the hypothalamus to regulate the hormonal and neuronal response.

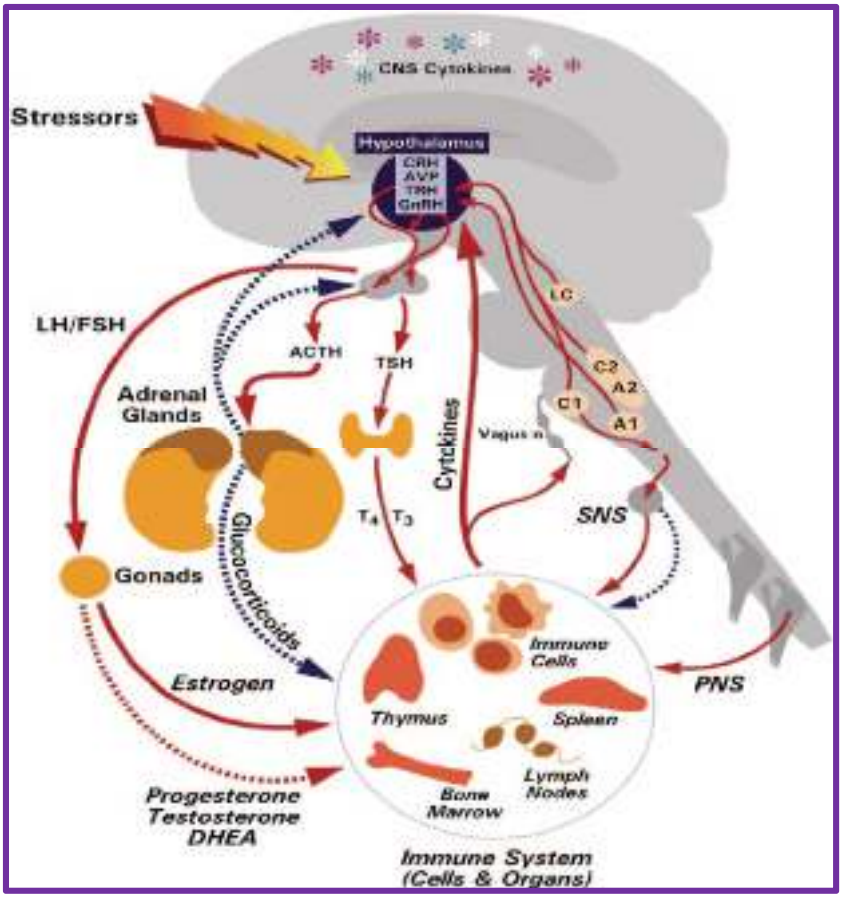


Figure 5: The HPA axis and its bidirectional communication between the ANS, and the IS **(Tait et al., 2008)**. Dotted lines represent negative regulatory pathways, and solid lines represent positive regulatory pathways. A1, C1, A2, C2: Brainstem adrenergic nuclei, AVP:

arginine vasopressin, DHEA, dehydroepiandrosterone, FSH: follicle-stimulating hormone, GnRH, gonadotropin-releasing hormone; LC, locus ceruleus; LH, luteinizing hormone, PNS, peripheral nervous system, T3: tri-iodothyronine, T4: thyroxine, TRH: thyrotropin-releasing hormone, TSH: thyroid-stimulating hormone, Vagus n.: vagus nerv

II.2.2. Different stress signals on neuro-immune endocrine axis

II.2.2.1. Anxiety and depression

The organism must maintain a complex dynamic equilibrium, or homeostasis, which is constantly challenged by internal or external adverse forces termed stressors. Stress occurs when homeostasis is threatened; homeostasis is re-established by various physiological and behavioral adaptive responses **(Chrousos, 2009)**. Anxiety was defined as a future-oriented mood state associated with preparation for possible upcoming negative events, the symptoms of anxiety include worry (verbal-subjective), avoidance (overt motor acts), and muscle tension (somato-visceral activity) **(Craske et al., 2009)**. Depression is characterized by a metabolic nervous disorder. The concentration of neurotransmitters (serotonin, norepinephrine, and dopamine) is in rupture of balance, generally due to a lasting hyperactivity of stress hormones. The main symptoms of depression are mood gloomy or the feeling of inner emptiness, exhaustion (burn-out), overwork, inner turmoil, thinking and sleeping disorders **(Keck, 2017)**. Depression include disruptive mood dysregulation, major depressive disorder, persistent depressive disorder, premenstrual dysphoric disorder, depressive disorder induced by substances/drugs, and depressive disorder due to other medical conditions. The common symptoms of these conditions is the presence of sad, empty, or irritable mood, which together with specific cognitive and somatic symptoms lead to significant distress or impairment in functioning **(Ruscio & Khazanov, 2015)**. Anxiety and depressive disorders often occur as comorbid illnesses and share many common symptoms. Risk factors for these disorders most likely include interactions of environmental and genetic factors **(Pollack, 2005)**.

II.2.2.2. Inflammation

The inflammation term is taken from the Latin word “inflammare” (to burn) **(Abdulkhaleq et al., 2018)**. Inflammation is an innate complex mechanism of defense and a necessary component of the body response to biological, chemical, or physical stimuli. This mechanism is non-specific and immediate. There are five fundamental signs of inflammation

including: heat (calor), redness (rubor), swelling (tumor), pain (dolor), and loss of function (functio laesa). Inflammation can be classified into three types based on the time of the process that responds to the injurious agent; acute occurs immediately after injury and lasts for few days, chronic inflammation that may last for months or even years when acute inflammation fails to settle, and subacute which is a transformational period from acute to chronic which lasts from 2 to 6 weeks **(Hannoodee, 2021)**. In the acute inflammation, platelets and granulocytic cells such as basophils, mast cells, neutrophils, and eosinophils are activated to produce and release soluble mediators that stimulate and regulate the inflammatory response (Figure 6). Neutrophils, also named polymorphonuclear neutrophils (PMNs) are the primary cellular mediators of acute inflammatory response. Their granules contain a variety of enzymes, peptides, and proteins and can also rapidly release reactive oxygen species (ROS) (respiratory burst). These serve to destroy and digest organisms and foreign material following phagocytosis, but may also be released and damage host tissues at the inflammatory site **(Germolec et al., 2018)**. Cytokines have important effects in inflammatory responses, IL-1β, IL-8, TNF-α, IL-6, and IL-12 are the most remarkable secretions included and known as pro-inflammatory cytokines. These cytokines affect other organs, especially the brain and liver, resulting in a systemic immune response called the acute-phase response **(Abdulkhaleq et al., 2018)**.

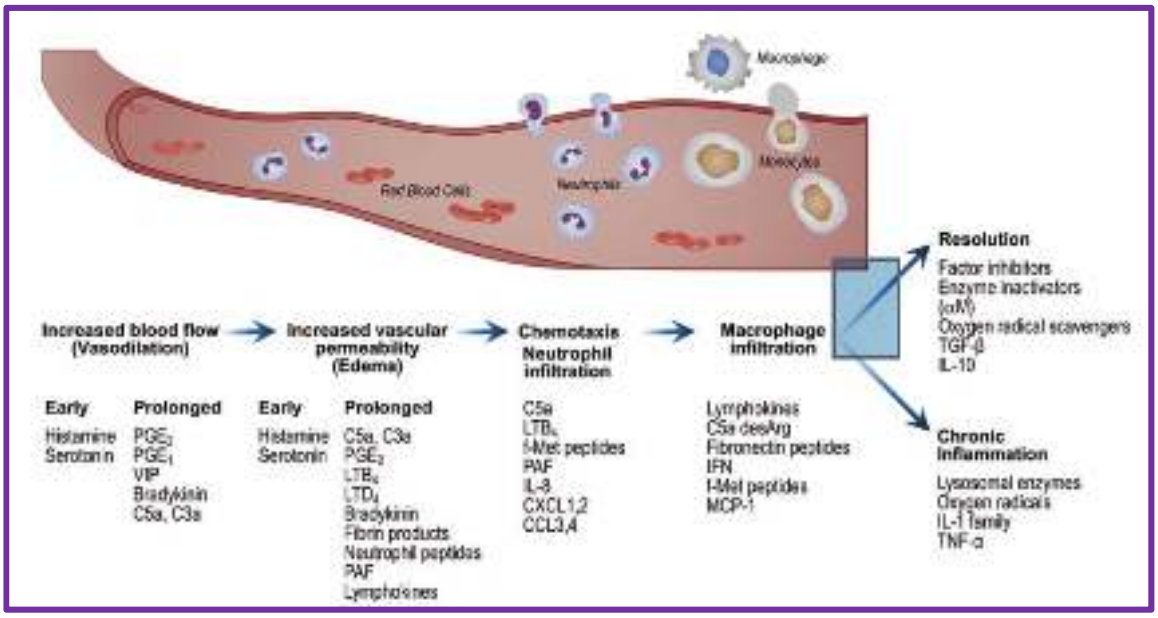


Figure 6: Mediators of inflammation **(Germolec et al., 2018)**.

This figure (6) summarizes the roles of the various mediators important in the process of inflammation from the acute to chronic phase. Abbreviations: PGE prostaglandin E, VIP

vasoactive intestinal polypeptide, LTB leukotriene B, LTD leukotriene D, PAF platelet-activating factor, CXCL chemokine (C-X-C motif) ligand, CCL CC chemokine ligand, MCP monocyte chemo-attractant protein, TGF transforming growth factor.

II.2.2.3. Oxidative stress

OS is explained as an imbalance between prooxidants and antioxidants in favor of the prooxidants, resulting in a disruption of redox signaling and control and/or molecular damage. In the open metabolic system, a steady-state redox balance is maintained at a given set point, which provides a basal redox tone, and that a deviation from the steady-state redox balance is considered a stress, initiating a stress response. Implicit in the definition of OS is that a deviation to the opposite side of the balance is “reductive stress”, and that there are physiological deviations, “oxidative eustress”, and supra-physiological deviations, “oxidative distress”. Oxidative eustress is an essential part of redox control and physiological redox signaling. This concept overlaps with that of redox homeostasis as the “golden mean”**(Sies, 2020)**.

Several chronic diseases are initiated by an increase in intracellular levels of free radicals. Elementary amounts of ROS are essential for normal physiological processes; whereas, excessive generation of free radicals causes tissue oxidative damage. OS induced by ROS engender an inflammatory state. Biomarkers are necessary for the evaluation of an OS state, the most common are listed in table 3. OS plays a critical role in aging, obesity, fatty liver disease, type 2 diabetes mellitus, depression, neurodegeneration, immune and endocrine dysfunction and cancer **(Guo et al., 2020)**.

Table 3: Oxidative stress biomarkers **(Tsukahara, 2007)**.

Formation of modified molecules by reactive oxygen species
Lipid peroxidation: malondialdehyde (MDA) malondialdehyde-lysine (MDA-Lys), 4-hydroxy-2-nonenallysine, acrolein-lysine, F2-isoprostan
Oxidative DNA damage: 8-hydroxy-2'-deoxyguanosine
Glyco-oxidation: carboxymethyl-lysine, pentosidine, argpyrimidine,methylglyoxal
Nitro-oxidation: nitrotyrosine, nitrite/nitrate
Others: o,o'-dityrosine, ortho-tyrosine, bilirubin oxidative metabolites, dehydroascorbate, oxidized glutathione, thiobarbituric acid reactive substances
Antioxidant enzymes and molecules
Enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), thioredoxin reductase, heme oxygenase
Proteins: albumin, ferritin, transferrin, lactoferrin, cerulolasmin, thioredoxin, L-type fatty acid binding protein
Low molecular weight molecules: bilirubin, tocopherols, carotenoids, ubiquinol/ ubiquinone, ascorbate, glutathione (GSH), cysteine, urate, nitrite/nitrate, selenium

II.3. *Ephedra alata* monjauzeana

II.3.1. Taxonomy

Ephedra alata monjauzeana is classified according to the following taxonomic levels:

Kingdom	**Plantae**
Phylum	**Tracheophyta**
Class	**Gnetopsida**
Order	**Ephedrales**
Family	**Ephedraceae**
Genus	***Ephedra* Tourn. ex L.**
Species	***Ephedra alata* Decne.**
Sub species	***Ephedra alata* subsp. *monjauzeana* Dubuis & Faurel (Figure 7)**

Figure 7: *Ephedra alata subsp. monjauzeana* Dubuis & Faurel **(Chagnoux S ., 2023)**.

II.3.2. Description

II.3.2.1. Range Description:

Recorded from North Africa to the Arabian Peninsula, and also in Mauritania. Occurs at an elevation of 50 m up to 1,200 m.

II.3.2.2. Country Occurrence:

Native: Algeria; Chad; Egypt (Egypt (African part), Sinai); Islamic Republic of Iran; Iraq; Jordan; Lebanon; Libya; Mali; Mauritania; Morocco; Saudi Arabia; Somalia; Syrian Arab Republic; Tunisia **(GBIF, 2019)**.

II.3.2.3. Habitat and Ecology

A robust xerophytic shrub forming dense clumps. Found on sandy calcareous soil, gravely/rocky soil or clay soil in arid environments often near Wadis, in Wadi-beds, or on shifting sand dunes. Often with Haloxylon association. Sometimes a dominant component of the "végétation contractée". Diaspores are dispersed by the wind as it has wing-like bracts. Flowering/fruiting time is from March to May **(Bell & Bachman, 2011)**.

Dioecious or monoecious shrubs or lianas. Leaves opposite, reduced, fused into a sheath at the base. Axillary male catkins, with 2-6 anthers on a common filament. Female flowers solitary or grouped 2-5, terminal, surrounded by 2-4 pairs of imbricated bracts. False fruit (galbule), consisting of accrescent bracts. Sessile anthers arranged in 2 groups of 4 on 2 staminal columns. Galbula with truncated bracts at the base. RR: SS. M'Zab **(Quézel and Santa, 1962)**.

II.3.3. Medicinal values

Ephedra alata, a medicinal plant belonging to the Ephedraceae family has been commonly used for a long time in traditional medicine in china and the most Arabian country for a variety of medicinal purposes, including treatment of allergies, asthma, chills, colds, coughs, edema, fever, flu, headaches, and nasal congestion **(Mighri et al., 2019)**. *Ephedra* was known as Ma-huang, whose literal translation means ‘hemp yellow’, discovered the first time as a Shrub that has been known in China for at least 5,000 years. Ma-huang has a warm nature, and it belongs to lung and bladder meridians for sweating and dispelling cold, relieving asthma and cough, inducing diuresis, and alleviating edema, and it is used to treat typhia, and lung diseases **(Miao et al., 2020)**. *Ephedra* acts as a central nervous excitant as a result of the rapid

passage of ephedrine through the BBB. The pure alkaloid ephedrine was first isolated and characterized by Nagai in 1885. This stimulates neurons in the limbic system, which also control part of the hypothalamus (supporting a variety of functions, including emotion) **(Lee, 2011)**. Stems from *Ephedra alata* Decne were chewed for treatment of bacterial and fungal infections **(Achraf et al., 2022)**. Diabetes protective activity, anti-obesity, wound healing, antiviral and anti-tumor effects were also recorded in many studies **(Gonz et al., 2020)**.

III Materials and Methods

The detail of chemicals and reagents of all experiments are given in appendix 1.

III.1. The phytochemistry study of *Ephedra alata* monjauzeana

III.1.1 Aim and objective

This part preceded the main experimental part of the work, in order to confirm the choice of the plant and to ensure that is the adequate candidate plant capable of engendering a preventive effect against the impact of pesticide. In this section of work, we have elaborated a chemical characterization of the crude extract that generally include the majority of metabolites, followed by a series of biological activities as preliminary tests that gave sufficient information about the efficiency of the plant. All the procedures are recapitulated, in figure 9, page 31.

III.1.2. Plant material and extraction procedure of *Ephedra alata* monjauzeana crude extract (EamCE)

The plant was collected during the flowering season (April) from the region of Beni Abes, Bechar. According to the phenotypical features described by Dubuis and Faurel (1957) **(IPNI, 2020)**, the plant was recognized and authenticated by Dr. Hani BOUYAHMED. Thereafter, the aerial parts of *Ephedra alata* monjauzeana were dried in obscurity at room temperature, and grinded into a fine powder using Microfine grinder Merke IK MF 10 Basic Staufen (DE) Germany. The EamCE was obtained by means of maceration with a mixture of methanol/water (80:20, *v*/*v*), under constant stirring and left overnight in the dark. The maceration exudate was filtered, and the recovered solution was then evaporated under vacuum using a rotary evaporator at 35 °C. The process was repeated every 24 h, three times. The EamCE was dissolved in a small quantity of methanol/water (80:20, *v*/*v*) and eventually filtered through a 0.2 µm filter before its analysis.

III.1.3. Chemical characterization

We have used to characterize the EamCE the liquid chromatography coupled to tandem mass spectrometry (LCMS/MS). The analysis were performed with an Agilent 1200 series rapid resolution (Agilent Technologies, Palo Alto, CA, USA) supplied by a binary pump, an auto sampler, and a diode array detector (DAD), using a quadrupole time of flight mass spectrometry analyzer (QTOF, model 6540 Agilent Ultra High Definition Accurate Mass QTOF), equipped with an electrospray ionization interface (ESI, model Agilent Dual Jet Stream interface).The

flow amount was adjusted at 0.80 mL/min throughout the gradient. Then, 10 µL of the EamCE solution (20.000 mg/L) was injected. Separation was executed on a 150 × 4.6 mm, 1.8 µm, Zorbax Eclipse Plus C18 column (Agilent Technologies) at room temperature. Gradient elution was run, utilizing as eluent A: water with 0.1% formic acid and as eluent B: acetonitrile. The following multistage linear gradient was applied: 0 min, 5% B; 45 min, 100% B; 55 min, 5% B; and, finally, a conditioning cycle of 5 min, with the same conditions for the next analysis. The separated compounds were monitored in sequence first with the DAD and then with a mass spectrometry detector spectra that were acquired over a mass range from m/z 70 to 1100 operating in negative ionization mode. Internal mass correction was achieved with an unceasing infusion of Agilent TOF mixture consisting of trifluoracetic acid, ammonium salt, and hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine. All spectra were calibrated prior to phytochemical identification. The detection window was set to 100 ppm. The MS and MS/MS data were processed using the Mass Hunter Qualitative Analysis B.06.00 software (Agilent Technologies) that yielded a list of eventual elemental formulas.

III.1.4. Total phenolic content (TPC) and total flavonoid content (TFC) assessment

III.1.4.1.TPC dosage

The TPC was assessed spectrophotometrically according to Folin Ciocalteu method, modified by **(Singleton & Rossi, 1965; Müller et al., 2010)**. The quantification was expressed as micrograms of gallic acid equivalents per milligram of extract (µg GAE/mg).

III.1.4.2. TFC dosage

The TFC was assessed spectrophotometrically following the method described by **(Topçu et al., 2007)**. The quantification was expressed as micrograms of quercetin equivalents per milligram of extract (µg QE/mg).

III.1.5. Biological activities of EamCE

III.1.5.1. Antioxidant activity

The antioxidant activity of EamCE was evaluated by ten different assays. All assays were realized in 96-well microplates, and the absorbance measurements were realized by Multimode Plate Reader, EnSpire, PerkinElmer, Waltham (US) United States of America.

BHA, BHT, α-Tocopherol, ascorbic acid, tannic acid, gallic acid, quercetin were used as standards (positive controls) to estimate the relative extract activity. The EamCE solution was prepared at seven different concentrations (800, 400, 200, 100, 50, 25, 12.5 µg/mL) and every assay was realized in triplicate. The results were expressed as concentrations of 50% inhibition (IC50) and of absorbance at 0.5 (A0.5), that are able to inhibit/ chelate 50% or to reduce the absorbance to 0.5 of the radical or the formation of metallic complexes/cations.

a. ABTS radical (ABTS•+) scavenging ability

The ABTS•+ scavenging ability was performed spectrophotometrically according to the modified method of **(Re et al., 1999)**. Firstly, the ABTS•+ was generated as follows: 2 mM of ABTS was dissolved in H_2O with 2.45 mM of $K_2S_2O_8$, and the mixture was conserved at ambient temperature for 16 h in obscurity. Secondly, 160 µL of diluted ABTS•+ solution (delivering an absorbance value of 0.700 ± 0.025 at 734 nm) was added to 40 µL of EamCE solution. Thereafter, the microplate was incubated for 10 min before measuring the absorbance at 734 nm. The equation below (*) was used to calculate the inhibition percentage of ABTS radical, and results were presented as IC50 values.

$$I\% = \left(\frac{Ac - As}{Ac}\right) \times 100 \quad (*)$$

I: inhibition

Ac: control's absorbance

As: sample's absorbance

b. DPPH radical (DPPH•) Scavenging capacity

The scavenging capacity of the stable DPPH• free radical was indicated by the adjusted procedure of **(Blois, 1958)**: it consists of adding 160 µL of DPPH solution (1 mM) in reaction with 40 µL of the EamCE solution; then, the absorbance of mixture was measured at 517 nm after 30 min of incubation in the dark. Results were provided as IC50 values, and the I% were calculated using the above formula (*).

c. Superoxide alkaline DMSO activity

The superoxide radical was produced as described **(Rao, 1990)**: in brief, 30 µL of NBT (1 mg/mL) and 40 µL of sample were added to 130 µL alkaline DMSO (1 mL DMSO, 5 mM

NaOH, 100 µL H_2O). The absorbance of the reaction mixture was measured at 560 nm, and results were provided as IC50 values.

d. Ferric reducing ability of plasma (FRAP)

To assess the ferric reducing power effect, 10 µL of the EamCE solution were added to 40 µL of 0.2 M phosphate buffer (pH 6.6) and 50 µL of $K_3[Fe(CN)_6]$ (1%), incubated for 20 min at 50 °C. Later, 50 µL of TCA (10%) and 10 µL of $FeCl_3$ (0.1%) were added before measuring the mixture's absorbance at 700 nm. The results were given as the EamCE concentration, giving an absorbance 0.5 (A0.5) **(Oyaizu, 1986)**.

e. β-Carotene/Linoleic acid bleaching activity

Proceeded as described by **(Marco, 1968)** with minor changes: 0.5 mg of β-carotene, 1 mL of chloroform, 25 µL of linoleic acid, and 200 µL of tween 40 were added, forming an emulsified mixture. Then, it was evaporated under vacuum, 50 mL of H_2O_2 (30%) were added later, with vigorous shaking. The absorbance at 470 nm was checked to give a value between (0.8–0.9). Next, 160 µL of the prepared β-carotene was added to 40 µL of EamCE solution. The 0 min (t0) and 120 min (t120) time absorbances were measured, and the results were given as IC50 values, according to the following equation:

$$I\% = 1 - \left(\frac{As(t0) - As(t120)}{Ac(t0) - Ac\ (t120)}\right) \times 100$$

As is the absorbance of the tested sample and Ac is the absorbance of control (methanol).

f. Cupric reducing antioxidant capacity (CUPRAC)

The assay was described by **(Apak et al., 2004)** and was performed with no modifications. In brief, 10 mM of $CuCl_2$ (50 µL), 7.5 mM of neocuproine in ethanol (50 µL), and 1 M of CH_3COONH_4 (60 µL) with 40 µL of the EamCE solution, were added simultaneously to generate the reaction. Then, the mixture was incubated for 1 h before measuring the absorbance at 450 nm. The result was given as A0.5 value.

g. Hydroxyl radical scavenging assay

The assay was performed according to the modified method **(Smirnoff and Cumbes, 1989)**. Initially, 40 µL of the EamCE solution was mixed with 80 µL of salicylic acid (3 mM),

24 µL of $FeSO_4$ (8 mM), and 20 µL of H_2O_2 (20 mM). The mixture was incubated for 30 min at 37 °C, and 36 µL of H_2O was added later. The absorbance was measured immediately, at 510 nm. The result was given as IC50 value.

h. O-Phenanthroline assay

As proceeded by **(Szydłowska-Czerniak et al., 2008)**, the reaction mixture held 30 µL of o-phenanthroline (0.5% in methanol), 50 µL of $FeCl_3$ 0.2%, 110 µL of methanol and 10 µL of the EamCE solution. Next, it was incubated for 20 min at 30 °C before measuring the absorbance at 510 nm. The result was given as A0.5 value.

i. Galvinoxyl radical (GOR) scavenging assay

The procedure consisted of adding 160 µL of galvinoxyl (0.1 mM) in methanol to 40 µL of the EamCE solution, followed by an incubation of 120 min, and then the absorbance was read at 428 nm. The result was given as IC50 value **(Shi et al., 2001)**.

j. Silver nanoparticle based method

The reduction of Ag+ to spherical silver nanoparticles (SNPs) was developed by **(Özyürek et al., 2012)**. Firstly, 130 µL of SNP solution (prepared by heating 50 mL of silver nitrate ($AgNO_3$) (1 mM) for 10 min; then, 5 mL of $Na_3C_6H_5O_7$ (1%) was added drop by drop until a pale-yellow color was obtained) and 50 µL of H_2O were added to 20 µL of the EamCE solution. The microplate was incubated for 30 min at 25 °C, and the absorbance was read at 423 nm. The result was given as A0.5 value.

III.1.5.2. Anti-inflammatory activity

The anti-inflammatory activity was evaluated by means of two different methods, heat induced hemolysis test (*in vitro)* and anti paw edema test (*in vivo*).

a. Heat induced hemolysis

Firstly, an erythrocyte suspension was prepared as follows: a total human blood was obtained from a safe donor, centrifuged for 5 min at 3000 rpm in heparinized centrifuge tubes. Then, the suspension was washed three times with an equivalent volume of NaCl 0.9%. Subsequently, it was diluted to obtain a suspension of 10% (v/v) in an isotonic buffer solution (10 mM phosphate buffer, pH 7.4: 0.2 of NaH_2PO_4, 1.15 of Na_2HPO_4 and 9.0 NaCl (g/L)). The

procedure consists of adding 0.05 mL of the erythrocyte suspension and 0.05 mL of the EamCE solution mixed with 2.95 mL phosphate buffer (pH 7.4). The conical tubes were incubated at 54 °C for 20 min in a shaking water bath. Once this was done, they were centrifuged at 2500 rpm for 3 min, and the absorbance of the supernatant was measured at 540 nm against a control using phosphate buffer. Diclofenac was used as a reference compound **(Gunathilake et al., 2018)**.The inhibition percentage of EamCE was recorded according to the equation below:

$$\boldsymbol{Inhibition\ \%\ of\ hemolysis = 100 - (1 \times \frac{C}{S})}$$

C: absorption of the control.

S: absorption of test sample mixture.

b. Anti paw edema

Adult albino wistar male rats were obtained from Algiers Pasteur Institute. The animals were maintained under normal laboratory condition of humidity (50%), temperature (23 ± 2°C), a 12 h light/dark cycle, and allowed free access to nutriments and water ad libithum. The animals were randomly divided into 5 groups (n = 5). At first, the volume of intact rats paw was measured in all groups using water displacement plethysmometer. By an intraperitoneal administration, the first group served as a negative control (normal saline 5 mL/kg bw) while the second group that represented a positive control was treated with a reference anti-inflammatory drug (diclofenac 25 mg/kg bw), the third, the fourth and fifth groups received different doses of EamCE (25, 200 and 400 mg/kg bw) and the fifth group received aspirin (300 mg/kg bw) as a positive control group, respectively. Edema was induced on the right hind paw of the rat by a subplantar injection of 0.1 mL of formalin (1 %) 30 min after drug administration. Swelling of formalin-injected foot was measured hourly; from the first to the fifth hour after the formalin injection, using the water displacement plethysmometer **(Piovezan et al., 1997; Agnel Arul John & Shobana, 2012)**. The anti-inflammatory ability to suppress paw inflammation was expressed as a percent of paw edema according to the following equation:

$$\boldsymbol{Edema\ \% = \frac{(VF - VI)}{VI} \times 100}$$

VI: intimal volume of edema (before injection of formalin).

VF: final volume of edema (after injection of formalin).

III.1.5.2. Toxicity and cytotoxicity investigations of EamCE

The toxicity of EamCE was evaluated by various methods namely:

a. **Acute oral toxicity (mice)**

b. **Cytotoxicity on cancer cell lines (human)**

a. Acute oral toxicity

The acute oral toxicity was determined in albinos mice *Mus musculus* of both sexes according to the experimental protocol described in guideline code 423 **(OECD, 2001)**. In order to reveal if the used dose is toxic or not. Four groups of mice were prepared as elucidated in figure 8, the mice were purchased from Algiers Pasteur Institute. The mice were fasted for 12 h before the start of the experiment, they were weighed before administration. A volume of 0.5 ml of EamCE (2000 mg/kg) was administered through enteral route, the same volume of physiological water (NaCl) 0.9% was given to the control. After treatment, observations related to mortality rate, weight variations, physical and behavioral changes (tremor, convulsion, salivation, diarrhea, lethargy, sleep and coma) were noted daily until the 14th day of the experiment. At the end of the experiment, each mouse was weighed, euthanized and then subjected to an autopsy. Attention focused on the presence or absence of hemorrhage, cysts and/or organ swelling. Vital organs (heart, liver and kidney) were weighed and compared with those of control.

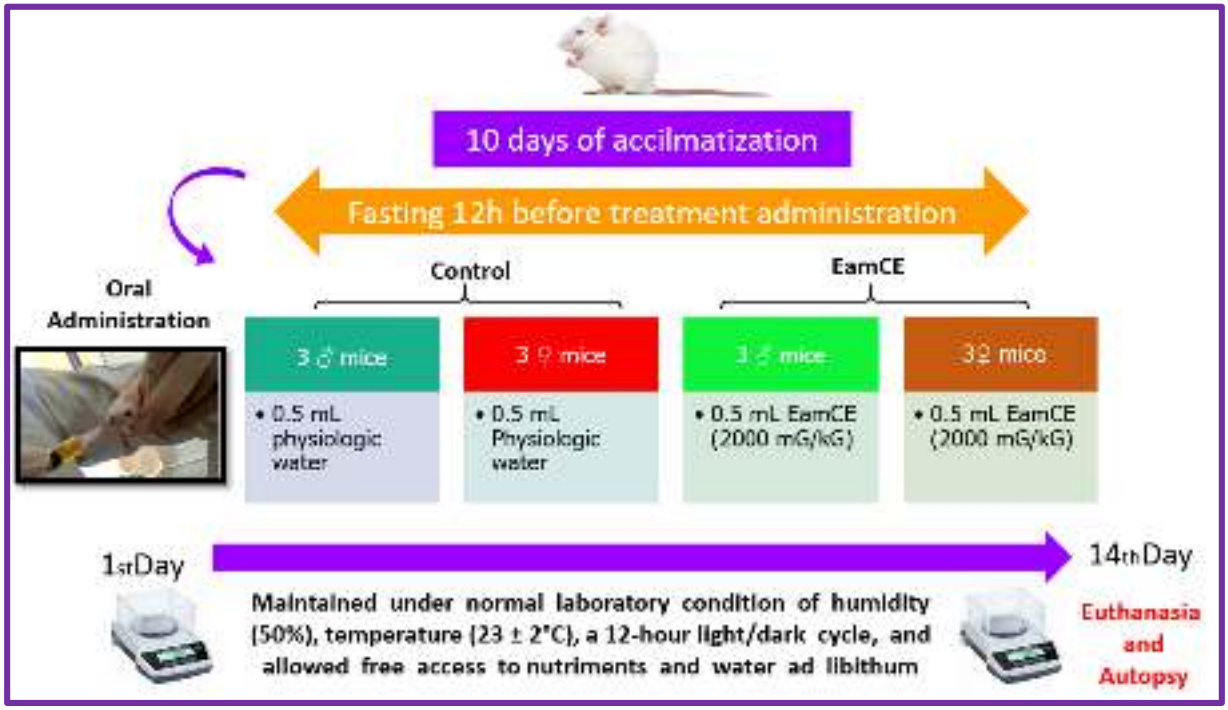


Figure 8: Experimental plan of acute oral toxicity

b. Cytotoxicity on human cancer cell lines

- **Cell culture**

Hep2 (Human epithelial type 2 (laryngeal carcinoma)) and Rd (rhabdomyosarcoma) cells were kindly provided by Pasteur Institute, Algiers, Algeria. The cells were grown and maintained in DMEM supplemented with 10% (v/v) fetal calf serum and 1% (v/v) antibiotic–antimycotic in a 37 °C, 5% CO_2 humidified atmosphere. The cells were harvested every 3 days. After thawing, the cells were kept in normal culture conditions for 10 days before experiments.

- **Cytotoxicity assessment**

Hep2 and Rd cells were incubated with different concentrations (15.625, 31.25, 62.5, 125, 250, 500 µg/mL) of the EamCE for 48 h and 72 h periods. Hep2 and Rd cell viability was assessed by the MTT assay **(Qadir et al., 2014)**, where 100 µL of MTT was added and incubated at 37 °C for 4 h. The insoluble formazan was dissolved in 100 µL of DMSO. The absorbance was measured at 490 nm and at two different time (t = 48 h and t = 72 h). The experiment and measurements were performed in triplicate. The cytotoxic effect was determined using the below formula:

$$\textbf{\textit{Cytotoxic effect}}\ (\%\ \textbf{\textit{Cell inhibition}}) = 1 - \left(\frac{\textbf{\textit{Ac}}}{\textbf{\textit{As}}}\right) \times 100$$

Ac: absorbance of the control.

As: absorbance of the sample.

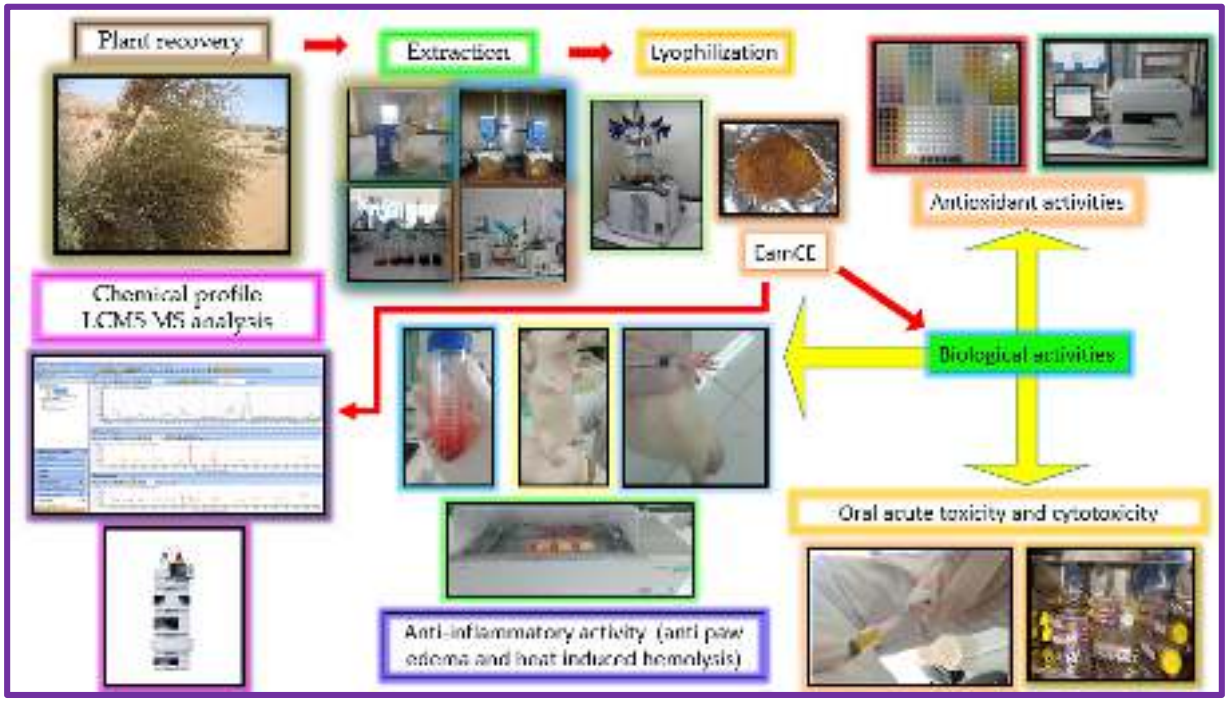


Figure 9: Summary of the phytochemistry study of EamCE (personal photos).

III.2. Pirimicarb toxicity on the neuro-immune endocrine system and the preventive effect of EamCE

The main purpose of this thesis is to evaluate the impact of pirimicarb (a carbamate pesticide) on the perturbation of neuro-immune endocrine homeostasis. To achieve this goal, an *in vivo* protocol was implemented in order to provoke a sub-acute toxicity by the effect of pirimicarb and to evaluate its impact on: behavior, neurological and reproductive tissue integrity, immune cells and cytokine activation, corticoid release and OS status. Moreover, an *in vitro* test was performed to assess the toxicity of pirimicarb on neutrophils. As well, the preventive effect of EamCE was assessed.

III.2.1. Pirimicarb induced sub acute toxicity

III.2.1.1. Animals

Twenty-four albino wistar male rats were purchased from Algiers Pasteur Institute, weighting 190-230 g and underwent an acclimatization period of 15 days before beginning the experiment. The rats were kept at 22±2 °C and 50–60% humidity under a light/dark cycle of 12 h and had free access to standard commercial pelleted feed (supplied by "Office national des aliments du bétail-ONAB-" Guelma, Algeria) and clean tap water ad libitum.

III.2.1.2. Experimental design

In order to induce a sub acute toxicity, we have followed different protocols **(Rai & Sharma, 2007; Chahal et al., 2015; Almeida et al., 2019)** to generate our own. The rats were subdivided in four groups (6 rats/each) and have received distinct treatments by oral gavage: G1: deionized water; G2: 200 mg/kg of EamCE; G3: 14.5 mg/kg (1/10 of LD 50 (145 mg/kg)) of pirimicarb **(Cambon et al., 1979; Zhou et al., 1996; Hardt et al., 1999; Wang et al., 2014)** and G4: 14.5 mg/kg (1/10 of LD 50 (145 mg/kg)) of pirimicarb + 200 mg/kg of EamCE (the EamCE was administrated 1 h before administrating pirimicarb). These daily repeated doses were given for a period of 28 days. During this period of treatment, the consumption of food and water of each group was daily tracked and the body weight of rats was measured once every two days. Thereafter, the animals were subject to three successive days of behavioral examination. Subsequently, animals were euthanized by cervical dislocation; blood tissue and organs (brain and testis) were collected for carrying out further investigations (Figure10). This study was evaluated and approved by the institutional ethic committee of the CRBt (ethical approval reference: N07KH-2021/2023/CCE, appendix 2).

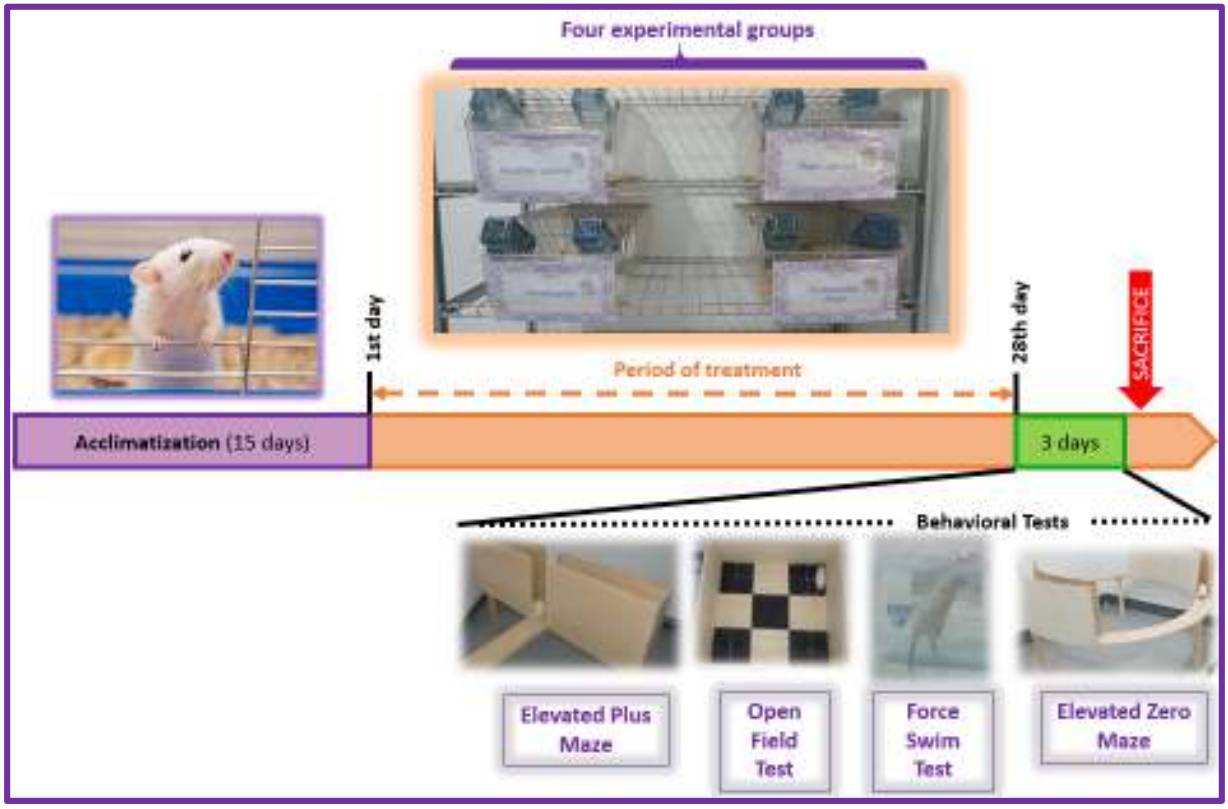


Figure 10: Experimental design of pirimicarb induced sub acute toxicity on rat wistar.

III.2.1.3. Behavioral study

a. Force swim test (FST)

The FST is generally used in rats as a preclinical model to predict and validate the potency of antidepressants **(Borsini & Meli, 1988)**. However, in our investigation, the objective is to check the eventual depressant effect of pirimicarb. To that end, we performed the FST **(Porsolt et al., 1978)** with slight modification; we omitted the pre-test which enables to induce a stress state in rats. Considering that pirimicarb is the stressful agent in this experiment and in order to avoid a further stress. So, the rats were immediately immersed in an aquarium (54 cm high by (34 – 60 cm) base area; these dimensions guarantee that the animal cannot escape by clinging to the edges of the device) for five minutes. The behavior of the rat in the device was filmed using a video camera. The aquarium was filled with warm water (26 °C) up to a height of 40 cm, for ensuring that the animal would not use its lower limbs to stay on the surface and therefore be forced to swim. Thereafter, we analyzed the sequences and recorded the immobility, swimming and climbing (escalation) time.

b. Open field test (OFT)

The locomotor ability was evaluated employing a device consisting of a rectangular wooden enclosure 1 m in diameter and 50 cm high, divided into a central part and six peripheral parts having the same size. The central part is serving as a starting point for the rats in each test. The rats are positioned in the central part and leaved in the device for 10 min. Locomotion was evaluated by recoding the total distance traveled, the number of entries in the central part and the number of redress. These cumulative indices gave us the total locomotion index for rats in the device **(Breed & Moore, 2021)**.

c. Elevated plus maze (EPM)

The EPM apparatus was in a form of a cross raised to a height of 40-60 cm from the ground. It consisted of a central part (10 × 10 cm), and two open protected arms without walls (50 × 10 × 50 cm) which oppose two other arms with closed walls, which are perpendicular to the open protected arms. The test lasts 5 min and begins when the rat is placed in the center of the maze, facing an open arm. An animal that explored within the open arms was described as being "slightly anxious" and an animal that remained confined in the closed arms of the device was described as being "anxious" **(Pellow et al., 1985; Richard, 1987).**

d. Elevated Zero maze (EZM)

An elevated annular runway with alternating open and enclosed quadrants (105 cm diameter, 10 cm width) was used. 65 cm above from the ground level, divided equally into four areas; this updated device helped to remove any uncertainty of interpretation regarding the time lost on the central square of the traditional design and allowed uninterrupted exploration **(Shepherd et al., 1994)**. Latency to enter into an open section, time spent in the open sections, number of entries in the open sections and number of head-dips were measured for 5 min **(Díaz-Morán et al., 2014)**.

III.2.1.4. Oxidative stress parameters

a. Tissue homogenate

The organs were immediately collected, washed using 0.9% NaCl solution and weighed; 1 g of each organ was put in 2 mL of TBS (Tris-buffered saline): Tris 50 mM, NaCl 150 mM, adjusted to pH = 7.4 with HCl 1M. The mix was homogenized using a "SONICS, Vibra-Cell

VX 130" sonificator, under ice-cold conditions. Homogenates were centrifuged at 3000×g for 30 min at 4 °C. The supernatants were then aliquoted and stored at -20 °C.

b. Protein titration

Proteins from tissue homogenates were quantified spectrophotometrically at 595 nm according to the modified method of Bradford **(Kruger, 1994)**, using bovine serum albumin (BSA) as standard.

c. Malondialdehyde

The evaluation of lipid peroxidation levels was accomplished by detecting the value of MDA in organ homogenates. MDA reacts with thiobarbituric acid as a reactive substance to generate a red-colored complex. The procedure involved combining 500µL of tissue homogenate with 1 mL of TCA -TBA - HCl (15 %, 0.375 %, 0.25 N) and mixing thoroughly. The mixture was heated in a boiling water bath for 15 min. Then, the flocculent precipitate was removed by centrifuging at 1000 *g* for 10 min and the absorbance was measured at 535 nm **(Buege & Aust, 1978)**. The concentration of MDA is expressed in nano moles per milligram of protein (nmol/mG protein) and calculated according to the following equation:

$$\boldsymbol{MDA(nmol\ /mGprotein) = \frac{DO \times 10^6}{E \times X \times L \times Fd}}$$

OD: Optical density read at 530 nm.

E: Molar extinction coefficient of MDA = $1.56.10^5$. M^{-1}. cm^{-1}.

L: Optical path length

X: Protein concentration of the extract (mG/mL).

Fd: Dilution factor = 0.2083.

d. Glutathione

GSH levels of organ homogenates were measured by employing a colorimetric technique based on the oxidation of GSH by DTNB, which generates a yellow color according to the Elman method **(Ellman, 1959)**. Tissue homogenate (800 µL) was added to 100 uL of sulfosalicylic acid (0.25%) and left for 15 min in an ice bath. After centrifugation at 1000 rpm for 15 min, 500 µL of supernatant was collected and added to 1 mL of tris-EDTA buffer (0.4

M HCl, 0.02 M EDTA, pH 9.6) and 25 µL of DTNB (0.01 M). After shaking and incubation for 5 min, the absorbance was recorded at 412 nm. The concentration of GSH is expressed in micro moles per milligram of protein (nmol/mG protein) and calculated according to the following equation:

$$\boldsymbol{GSH}(\mu\boldsymbol{mol/mG\ protein}) = \frac{\boldsymbol{OD \times L \times 1.525}}{\boldsymbol{13.1 \times 0.8 \times 0.5 \times mG\ protein}}$$

OD: Optical Density.

L: Optical path length

1.525: Total volume reaction mixture used (0.5 ml supernatant + 1 ml Tris-EDTA 0.025 ml DTNB).

13100: Molar extinction coefficient of GSH ($M^{-1}.cm^{-1}$) at 412 nm.

0.8: Volume of tissue homogenate

e. Superoxide dismutase

The SOD activity was estimated according to the Marklund procedure **(Marklund & Marklund, 1974)** with slight changes. The method was based on inhibition of the auto-oxidation of pyrogallol by SOD. An 850 µL quantity of tris HCl buffer (50 mM, pH = 8.2) was added, followed by 100 µL of EDTA (10 mM). Then, the reaction was started by the addition of 50 µL of pyrogallol (2.5 mM in 10 mM HCl). The absorbance reading was taken at 420 nm every minute for 3 min in the presence or absence of 20 µL of tissue homogenate sample. SOD activity was expressed as U/mg protein. One unit of SOD activity (U) was determined as the amount of enzyme required to inhibit 50% of pyrogallol autoxidation.

The enzymatic activity of the SOD is assessed according to the equation below:

$$\boldsymbol{Total\ inhibition} = \frac{\boldsymbol{OD\ of\ blank - OD\ of\ sample}}{\boldsymbol{OD\ of\ blank}} \times \boldsymbol{100}$$

$$\boldsymbol{SOD\ (U/mG\ protein)} = \frac{\boldsymbol{Total\ inhibition}}{\boldsymbol{n \times 50}}$$

n: mg of protein present in the sample used volume.

f. Catalase

CAT activity was evaluated following the procedure of **(Aebi, 1995)**. In brief, 983.5 µL of H_2O_2 (10 mM, prepared in 50 mM phosphate buffer ((KH_2PO_4, Na_2HPO_4), pH=7.2) was added to 16.5 µL of tissue homogenate. The reaction was based on the disappearance of hydrogen peroxide, and the decrease in absorbance was monitored for 30 s at 240 nm.

$$\boldsymbol{Cat}(\mu\boldsymbol{mol\ H2O2\ /min/mG\ protein}) = \frac{\Delta\ \boldsymbol{OD/min}}{\boldsymbol{\varepsilon \times L \times n}}$$

ε: Molar extinction coefficient of H_2O_2 :43.6 M^{-1} . cm^{-1}

L: Optical path length

n: mg of protein present in the sample used volume

III.2.1.5. Cortisol and testosterone titers

Serum titers of cortisol and testosterone were quantified using Abbott Alinity automaton with their respective specific kits (Alinity 08P3320, Alinity 07P6821).

III.2.1.6. IL-1β titration and quantification in brain and plasma

The level of Il-1β was quantified from brain homogenates and plasma samples, using Rat IL-1 ELISA Kit, E-EL-R0012 (Elabscience Biotechnology Inc.: Houston, TX, USA). The generation of the standard curve and quantification steps were executed according to the manufacturer's handbook (appendix 3). The used ELISA kit is based on the sandwich-ELISA principle and the summary of the procedure is demonstrated in figure 11.

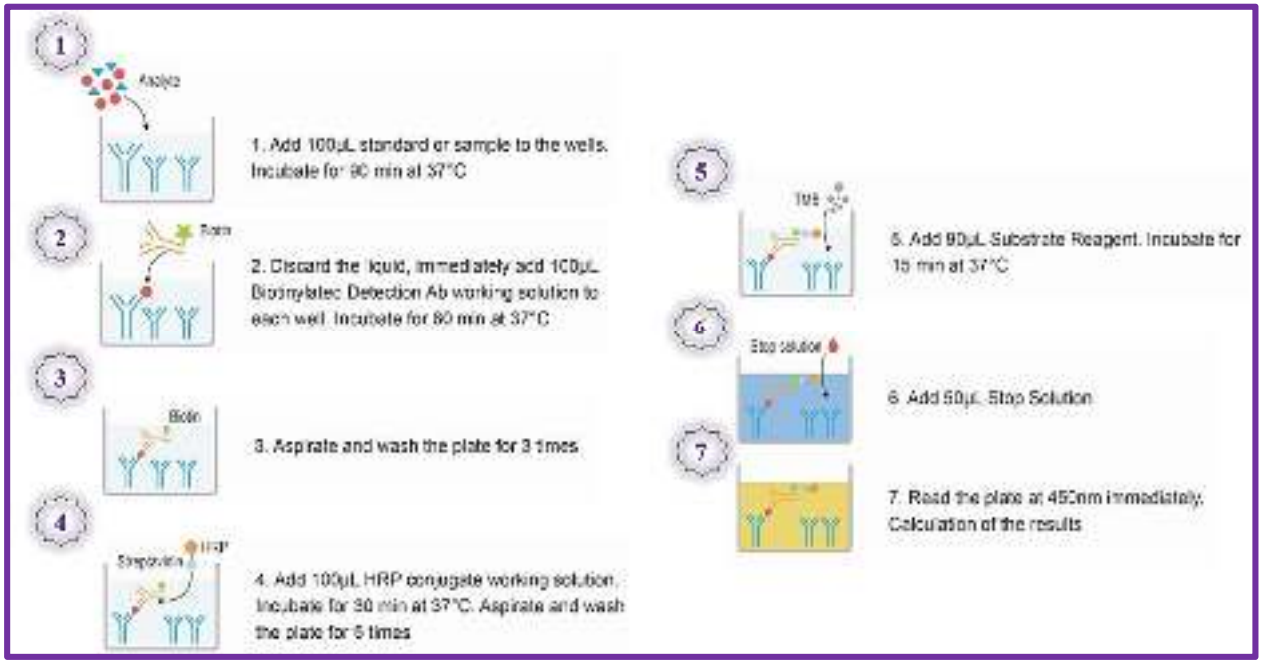


Figure 11: The procedure assay of IL1-β quantification by ELISA sandwich.

III.2.1.7. Assessment of hematological parameters

The count of blood parameters was performed using an automated hematology analyzer (Mindray BC-3000 Plus). The blood samples of rats were collected into EDTA-tubes. We have taking in consideration the following parameters: white blood cells, red blood cells, lymphocytes, monocytes, granulocytes, platelets.

III.2.1.8. Histopathological examination

No treatment-related deaths were evident. Sacrificed rats were subjected to a full necropsy examination. Organs were then removed and examined for any gross lesion after being rinsed with NaCl (0.9%) solution thoroughly and properly. Then, they were immediately fixed in formaldehyde solution (10%). Tissue samples (brain and testis) were routinely processed through an automatic tissue processor. After that, the tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) according to the technique described by **(Kim, 2019)**. The main steps are illustrated in figure 12 (personal photos). Photomicrographs of selected lesions were taken using an optical microscope with an integrated camera (BioBlue Euromex (EU 2131898)) and treated by Image Focus plus V2.

Figure 12: Basic steps of tissue processing for the histological study (personal photos).

III.2.1.9. Tracking pirimicarb traces in brain and testis tissues

a. Pirimicarb extraction

A 0.33 g tissue fragment from each animal target organ (brain and testis) was mixed and stirred vigorously with 10 mL of distilled water, 10 mL of acetonitrile, 4 g of $MgSO_4$ and 4 g of NaCl. After that, it was centrifuged at 4500 rpm, 15 °C for 5 min. The supernatants were collected, then, 2 g of $MgSO_4$ and 25 mg of activated charcoal were added. The newly constituted mixture was centrifuged under the same previous conditions, and the supernatants were recovered, filtered through a 0.22 µM filter and evaporated. Next, the extract was dissolved with a small quantity of acetonitrile and stored at 20°C before analysis **(Anastassiades et al., 2003)**.

b. LS-MS/MS Analysis

The tissue extracts were analyzed using the LC-MS/MS method in multiple reaction monitoring (MRM) mode. The analysis was performed using UPLC-ESI-MS-MS Shimadzu 8040 Ultra-High sensitivity with UFMS technology and equipped with binary bump Nexera XR LC-20AD. The ESI conditions were as follows: CID gas, 230 KPa; conversion dynode, 6.00 Kv; interface temperature, 350°C; DL temperature, 250°C; nebulizing gas flow, 3.00 L/min; heat block, 400°C; and drying gas flow, 15.00 L/min. The MRM transition was accessed from Shimadzu Pesticide MRM Library Support for LC/MS/MS. The pump mode was isocratic, and the mobile phase contained: 15% A water, 0.1% formic acid, and 85% B acetonitrile. The flow rate was: 0.2 mL/min and the injected volume of extracts was 5µL, using a Restek column of force C18 1.8m 50 x 2.1 mm.

III.2.2. Pirimicarb cytotoxicity on neutrophils and preventive effect of EamCE

III.2.2.1. Neutrophils isolation

Neutrophils were isolated from freshly heparinized blood of three healthy volunteers according to **(Bouriche & Arnhold, 2010)**. The neutrophils were isolated by centrifugation on a Ficoll-Hypaque gradient density after sedimentation enhanced by dextran. The remaining erytrocytes were waived by means of hypotonic lysis. Neutrophils are preserved in HBSS and kept on ice for further use.

III.2.2.2. Trypan blue exclusion to estimate cytotoxic effect

The cytotoxic effect of pirimicarb and the eventual preventive effect of EamCE were estimated by recording neutrophils viability %. After a cool incubation in water bath at 37 °C during 10 min of different mixtures composed of pirimicarb or EamCE and pirimicarb + EamCE (at different concentrations: 50, 75, 100µg) **(Soares et al., 2016; Aliyu, 2020)** with neutrophils suspension, the trypan blue exclusion test **(Lucisano-Valim et al., 2002)** was used to count viable cells. This procedure was effected using Neubauer haemotocytometer and observed under Nikon TS2-S-SM inverted microscope at x10. The cytotoxic effect was estimated according to viability percentage that was calculated following this formula:

$$\textbf{\textit{Viability}}\ \% = \frac{\textbf{\textit{number of viable cells}}}{\textbf{\textit{total number of cells}}} \times \mathbf{100}$$

III.3. Statistical study

The variance analysis of the obtained results was conducted on XLSTAT Version 2016. 02. 28451 using ANOVA, the significance of differences was checked using Tukey's HSD test. Values with different subscripts (a, b, c, d, e) in the same parameter were significantly different compared with the control (*** $p \leq 0.001$ = very highly significant, ** $p \leq 0.01$ = highly significant, * $p \leq 0.05$ = significant), and those with the same subscripts were not significantly different ($p > 0.05$). For the results of *in vivo* sub acute toxicity of pirimicarb in rats, values with different subscripts (a, b, c, d, e) in the same parameter were significantly different compared with the healthy group G1.

IV Results and Discussion

IV.1. The phytochemistry study

IV.1.1. LC-MS/MS analysis

The LC-MS/MS analysis of the EamCE has given a base peak chromatogram (BPC) in the negative ionization mode that is demonstrated in figure 13.

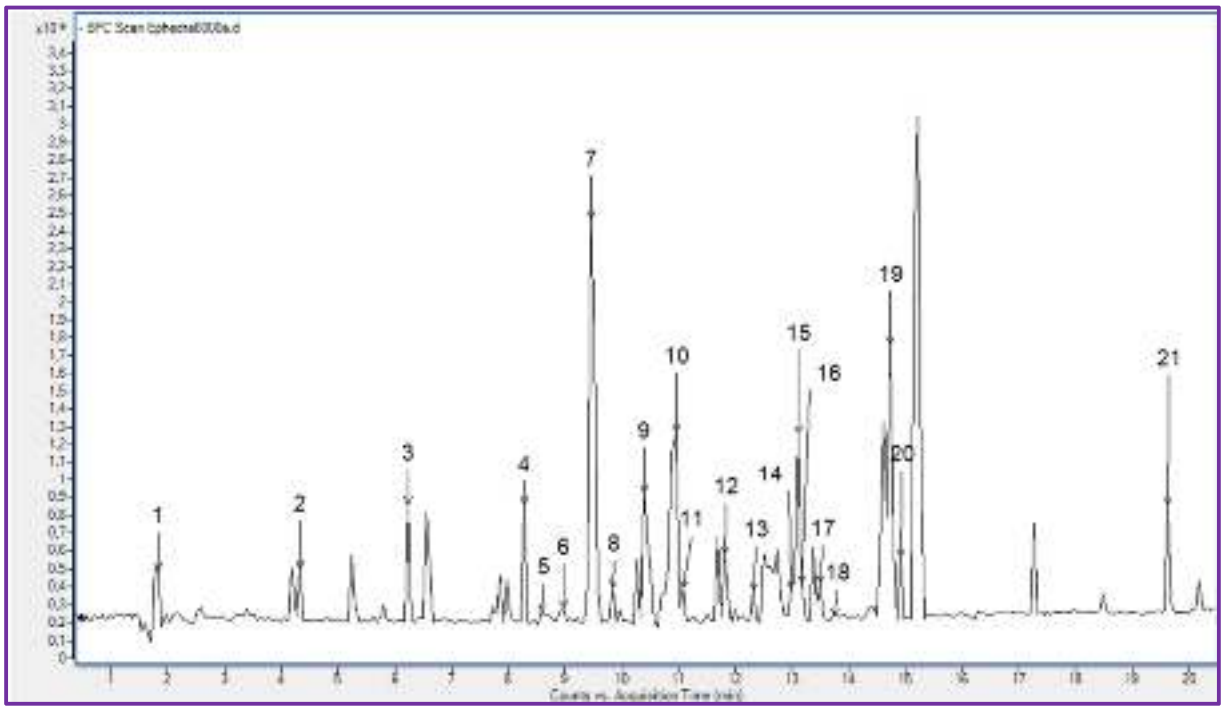


Figure 13: The base pick chromatogram of EamCE

A list of the molecular ions ([M −H]−) found in the EamCE and tentative identification of each of them is provided in table 3. Based on their MS and MS/MS data, twenty-one compounds were recognized and characterized as listed in table 3.

Table 4: Identified flavonoids and phenolic acids in EamCE

Peak	Compounds	Rt (min)	Molecular formula	m/z experimental	m/z calculated	Ionization mode	Error	Major fragments m/z (intensity %), reference
1	Caffeic acid	1.90	$C_6H_{12}O_6$	179.0558	179.0561	N	1.82	135 (6.7), **(Ben Mohamed et al., 2018)**
2	Gallic acid	4.37	$C_7H_6O_5$	169.0143	169.0142	N	-0.39	125(85), 79(100), **(Danciu et al., 2018)**
3	(epi)gallocatechin	6.238	$C_{15}H_{14}O_7$	305.0668	305.0667	N	-0.29	125 (3.69), (**Lv et al., 2015)**
4	Catechin-O-hexoside	8.222	$C_{21}H_{24}O_{11}$	451.1243	451.1246	N	0.51	289 (10.41), **(Ziani et al., 2019)**
5	*o*-Coumaric acid glucoside	8.594	$C_{15}H_{18}O_8$	325.0928	325.0929	N	3.32	290 (15.02), 145(8.31), 93 (57.82), **(Lv et al., 2015)**
6	Quercetin 3-O-rhamnoside-7-O-glucoside	8.718	$C_{27}H_{30}O_1$	609.1465	609.1461	N	-0.53	462 (1.82**), (Aguiar et al., 2019)**
7	Apigenin-6,8-C-dihexoside	9.462	$C_{27}H_{30}O_{15}$	593.1523	593.1512	N	-1.64	473 (53), 383 (26), 353 (44), **(Ziani et al., 2019)**
8	Epicatechin	9.895	$C_{15}H_{14}O_6$	289.0718	289.0718	N	0.23	245 (3,8), **(Zengin et al., 2020)**
9	Apigenin 6-C-pentoside-8-C-hexoside	10.39	$C_{26}H_{28}O_{14}$	563.1413	563.1413	N	-0.99	473 (1,1), **(Aguiar et al., 2019)**
10	Rutin	10.94	$C_{27}H_{30}O_{16}$	609.1468	609.1468	N	-1.05	300 (26), **(Shen et al., 2019)**
11	myricetin-*O*-hexoside	11.507	$C_{21}H_{20}O_{13}$	479.0835	479.0831	N	-0.55	317 (6.45), **(Ziani et al., 2019)**

12	Quercetin-O-rhamnoside	11.817	$C_{21}H_{20}O_{11}$	447.0938	447.0933	N	-1.14	300 (3.61), 173 (11.85), 111 (10.77), **(Radenkovs et al., 2018)**
13	Hyperoside	12.313	$C_{21}H_{20}O_{12}$	463.0885	463.0882	N	-0.36	300 (4.31), 271 (1.09), **(Shen et al., 2019)**
14	Luteolin 8-C-glucoside	12.994	$C_{21}H_{20}O_{11}$	447.0935	447.0938	N	-0.38	429(1.82) , **(Aguiar et al., 2019)**
15	Quercetin-3-O-galactoside	13.118	$C_{21}H_{20}O_{12}$	463.0888	463.0882	N	-1.25	301 (4.54), **(Pawlowska et al., 2010)**
16	Verbascoside	13.181	$C_{27}H_{28}O_{17}$	623.1256	623.1254	N	-0.26	461(4.74), **(Ben Mohamed et al., 2018)**
17	Isorhamnetin-3-O-glucoside	13.490	$C_{22}H_{22}O_{12}$	477.1043	477.1038	N	-0.6	300(5.18), **(Shen et al., 2019)**
18	Naringenin-O-hexoside	13.676	$C_{21}H_{22}O_{10}$	433.1134	433.114	N	0.91	271(15.2), **(Ziani et al., 2019)**
19	Kaempferol rhamnoside	14.792	$C_{21}H_{20}O_{10}$	431.0986	431.0984	N	-0.5	285(4.74), **(Lv et al., 2015)**
20	Quercetin-3-O-glucoside	14.91	$C_{21}H_{20}O_{12}$	463.0883	463.0882	N	-0.02	300 (39), **(Schieber et al., 2005)**
21	Luteolin	19.68	$C_{15}H_{10}O_{6}$	285.0406	285.0405	N	-0.57	133 (2), **(Ziani et al., 2019)**

The characterized compounds of the EamCE are phenolic molecules; we categorize them into three different classes: phenolic acids, phenylpropanoids, and flavonoids. Caffeic acid, gallic acid and O-coumaric acid glucoside (glycosylated) are phenolic acids. Verbascoside is the only found compound that appertain to phenylpropanoid family. The rest of the compounds (seventeen) were identified as flavonoids with four distinct subclasses: flavonols, flavones, flavan-3-ols and flavanones. Indeed, we have determined the presence of myricetin-O-hexoside, quercetin-O-rhamnoside, hyperoside, quercetin-3-O-galactoside, isorhamnetin-3-O-glucoside, kaempferol rhamnoside, quercetin-3-O-glucoside. These latter are classified as glycosylated flavonols. Two compounds of di-glycosylated flavonols were also found: quercetin 3-O-rhamnoside-7-O-glucoside and rutin. Besides, the recognized flavones are distinguished by the number of carbohydrates attached to their structure. We have determined two di-glycosylated compounds namely: apigenin-6,8-C-dihexoside and apigenin 6-C-pentoside-8-C-hexoside, one glycosylated compound that was designated as luteolin 8-C-glucoside and one compound without a carbohydrate moleucle, which is the last flavone "luteolin". Likewise, the analysis has shown the presence of three flavan-3-ols that are recorded in peaks 3, 4, and 8, appropriately: (epi) gallocatechin, catechin-O-hexoside (glycosylated) and epicatechin. Only one structure was defined as flavanone "naringenin-O-hexoside".

Numerous researches have endorsed the presence of phenolic acid and flavonoid compounds in various *Ephedra* species, notably *Ephedra alata*. These compounds act as the main antioxidant potent agents and have approved their efficiency to many other biological activities **(Mighri et al., 2019; Soumaya et al., 2020; Elhadef et al., 2020)**. Flavonoids are the most common class of secondary metabolites within the genus of *Ephedra*. Over forty flavonoids have been classified as: flavonols, dihydro-flavonols, flavonones, flavanols, flavones, and anthocyanins. Notably, flavones and their glycosides, as well as flavonols and their 3-O-glycosides constituents, are the most common flavonoids in *Ephedra* **(Zhang et al., 2018)**. It has been revealed in *Ephedra* species that certain glycans (ephedran A, B, C, D, and E) are enclosed in the aerial parts, and diverse flavanols were identified as components of twigs and barks **(EFSA, 2013)**. Furthermore, additional secondary metabolites originating from other *Ephedra* species include alkaloids, amino acids and derivatives, volatiles, and phenolic compounds. The alkaloids were of significant biological relevance, noting: ephedrine, pseudoephedrine, norephedrine, norpseudoephedrine, methylephedrine, and methylpseudoephedrine. More alkaloids have been detected in some Eurasian *Ephedra* species,

such as: ephedroxane and macrocyclic spermidines called ephedradine A–D **(Ibragic & Sofić, 2015)**; nevertheless, they were not revealed using the negative ionization mode.

IV.1.2. TPC and TFC dosage

The quantification of EamCE contents in polyphenols and flavonoids is demonstrated in table 5 and calibration curves of gallic acid and quercetin are shown in appendix 4.

Table 5: TPC and TFC quantification of EamCE.

TPC (µg GAE/mg)	TFC (µg QE/mg)
235.62 ± 2.03	23.61±0.14

TPC is expressed as µg gallic acid equivalents/mg of extract (µg GAE/mg). TFC is expressed as µg quercetin equivalents/mg of extract (µg QE/mg).

The TPC and TFC analyses have shown that the EamCE possesses a considerable amount of phenolic and flavonoid compounds, which are higher than the ones found in other studies of the same plant species **(Sioud et al., 2020)**. Recently, a methanolic extract of *Ephedra alata* from the Algerian Sahara has exhibited practically the same values; 214.92 mg GAE/g for TPC and 30.74 mg Catechin/g extract for TFC **(Boussena et al., 2022)**.

IV.1.3. Antioxidant activities

Results of *in vitro* antioxidant tests are reported as the mean values ± SD of three measurements; the IC50 and A0.5 values were calculated by linear regression analysis and are summarized in table 6.

Table 6: Antioxidant activities of EamCE (IC50 and A0.5 values).

IC50						
Products	**DPPH**	**ABTS**	**Beta Carotene**	**DMSO Alcalin**	**GOR**	**Hydroxyl Radical**
EamCE	32.49 ± 0.49[a]	11.77 ± 0.81[a]	380.96 ± 0.93[a]	15.31 ± 0.91	31.38 ± 0.56[a]	163.32 ± 1.39[a]
BHT *	12.99 ± 0.41[b]	1.29 ± 0.30 [b]	1.05 ± 0.03 [b]	NT	5.38 ± 0.06[b]	NT
BHA *	6.14 ± 0.41[c]	1.81 ± 0.10 [c]	0.91 ± 0.01 [c]	NT	3.32 ± 0.18[c]	NT
α-Tocopherol *	13.02 ± 5.17[d]	NT	NT	<3.125	NT	NT
Ascorbic Acid *	NT	NT	NT	NT	5.02 ± 0.01[d]	32.33 ± 1.17[b]
Tannic Acid *	NT	NT	NT	<3.125	NT	NT
Trolox *	5.12 ± 0.21[e]	3.21 ± 0.06[d]	NT	NT	4.31 ± 0.05[e]	NT

A 0.5				
Products	**CUPRAC**	**FRAP**	**SNP**	**Phenonthroline**
EamCE	25.71 ± 1.66 [a]	38.57 ± 1.44 [a]	30.97 ± 0.87 [a]	17.11 ± 0.30 [a]
BHT *	8.97 ± 3.94 [b]	NT	NT	2.24 ± 0.17 [b]
BHA *	5.35 ± 0.71 [c]	NT	NT	0.93 ± 0.07 [c]
α-Tocopherol *	NT	34.93 ± 2.38 [b]	NT	NT
Ascorbic Acid *	8.31 ± 0.15 [d]	6.77 ± 1.15 [c]	7.14 ± 0.05 [b]	3.08 ± 0.02 [d]
Tannic Acid *	NT	5.39 ± 0.91 [d]	NT	NT
Trolox *	8.69 ± 0.14 [e]	5.25 ± 0.20 [e]	34.17 ± 1.23 [c]	5.21 ± 0.27 [e]

* Standard compounds. NT: not tested. IC50 and A0.50 values are defined as the concentration of 50% inhibition percentages and the concentration at 0.50 absorbance, respectively. IC50 and A0.50 were calculated by linear regression analysis and expressed as mean ± SD (n = 3). The values with different superscripts (a, b, c, d, e) in the same columns are significantly different ($p < 0.05$).

The plausible antioxidant potential of a bioactive sample can be evaluated by various *in vitro* assays based on the inhibition ability of consistent free radicals **(Luximon-Ramma et al., 2002)**. Therefore, we have established different antioxidant assays which are direct, indirect, or competitive procedures. The reaction principle was based on reduction, chelation, or inhibition. We believe that testing a dozen method and having positive results confirm strongly the antioxidant potential of EamCE. Effectively, the EamCE has provided a very powerful antioxidant activity, the IC50 and A 0.5 concentrations shown in table 6 were relatively low and approximately the same order of magnitude as the standard ones (except for the β-carotene bleaching and hydroxyl scavenging assays). From the knowledge that, EamCE is a set of molecules, and if we presume that the generated activity goes to the effect of a single molecule, we could say that this molecule is very potent since it is not pure and that the other molecules could be antagonist. Otherwise, we could also hypothesize that the measured activity was the result of synergic and combined effect of many molecules. The EamCE demonstrated a strong antioxidant capacity with lower DPPH IC50 (µg/mL) and FRAP A0.5 (µg/mL) values compared to other extracts evaluated in previous studies, from *Ephedra alata*, *Ephedra alata Campylopoda Fragilis*, *Ephedra Procera fisch. et may*, and *Ephedra sarcocarpa* species. Indeed, DPPH IC50 from *Ephedra alata* extract was: >1000, 83.07 ± 0.2 **(Soumaya et al., 2020)**, 330 ± 0.004, 454 ± 0.008, 180 ± 0.002, 176 ± 0.002 **(Mighri et al., 2019)**, 450 ± 7, 540 ± 3.455 **(Ziani et al., 2019)**. DPPH IC50 (µg/mL) from extracts *Ephedra alata* Campylopoda Fragilis was: 125 ± 4.4, 150 ± 5.1, 300 ± 4.4 **(Kallassy et al., 2017).** DPPH IC50 (µg/mL) from *Ephedra Procera fisch. et may* extract was 300 ± 4.4, 150 ± 5.1, 125 ± 4.4 **(Kallassy et al., 2017)**, **(Dehkordi et al., 2015)**; and DPPH IC50 (µg/mL) from *Ephedra sarcocarpa* extract was 5300 ± 0.027 **(Rustaiyan et al., 2011)**. In addition, FRAP A0.5 (µg/mL) from *Ephedra alata* plant extracts was 108 ± 1 **(Elhadef et al., 2020),** 377 ± 4 **(Ziani et al., 2019)**. Regarding, earlier antioxidant studies of *Ephedra alata* species or other species, none of them has used ten different antioxidant methods to demonstrate how much powerful and bioactive is the extract

of interest. The exhibited prominent antioxidant activity of EamCE enables us to consider it as a very promising product that could be involved in the resolution of many physiological disorders related to oxidant/antioxidant imbalances and OS that induce the development of other serious health issues.

IV.I.4. Anti-inflammatory activity

IV.1.4.1. Heat induced hemolysis

The anti-inflammatory potential of EamCE in heat induced hemolysis assay was evaluated by calculating the inhibition percentage of hemolysis; the results are recorded in table 7, where the EamCE has given a favorable activity with a high inhibition almost 80% at the highest applied concentration 65.5 µg/mL.

Table 7: Heat induced hemolysis (percentage inhibition of hemolysis).

Concentration (µg/mL)	Diclofenac % Inhibition	EamCE% Inhibition
65.5	73.87 ± 1.86 ^{a}	76.76 ± 0.15 ^{a}
32.75	72.05 ± 0.64 ^{a}	76.11 ± 2.08 ^{b}
16.375	72.03 ± 0.49 ^{a}	74.92 ± 1.21 ^{b}
8.1875	70.23 ± 0.99 ^{a}	71.03 ± 1.38 ^{b}

The inhibition % of hemolysis is expressed as mean ± SD (n = 3). The values with different superscripts (a, b) in the same line are significantly different ($p \leq 0.05$), and the values with the same superscripts (a, a) are not significantly different ($p > 0.05$).

IV.1.4.2. Anti paw edema

The anti-inflammatory effect in anti paw edma was expressed as a percentage of edma. The administration of EamCE in parallel of provoked paw edema, has eminently reduced the percentage of edema comparatively to diclofenac effect, the effect of EamCE after 4 h (240 min) was better than diclofenac effect at 200 mg/kg and 400 mg/kg as shown in figure 14.

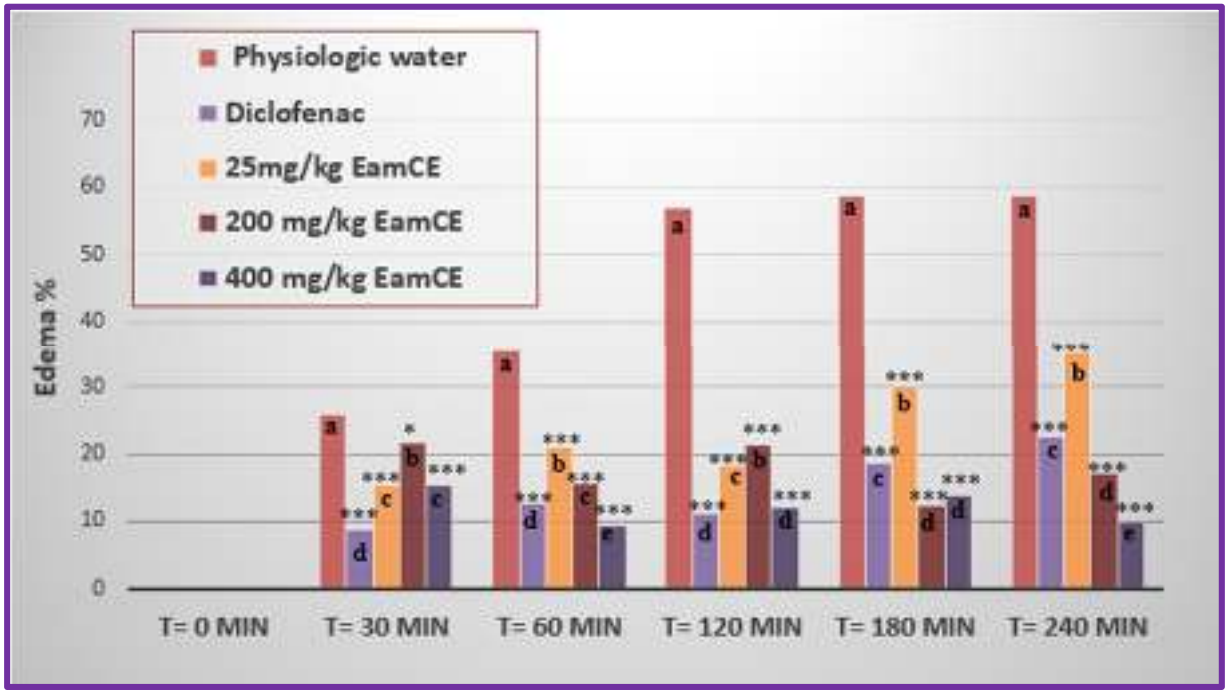


Figure 14: Anti paw edema effect of EamCE

The percentage of edema is expressed as mean ± SD (n = 5). The values with different superscripts (a, b, c, d, e) are significantly different ($p \leq 0.05$), and the values with the same superscripts are not significantly different ($p > 0.05$). *** $p \leq 0.001$ = very highly significant, ** $p \leq 0.01$ = highly significant, and * $p \leq 0.05$ = significant.

Inflammation and edema are coupled physiological processes associated with tissue damage arising due to sustained mechanical loading **(Van Damme et al., 2020)**. Researchers are constantly searching for new anti-inflammatory compounds without adverse effects for the treatment of inflammation. Natural polyphenolic compounds are consumed in large amounts in the daily diet and can play an important role in the treatment of diseases **(Soyocak et al., 2019).** Recent findings have demonstrated that quercetin-rich methanol extract of *Ephedra ciliata* has an anti-inflammatory activity, which promoted the healing of wounds in two different models, and, at cytokine reduced amount, the downregulation of TNF-α was suggested as the inducer factor of the anti-inflammatory and wound healing activity **(Yaseen et al., 2020)**. Crucial compounds of *Ephedra*, including quercetin, luteolin, kempferol, naringenin, and beta-sitosterol, were identified in treating asthma by inhibiting the expression of many inflammatory targets, SELE, IL-2, and CXCL10, at mRNA and protein levels; these substances are involved

in the biological processes of immune response, inflammatory response, cell-cell signaling, and response to lipopolysaccharide **(Huang et al., 2020)**.

The EamCE gave an outstanding anti-inflammatory effect in the current survey. In heat induced hemolysis approach, the EamCE provided a convincing anti-inflammatory ability. The inhibition percentage of EamCE was very high, 76.76 ± 0.15%, even in at low concentration (65.5 µg/mL), this effect was better than that of diclofenac: 73.87 ± 1.86 (notably with the same used concentration). Additionally, the effect was not reduced, even with low concentration of EamCE (71.03 ± 1.38 % at 8.1875 µg/mL), as it is shown in table 7.

Likewise, the EamCE exhibited an interesting preventive response from induced paw edema with the three tested concentrations. Although, 200mg/kg and 400mg/kg of EamCE were more effective than diclofenac (25 mg/kg), giving a quite reduced edema percentage after 240 min: 17.10 ± 0.61, 9.91 ± 0.79, respectively. The richness of plants in term of phenolic compounds, saponins, tannins and triterpenes, provide them anti-inflammatory properties **(Reis Nunes et al., 2020)**. *Ephedra alata* Decne belongs to plants with high amount of these metabolites **(Jaradat et al., 2015; Ziani et al., 2019)**.

IV.1.5. Toxicity and cytotoxicity

IV.1.5.1. Acute oral toxicity

The daily monitoring of mice has not registered any mortality neither in the control groups nor in the treated ones. No sign of particular behavior or visible change (diarrhea, anorexia, loss of hair, etc.) was noticed. The panderal change of all mice, before and after administration of the EamCE dose, are represented in table 8.

Table 8: Weight body and relative organ weight records.

Experimental groups		Weight at day 1 (g)	Weight at day 14 (g)
Control groups	**Male mice**	25.72 ± 0.91[a]	26,89 ± 1.43[a]
	Female mice	24.13 ± 0.68[a]	25,83 ± 0.89[a]
EamCE treated groups	**Male mice**	25.94 ± 1.51[a]	28.94 ± 0.72[a]
	Female mice	25.03 ± 0.61[a]	27.07 ± 0.55[a]

Relative organ weight (g)				
Experimental groups		**Liver**	**Heart**	**Kidney**
Control groups	**Male mice**	1.61 ± 0.31[a]	0.21 ± 0.11[a]	0.45 ± 0.22[a]
	Female mice	1.96 ± 0.18[a]	0.25 ± 0.59[a]	0.46 ± 0.53[a]
EamCE treated groups	**Male mice**	1.78 ± 0.29[a]	0.23 ± 0.41[a]	0.43 ± 0.62[a]
	Female mice	1.98 ± 0.43[a]	0.26 ± 0.32[a]	0.47 ± 0.14[a]

The values with the same superscripts are not significantly different ($p > 0.05$). In each group (n=3).

The weight of mice (male and female) treated with 2000 mg/kg of EamCE did not show any significant modification during the fourteen days after administration. The EamCE could be designated as non-toxic and relatively harmless product according to the tabulation toxicity classes **(Hodge & Sterner, 1949)**. The autopsy realized at the end of the experiment did not revealed any installation of cysts, hemorrhage or bleeding.

Visual examination of vital organs (kidneys, liver and heart) demonstrated that these organs have no morphological changes compared to those of the control. Moreover, the evaluation of the relative weight of each vital organ did not record any difference between the control and treated groups in both male and female mice.

IV.1.5.2. Cytotoxicity on cancer cell lines

The EamCE cytotoxicity was assessed by the inhibition % of HEP2 and RD cell lines. Testing different concentration, the highest one (500 µg/mL) has given a very reduced rate of inhibition after 48 h and even after 72 h, this rate has not been enhanced significantly (Table 9). This observation concerning EamCE effect is valid for both cell lines.

Table 9: The EamCE percentage inhibition of HEP2 and RD cell lines.

	HEP2 (% Inhibition)		**RD (% Inhibition)**	
Concentration (µg/mL)	**48h**	**72h**	**48h**	**72h**
500	25.25 ± 0.06 [a]	28.56 ± 0.05 [a]	7.20 ± 0.04 [a]	12.56 ± 0.45 [a]
250	22.02 ± 0 [b]	22.87 ± 0.05 [b]	6.4 ± 0.21 [b]	9.67 ± 0.23 [b]
125	19.78 ± 0.04 [c]	19.32 ± 0.04 [c]	3.02 ± 0.06 [c]	5.11 ± 0.94 [c]
62.5	6.74 ± 0.05 [d]	10.8 ± 0.08 [d]	1.22 ± 0.05 [d]	3.42 ± 0.12 [d]
31.25	2.81 ± 0 [e]	10.23 ± 0.05 [e]	0 [e]	1.11 ± 0.02 [e]
15.625	0 [f]	9.52 ± 0.07 [f]	0 [f]	0 [f]

IC50 are expressed as mean ± SD (n = 3). The values with different superscripts (a, b, c, d, e, f) in the same columns are significantly different ($p \leq 0.05$).

The relationship between free radical scavenging ability and cytotoxic effect of plant extracts was directly proportional. The screening of extracts from 57 different plant for their antioxidant ability has revealed that, IC50 ≤10 µg/mL enhanced the increase of cytotoxicity. **(Sammar et al., 2019)**. Some compounds isolated from *Ephedra* extracts of different species, such as herbacetin, ephedrine alkaloids, and oligomeric proanthocyanidins, exerted a powerful anti-proliferative potential against different cancer cell lines **(Zhang et al., 2018)**. The criteria of cytotoxic activity for crude extracts, as handled by the American National Cancer Institute, is an IC50 < 30 µg in the preliminary assay **(Itharat et al., 2004)**. Consequently, we consider

that the EamCE is not an interesting anticancer agent, contrary to other crude extracts from E. alata (possessing a powerful anticancer activity) **(Sioud et al., 2020; Soumaya et al., 2020)**. The EamCE has given an inhibition percentage of 20%, at the highest used concentration (500 µg/mL), the effect remained the same even after 72 h of interaction (Table 9). This is maybe due to the lacking of specific Ephedra alkaloids or other metabolites, that are the inducer agents of cell death metabolic pathways occurring in another particular Ephedra species **(Ibragic & Sofić, 2015)**.

IV.2. Pirimicarb toxicity on neuro-immune endocrine system and preventive effect of EamCE

IV.2.1. Pirimicarb induced sub acute toxicity

IV.2.1.1. Behavioral checking

a. Force swim test

Rats of G3 spent the majority of time being immobile (more than 69%) compared with control groups (G1 and G2) and G4 (treated with EamCE), in which a moderate amount of time was spent in swimming. Only 24.167 s was spent in escalation attempts; however, rats of healthy groups spent the most time trying to climb (236.833 s (G1) and 226.500 s (G2)), as shown in figure 15.

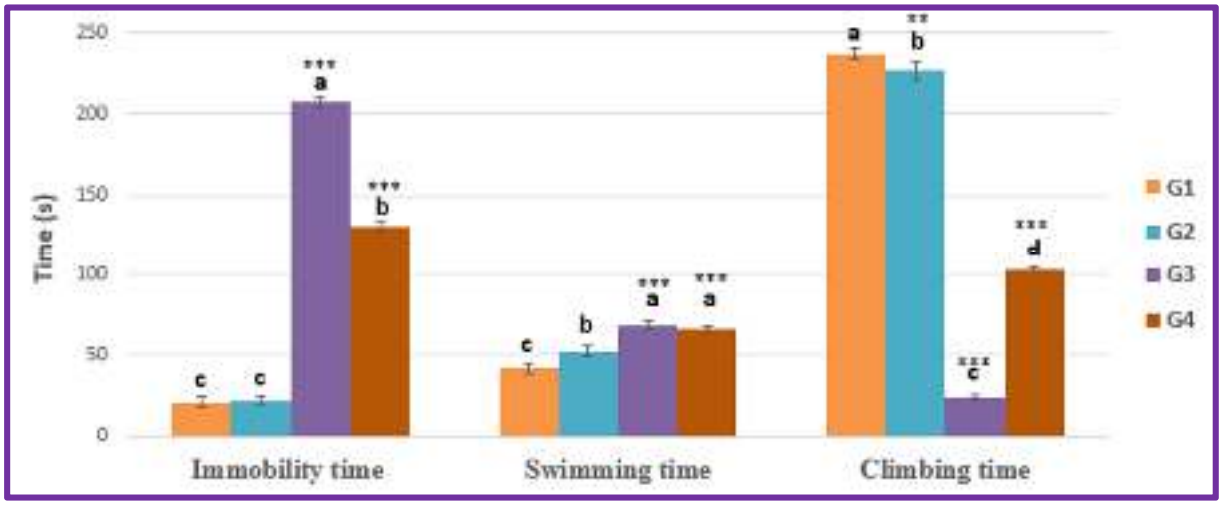


Figure 15: Evaluation of force swim test parameters.

G1: negative control, G2: negative control of EamCE, G3: pirimicarb, G4: EamCE + pirimicarb. The FST parameters included immobility, swimming, and escalation times; the histogram in figure 15 describes their variation between different experimental groups. Time was recorded in seconds (s), the values are expressed as mean ± SD (n = 6). *** $p \leq 0.001$ = very highly significant, ** $p \leq 0.01$ = highly significant, and those with the same subscripts were not significantly different ($p > 0.05$).

b. Open field test

Statistical analysis of OFT parameters demonstrated that variations among groups were highly significant ($p \leq 0.001$). The total distance traveled by rats in the healthy control (G1),

EamCE control (G2), and pirimicarb + EamCE (G4) groups was higher compared with the pesticide group (G3) (501.167 ± 3.656, 460.833 ± 3.817, 204.500 ± 3.271) vs. (94.000 ± 4.382) (Figure 16). Conversely, locomotor activity was significantly decreased in the group treated with pirimicarb, compared with the other control groups, and the group treated with the pirimicarb + EamCE complex.

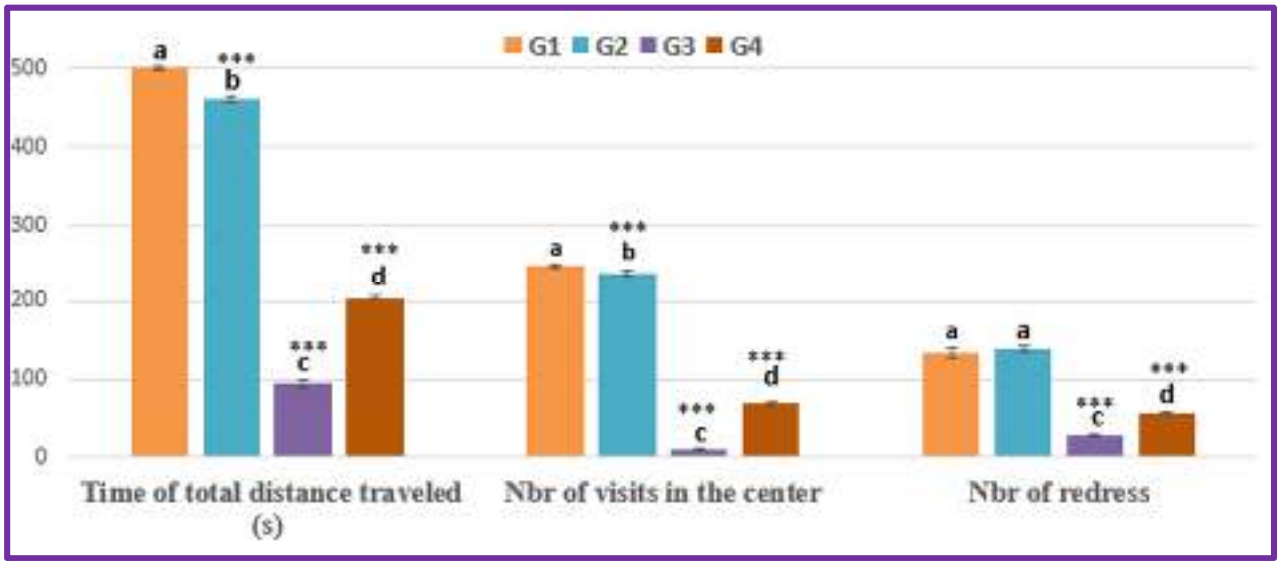


Figure 16. Evaluation of open field test parameters.

G1: negative control, G2: negative control of EamCE, G3: pirimicarb, G4: EamCE + pirimicarb. Rats were evaluated by measuring the duration of the total distance traveled, the number (nbr) of visits in the center of the device, and the nbr of redress. Time was recorded in seconds (s), the values are expressed as mean ± SD (n = 6). *** $p \leq 0.001$ = very highly significant, and those with the same subscripts were not significantly different ($p > 0.05$).

c. Elevated plus maze

The locomotor activity of healthy control groups (G1 and G2) was higher than that of the group treated with pirimicarb; this was demonstrated by the total number of visits (11.167 ± 0.983 vs. 3.333 ± 0.816), (7.500 ± 1.049 vs. 3.333 ± 0.816). The same result was noted in rats treated with pirimicarb + EamCE, which showed high locomotor activity compared with rats treated with pirimicarb only (5.167 ± 1.472 vs. 3.333 ± 0.816). Finally, we noted that rats of healthy control, rats treated with EamCE and rats treated with pirimicarb + EamCE complex, spent the majority of time in the open arms of the device; this was well illustrated by the

percentage of the time spent in the open fields (OF) of the device, as shown in figure 17 (83.665 ± 0.731, 90.757 ± 1.244, 90.757 ± 1.244). At the same time, rats of pirimicarb group spent the majority of their time (a percentage of 98.28 ± 0.509) in the closed fields (CF) of the device and a percentage of only 1.720 ± 0.491 in the open parts (Figure 17).

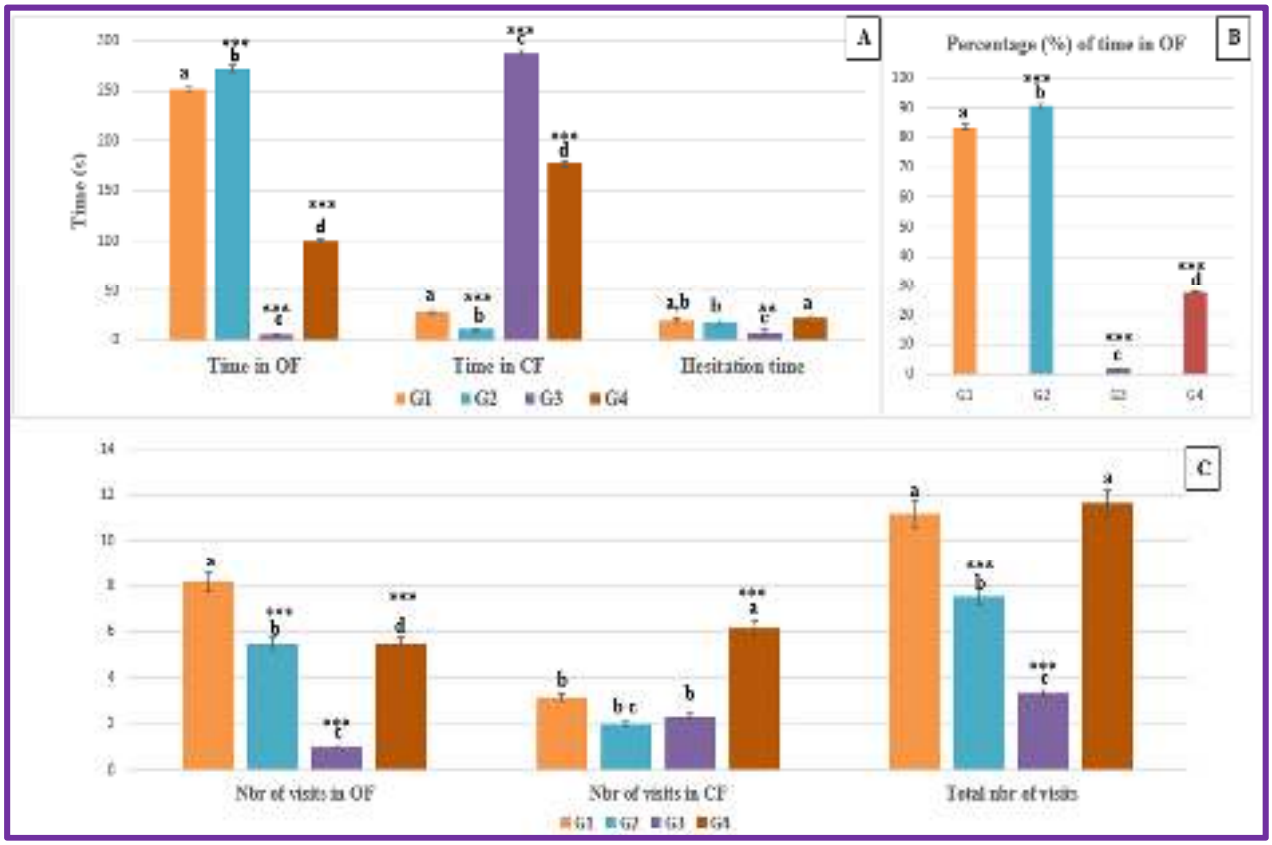


Figure 17: Evaluation of elevated plus maze parameters.

G1: negative control, G2: negative control of EamCE, G3: pirimicarb, G4: EamCE + pirimicarb. For the EPM, several parameters were taken in consideration, including time spent in open fields (OF) and in closed fields (CF), hesitation time (A). Percentage of time in OF (B). The number of visits in different compartments of the apparatus (C). Time was recorded in seconds (s), the values are expressed as mean ± SD (n = 6). *** $p \leq 0.001$ = very highly significant, ** $p \leq 0.01$ = highly significant, and those with the same subscripts were not significantly different ($p > 0.05$).

d. Elevated zero maze

The latency of entering the open section for rats of healthy control groups (G1 and G2) was lower than that of rats treated with pirimicarb (51.50 ± 1. 36 vs. 256.167 ± 0.983); (35.16

± 1.47 vs. 102.333 ± 4.32). In contrast, the time spent in the open section was very high for healthy controls compared with the group treated with pirimicarb. In addition, the number of entries into the open section (EOS) and the number of head dips (HD) were most notable in the healthy groups, G1 (EOS): 8.16 ± 0.4, G2 (EOS): 5.5 ± 0.83, G1 (HD): 31.83 ± 1.32, G2 (HD): 45.16 ± 0.40 and moderate in G4 animals treated with (pirimicarb + EamCE) complex, EOS: 3.5 ± 0.83, HD: 19.16 ± 1.32. However, both parameters values were limited in the pirimicarb group, EOS (1.66 ± 0.81), HD (3.5 ± 0.83), (Figure 18).

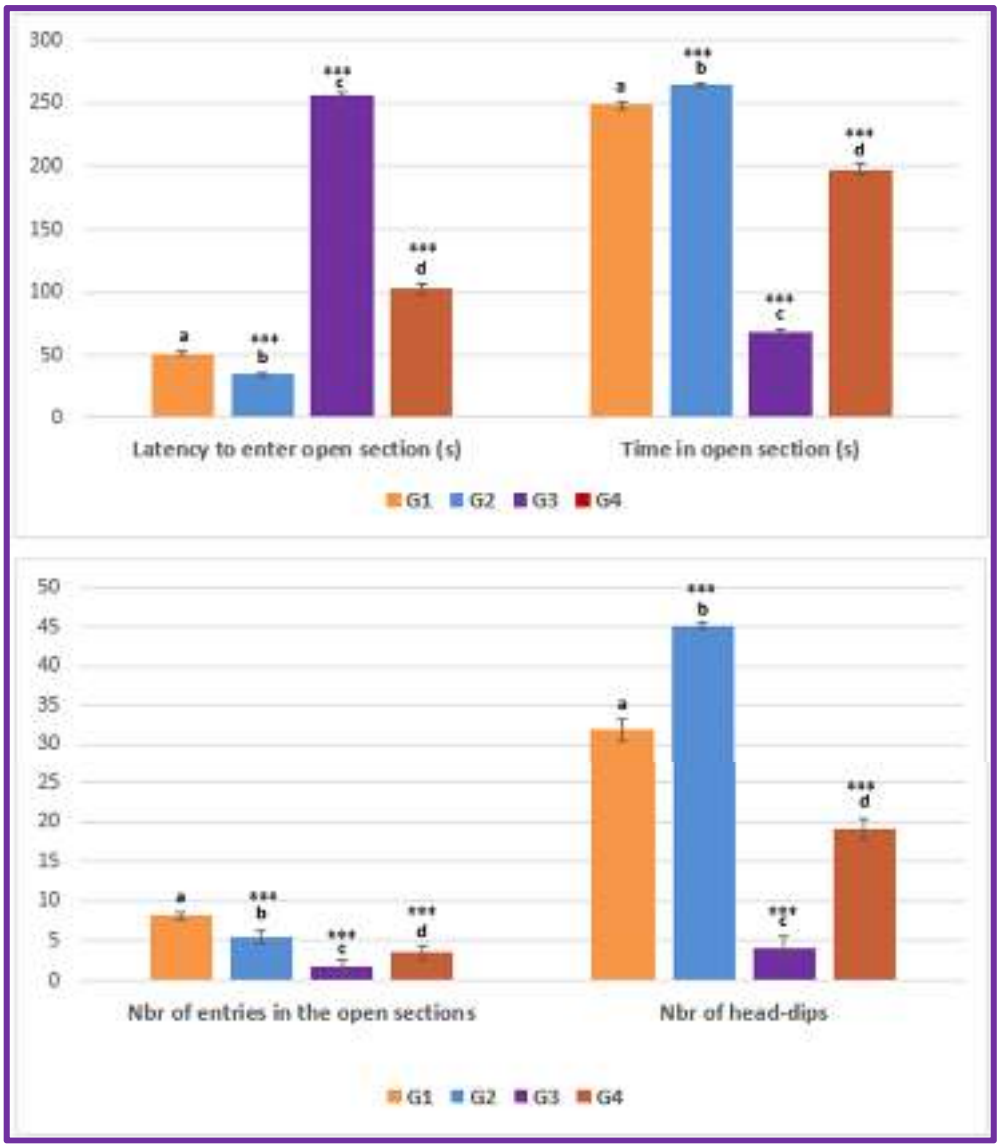


Figure 18: Evaluation of elevated zero maze parameters

G1: negative control, G2: negative control of EamCE, G3: pirimicarb, G4: EamCE + pirimicarb. In this trial, we recorded the latency time and the duration spent in the open section

of the device in seconds (s); we also assessed the number of entries in the open section and the number of head-dips. The values are expressed as mean ± SD (n = 6).*** $p \leq 0.001$ very highly significant, and those with the same subscripts were not significantly different $p > 0.05$.

Behavioral trials in rodents are utilized to estimate neurological traits and events, such as locomotor activity, depression-like behavior, socialization, memory, and other traits **(Uslu, 2021)**.The EPM and the OFT are some of the most widely employed procedures to investigate anxiety-like behavior in rats **(Knight et al., 2021)**. Moreover, the EZM has been proposed and validated as an apparatus for measuring anxiety status; however, the OFT has long been used for evaluating anxiety/fearfulness as well as locomotor/exploratory activity **(Díaz-Morán et al., 2014)**. Likewise, the FST is also one of the most commonly used tests to assess depressive-like behavior in animal models **(Bogdanova et al., 2013)**. The results obtained in the current study revealed the negative impact of pirimicarb on the behavior status of rats. In fact, in the group of rats fed with pesticide (G3), the reduced total traveled distance, the minimized number of visits to the central area, and the increased duration of stay in the periphery zone and device corners indicated significantly decreased locomotor activity compared with control groups. Also, we noticed that the action of EamCE was clearly ameliorative in G4. In addition, induced anxiety in the rats of G3 was significantly linked to reduced time spent in the open fields; increased time spent in the closed fields; fewer total number of entries into the open section; reduced frequency of head dipping; and extended latency to enter into the open section. Further, the climbing ability in FST was very feeble in affected rats of G3; they spent the majority of their time immobile. These deficiencies reveal clearly the diminution of the cognitive and locomotor competencies that would be sufficient to confirm the depressive effect of pirimicarb. Various negative effects on neuro-behavior have been previously recorded subsequent to exposure to a combination of different pesticides, even with safe doses **(Sergievich et al., 2020)**. In the current study, this depressive effect was thoroughly reduced by the preventive activity of EamCE in all the recorded behavioral parameters, with an obvious tendency to approach to the values registered in the control groups. In fact, physical and cognitive performance in an anterior study was experimentally enhanced by the action of the major component ephedrine that was isolated from *Ephedra alata* plant, additionally to its many other potent pharmacological effects, notably for treating narcolepsy and depression **(Lieberman, 2001)**.

IV.2.1.2. Food and water consumption

The daily tracking of food and water intake of rats per each group is illustrated in figure 19. Both graphics (A and B) showed that G3 has recorded the most important consumption either for food or for water and during the whole period of treatment.

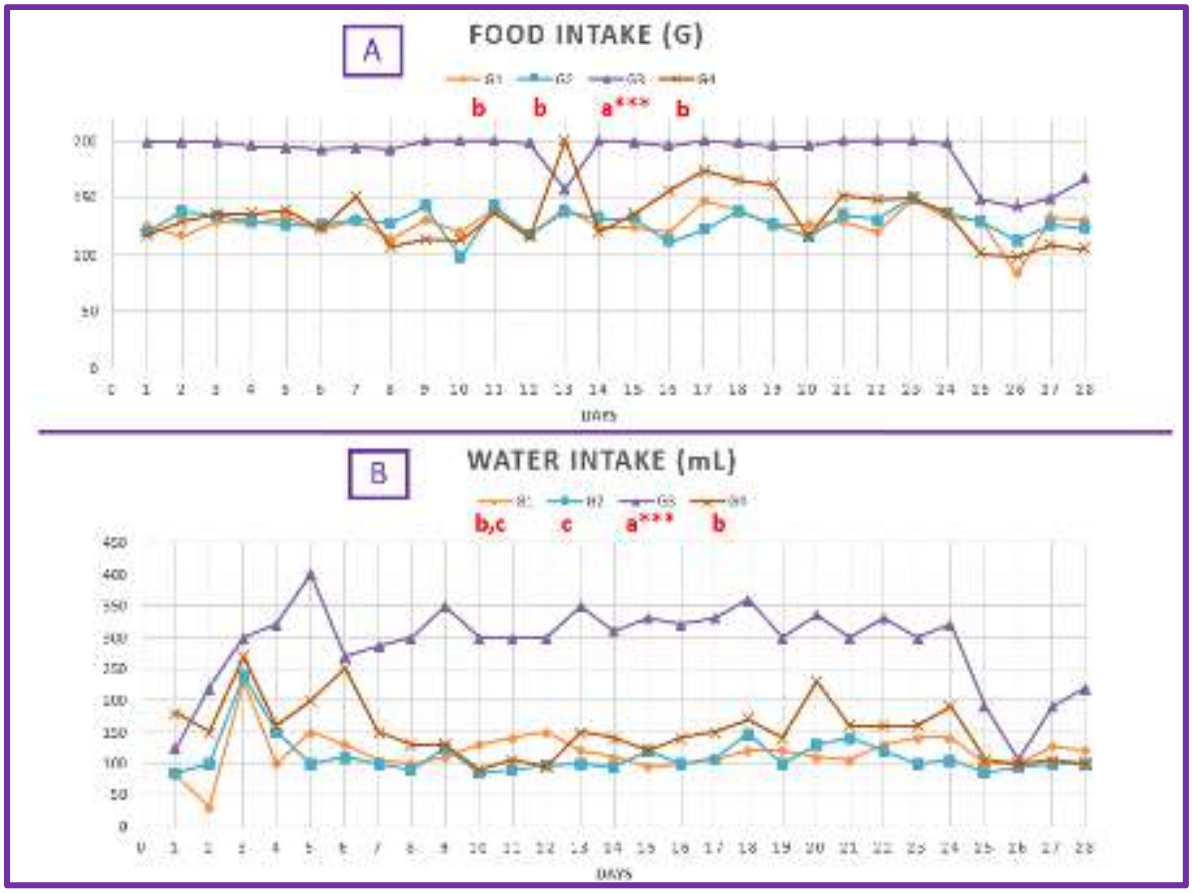


Figure 19: Recording of Food and water consumption

G1: negative control, G2: negative control of EamCE, G3: pirimicarb, G4: EamCE + pirimicarb. The values are expressed as mean ± SD (n = 6).Values with different subscripts were significantly different compared with the healthy group G1 (*** p≤ 0.05 = very highly significant), and those with the same subscripts were not significantly different (p > 0.05).

IV.2.1.3. Body weight fluctuations

The recording of body weight from the beginning of the experiment until its end, allowed us to fellow its change and to compare its variance among experimental groups. Besides, it enables to estimate the loss or the gain of weight in each group and to notice the

influence of pirimicarb and EamCE. The results showed that all rats gained weight and those of G3 presented the most elevated value; 79.36±12.03 (g) as indicated in table 10. However, the variance of relative weight of both brain and testis through the four experimental groups was not significant (p >0.05).

Table 10: Body weight and relative organ weight changes

Experimental groups	Gain of bw (g)	Relative weight (g/100 g of bw)	
		Brain	Tesis
G1	58.8 ± 9.31 [b]	0.74 ± 0.06 [a]	1.29 ± 0.24 [a]
G2	45.48 ± 10.68 [b]	0.79 ± 0.05 [a]	1.25 ± 0.30 [a]
G3	79.36 ± 12.03 [a] *	0.71 ± 0.12 [a]	1.26 ± 0.16 [a]
G4	41,55 ± 11.71 [b]	0.83±0.04 [a]	1.39 ± 0.10 [a]

G1: negative control, G2: negative control of EamCE, G3: pirimicarb, G4: EamCE + pirimicarb. The values are expressed as mean ± SD (n = 6). Values with different subscripts (a, b) in the same column were significantly different compared with the healthy group G1 (* $p \leq 0.05$ = significant), and those with the same subscripts (a) were not significantly different ($p > 0.05$).

The results of food and water consumption are positively correlated to those of the assessment of weight gain. In effect, the excessive consumption of food/water in G3 (190.66 ± 17.35 g / 290.37 ± 67.13 mL) compared to the reduced consumption in G1,G2 and G4 (126.95 ± 12.32g / 117.74 ± 32.54 mL, 128.42 ± 10.94 g/ 111.81 ± 30.82 mL, 134.74 ± 23.81 g/ 152.96 ± 44.54 mL; respectively); was associated to the elevation of weight gain in G3 relatively to the reduced weight gain of the other groups (Table 10). A systematic review has reported that at different stages of life, exposure to organophosphate, organochlorine, pyrethroid, neonicotinoid, and carbamate, as well as a combined pesticide has direct effects on body weight gain and on obesity in both humans and experimental animals **(Pinos et al., 2021)**. Likewise, a previous study concerning the impact of chlorpyrifos pesticide intake in mice has found that chlorpyrifos impaired intestinal integrity to promote more LPS bacteria entry into the body

resulting in low-grade inflammation, changing microbiota, which ultimately led to insulin resistance and obesity **(Liang et al., 2019)**. Another survey, conducted on 6770 subjects aged from 6 to 19 years, has revealed a positive dose-dependent relationship between urinary levels of dichlorophenol pesticide and obesity **(Twum & Wei, 2011)**. Our finding, declare that pirimicarb has installed a stressful state leading to anxiety and depression, this stress could be the main factor that provoked the gain of weight in rats of G3. Many studies, endorsed our speculations and deducing. The increased intensity of perceived stress experienced by individuals in modern society affects feeding behavior. Sadness favored eating of high fat/sweet, hedonically rewarding foods, whereas intake during a happy state favored dried fruit. Stress also induces secretion of glucocorticoids, which increases motivation for food, and insulin, which promotes food intake and obesity **(Mary F.Dallman, 2010)**. Stress can disrupt activity patterns, whether by decreasing physical activity or by increasing sedentary behavior. It is also known as a disrupter of sleep, it particulary reduces the sleep duration. In fact, insufficient sleep, ties to a higher tendency of obesity **(Tomiyama, 2019)**. Several researches have approved the anti-diabetic and anti-obesity virtues of *Ephedra alata Decne* **(Jaradat et al., 2021; Tiss et al., 2022)**. There is a positive correlation between food-rich flavonoids and polyphenols and obesity and type-2 diabetes prevention. Cafeic acid, apigenin 7-O-glucoside, apigenin, rutin, luteolin7-O-glucoside, p-Coumaric acid, gallic acid, vanilic acid, rutin, quercetin, cinnamic acid, apigenin-8-C-glucoside, epi-catechin, quercetin-3-O-glucuronide were found in abundance in *Ephedra alata Decne*, and have exhibited an inhibitory potential of intestinal and pancreatic lipase activity. Caffeic acid, apigenin and gallic acid have shown a preventive ability against atherosclerosis and hyperglycemia through inhibition of α-amylase, stimulation of insulin secretion, prevention of insulin resistance, reduction of body weight gain and adipose tissue weight **(Saidi et al., 2022)**. The most of the potent anti-diabetic and anti-obesity cited metabolites were identified in EamCE. Effectively, our extract has eminently reduced the gain of weight in G4 treated with both pirimicarb and EamCE, from 79.36 ± 12.03 g (G3 treated only with pirimicarb) to 41,55 ± 11.71 g.

IV.2.1.4. Assessment of oxidative stress parameters

The tissue homogenates from brain and testis had highly significant differences in the variation of protein concentrations ($p \leq 0.001$) (Table 11).

Table 11: Protein concentrations in the brain and testis tissues.

Protein concentration (mg/mL)	Brain	Testis
G1	$(2.372 \pm 0.001)^{a}$	$(1.844 \pm 0.003)^{a}$
G2	$(1.714 \pm 0.003)^{b***}$	$(2.053 \pm 0.002)^{b***}$
G3	$(1.983 \pm 0.003)^{c***}$	$(2.254 \pm 0.002)^{c***}$
G4	$(1.829 \pm 0.002)^{d***}$	$(2.042 \pm 0.002)^{d***}$

G1: negative control, G2: negative control of EamCE, G3: pirimicarb, G4: EamCE + pirimicarb. **.** The values are expressed as mean ± SD (n = 6). *** $p \leq 0.001$ = very highly significant, and those with the same subscripts were not significantly different ($p > 0.05$).

MDA concentration was increased in G3, its value gradually decreasing from G4, to G2, to G1. Concentrations of GSH and CAT differed among the groups, being high in G1 and G2, moderately elevated in G4, and decreased in G3. The total findings are summarized in figure 20.

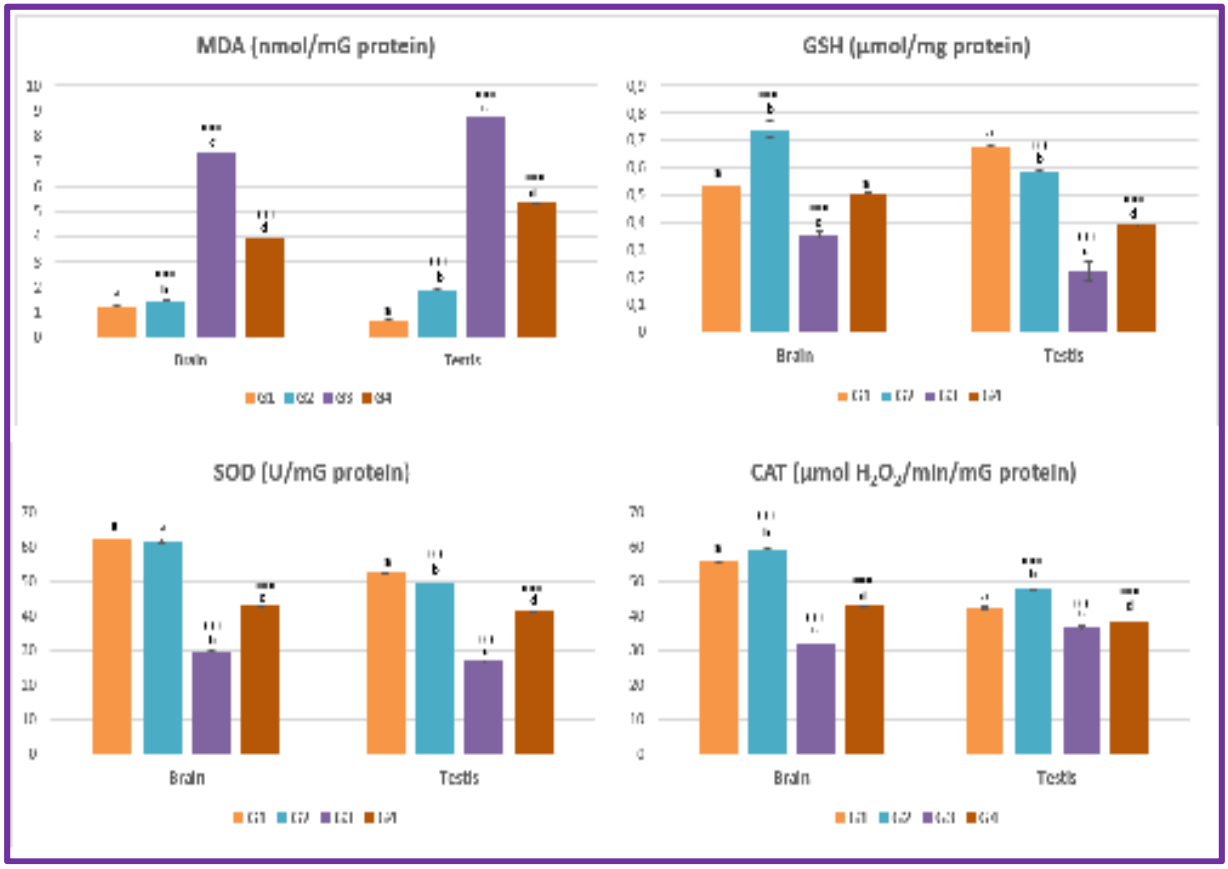


Figure 20: Assessment of oxidative stress parameters

G1: negative control, G2: negative control of EamCE, G3: pirimicarb, G4: EamCE + pirimicarb. The figure demonstrates the concentration of: MDA, GSH, SOD and CAT in tissue homogenates of brain and testis. The values are expressed as mean ± SD (n = 6).

*** $p \leq 0.001$: very highly significant, and those with the same subscripts were not significantly different ($p > 0.05$).

The outcomes of OS assessment in our study illustrated the genesis of a substantial profile of OS, affecting both tissues of interest the brain and testis. Certainly, we refer to the samples from the pirimicarb treated group of rats. Lipids are susceptible to oxidation, and lipid peroxidation products are potential biomarkers for OS status and its related diseases *in vivo*. Lipid peroxidation products in biological samples have been widely assessed; aldehydes such as MDA have been considered as a marker of lipid peroxidation *in vivo* **(Niki, 2008)**. Undoubtedly, the concentration of MDA as an OS indicator was increased in G3 rats exposed to pesticide, compared to the reduced concentration in G4, G2 and G1. A review work has

reported that disturbances of MDA, GSH and SOD were associated with recurrent depressive disorder and lowered levels have been found in depressed patients compared with healthy volunteers **(Lopresti et al., 2014).** Several investigations have been performed to evaluate the toxic effect of various types of pesticide and prove their ability to induce OS, as prominently indicated by low titers of antioxidant factors such as GSH, SOD, and CAT and high concentrations of MDA **(Possamai et al., 2007; Uchendu et al., 2018)** in brain tissue **(Rai & Sharma, 2007; Jafari et al., 2012; Singh et al., 2014)** and the male reproductive system **(Attia et al., 2012; Abarikwu et al., 2015; Avdatek et al., 2018)**, in the context of provoking neuro and reproductive male toxicities. ROS are released as a result of normal cellular metabolism at low-to-moderate rates; they react normally in physiological cell processes, but higher amounts produce detrimental changes in cell elements, such as lipids, proteins, and DNA. The alteration of the oxidant/ antioxidant balance in favor of oxidants is called "OS". It may contribute to several health issues, including cancer, neurological disorders, hypertension, ischemia, diabetes, acute respiratory distress syndrome, etc **(Birben et al., 2012)**. We suggest that pirimicarb exerted an inhibitory effect towards the antioxidant enzymes such as SOD and CAT, which may have resulted in an increase in ROS amounts (resulting even from pirimicarb metabolism) that in turn caused the elevation of MDA, the decrease in GSH, and the promotion of toxicological manifestations. A previous study reported changes in human red blood cell antioxidant enzymes in subjects with long-term exposure to pesticides. The most important finding was the reduction in SOD and CAT activities, with significant lower levels compared with controls in both the long and the short duration of pesticide exposure **(López et al., 2007)**.

Nevertheless, the authors strongly presume that, EamCE enhanced the antioxidant potential of rats fed with the extract and protected them from damage caused by pirimicarb, notably the OS engendered in G4 rats. *Ephedra alata* has been widely exploited in different biological investigations. Unquestionably, the *in vitro* and *in vivo* antioxidant, anti-inflammatory, analgesic, antipyretic, antidiabetic, antihypolipidemic, antihemolytic, and antithrombotic activities of *Ephedra alata* extracts were efficiently performed, being rich in terms of polyphenol and flavonoid content **(Jaradat et al., 2015; Al-Rimawi et al., 2017; Ben Lamine et al., 2019; Chouikh, 2020; Soumaya et al., 2020; Tiss et al., 2022; Zerargui et al., 2022)**. Antioxidant phytochemicals, such as polyphenols and flavonoids, induce the high expression and activation of antioxidant enzymes, namely, CAT, SOD, glutathione peroxidase, and glutathione reductase. These plant components have electrophilic activity and can favor

antioxidant enzymes via the Kelch-like ECH-associated protein 1-NF-E2-related factor-2 pathway and antioxidant, responsive elements **(Han et al., 2016)**.

IV.2.1.5. Serum titer of cortisol

The concentration of cortisol showed a noticeable elevation in the pirimicarb group compared with the other groups (G3: 2.070 ± 0.118 > G4: 1.353 ± 0.053 > G2: 0.977 ± 0.038 > G1: 0.996 ±0.006), as presented in figure 21.

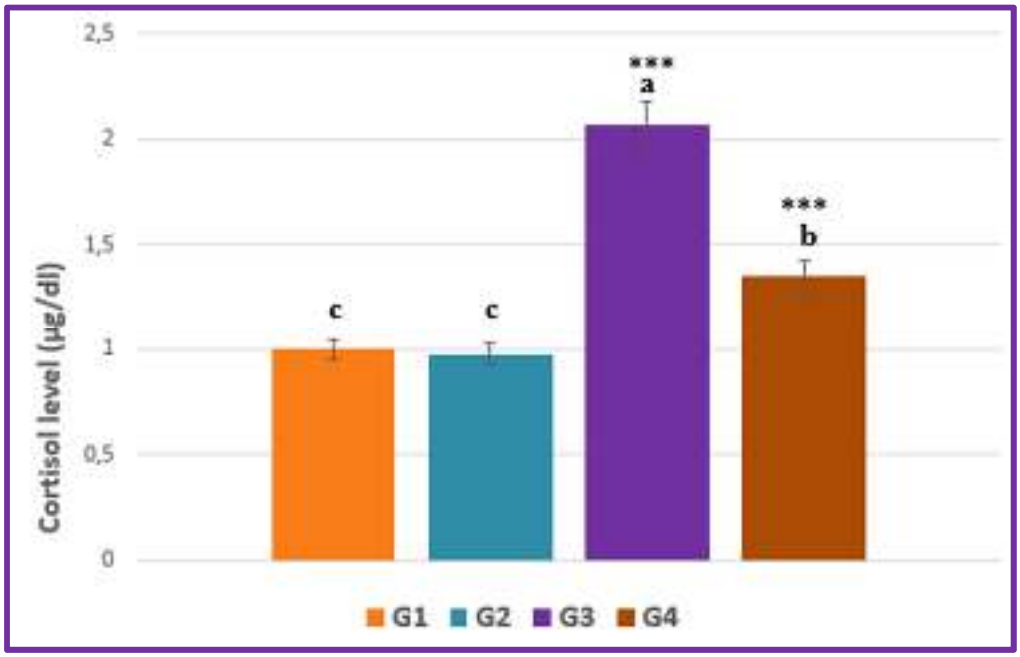


Figure 21: Serum titer of cortisol

G1: negative control, G2: negative control of EamCE, G3: pirimicarb, G4: EamCE + pirimicarb. The values are expressed as mean ± SD (n = 6). *** p ≤0.001 = very highly significant, and those with the same subscripts were not significantly different (p > 0.05).

Depressive and anxiety-like behaviors trigger the secretion of glucocorticoids **(Steckler et al., 1999; Fodor et al., 2016)**. Upon exposure to persistent or repetitive stress, this leads to already sensitive stress pathways to become markedly hyperactive and, consequently, increases in cortisol secretion persist, which may cause alterations in glucocorticoid receptors and therefore contribute to the pathogenesis of mood and anxiety disorders **(Mello et al., 2003)**. Our findings appear to endorse the latter point; serum titer of cortisol was high in anxious and depressive rats those treated with pirimicarb that acted as a chemical stressor agent.

IV.2.1.6. IL-1β quantification

The quantification of IL-1β in brain homogenate and plasma samples showed a significant increase in rats of G3 treated with pirimicarb (478.66 ± 1.43 pg/mL and 504.66 ± 0.22 pg/mL, respectively), compared with basic levels of IL-1β registered in samples of healthy rats (G1: 150 ± 0.68 pg/mL in brain homogenate and 155 ± 0.51 pg/mL in plasma). Concerning rats of G2 and G4, the levels of IL-1β were nearer to the basic values in G1 and were significantly reduced compared with the levels recorded in G3 (Figure 22). The calibration standard curve (concentration of IL-1β (pg/mL) vs. OD) is represented in appendix 4.

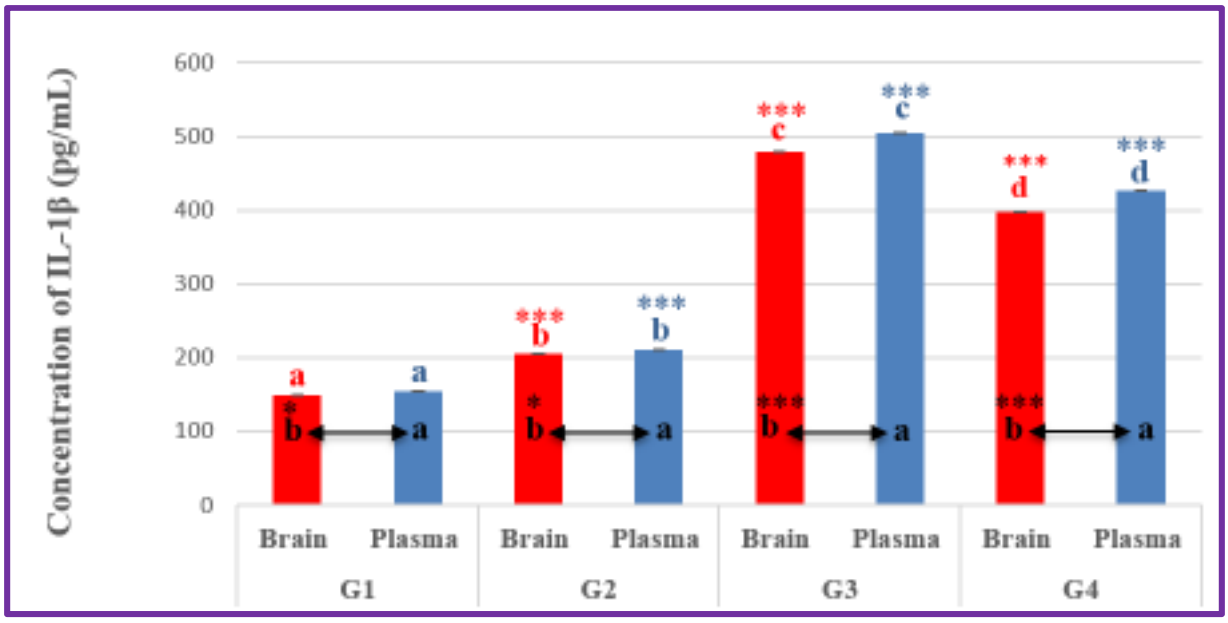


Figure 22: Quantification and titration of IL-1β in brain and plasma.

G1: negative control, G2: negative control of EamCE, G3: pirimicarb, G4: EamCE + pirimicarb. The values are expressed as mean ± SD (n = 6). In Figure 22, the red subscripts and asterisks indicate statistical comparisons of significance between IL-1β levels in brain homogenate of different groups (G1, G2, G3, G4). The blue subscripts and asterisks indicate statistical comparisons of significance between IL1-β levels in plasma of the different groups. Black subscripts and asterisks indicate statistical comparisons between IL-1β levels in the brain homogenate and plasma of each group. *** $p \leq 0.001$ = very highly significant, * $p \leq 0.05$ = significant, and those with the same subscripts were not significantly different ($p > 0.05$).

The brain has long been considered a privileged organ, from an immunological point of view, since the BBB and its tight junctions prevent the transmigration of systemic immune cells **(Laflamme & Rivest, 2001)**. Cytokines are chemical messengers that stimulate the HPA axis when the organism is under stress or an infection, acting as endocrine factors to regulate hormone secretion and feedback control of the HPA axis.

They transmit information from the immune compartments to the central nervous system as immunotransmitters and function in immunomodulatory neuroendocrine circuits **(Haddad et al., 2002)**. Systemic immunological stressors elicit extended activation of the HPA axis, mainly due to the release of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) from stimulated peripheral immune cells **(Beishuizen & Thijs, 2003)**. There is ample evidence to support the association between increased cortisol and pro-inflammatory cytokines (IL-1, IL-6, IL-8, TNF-α) in negative mood conditions, stress levels, anxiety, and depression **(Wahyuni et al., 2022)**. IL-1β is a major cytokine involved in monocyte activation and activation of pro-inflammatory signaling pathways in peripheral tissues and brain **(Hwang, 2012)**. IL1-β stimulates the production of mesencephalic astrocyte-derived neurotrophic factor (MANF), through its specific receptor type 1 (IL-1 R1) **(Nguyen et al., 2002).** Astrocytes are usually described as maintenance cells that participate indirectly in nerve transmission. They are now known to participate in the inflammatory response in acute brain injury by modifying their morphological and functional phenotype; by expressing the major histocompatibility complex, cytokines, and chemokines; and by producing NO via over-expression of inducible NO synthase (iNOS) **(Dong & Benveniste, 2001)**. More than that, IL-1 aggravates brain damage and its pharmacological blockade or transgenic mutation of the IL-1 receptor reduces the size of the lesion and the behavioral dysfunction **(Basu et al., 2005)**. Given the above, we assume that the finding of high levels of IL-1β in plasma and, notably, in brain homogenate of the pirimicarb exposed animals had a direct and/or indirect action on the activation of HPA axis, the elevated titer of cortisol, the neurobehavioral disturbances and observed neurodegeneration. Vice versa, we strongly assert that cortisol enhanced the production of IL-1β from peripheral immune cells; this correlation has previously been proven **(Yeager et al., 2018).** Regarding the role of EamCE and the reduced release of IL-1β, we do associate this with the anti-inflammatory effect of the plant **(Hass et al., 2017; Soumaya et al., 2020)**. Importantly, *Ephedra alata* Decne has already shown the ability to reduce the production of pro-inflammatory cytokines **(Kmail, 2017)**.

IV.2.1.7. Assessment of hematological parameters

The count of blood elements namely: white blood cells, red blood cells, lymphocytes, monocytes, granulocytes, platelets of rats from each experimental group are reported in table 12. The results showed a decrease in lymphocytes and granulocytes in rats of G3 comparing with those of the other groups. Per contra, there was a remarkable increase in monocytes, and a very important rise in platelets of G3 relatively to G1, G2 and G4. Red blood cells remained with no significant variance between groups.

Table 12: Hematological parameters assessment.

	Concentration (10^9 cell/L)			
Hematological parameters	**G1**	**G2**	**G3**	**G4**
White blood cells	11.12 ± 0.19^{a}	11.03 ± 0.11^{a}	$6.3 \pm 0.17^{c***}$	$8.9 \pm 0.13^{b***}$
Lymphocytes	6.3 ± 0.04^{a}	$6.21 \pm 0.13^{b***}$	$3.67 \pm 0.18^{d***}$	$5.43 \pm 0.21^{c***}$
Monocytes	1.00 ± 0.24^{d}	$1,11 \pm 0.18^{c***}$	$2.3 \pm 0.22^{a***}$	$1.62 \pm 0.13^{b***}$
Granulocytes (cells/L)	3.7 ± 0.32^{a}	$3.56 \pm 0.20^{b***}$	$1.07 \pm 0.05^{d***}$	$3.34 \pm 0.16^{c***}$

Red blood cells (10^{12} cell/L)			
G1	**G2**	**G3**	**G4**
$8.12 \pm 0.03^{a,b}$	8.21 ± 0.02^{a}	8.10 ± 0.04^{b}	8.06 ± 0.04^{b}

Platelets (10^3 /µL)			
G1	**G2**	**G3**	**G4**
750 ± 3.76^{d}	$787 \pm 2.93^{c***}$	$987 \pm 3.55^{a***}$	$810 \pm 3.01^{b***}$

G1: negative control, G2: negative control of EamCE, G3: pirimicarb, G4: EamCE + pirimicarb. The values are expressed as mean ± SD (n = 6). *** $p \leq 0.001$ = very highly significant, and those with the same subscripts were not significantly different ($p > 0.05$).

The impact of pirimicarb on leucocytes was manifested by a serious lymphopenia and agranulocytosis, the level has been decreased from $6.3*10^9$ cell/L to $3.67*10^9$ cell/L for lymphocytes and from $3.7*10^9$ cell/L to $1.07*10^9$ cell/L for granulocytes. Differences in white blood cells amount were found to be reliable markers of stress caused by environmental factors. Lymphopenia has been reported as a consequence of pesticide exposure and accompanied by concurrent augmentation in monocytes occurring as a response to stress induced by pesticide exposure **(Lushchak et al., 2018)**. This could be interpreted according to an earlier study, by the possible involvement of free radicals and OS provoked under the effect pesticide in the induction of immunocytotoxicity **(Koner et al., 1998)**. Otherwise, Pirimicarb has increased the level of monocytes from $1.00*10^9$ cell/L to $2.3*10^9$ cell/L. Studies conducted in rodents asserted that stress increases the production of inflammatory cytokines such as IL-1β or IL-6 by spleen cells and peritoneal macrophages. In addition to potentiating the production of inflammatory cytokines, stress can decrease the sensitivity of macrophages to glucocorticoids. In humans, an acute stress involves catecholamines like noradrenaline via receptors α1- and β-adrenergics to activate the NF-κB pathway in monocytes and induce the expression of inflammatory cytokines genes. The stress could also exacerbate the release of TNF-α, IL-1β and IL-6 by macrophages by the induction of local release of CRH via free nerve endings. Indeed, we assume that the elevation in IL-1β levels was in parallel of the elevated amount of monocytes caused by pirimicarb induced stress **(Merlot, 2004)**.

Platelets have also registered a noticeable raise ($987*10^3$/µL vs. $750*10^3$/µL) due to pirimicarb impact. This result is in agreement with the study of a group of veterinarian researchers that have obtained a significant increase in the number of platelets in chickens after a pesticide (thiram) intake, owing to the increase expression of thrombopoietin mRNA in the dysfunction liver **(Huang et al., 2019)**. Similarly, our finding is in the same line as an Algerian survey conducted on fifty farmers using chemical pesticides and 60 unexposed control. Changes in hemostasis have been noted in farmers using pesticides comparing to control individuals; a procoagulant state was observed (increase in clotting factors such as fibrinogen and prothrombin) accompanied with an evident state of OS (diminishing in vitamins C, E, and GSH levels and in catalase and SOD activities that may be due to their excessive use to struggle with ROS). Inflammation was also associated to precedent changes with high plasma CRP level **(Madani et al., 2016)**.

IV.2.1.8. Photomicrographs of histologic study and testosterone titer

Photomicrographs of brain sections, specifically of the cerebral cortex region are demonstrated in figure 23, corresponding to rats from different experimental groups. As well as, testis photomicrographs that are presented in figure 24, and that showed histological changes in the testis of rats from the four studied groups. The testosterone titer (Figure 25) was lowest in the pirimicarb group and was at its highest level in the group of rats treated with EamCE (G1: 4.908 ± 0.898, G2: 7.850 ± 0.388, G3: 0.809 ± 0.334, G4: 2.681 ± 0.166).

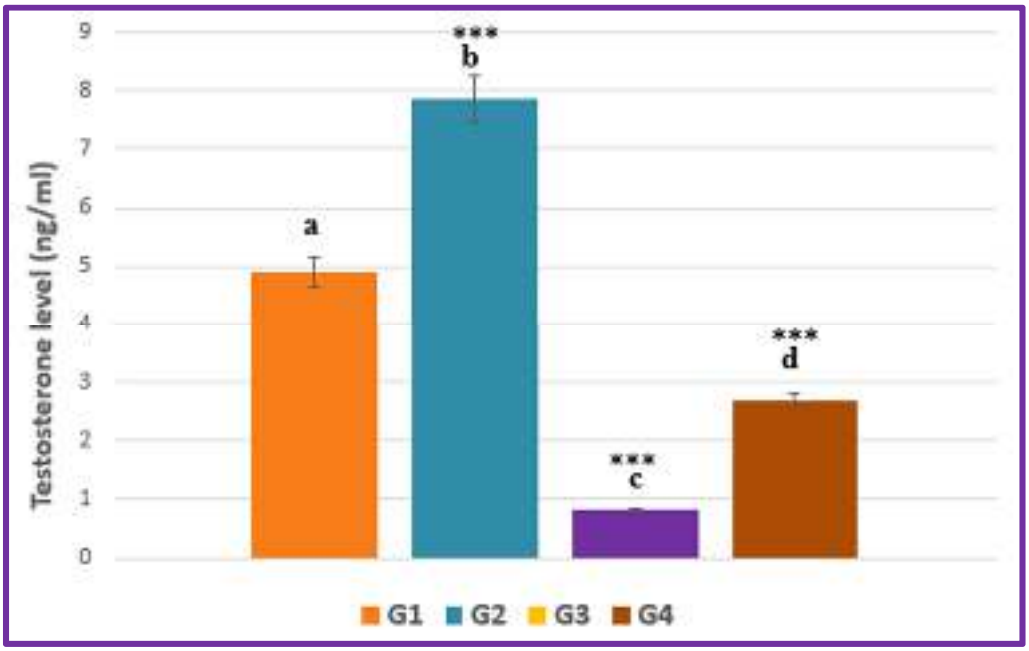


Figure 25: Serum titer of testosterone

G1: negative control, G2: negative control of EamCE, G3: pirimicarb, G4: EamCE + pirimicarb. The values are expressed as mean ± SD (n = 6). *** $p \leq 0.001$ = very highly significant, and those with the same subscripts were not significantly different ($p > 0.05$).

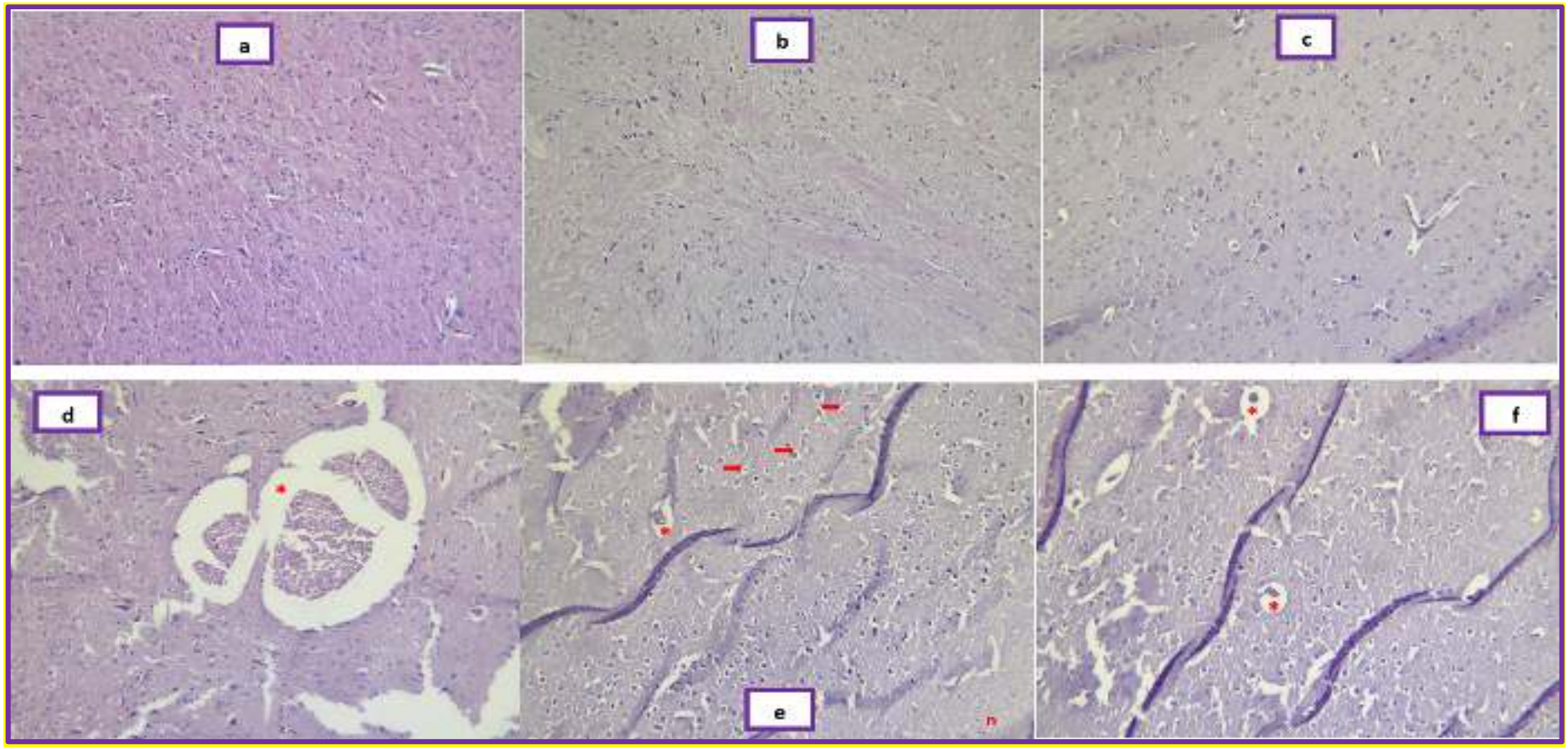


Figure 23: Photomicrographs of cerebral cortex sections. Images (a,b) are control groups G1 and G2 showing normal neuroglial cell arrangement. G3 is represented in images (d,e,f) showing an area of liquefactive necrosis (n), shrunken nerve cells with dark nuclei and surrounded by vacuoles (red arrows), vascular congestions (asterisks) and vacuolization. Image (c) shows a G4 section with almost normal morphological appearance of nerve cells with less fine vacuolization (H&E, X 100)

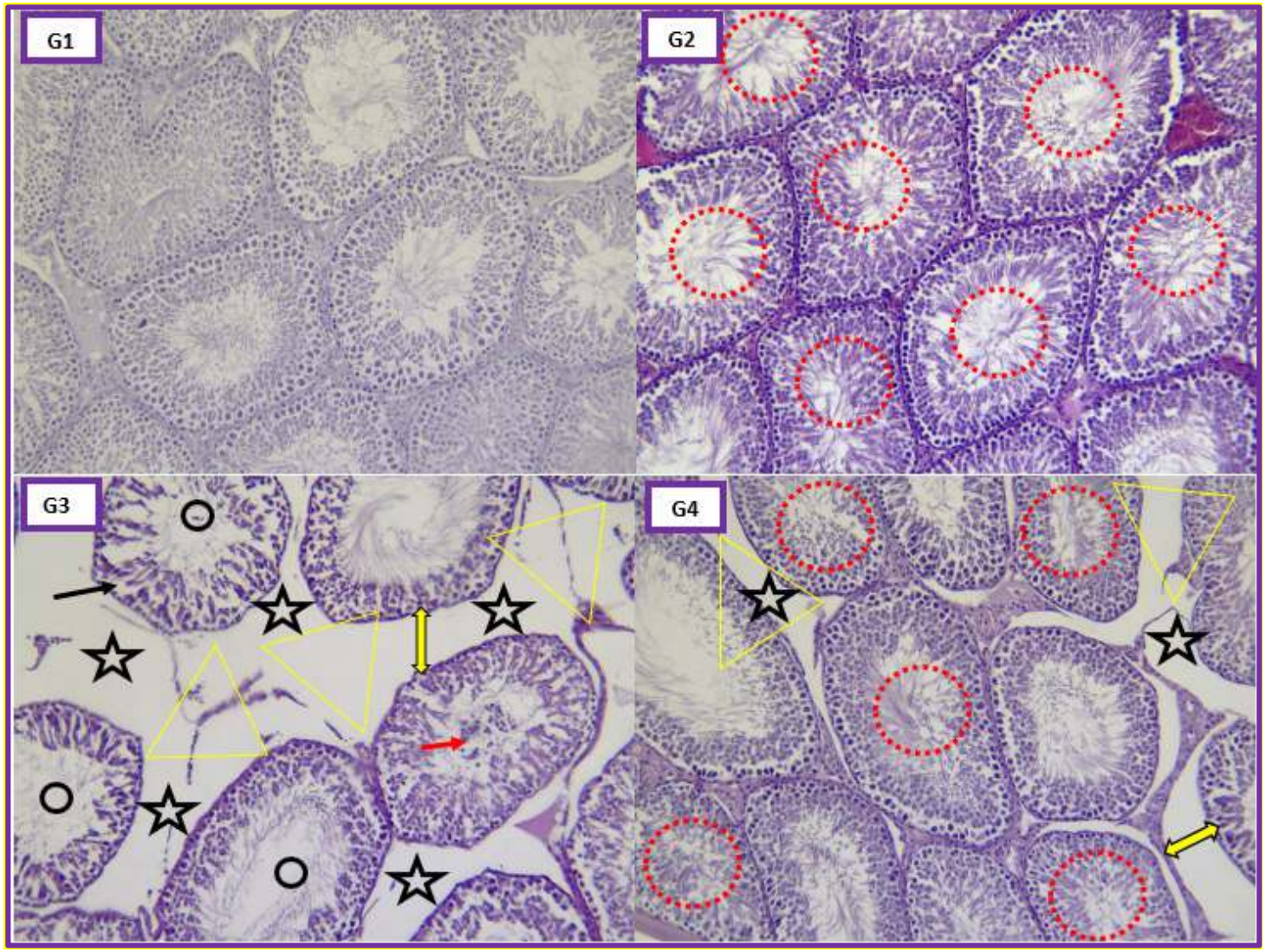


Figure 24: . Photomicrographs of testis sections. Control group rats (G1) showed typical organization of seminiferous tubules. EamCE (200 mg/kg) treated rats (G2) showed important spermatogenesis (red circle), normal testicular architecture and normal Leydig cell appearance. Rats treated with 145 mg/kg of pirimicarb (G3) exhibited degeneration of germinal and Sertoli cells (black arrow); absence of Leydig cells (black star); loss of interstitial tissue (yellow triangle); an abnormal increase in intertubular spaces (yellow double arrow); spermatocytic maturation arrest (black circle); lumen-devoid spermatozoa (black circle) and the formation of many exfoliated cells (red arrow). Rats treated with 145 mg/kg of pirimicarb and EamCE (200 mg/kg) (G4) showing an increase in spermatogenesis (red circle) and an improvement in general appearance almost comparable to the control group, except for partial loss of Leydig cells (black star) and interstitial tissue (yellow triangle) (H&E, X 100).

Histological examination of rats in control groups G1 and G2 showed brain sections with normal histological architecture with no lesions (Figure 23: a,b). In the brain sections of pirimicarb treated rats, the parenchymatous cells of the cerebral cortex showed degeneration and liquefactive necrosis, characterized by partial or complete dissolution of dead tissue and transformed into a liquid, viscous mass **(Adigun et al., 2020)**. Prominent vacuolization was also identified; this was previously considered a side effect of the action of cytotoxic factors and its accumulation an important initiating event, causing metabolic alterations or stress responses that lead to cell death **(Shubin et al., 2016)**. In addition, some glial cells showed pyknotic nuclei and numerous congested blood vessels (Figure 23: d, e, f); these deleterious aspects have been determined in numerous neurotoxicity studies induced by pesticides or other toxic chemicals **(Menze et al., 2012; Elshama et al., 2017; Madkour et al., 2021)**. In contrast, the brain sections of G4 rats showed almost normal morphological appearance of nerve cells with less fine vacuolization.

No pathological changes were observed in testicular sections of untreated control animals. Indeed, microscopic examination of testicular tissue of rats in the control group showed normal histological architecture with normal seminiferous tubules showing all cell layers of germinal cells and a spermatozoa-filled lumen (Figure 24, G1). However, EamCE (200 mg/kg) treated rats (G2) showed an increase in the number of spermatozoa and round spermatids (Figure 24, G2). Testes of rats treated with 147 mg/kg of pirimicarb (G3) showed marked histological changes characterized by reduced diameter; a markedly reduced number of spermatozoa; seminiferous tubules showing a disrupted germinal epithelium with various degree of atrophy; degeneration and necrosis with a prominent increase in interstitial space; and a reduced number of Leydig cells (Figure 24, G3). Rats receiving pirimicarb with EamCE (G4) showed a tendency towards a return to normal testicular histology indicated by a markedly developed germinal epithelium, and with almost a normal number of Sertoli cells and Leydig cells (Figure 24, G4). Moreover, the presence of spermatozoa was observed in the lumen of seminiferous tubules.

The ongoing hispathological manifestations were accompanied by a raised level of cortisol and a decline in the testosterone hormone. Previously, researchers have found that long-term exposure to deltamethrin pesticide caused alteration of reproductive hormones, including serious dysfunction of testicular tissue in which cortisol and testosterone levels were inversely

proportional **(Maksoud et al., 2020)**. An earlier study that corroborates our findings on the potential role of carbamate pesticides interference on the natural hormones of the hypothalamic–pituitary–thyroid that result in disturbances of the male reproductive system. It has been reported that there is evidence that exposure to carbamates leads to lower levels of GnRH, LH and/or FSH, thus compromising steroid genesis and spermatogenesis. Furthermore, various histologic alterations have been demonstrated in the testis along with deficiency of male reproductive capacity **(Moreira et al., 2022)**.

The preventive effect of EamCE regarding testicular tissue integrity and boosting fertility via enhancing spermatogenesis and increasing testosterone level, could probably be explained by the effect of saponins **(Dbeibia et al., 2022; Hibi et al., 2022)** and ephedrine alkaloids **(Ibragic & Sofić, 2015; Sioud et al., 2020)** that are contained in the plant crude extract. In effect, saponins have been known as substance responsible for, and enhancers of, an aphrodisiac effect due to their ability to increase androgen hormones **(Yakubu et al., 2005; Sumalatha & Kumar, 2010)**.

In addition, we also observed, from a few days after starting the experiment, and frequently until the day of sacrifice, some sexual behaviors in the G2 (EamCE control) fed rats, such as mounting and interomission, that strongly explain the presumed fertilizing and aphrodisiac effects of EamCE.

IV.2.1.9.LC-MS/MS analysis of brain and testis extracts

The LCMS/MS analysis of extracts from brain and testis determined the presence of pirimicarb in all the tested extracts, the peaks for pirimicarb and its ion products (Table 13) are well represented in the chromatograms in figure 26: A–D, corresponding to brain and testis extracts from rats of G3 and brain and testis extracts from rats of G4. The LCMS/MS data are provided in table 13. The structure of ion products is described in appendix 5.

Table 13: LCMS/MS data of pirimicarb.

Compound	Formula	M	MH^+	Ioniza-tion Mode	MRM Transition	Collision Energy (v)	Rt Min
Pirimicarb	$C_{11}H_{18}NO_2$	238.1430	239.15	ESI+	72.1	−22	0.8
					182.2	−17	0.8

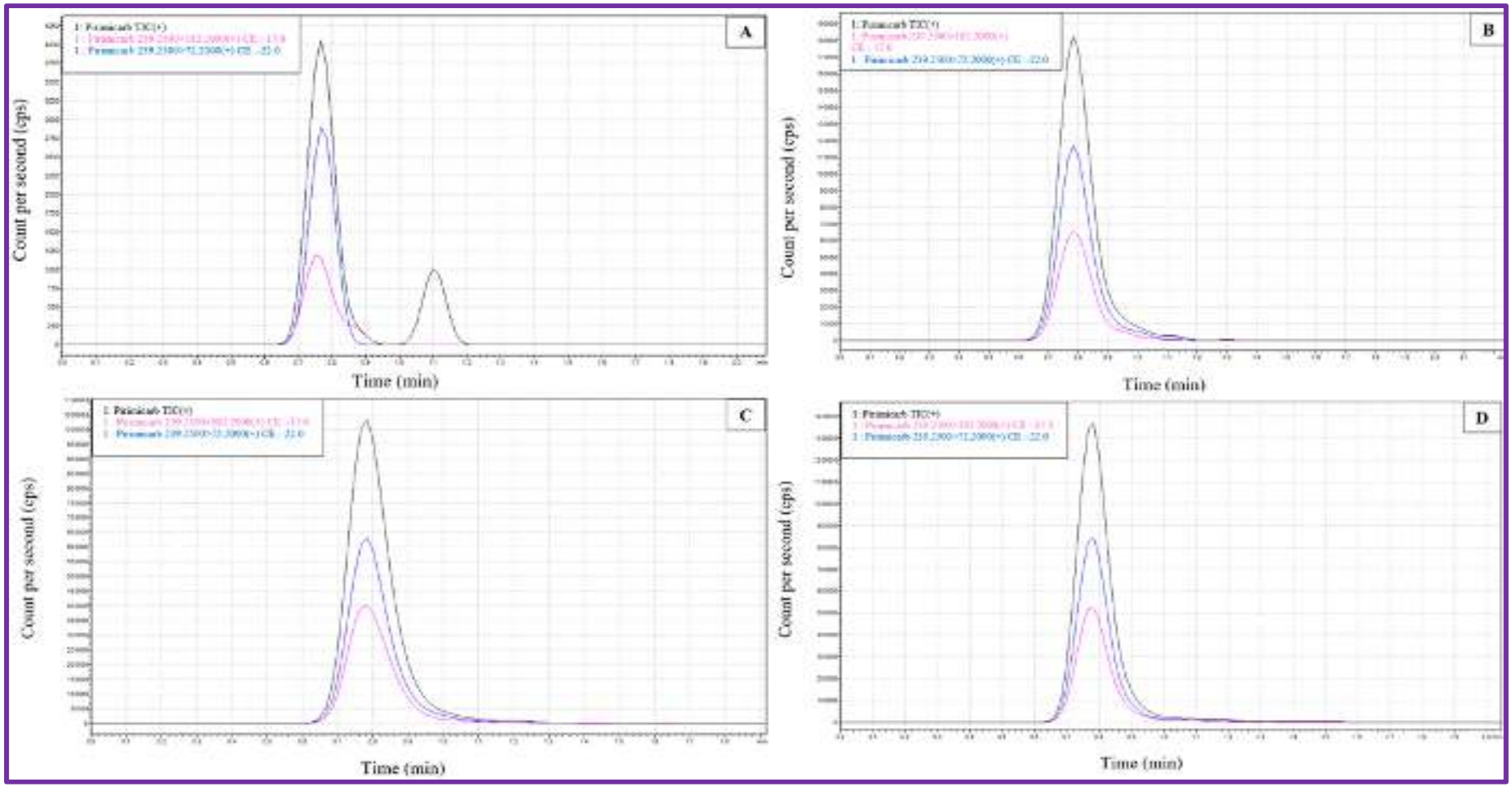


Figure 26: Representative chromatograms of pirimicarb from extracted samples.

(A)Chromatogram of pirimicarb from brain extracts of G3 rats; (B) chromatogram of pirimicarb from testis extracts of G3 rats; (C) chromatogram of pirimicarb from brain extracts of G4 rats; (D) chromatogram of pirimicarb from testis extracts of G4 rats.

Contrary to organophosphate poisoning, carbamate poisoning normally starts to diminish within a few hours and disappears after 24 h, usually without any permanent sequelae. Carbamates commonly do not traverse the BBB as easily as organophosphates; as such, brain injuries with carbamates occur with a lower frequency and are less severe than with organophosphates **(Rosman et al., 2009)**. In another study, it was reported that CNS symptoms are not particularly noticeable in carbamate poisoning due to the poor permeability of these compounds across the BBB **(Chahal et al., 2015)**. A molecule can be totally (100%) absorbed from a given formulation; however, it may have low bioavailability before being broken down after absorption **(Toutain & Bousquet-Mélou, 2004)**. Pesticides are generally distributed in the organism due to their ability to bind with plasma proteins, blood cells, and lipids in various organs and peripheral tissues. The binding potential is determined by the lipophilicity, which increases the pesticide's successive bioaccumulation. Thus, the lipophilicity of compounds can truly alter their bioavailability **(Kalyabina et al., 2021)**. According to the Swiss ADME prediction online platform **(*SwissADME*, 2023)**, pirimicarb has a lipophilicity with a log Po/w of 3.39 and with a score of bioavailability equal to 0.55, which are sufficient values for a molecule to accumulate and engender its different modes of action. LCMS/MS-MRM analysis in the current study has effectively allowed us to confirm simulation data and find pirimicarb in brain and testis tissues from animals orally administered pirimicarb (rats in G3 and G4). The revelation of pirimicarb in brain and testis disclose that this molecule is resistant to different mechanisms of biotransformation and metabolism in the rat organism; in this case, pirimicarb bioavailability increased and the molecule was able to infiltrate many types of tissue. Renal excretion of unchanged drug has only a modest role in the complete elimination of many chemicals as long as lipophilic compounds filtered through the glomerulus are mostly reabsorbed into the systemic circulation during passage through the renal tubules **(Skinner et al., 2018)**.

IV.2.2. Pirimicarb cytotoxicity on human neutrophils and preventive effect of EamCE

The EamCE has enhanced the viability % of neutrophils from 74.44 ± 2.47 to 94.09 ± 1.19 when EamCE was applied at 100 µg/ml , to 85.57 ± 1.17 when EamCE was applied at 75 µg/ml to and to 81.69±1.55 when EamCE was applied at 50 µg/ml. Although, pirimicarb has reduced the viability % of neutrophils with varied cytotoxic potency inversely proportional to the applied concentration. The viability % of neutrophils reaches a rate of 74.44 ± 2.47 % when pirimicarb was applied at 100 µg/ml, it has been enhanced with decreased concentrations of pirimicarb as demonstrated in table 14.

Table 14: EamCE preventive effect against pirimicarb cytotoxicity assessed by % of neutrophils viability

	Neutrophils viability %
Control	(99.30 ± 0.23) ^{a}
EamCE 100 µg/mL	(99.50 ± 0.44) ^{a}
EamCE 75 µg/mL	(99.03 ± 0.65) ^{a}
EamCE 50 µg/mL	(98.70 ± 0.57) ^{a}
Pirimicarb 100 µg/ml	(74.44 ± 2.47) ^{e***}
Pirimicarb 75 µg/ml	(79.08 ± 0.28) ^{d***}
Pirimicarb 50 µg/ml	(82.08 ± 0.78) ^{d***}
Pirimicarb 100 µg/ml+EamCE 100 µg/ml	(94.09 ± 1.19) ^{b**}
Pirimicarb 100 µg/ml+EamCE 75 µg/ml	(85.57 ± 1.17) ^{c***}
Pirimicarb 100 µg/ml+EamCE 50 µg/ml	(81.69 ± 1.55) ^{d***}

** $p \leq 0.01$ = highly significant , *** $p \leq 0.001$ = very highly significant, and those with the same subscripts were not significantly different ($p > 0.05$).

In our *in vivo* experience, blood analysis of rats fed with pirimicarb conceded that the level of granulocyte and lymphocytes was critically lowered. We have carried the current experiment to approbate the cytotoxic effect of pirimicarb on immune cells. Neutrophils are the main pathogen-combating immune cells in humans and the majority of animals. Neutrophils have the ability of recruitment to the site of infection, recognition and phagocytosis of microbes and different mechanism of killing pathogens , including the release of ROS, the secretion of antimicrobial peptides, and expulsion of nuclear substances to form neutrophil extracellular traps **(Mayadas et al., 2014)**. Pesticides are recognized to engender toxicological effects with various action modes to provoke OS, mitochondrial dysfunction and endoplasmic reticulum stress in living organisms. Pesticides like as atrazine, organophophorus, carbamates, and pyrethroids were approved to inhibit the survival and growth of leukocytes by causing apoptosis or cell cycle arrest and interfering with the specific immunological functions of each type of immune cells **(Lee & Choi, 2020)**. The function of neutrophil was examined in 40 workers exposed to carbamate and organophophate insecticides by checking phagocytosis and intracellular killing of Candida albicans. As a result, an important reduction in killing ability of neutrophils towards Candida albicans, this finding insures that exposure to carbamates and organophophates pesticides would altered neutrophil function **(Queiroz et al., 1999)**. Decently, in this survey, pirimicarb has induced the reduction of neutrophils viability with a percentage of 74.44 ± 2.47 at 100µg/ml, 79.08 ± 0.28 at 75µg/ml and 82.08 ± 0.78 at 50 µg/ml, which means less is the concentration applied, less is the cytotoxic effect. Whereas, the combination of pirmicarb with EamCE has enhanced the viability % of neutrophils even when pirimicarb was applied at the highest dose (100 µg/mg), this, was registered at 100 and 75 µg/ml of EamCE (94.09 ± 1.19, 85.57 ± 1.17), 50µg/ml has exhibited a moderate preventive effect (81.69 ± 1.55). By dint of the non-cytotoxic effect of EamCE at different concentration, confirmed with typical viability % of neutrophils (99.50 ± 0.44, 99.03 ± 0.65, 98.70 ± 0.57), and due to its enhanced effect as a complex with pirimicarb, we declare its preventive activity against cytotoxic mechanisms.

In spite of all, we schematized the general impact of pirimicarb on the neuro-immune-endocrine axis referring to the reached outcomes and the suggested action mode of EamCE in figure 27.

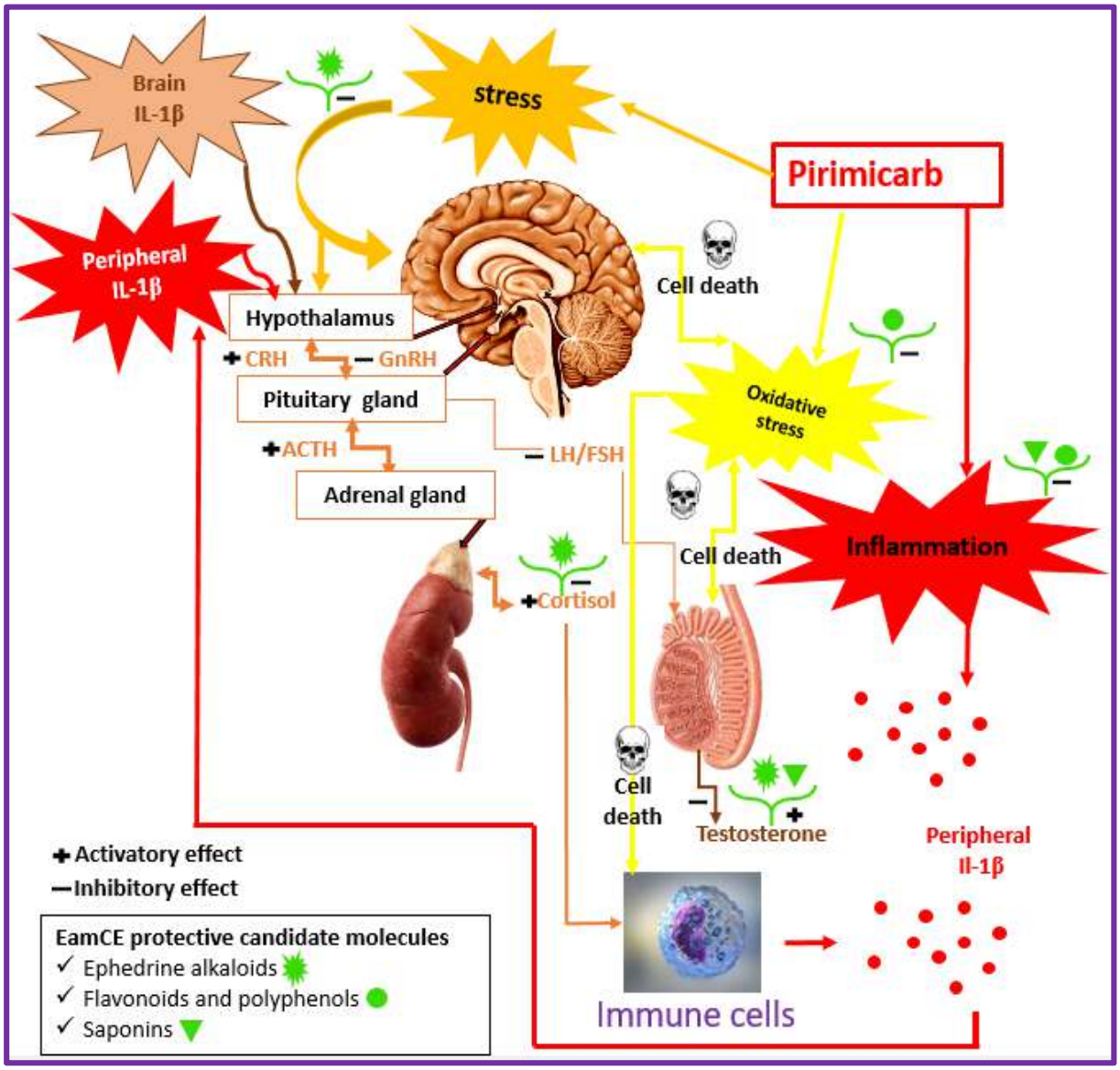


Figure 27: Proposed mechanisms elucidating how EamCE could prevent pirimicarb-induced damage.

Pirimicarb introduced a stressful status; this stress is responsible of the induction of behavioral troubles and activation of the HPA axis via stimulating the hypothalamus to secrete the CRH; this hormone influence on the pituitary gland to release more ACTH and to down

regulate the GnRH. Besides, decreased levels of GnRH; downregulates the release of LH and FSH from the pituitary gland. The down regulation of sexual hormones and the up regulation of ACTH lead to low amount of testosterone and to high titer of cortisol. Cortisol has a repercussion on the immune cells, it activates them to secrete more IL-1β; increased peripheral IL-1β concentrations attain the brain with synergy of local produced IL-1β activate the secretion of cortisol via specific receptors.

Furthermore, pirimicarb induces OS in the brain, testis and immune cells (provoking cell death and serious lesions), causing inflammatory status advantaging the release of IL-1β from monocytes.

The EamCE has a general preventive activity due its different metabolites, mainly; polyphenols, flavonoids, ephedrine alkaloids and saponins. Each type of metabolite has its specific mode of action and level of interaction.

V Conclusions and Perspectives

The backgrounds regarding the richness of *Ephedra alata* species in terms of different metabolites, its medical application and traditional use worldwide has eased our choice to use it as a preventive agent in our study. The outcomes of *Ephedra alata* monjauzeana chemical screening and biological virtues have highlighted the sub species as a potent and a promising natural source to protect from many troubles and health issues.

In effect, the abundance of metabolites (polyphenols and flavonoids) in EamCE, its impressive antioxidant and anti-inflammatory potentials, as well as its inoffensive and no toxic feature, which were proven in our *in vitro* and *in vivo* investigations; endorsed earlier findings and approbated the choice of the plant.

Pesticides are hazardous products and well known to provoke toxicological effects for workers in the area of their production or application. The general public is not covered and could be exposed by means of contaminated water and food.

Indeed, the impact of pirimicarb pesticide in wistar rats gave us sufficient insights about its toxicity on the neuro-immune endocrine system. The revealed accumulation of pirimicarb in the brain and testis tissues interpreted its ability of behavior and mood perturbation, induction of OS and inflammation being the cause of important lesions in brain and testis tissues, lymphopenia and agranulocytosis. These disturbances are typical for the initiation of dementia and infertility. Long-term pirimicarb intake or exposure could lead to more severe complications notably cancer malignancy and life loss.

The crude extract from *Ephedra alata* monjauzeana offered significant prevention from pirimicarb damage due to its antioxidant and anti-inflammatory properties that prevent deleterious impacts of oxidative stress, induction of apoptosis and inflammatory responses. In addition to its fertilizing potentials which enhanced secretion of androgenic hormones and spermatogenesis. Likewise, this plant revealed its euphoric ability that dealt with stress and prevent anxiety and depression.

In future projects, we plan:

1. To seek pirimicarb circulation in the organism, and to determine its precise amounts in each compartment of detection.
2. To determine the period of complete elimination of pirimicarb from the organism, counting from the day of its intake arrest.
3. To elucidate the molecular mechanism of action and to carry a comparative study between male and female rats.
4. To carry an investigation concerning the EamCE effect: in order to identify if it was generated due to the synergic effect of many molecules or specific individual molecule.
5. To purify the beneficial molecule(s) from *Ephedra alata* monjauzeana and to elaborate a dietary supplement contributing to preserve moral health and fertility.

References

A

Abarikwu, S. O., Akiri, O. F., Durojaiye, M. A., & Adenike, A. (2015). Combined effects of repeated administration of Bretmont Wipeout (glyphosate) and Ultrazin (atrazine) on testosterone, oxidative stress and sperm quality of Wistar rats. *Toxicology Mechanisms and Methods*, *25*(1), 70–80. https://doi.org/10.3109/15376516.2014.989349

Abdulkhaleq, L. A., Assi, M. A., Abdullah, R., Zamri-Saad, M., Taufiq-Yap, Y. H., & Hezmee, M. N. M. (2018). The crucial roles of inflammatory mediators in inflammation: A review. *Veterinary World*, *11*(5), 627–635. https://doi.org/10.14202/ vetworld .2018. 627-635

Abubakar, Y., Tijjani, H., Egbuna, C., Adetunji, C. O., Kala, S., Kryeziu, T. L., & Patrick-Iwuanyanwu, K. C. (2019). Pesticides, history, and classification. *Natural Remedies for Pest, Disease and Weed Control*, 29–42. https://doi.org/10.1016/B978-0-12-819304-4.00003-8

Achraf, K., Kheira, M., Asma, C., Majida, A., & Abdellah, M. (2022). Phytochemical Content, Antioxidant and Antifungal Activities of Ephedra alata Decne. Aerial Parts Grown Wild in South-west Algeria. *Indian Journal Of Agricultural Research*, *57*(2), 164–169. https:// doi.org/10.18805/IJARe.AF-728

Adigun, R., Basit, H., & Murray, J. (2020). Cell Liquefactive Necrosis. In *StatPearls*, 1–7. http://www.ncbi.nlm.nih.gov/pubmed/28613685

Aebi, H. (1995). Catalase. *Nippon Rinsho. Japanese Journal of Clinical Medicine*, *53*(1), 358–360. https://doi.org/10.1086/330448

Agnel Arul John, N., & Shobana, G. (2012). Anti-inflammatory activity of Talinum fruticosum L. on formalin induced paw edema in albino rats. *Journal of Applied Pharmaceutical Science*, *2*(1), 123–127.

Aguiar, A. C. de, da Fonseca Machado, A. P., Figueiredo Angolini, C. F., de Morais, D. R., Baseggio, A. M., Eberlin, M. N., Maróstica Junior, M. R., & Martínez, J. (2019). Sequential high-pressure extraction to obtain capsinoids and phenolic compounds from

biquinho pepper (Capsicum chinense). *Journal of Supercritical Fluids*, *150*, 112–121. https://doi.org/10.1016/j.supflu.2019.04.016

Al-Rimawi, F., Abu-Lafi, S., Abbadi, jehad, Alamarneh, A. A. ., Sawahreh, R. A., & Odeh, I. (2017). Analysis of Phenolic and Flavonoids of Wild Ephedra Alata Plant Extracts By Lc/Pda and Lc/Ms and Their Antioxidant Activity. *African Journal of Traditional, Complementary and Alternative Medicines*, *14*(2), 130–141. https://doi.org/10.21010/ajtcam.v14i2.14

Ali, S., Ullah, M. I., Sajjad, A., Shakeel, Q., & Hussain, A. (2021). Environmental and Health Effects of Pesticide Residues. *Sustainable Agriculture Reviews, 48*, 311–336. https://doi.org/10.1007/978-3-030-54719-6_8

Almeida, A., Frinhani, A., Leite, G., Marques, M., Simões, I., Felippe, A., Sampaio, V., Coelho, T., Aparecida, M., Andrea, T., Gomes, R., & Pires, W. (2019). Neurotoxicology and Teratology Atropine counteracts the depressive-like behaviour elicited by acute exposure to commercial chlorpyrifos in rats. *Neurotoxicology and Teratology*, *71*(November 2018), 6–15. https://doi.org/10.1016/j.ntt.2018.11.002

Anastassiades, M., Lehotay, S. J., Štajnbaher, D., & Schenck, F. J. (2003). Fast and Easy Multiresidue Method Employing Acetonitrile Extraction/Partitioning and "Dispersive Solid-Phase Extraction" for. *Journal of Aoac International*, *86*(2), 412–431.

Antonio F.Hernández. (2021). Food safety_ pesticides - ScienceDirect. In Encyclopedia of Human Nutrition (4th ed., pp. 375–388). Elsevier. https://doi.org/https:// doi.org/10.1 016/ B978-0-12-821848-8.00042-1

Apak, R., Güçlü, K., Özyürek, M., & Karademir, S. E. (2004). Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. *Journal of Agricultural and Food Chemistry*, *52*(26), 7970–7981. https://doi.org/10.1021/jf048741x

Attia, A. A., ElMazoudy, R. H., & El-Shenawy, N. S. (2012). Antioxidant role of propolis extract against oxidative damage of testicular tissue induced by insecticide chlorpyrifos in rats. *Pesticide Biochemistry and Physiology*, *103*(2), 87–93.

https://doi.org/10.1016/j.pestbp.2012.04.002

Avdatek, F., Birdane, Y. O., Türkmen, R., & Demirel, H. H. (2018). Ameliorative effect of resveratrol on testicular oxidative stress, spermatological parameters and DNA damage in glyphosate-based herbicide-exposed rats. *Andrologia*, *50*(7), 1–8. https://doi.org/10.1111/and.13036

B

Barzegar, F., Kamankesh, M., & Mohammadi, A. (2021). Recent Development in Formation, Toxic Effects, Human Health and Analytical Techniques of Food Contaminants. *Food Reviews International*, *32*(2), 1157-1183. https://doi.org/10.1080/87559129.2021.1929303

Basu, A., Lazovic, J., Krady, J. K., Mauger, D. T., Rothstein, R. P., Smith, M. B., & Levison, S. W. (2005). Interleukin-1 and the interleukin-1 type 1 receptor are essential for the progressive neurodegeneration that ensues subsequent to a mild hypoxic/ischemic injury. In *Journal of Cerebral Blood Flow and Metabolism* 25 (1), 17–29. https://doi.org/10.1038/sj.jcbfm.9600002

Bayramgürler, D., Karson, A., Özer, C., & Utkan, T. (2013). Effects of long-term etanercept treatment on anxiety-and depression-like neurobehaviors in rats. *Physiology & behavior*, 119, 145-148.

Beishuizen, A., & Thijs, L. G. (2003). Endotoxin and the hypothalamo-pituitary-adrenal (HPA) axis. *Journal of Endotoxin Research*, *9*(1), 3–24. https://doi.org/10.1179/096805103125001298

Bell, A & Bachman, S. (2011). Ephedra alata L. In *The IUCN Red List of Threatened Species* . https://doi.org/http://dx.doi.org/10.2305/IUCN. UK.2011 2.RLTS.T201688A9165505. en

Ben Lamine, J., Boujbiha, M. A., Dahane, S., Cherifa, A. Ben, Khlifi, A., Chahdoura, H., Yakoubi, M. T., Ferchichi, S., El Ayeb, N., & Achour, L. (2019). α-Amylase and α-glucosidase inhibitor effects and pancreatic response to diabetes mellitus on Wistar rats of

Ephedra alata areal part decoction with immunohistochemical analyses. In *Environmental Science and Pollution Research*, 26(10), 9739–9754. https://doi.org/10.1007/s11356-019-04339-3

Ben Mohamed, M., Guasmi, F., Ben Ali, S., Radhouani, F., Faghim, J., Triki, T., Kammoun, N. G., Baffi, C., Lucini, L., & Benincasa, C. (2018). The LC-MS/MS characterization of phenolic compounds in leaves allows classifying olive cultivars grown in South Tunisia. *Biochemical Systematics and Ecology*, *78*(2), 84–90. https://doi.org/10.1016/j.bse.2018.04.005

Birben, E., Sahiner, U., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative Stress and Antioxidant Defense Mechanism in. *WAO Journal*, *22*(96), 9–19. https://waojournal.biomedcentral.com/track/pdf/10.1097/wox.0b013e3182439613

Blair, A., Ritz, B., Wesseling, C., & Freeman, L. B. (2015). Pesticides and human health. *Occupational and Environmental Medicine*, *72*(2), 81–82. https://doi.org/10.1136/oemed-2014-102454

Blalock, J. E. (1984). The immune system as a sensory organ. In *Journal of immunology (Baltimore, Md. : 1950)*, 132 (3), 1067–1070. http://www.ncbi.nlm.nih.gov/pubmed /6363533

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, *181*(4617), 1199–1200. https://doi.org/10.1038/1811199a0

Bogdanova, O. V., Kanekar, S., D'Anci, K. E., & Renshaw, P. F. (2013). Factors influencing behavior in the forced swim test. In *Physiology and Behavior*, 118, 227–239. https://doi.org/10.1016/j.physbeh.2013.05.012

Borsini, F., & Meli, A. (1988). Is the forced swimming test a suitable model for revealing antidepressant activity. *Psychopharmacology*, *94*(2), 147–160. https://doi.org/10.1007/BF00176837

Bouriche, H., & Arnhold, J. (2010). Effect of Cleome arabica leaf extract treated by naringinase on neutrophil degranulation. *Acta Horticulturae*, *854*, 15–22.

https://doi.org/10.17660/ActaHortic.2010.854.1

Boussena, A., Bahri, F., Bouyahyaoui, A., Kouidri, M., & Meziane, M. (2022). Screening of Phytochemical, Evaluation of Phenolic Content, Antibacterial and Antioxydant Activities of Ephedra Alata From the Algerian Sahara. *Journal of Applied Biological Sciences E*, *16*(2), 220–229. https://doi.org/10.5281/zenodo.6590258

Breed, M. D., & Moore, J. (2021). Animal Behavior. In *Animal Behavior*. https://doi.org/10.1016/B978-0-12-819558-1.00019-1

Buege, J. A., & Aust, S. D. (1978). Biomembranes - Part C: Biological Oxidations. *Methods in Enzymology*, *52*, 302–310.

C

Cambon, C., Declume, C., & Derache, R. (1979). Effect of the insecticidal carbamate derivatives (carbofuran, pirimicarb, aldicarb) on the activity of acetylcholinesterase in tissues from pregnant rats and fetuses. In *Toxicology and Applied Pharmacology* , *49*(2), 203–208. https://doi.org/10.1016/0041-008X(79)90242-4

Chagnoux S . (2023). The vascular plants collection (P) at the Herbarium of the Muséum national d'Histoire Naturelle (MNHN - Paris). *MNHN - Museum National d'Histoire Naturelle. Occurrence Dataset /Www.Gbif.Org/Occurrence/437873080*, *Version 69*. https://doi.org/https://doi.org/10.15468/nc6rxy accessed via GBIF.org on 2023-04-07.

Chahal, K. S., Prakash, A., & Majeed, A. B. A. (2015). The role of multifunctional drug therapy against carbamate induced neuronal toxicity during acute and chronic phase in rats. *Environmental Toxicology and Pharmacology*, *40*(1), 220–229. https://doi.org /10.1016/j.etap.2015.06.002

Chouikh, A. (2020). Phytochemical profile, antioxidant, analgesic and hypolipidaemic effects of ephedra alata decne. Female cones extract. *Farmacia*, *68*(6), 1011–1020. https://doi.org/10.31925/farmacia.2020.6.7

Chrousos, G. P. (2009). Stress and disorders of the stress system. *Nature Reviews Endocrinology*, *5*(7), 374–381. https://doi.org/10.1038/nrendo.2009.106

Corsini, E., Liesivuori, J., Vergieva, T., Van Loveren, H., & Colosio, C. (2008). Effects of pesticide exposure on the human immune system. In *Human and Experimental Toxicology*, *27* (9), 671–680. https://doi.org/10.1177/0960327108094509

Craske, M. G., Rauch, S. L., Ursano, R., Prenoveau, J., Pine, D. S., & Zinbarg, R. E. (2009). What is an anxiety disorder. *Depression and Anxiety*, *26*(12), 1066–1085. https://doi.org/10.1002/da.20633

D

Danciu, C., Muntean, D., Alexa, E., Farcas, C., Oprean, C., Zupko, I., Bor, A., Minda, D., Proks, M., Buda, V., Hancianu, M., Cioanca, O., Soica, C., Popescu, S., & Dehelean, C. A. (2018). Phytochemical Characterization and Evaluation of the Antimicrobial, Antiproliferative and Pro-Apoptotic Potential of Ephedra alata Decne. Hydroalcoholic Extract against the MCF-7 Breast Cancer Cell Line. *Molecules (Basel, Switzerland)*, *24*(1). https://doi.org/10.3390/molecules24010013

Dar, M. A., Kaushik, G., & Villareal Chiu, J. F. (2019). Pollution status and bioremediation of chlorpyrifos in environmental matrices by the application of bacterial communities: A review. *J Environ Manage*, 1(239), 124 -136. https://doi.org/10.1016/B978-0-12-818095-2.00002-3

Dbeibia, A., Taheur, F. Ben, Altammar, K. A., Haddaji, N., Mahdhi, A., Amri, Z., Mzoughi, R., & Jabeur, C. (2022). Control of Staphylococcus aureus methicillin resistant isolated from auricular infections using aqueous and methanolic extracts of Ephedra alata. In *Saudi Journal of Biological Sciences* 29 (2), 1021–1028. https://doi.org/10. 1016/ j.sjbs.2021.09.071

Dehkordi, N. V., Kachouie, M. A., Pirbalouti, A. G., Malekpoor, F., & Rabei, M. (2015). Total phenolic content, antioxidant and antibacterial activities of the extract of Ephedra procera fisch. et mey. *Acta Poloniae Pharmaceutica - Drug Research*, *72*(2), 341–345.

Díaz-Morán, S., Estanislau, C., Cañete, T., Blázquez, G., Ráez, A., Tobeña, A., & Fernández-Teruel, A. (2014). Relationships of open-field behaviour with anxiety in the elevated zero-maze test: Focus on freezing and grooming. *World Journal of Neuroscience*,

04(01), 1–11. https://doi.org/10.4236/wjns.2014.41001

Dong, Y., & Benveniste, E. N. (2001). Immune function of astrocytes. *Glia*, *36*(2), 180–190. https://doi.org/10.1002/glia.1107

E

EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS). (2013). Scientific Opinion on safety evaluation of Ephedra species for use in food. *European Food Safety Authority,* 11(11), 3467.

Elhadef, K., Smaoui, S., Fourati, M., Ben Hlima, H., Chakchouk Mtibaa, A., Sellem, I., Ennouri, K., & Mellouli, L. (2020). A Review on Worldwide Ephedra History and Story: From Fossils to Natural Products Mass Spectroscopy Characterization and Biopharmacotherapy Potential. In *Evidence-based Complementary and Alternative Medicine*, 2022, 22. https://doi.org/10.1155/2020/1540638

Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics*, 82(1), 70–77. https://doi.org/10.1016/0003-9861(59)90090-6

Elshama, S. S., El-Kenawy, A. E. M., & Osman, H. E. H. (2017). Histopathological study of zinc oxide nanoparticle-induced neurotoxicity in rats. *Current Topics in Toxicology*, *13*, 95–103.

F

Fodor, A., Kovács, K. B., Balázsfi, D., Klausz, B., Pintér, O., Demeter, K., Daviu, N., Rabasa, C., Rotllant, D., Nadal, R., & Zelena, D. (2016). Depressive- and anxiety-like behaviors and stress-related neuronal activation in vasopressin-deficient female Brattleboro rats. *Physiology and Behavior*, *158*(2016), 100–111. https://doi.org/10.1016/j.physbeh.2016.02.041

G

GBIF. (2019). GBIF: the global biodiversity information facility.

Germolec, D. R., Shipkowski, K. A., Frawley, R. P., & Evans, E. (2018). Markers of

inflammation. *Methods in Molecular Biology*, *1803*, 57–79. https://doi.org/10.1007/978-1-4939-8549-4_5

Gonz, D. E., Escobedo-moratilla, A., Flores, J., Hidalgo-figueroa, S., Mart, N., Ram, A., Trujillo, J., & Bautista, E. (2020). A Review of the Ephedra genus : Distribution , Ecology. *Molecules*, *25*(14):3283. https://doi: 10.3390/molecules25143283.

Gunathilake, K. D. P. P., Ranaweera, K. K. D. S., & Rupasinghe, H. P. V. (2018). In vitro anti-inflammatory properties of selected green leafy vegetables. *Biomedicines*, *6*(4), 1–10. https://doi.org/10.3390/biomedicines6040107

Guo, Q., Li, F., Duan, Y., Wen, C., Wang, W., Zhang, L., Huang, R., & Yin, Y. (2020). Oxidative stress, nutritional antioxidants and beyond. *Science China Life Sciences*, *63*(6), 866–874. https://doi.org/10.1007/s11427-019-9591-5

Gupta, R. C. (2014). Carbamate Pesticides. *Encyclopedia of Toxicology: Third Edition*, 661–664. https://doi.org/10.1016/B978-0-12-386454-3.00106-8

H

Haddad, J. J., Saadé, N. E., & Safieh-Garabedian, B. (2002). Cytokines and neuro-immune-endocrine interactions: A role for the hypothalamic-pituitary-adrenal revolving axis. *Journal of Neuroimmunology*, *133*(1–2), 1–19. https://doi.org/10.1016/S0165-5728(02)00357-0

Hannoodee, S., & Nasuruddin, D. N. (2021). Acute inflammatory response.

Han, K. H., Hashimoto, N., & Fukushima, M. (2016). Relationships among alcoholic liver disease, antioxidants, and antioxidant enzymes. *World Journal of Gastroenterology*, *22*(1), 37–49. https://doi.org/10.3748/wjg.v22.i1.37

Hardt, J., Appl, U., & Angerer, J. (1999). Biological monitoring of exposure to pirimicarb: Hydroxypyrimidines in human urine. *Toxicology Letters*, *107*(1–3), 89–93. https://doi.org/10.1016/S0378-4274(99)00035-1

Hashimi, M. H., Hashimi, R., & Ryan, Q. (2020). Toxic Effects of Pesticides on Humans,

Plants, Animals, Pollinators and Beneficial Organisms. *Asian Plant Research Journal*, *5(4),* 37–47. https://doi.org/10.9734/aprj/2020/v5i430114

Hass, U., Christiansen, S., Axelstad, M., Scholze, M., & Boberg, J. (2017). Combined exposure to low doses of pesticides causes decreased birth weights in rats. In *Reproductive Toxicology* 72, 97–105. https://doi.org/10.1016/j.reprotox.2017.05.004

Hassaan, M. A., & El Nemr, A. (2020). Pesticides pollution: Classifications, human health impact, extraction and treatment techniques. *Egyptian Journal of Aquatic Research*, *46*(3), 207–220. https://doi.org/10.1016/j.ejar.2020.08.007

Hernández, A. F., Parrón, T., Tsatsakis, A. M., Requena, M., Alarcón, R., & López-Guarnido, O. (2013). Toxic effects of pesticide mixtures at a molecular level: Their relevance to human health. *Toxicology*, *307(5)*, 136–145. https://doi. org/10.1016/ j.tox.2012.06.009

Hibi, Z., A. Makhloufi, & R.Azzi. (2022). Ethnobotanical, phytochemical characterization and biological activities of Ephedra alata Decne extracts, growing wild in Bechar region, south west of Algeria. *South Asian Journal of Experimental Biology*, *12*(1), 35–45. https://doi.org/10.38150/sajeb.12(1).p35-45

Hodge, H. C., & Sterner, J. H. (1949). Tabulation of Toxicity Classes. *American Industrial Hygiene Association Quarterly*, *10*(4), 93–96. https://doi.org/10.1080/00968204909 344159

Horsak, R. D., Bedient, P. B., Hamilton, M. C., & Thomas, F. Ben. (1964). Pesticides. *Environmental Forensics: Contaminant Specific Guide*, 143–165. https://doi.org/10.1016/B978-012507751-4/50030-6

Huang, S. C., Li, L., Rehman, M. U., Gao, J. dong, Zhang, L. hong, Tong, X. le, Waqas, M., & Li, J. kui. (2019). Tibial growth plate vascularization is inhibited by the dithiocarbamate pesticide thiram in chickens: potential relationship to peripheral platelet counts alteration. In *Environmental Science and Pollution Research*, 26 (36), 36322–36332). https://doi.org/10.1007/s11356-019-06664-z

Huang, X. F., Cheng, W. Bin, Jiang, Y., Liu, Q., Liu, X. H., Xu, W. F., & Huang, H. T. (2020). A network pharmacology-based strategy for predicting anti-inflammatory targets of ephedra in treating asthma. *International Immunopharmacology*, *83*(12), 1567-5769. https://doi.org/10.1016/j.intimp.2020.106423

Hwang, D. H. (2012). Dietary Modulation of Inflammation. *Encyclopedia of Human Nutrition*, 74–78. https://doi.org/10.1016/B978-0-12-375083-9.00161-6

I

Ibragic, S., & Sofić, E. (2015). Chemical composition of various ephedra species. In *Bosnian Journal of Basic Medical Sciences*, 15 (3), 21–27. https://doi.org/ 10.17305/bjbms. 2015.539

IPNI, I. P. N. I. (2020). International Plant Names Index. In *Checklist dataset https://doi. org/10.15468/uhllmw accessed via https://www. gbif. org on* (pp. 9–30).

Itharat, A., Houghton, P. J., Eno-Amooquaye, E., Burke, P. J., Sampson, J. H., & Raman, A. (2004). In vitro cytotoxic activity of Thai medicinal plants used traditionally to treat cancer. *Journal of Ethnopharmacology*, *90*(1), 33–38. https://doi.org/ 10.1016/ j.jep.2003.09.014

J

Jafari, M., Salehi, M., Ahmadi, S., Asgari, A., Abasnezhad, M., & Hajigholamali, M. (2012). The role of oxidative stress in diazinon-induced tissues toxicity in Wistar and Norway rats. *Toxicology Mechanisms and Methods*, *22*(8), 638–647. https://doi.org/10.3109/15376516.2012.716090

Jaradat, N., Dacca, H., Hawash, M., & Abualhasan, M. N. (2021). Ephedra alata fruit extracts: phytochemical screening, anti-proliferative activity and inhibition of DPPH, α-amylase, α-glucosidase, and lipase enzymes. In *BMC Chemistry*, *15* (1), 41. https://doi.org/10.1186/s13065-021-00768-9

Jaradat, N., Hussen, F., & Ali, A. Al. (2015). Preliminary phytochemical screening, quantitative estimation of total flavonoids, total phenols and antioxidant activity of

Ephedra alata decne. *Journal of Materials and Environmental Science*, *6*(6), 1771–1778.

Joly, P. (2014). *Etude des effets de mélanges d ' herbicides employés sur le maïs , sur les communautés microbiennes édaphiques : approche en microcosmes*. (Doctoral dissertation, Clermont-Ferrand 2).

K

Kallassy, H., Fayyad-Kazan, M., Makki, R., El-Makhour, Y., Rammal, H., Leger, D. Y., Sol, V., Fayyad-Kazan, H., Liagre, B., & Badran, B. (2017). Chemical Composition and Antioxidant, Anti-Inflammatory, and Antiproliferative Activities of Lebanese Ephedra Campylopoda Plant. *Medical Science Monitor Basic Research*, *23*, 313–325. https://doi.org/10.12659/MSMBR.905056

Kalyabina, V. P., Esimbekova, E. N., Kopylova, K. V., & Kratasyuk, V. A. (2021). Pesticides: formulants, distribution pathways and effects on human health – a review. *Toxicology Reports*, *8*, 1179–1192. https://doi.org/10.1016/j.toxrep.2021.06.004

Kaphalia, B. S. (2011). Pancreatic Toxicology. *Encyclopedia of Environmental Health*, 315–324. https://doi.org/10.1016/B978-0-444-52272-6.00298-1

Kaushik, P., & Kaushik, G. (2007). An assessment of structure and toxicity correlation in organochlorine pesticides. *Journal of Hazardous Materials*, *143*(1–2), 102–111. https://doi.org/10.1016/j.jhazmat.2006.08.073

Keck, M. E. (2010). Depression. *Switzerland: Lundbeck (Schweiz) AG*.

Kim, K. H., Kabir, E., & Jahan, S. A. (2017). Exposure to pesticides and the associated human health effects. *Science of the Total Environment*, *575*, 525–535. https://doi.org/10.1016/j.scitotenv.2016.09.009

Kim, S. (2019). Bancroft's Theory and Practice of histological techniques eighth edition/S. Ki.

Kmail, A. (2017). In vitro evaluation of anti-inflammatory and antioxidant effects of Asparagus aphyllus L., Crataegus azarolus L., and Ephedra alata Decne.in monocultures and co-cultures of HepG2 and THP-1-derived macrophages. *Pharmacognosy Communications*,

7(1), 24–33. https://doi.org/10.5530/pc.2017.1.4

Knight, P., Chellian, R., Wilson, R., Behnood-Rod, A., Panunzio, S., & Bruijnzeel, A. W. (2021). Sex differences in the elevated plus-maze test and large open field test in adult Wistar rats. *Pharmacology Biochemistry and Behavior*, *204*, 173168. https://doi.org/10.1016/j.pbb.2021.173168

Koh, S. B., Kim, T. H., Min, S., Lee, K., Kang, D. R., & Choi, J. R. (2017). Exposure to pesticide as a risk factor for depression: A population-based longitudinal study in Korea. *Neurotoxicology*, 62, 181–185. https://doi.org/10.1016/j.neuro.2017.07.005

Koner, B. C., Banerjee, B. D., & Ray, A. (1998). Organochlorine pesticide-induced oxidative stress and immune suppression in rats. In *Indian Journal of Experimental Biology* (Vol. 36 (4), 395–398.

Kruger, N. J. (1994). The Bradford method for protein quantitation. *Methods in Molecular Biology (Clifton, N.J.)*, *32*, 9–15. https://doi.org/10.1385/0-89603-268-x:9

****L****

Laflamme, N., & Rivest, S. (2001). Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. *The FASEB Journal*, 15(1), 155–163. https://doi.org/10.1096/fj.00-0339com

Larramendy, M. L., & Soloneski, S. (Eds.). (2019). Pesticides: Use and Misuse and Their Impact in the Environment. BoD–Books on Demand (IntechOpen). https://doi.org/10.5772/intechopen.78909

Lee, G. H., & Choi, K. C. (2020). Adverse effects of pesticides on the functions of immune system. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 235, 108789. https://doi.org/10.1016/j.cbpc.2020.108789

Lee, M. R. (2011). The history of Ephedra (ma-huang). *Journal of the Royal College of Physicians of Edinburgh*, *41*(1), 78–84. https://doi.org/10.4997/JRCPE.2011.116

Leonard, B. E. (2018). Inflammation and depression: A causal or coincidental link to the

pathophysiology? *Acta Neuropsychiatrica*, *30*(1), 1–16. https://doi.org/ 10.1017/ neu.2016.69

Liang, Y., Zhan, J., Liu, D., Luo, M., Han, J., Liu, X., Liu, C., Cheng, Z., Zhou, Z., & Wang, P. (2019). Organophosphorus pesticide chlorpyrifos intake promotes obesity and insulin resistance through impacting gut and gut microbiota. *Microbiome*, 7 (1), 1-15. https://doi.org/10.1186/s40168-019-0635-4

Lieberman, H. R. (2001). The effects of ginseng, ephedrine, and caffeine on cognitive performance, mood and energy. *Nutrition Reviews*, *59*(4), 91–102. https://doi.org/10.1111/j.1753-4887.2001.tb06995.x

López, O., Hernández, A. F., Rodrigo, L., Gil, F., Pena, G., Serrano, J. L., Parrón, T., Villanueva, E., & Pla, A. (2007). Changes in antioxidant enzymes in humans with long-term exposure to pesticides. *Toxicology Letters* 171 (3), 146–153. https://doi.org/ 10.1016/j.toxlet.2007.05.004

Lopresti, A. L., Maker, G. L., Hood, S. D., & Drummond, P. D. (2014). A review of peripheral biomarkers in major depression: The potential of inflammatory and oxidative stress biomarkers. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *48*, 102–111. https://doi.org/10.1016/j.pnpbp.2013.09.017

Lucisano-Valim, Y. M., Kabeya, L. M., Kanashiro, A., Russo-Carbolante, E. M. S., Polizello, A. C. M., Azzolini, A. E. C. S., Silva, S. C., Lopes, J. L. C., De Oliveira, C. A., & Mantovani, B. (2002). A simple method to study the activity of natural compounds on the chemiluminescence of neutrophils upon stimulation by immune complexes. *Journal of Pharmacological and Toxicological Methods*, *47*(1), 53–58. https://doi.org/10.1016/S1056-8719(02)00206-X

Lushchak, V. I., Matviishyn, T. M., Husak, V. V., Storey, J. M., & Storey, K. B. (2018). Pesticide toxicity: A mechanistic approach. *Experimental and Clinical Sciences*, *17*, 1101–1136. https://doi.org/10.17179/excli2018-1710

Luximon-Ramma, A., Bahorun, T., Soobrattee, M. A., & Aruoma, O. I. (2002). Antioxidant activities of phenolic, proanthocyanidin, and flavonoid components in

extracts of Cassia fistula. *Journal of Agricultural and Food Chemistry*, *50*(18), 5042–5047. https://doi.org/10.1021/jf0201172

Lv, M., Chen, J., Gao, Y., Sun, J., Zhang, Q., Zhang, M., Xu, F., & Zhang, Z. (2015). Metabolomics based on liquid chromatography with mass spectrometry reveals the chemical difference in the stems and roots derived from Ephedra sinica. *Journal of Separation Science*, *38*(19), 3331–3336. https://doi.org/10.1002/jssc.201500529

M

M.U. Kalgo , K.M. Hamid , U. A. Muhammad , A. Balarabe , M. H. Yeldu , I.S. Yahaya , Z.M. Kalgo , B. Aliyu, Y. G. B. (2020). Effects Of Aqueous Stem Bark Extract Of Vitellaria Paradoxa On Human Neutrophil Function And Viability. *International Journal of Biological & Medical Research, 10*(3), 6782–6787.

Madani, F. Z., Hafida, M., Merzouk, S. A., Loukidi, B., Taouli, K., & Narce, M. (2016). Hemostatic, inflammatory, and oxidative markers in pesticide user farmers. *Biomarkers*, *21*(2), 138–145. https://doi.org/10.3109/1354750X.2015.1118545

Madkour, D. A., Ahmed, M. M., Orabi, S. H., Sayed, S. M., Korany, R. M. S., & Khalifa, H. K. (2021). Nigella sativa oil protects against emamectin benzoate-Induced neurotoxicity in rats. *Environmental Toxicology*, *36*(8), 1521–1535. https://doi.org/10.1002/tox.23149

Maksoud, H. A., Mahfouz, M., Soliman, M., Elharrif, M. G., Abbass, M., & El-Badry, M. (2020). Harmful effects of pyrethroid ester insecticide on the male reproductive system mainly through affecting testicular function and inflammatory markers. *Biocell*, *44*(1), 111–115. https://doi.org/10.32604/BIOCELL.2020.08399

Marco, G. J. (1968). *A rapid method for evaluation of antioxidants - Marco - 1968 - Journal of the American Oil Chemists' Society - Wiley Online Library*. American Oil Chemists' Society. https://doi.org/https://doi.org/10.1007/BF02668958

Marklund, S., & Marklund, G. (1974). Involvement of the Superoxide Anion Radical in the Autoxidation of Pyrogallol and a Convenient Assay for Superoxide Dismutase. *European*

Journal of Biochemistry, *47*(3), 469–474. https://doi.org/10.1111/j.1432-1033. 1974. tb03714.x

Mary F.Dallman. (2010). Stress-induced obesity and the emotional nervous system - ScienceDirect. In *Trends in Endocrinology and metabolism*, 21 (3), 159–165.

Mayadas, T. N., Cullere, X., & Lowell, C. A. (2014). The multifaceted functions of neutrophils. In *Annual Review of Pathology: Mechanisms of Disease*, 9, 181–218. https://doi.org/10.1146/annurev-pathol-020712-164023

Mello, A. D. A. F. De, Mello, M. F. De, Carpenter, L. L., & Price, L. H. (2003). Update on stress and depression : the role of the hypothalamic-pituitary-adrenal (HPA) axis Uma atualização sobre estresse e depressão : o papel do eixo. *Revista Brasileira de Psiquiatria*, *25*(4), 231–238.

Menze, E. T., Tadros, M. G., Abdel-Tawab, A. M., & Khalifa, A. E. (2012). Potential neuroprotective effects of hesperidin on 3-nitropropionic acid-induced neurotoxicity in rats. *NeuroToxicology*, *33*(5), 1265–1275. https://doi.org/10.1016/j.neuro.2012.07.007

Merlot, E. (2004). Conséquences du stress sur la fonction immunitaire chez les animaux d'élevage= Consequences of stress on immune function in farm animals. *Productions Animales*, *17*(4), 255–264. http://cat.inist.fr/?aModele=afficheN&cpsidt=16329514

Miao, S. M., Zhang, Q., BI, X. B., Cui, J. L., & Wang, M. L. (2020). A review of the phytochemistry and pharmacological activities of Ephedra herb. *Chinese Journal of Natural Medicines*, *18*(5), 321–344. https://doi.org/10.1016/S1875-5364(20)30040-6

Mighri, H., Akrout, A., Bennour, N., Eljeni, H., Zammouri, T., & Neffati, M. (2019). LC/MS method development for the determination of the phenolic compounds of Tunisian Ephedra alata hydro-methanolic extract and its fractions and evaluation of their antioxidant activities. *South African Journal of Botany*, *124*, 102–110. https://doi.org/ 10.1016/ j.sajb.2019.04.029

Moreira, S., Silva, R., Carrageta, D. F., Alves, M. G., Seco-Rovira, V., Oliveira, P. F., & de Lourdes Pereira, M. (2022). Carbamate Pesticides: Shedding Light on Their Impact

on the Male Reproductive System. *International Journal of Molecular Sciences*, *23*(15), 1–13. https://doi.org/10.3390/ijms23158206

Mössner, R., & Lesch, K. P. (1998). Role of serotonin in the immune system and in neuroimmune interactions. *Brain, Behavior, and Immunity*, *12*(4), 249–271. https://doi.org/10.1006/brbi.1998.0532

Müller, L., Gnoyke, S., Popken, A. M., & Böhm, V. (2010). Antioxidant capacity and related parameters of different fruit formulations. *LWT - Food Science and Technology*, *43*(6), 992–999. https://doi.org/10.1016/j.lwt.2010.02.004

Nguyen, M. D., Julien, J. P., & Rivest, S. (2002). Innate immunity: The missing link in neuroprotection and neurodegeneration? *Nature Reviews Neuroscience*, 3 (3), 216–227. https://doi.org/10.1038/nrn752

N

Niaounakis, M. (2017). Environmental, Social, and Economic Impacts. *Management of Marine Plastic Debris*, 57-126. https://doi.org/10.1016/b978-0-323-44354-8.00002-1

Niki, E. (2008). Lipid peroxidation products as oxidative stress biomarkers. *BioFactors*, *34*(2), 171–180. https://doi.org/10.1002/biof.5520340208

O

OECD, T. N. (2001). Acute Oral Toxicity: Acute Toxic Class Method. *Test Guideline*, 423.

Oyaizu, M. (1986). Studies on products of browning reaction. Antioxidative activities of products of browning reaction prepared from glucosamine. *The Japanese Journal of Nutrition and Dietetics*, *44*(6), 307–315. https://doi.org/10.5264/eiyogakuzashi.44.307

Özyürek, M., Güngör, N., Baki, S., Güçlü, K., & Apak, R. (2012). Development of a silver nanoparticle-based method for the antioxidant capacity measurement of polyphenols. *Analytical Chemistry*, *84*(18), 8052–8059. https://doi.org/10.1021/ac301925b

P

Pawlowska, A. M., Camangi, F., & Braca, A. (2010). Quali-quantitative analysis of flavonoids of Cornus mas L. (Cornaceae) fruits. *Food Chemistry*, *119*(3), 1257–1261. https://doi.org/10.1016/j.foodchem.2009.07.063

Pellow, S., Chopin, P., File, S. E., & Briley, M. (1985). Validation of open : closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *Journal of Neuroscience Methods*, *14*(3), 149–167. https://doi.org/10.1016/0165-0270(85)90031-7

Pinos, H., Carrillo, B., Merchán, A., Biosca-Brull, J., Pérez-Fernández, C., Colomina, M. T., Sánchez-Santed, F., Martín-Sánchez, F., Collado, P., Arias, J. L., & Conejo, N. M. (2021). Relationship between prenatal or postnatal exposure to pesticides and obesity: A systematic review. *International Journal of Environmental Research and Public Health*, *18*(13), 1–24. https://doi.org/10.3390/ijerph18137170

Piovezan, A. P., D'Orléans-Juste, P., Tonussi, C. R., & Rae, G. A. (1997). Endothelins potentiate formalin-induced nociception and paw edema in mice. *Canadian Journal of Physiology and Pharmacology*, *75*(6), 596–600. https://doi.org/10.1139/y97-057

Pirimicarb - PubChem. (2023). https://pubchem.ncbi.nlm.nih.gov/compound/ Pirimicarb# section=2D-Structure

Pollack, M. H. (2005). Comorbid anxiety and depression. *Journal of Clinical Psychiatry*, *66*(8), 22–29.

Porsolt, R. D., Anton, G., Blavet, N., & Jalfre, M. (1978). Behavioural despair in rats: A new model sensitive to antidepressant treatments. *European Journal of Pharmacology*, *47*(4), 379–391. https://doi.org/10.1016/0014-2999(78)90118-8

Possamai, F. P., Fortunato, J. J., Feier, G., Agostinho, F. R., Quevedo, J., Wilhelm Filho, D., & Dal-Pizzol, F. (2007). Oxidative stress after acute and sub-chronic malathion intoxication in Wistar rats. In *Environmental Toxicology and Pharmacology,* 23 (2), 198–204). https://doi.org/10.1016/j.etap.2006.09.003

Pundir, C. S., Malik, A., & Preety. (2019). Bio-sensing of organophosphorus pesticides: A review. *Biosensors and Bioelectronics*, *140*, 111348. https://doi.org/ 10.1016/

j.bios.2019.

Q

Qadir, M. I., Ali, M., & Ibrahim, Z. (2014). Anticancer activity of Morus nigra leaves extract. *Bangladesh Journal of Pharmacology*, *9*(4), 171–179. https://doi.org/ 10.3329/ bjp.v9i4.19783

Queiroz, M. L. S., Fernandes, M. D., & Valadares, M. C. (1999). Neutrophil function in workers exposed to organophosphate and carbamate insecticides. *International Journal of Immunopharmacology*, 21 (4), 263–270. https://doi.org/10.1016/S0192-0561(99)00006-5

Quézel, P., & Santa, S. (1962). Nouvelle Flore de l'Algérie et des Régions désertiques méridionales. Editions Du Centre National De La Recherche Scientifique, Tome 1.

R

Radenkovs, V., Püssa, T., Juhnevica-Radenkova, K., Anton, D., & Seglina, D. (2018). Phytochemical characterization and antimicrobial evaluation of young leaf/shoot and press cake extracts from Hippophae rhamnoides L. *Food Bioscience*, *24*, 56–66. https://doi.org/10.1016/j.fbio.2018.05.010

Rahal, A., Kumar, A., Singh, V., Yadav, B., Tiwari, R., Chakraborty, S., & Dhama, K. (2014). Oxidative stress, prooxidants, and antioxidants: The interplay. *BioMed Research International*, *2014*. https://doi.org/10.1155/2014/761264

Rai, D. K., & Sharma, B. (2007). Carbofuran-induced oxidative stress in mammalian brain. *Molecular Biotechnology*, *37*(1), 66–71. https://doi.org/10.1007/s12033-007-0046-9

Rani, L., Thapa, K., Kanojia, N., Sharma, N., Singh, S., Grewal, A. S., Srivastav, A. L., & Kaushal, J. (2021). An extensive review on the consequences of chemical pesticides on human health and environment. *Journal of Cleaner Production*, *283*, 124657. https://doi.org/10.1016/j.jclepro.2020.124657

Rao, A. (1990). Oxygen radical scavenging activity of curcumin. *International journal of pharmaceutics, 58*, 237–240.

Ravula, A. R., & Yenugu, S. (2021). Pyrethroid based pesticides–chemical and biological aspects. *Critical Reviews in Toxicology*, *51*(2), 117–140. https://doi.org/ 10.1080/ 10408444.2021.1879007

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*(9–10), 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3

Reis Nunes, C., Barreto Arantes, M., Menezes de Faria Pereira, S., Leandro da Cruz, L., De Souza Passos, M., Pereira de Moraes, L., Curcino Vieira, I. J., & Barros de Oliveira, D. (2020). Plants as Sources of Anti-Inflammatory Agents. *Molecules*, *25*(3726), 1–22.

Richard.G., L. (1987). The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology*, *92*(2), 180–185.

Rosman, Y., Makarovsky, I., Bentur, Y., Shrot, S., Dushnistky, T., & Krivoy, A. (2009). Carbamate poisoning: treatment recommendations in the setting of a mass casualties event. *American Journal of Emergency Medicine*, *27*(9), 1117–1124. https://doi.org/ 10.1016/ j.ajem.2009.01.035

Ruscio, A. M., & Khazanov, G. K. (2015). Anxiety and depression. *The Oxford Handbook of Mood Disorders*, 313–324. https://doi.org/10.1093/oxfordhb/9780199973965.013.27

Rustaiyan, A., Javidnia, K., Farjam, M. H., Aboee-Mehrizi, F., & Ezzatzadeh, E. (2011). Antimicrobial and antioxidant activity of the Ephedra sarcocarpa growing in Iran. *Journal of Medicinal Plants Research*, *5*(17), 4251–4255.

S

Sabarwal, A., Kumar, K., & Singh, R. P. (2018). Hazardous effects of chemical pesticides on human health–Cancer and other associated disorders. *Environmental Toxicology and Pharmacology*, *63*(September), 103–114. https://doi.org/10.1016/j.etap.2018.08.018

Saidi, S. A., Al-Shaikh, T. M., Alghamdi, O. A., & Hamden, K. (2022). Ephedra alata subsp.

alenda (Ephedraceae) leaf extracts: phytochemical screening, anti-diabetic, anti-obesity and anti-toxic activities on diabetic-induced liver-kidney-testes toxicities and inhibition of α-amylase and lipase enzymes. *Heliyon*, *8*(12), e11954. https://doi.org/ 10.1016/ j.heliyon.2022.e11954

Sammar, M., Abu-Farich, B., Rayan, I., Falah, M., & Rayan, A. (2019). Correlation between cytotoxicity in cancer cells and free radical-scavenging activity: In vitro evaluation of 57 medicinal and edible plant extracts. *Oncology Letters*, *18*(6), 6563–6571. https://doi.org/10.3892/ol.2019.11054

Schieber, A., Carle, R., Berardini, N., Mihalev, K., & Mollov, P. (2005). Flavonol Glycosides from Distilled Petals of Rosa damascena Mill. *Zeitschrift Fur Naturforschung - Section C Journal of Biosciences*, *60*(5–6), 379–384. https://doi.org/10.1515/znc-2005-5-602

Schwack, W., & Kopf, G. (1993). Photodegradation of the carbamate insecticide pirimicarb. *Zeitschrift Für Lebensmittel-Untersuchung Und -Forschung*, *197*(3), 264–268. https://doi.org/10.1007/BF01185283

Sergievich, A. A., Khoroshikh, P. P., Artemenko, A. F., Zakharenko, A. M., Chaika, V. V., Kodintsev, V. V., Stroeva, O. A., Lenda, E. G., Tsatsakis, A., Burykina, T. I., Agathokleous, E., Kostoff, R. N., Zlatian, O., Docea, A. O., & Golokhvast, K. S. (2020). Behavioral impacts of a mixture of six pesticides on rats. *Science of the Total Environment*, *727*, 138491. https://doi.org/10.1016/j.scitotenv.2020.138491

Sharma, A., Kumar, V., Shahzad, B., Tanveer, M., Sidhu, G. P. S., Handa, N., Kohli, S. K., Yadav, P., Bali, A. S., Parihar, R. D., Dar, O. I., Singh, K., Jasrotia, S., Bakshi, P., Ramakrishnan, M., Kumar, S., Bhardwaj, R., & Thukral, A. K. (2019). Worldwide pesticide usage and its impacts on ecosystem. *SN Applied Sciences*, *1*(11), 1–16. https://doi.org/10.1007/s42452-019-1485-1

Shen, J., Jia, Q., Huang, X., Yao, G., Ma, W., Zhang, H., Ouyang, H., & He, J. (2019). Development of a HPLC-MS/MS Method to Determine the 13 Elements of Semen Cuscutae and Application to a Pharmacokinetic Study in Rats. *Evidence-Based Complementary and Alternative Medicine*, *2019*. https://doi.org/10.1155/2019/6518528

Shepherd, J. K., Grewal, S. S., Fletcher, A., Bill, D. J., & Dourish, C. T. (1994). Behavioural and pharmacological characterisation of the elevated "zero-maze" as an animal model of anxiety. *Psychopharmacology*, *116*(1), 56–64. https://doi.org/10.1007/BF02244871

Shi, H., Noguchi, N., & Niki, E. (2001). Galvinoxyl method for standardizing electron and proton donation activity. *Methods in Enzymology*, *335*(1992), 157–166. https://doi.org/10.1016/S0076-6879(01)35240-0

Shubin, A. V., Demidyuk, I. V., Komissarov, A. A., Rafieva, L. M., & Kostrov, S. V. (2016). Cytoplasmic vacuolization in cell death and survival. *Oncotarget*, *7*(34), 55863–55889. https://doi.org/10.18632/oncotarget.10150

Sies, H. (2020). Oxidative stress: Concept and some practical aspects. *Antioxidants*, *9*(9), 1–6. https://doi.org/10.3390/antiox9090852

Singh, R., Firdous, S., & Sharma, P. (2014). Neurotoxic effect of cypermethrin and protective role of resveratrol in Wistar rats. *International Journal of Nutrition, Pharmacology, Neurological Diseases*, *4*(2), 104. https://doi.org/10.4103/2231-0738.129598

Singleton, V. L., & Rossi, J. A. J. (1965). *Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture,* 16 (3), 144–158.

Sioud, F., Amor, S., Toumia, I. ben, Lahmar, A., Aires, V., Chekir-Ghedira, L., & Delmas, D. (2020). A New Highlight of Ephedra alata Decne Properties as Potential Adjuvant in Combination with Cisplatin to Induce Cell Death of 4T1 Breast Cancer Cells In Vitro and In Vivo. *Cells*, *9*(2), 362. https://doi.org/10.3390/cells9020362

Skinner, B. W., Curtis, K. A., & Goodhart, A. L. (2018). Hypnotics and Sedatives. In *Side Effects of Drugs Annual*, 40. https://doi.org/10.1016/bs.seda.2018.06.002

Smirnoff, N., Cumbes, Q. J. (1989). Hydroxyl Radical Scavenging Activity Of Compatible Solutes. *Phytochemistry*, 28(4), 1057–1060. https://doi.org/10.1016/0031-9422(89) 801 82-7

Soares, T., Ribeiro, D., Proença, C., Chisté, R. C., Fernandes, E., & Freitas, M. (2016).

Size-dependent cytotoxicity of silver nanoparticles in human neutrophils assessed by multiple analytical approaches. *Life Sciences*, *145*, 247–254. https://doi.org/ 10.1016/ j.lfs.2015.12.046

Soumaya, B., Yosra, E., Rim, B. M., Sarra, D., Sawsen, S., Sarra, B., Kamel, M., Wissem, A. W., Isoda, H., & Wided, M. K. (2020). Preliminary phytochemical analysis, antioxidant, anti-inflammatory and anticancer activities of two Tunisian Ephedra species: Ephedra alata and Ephedra fragilis. *South African Journal of Botany*, *135*, 421–428. https://doi.org/10.1016/j.sajb.2020.09.033

Soumaya, B., Yosra, E., Rim, B. M., Sarra, D., Sawsen, S., Sarra, B., Kamel, M., Wissem, A. W., Isoda, H., & Wided, M. K. (2020). Preliminary phytochemical analysis, antioxidant, anti-inflammatory and anticancer activities of two Tunisian Ephedra species: Ephedra alata and Ephedra fragilis. In *South African Journal of Botany* 135 ,421–428. https://doi.org/10.1016/j.sajb.2020.09.033

Soyocak, A., Kurt, H., Cosan, D. T., Saydam, F., Calis, I. U., Kolac, U. K., Koroglu, Z. O., Degirmenci, I., Mutlu, F. S., & Gunes, H. V. (2019). Tannic acid exhibits anti-inflammatory effects on formalin-induced paw edema model of inflammation in rats. *Human and Experimental Toxicology*, *38*(11), 1296–1301. https://doi.org/10.1177/ 0960327119864154

Steckler, T., Holsboer, F., & Reul, J. M. H. M. (1999). Glucocorticoids and depression. *Bailliere's Best Practice and Research in Clinical Endocrinology and Metabolism*, *13*(4), 597–614. https://doi.org/10.1053/beem.1999.0046

Stoytcheva, M. (2012). Pesticides in the Modern World - Trends in Pesticides Analysis. In *Pesticides in the Modern World - Trends in Pesticides Analysis*. IntechOpen. https://doi.org/10.5772/701

Sumalatha, K., Kumar, S. A., & Lakshmi, S. M. (2010). Review on Natural Aphrodisiac Potentials To Treat sexual dysfunction. *International Journal of Pharmacy & Therapeutics*, *1*(1), 6–14.

SwissADME. (2023). http://www.swissadme.ch/index.php

Szydłowska-Czerniak, A., Dianoczki, C., Recseg, K., Karlovits, G., & Szłyk, E. (2008). Determination of antioxidant capacities of vegetable oils by ferric-ion spectrophotometric methods. *Talanta*, *76*(4), 899–905. https://doi.org/10.1016/j.talanta.2008.04.055

T

Tait, A. S., Butts, C. L., & Sternberg, E. M. (2008). The role of glucocorticoids and progestins in inflammatory, autoimmune, and infectious disease. *Journal of Leukocyte Biology*, *84*(4), 924–931. https://doi.org/10.1189/jlb.0208104

Tang, W., Wang, D., Wang, J., Wu, Z., Li, L., Huang, M., Xu, S., & Yan, D. (2018). Pyrethroid pesticide residues in the global environment: An overview. *Chemosphere*, *191*, 990–1007. https://doi.org/10.1016/j.chemosphere.2017.10.115

Terziev, V., & Petkova-Georgieva, S. (2020). The Health and Safety Problems According to the Pesticides Usage in the Ecosystem. Available at SSRN 3653283. https://doi.org/10.2139/ssrn.3653283

Tiss, M., Souiy, Z., Achour, L., & Hamden, K. (2022). Ephedra alata extracts exerts anti-obesity, anti-hyperglycemia, anti-antipyretic and analgesic effects. In *Nutrition and Food Science*; 52 (1), 119–128. https://doi.org/10.1108/NFS-03-2021-0086

Tomiyama, A. J. (2019). Stress and Obesity. *Annual Review of Psychology*, *70*, 703–718. https://doi.org/10.1146/annurev-psych-010418-102936

Topçu, G., Ay, M., Bilici, A., Sarikürkcü, C., Öztürk, M., & Ulubelen, A. (2007). A new flavone from antioxidant extracts of Pistacia terebinthus. *Food Chemistry*, *103*(3), 816–822. https://doi.org/10.1016/j.foodchem.2006.09.028

Torres-Palma, R. A., & Serna-Galvis, E. A. (2018). Sonolysis. In *Advanced Oxidation Processes for Wastewater Treatment: Emerging Green Chemical Technology*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-810499-6.00007-3

Toutain, P. L., & Bousquet-Mélou, A. (2004). Bioavailability and its assessment. *Journal of Veterinary Pharmacology and Therapeutics*, *27*(6), 455–466. https://doi.org/10.1111/j.1365-2885.2004.00604.x

Tudi, M., Li, H., Li, H., Wang, L., Lyu, J., Yang, L., Tong, S., Yu, Q. J., Ruan, H. D., Atabila, A., Phung, D. T., Sadler, R., & Connell, D. (2022). Exposure Routes and Health Risks Associated with Pesticide Application. *Toxics*, *10*(6), 1–23. https://doi.org/ 10.3390/ toxics10060335

Twum, C., & Wei, Y. (2011). The association between urinary concentrations of dichlorophenol pesticides and obesity in children. *Reviews on Environmental Health*, *26*(3), 215–219. https://doi.org/10.1515/REVEH.2011.029

U

Uchendu, C., Ambali, S. F., Ayo, J. O., & Esievo, K. A. N. (2018). Chronic co-exposure to chlorpyrifos and deltamethrin pesticides induces alterations in serum lipids and oxidative stress in Wistar rats: mitigating role of alpha-lipoic acid. *Environmental Science and Pollution Research*, *25*(20), 19605–19611. https://doi.org/10.1007/s11356-018-2185-x

USLU, Z. S. A. (2021). Recent advancements in behavioral testing in rodents. *MethodsX*, 8. https://doi.org/10.1016/j.mex.2021.101536

V

Van Damme, N., Van Hecke, A., Remue, E., Van den Bussche, K., Moore, Z., Gefen, A., Verhaeghe, S., & Beeckman, D. (2020). Physiological processes of inflammation and edema initiated by sustained mechanical loading in subcutaneous tissues: A scoping review. *Wound Repair and Regeneration*, *28*(2), 242–265. https://doi.org/10.1111/ wrr.12777

Verhoeven, W. M. A., Van der Heijden, F. M. M. A., Wijers, F. W. H. M., & Tuinier, S. (2005). Novel antipsychotics: Facts and fictions. *Clinical Neuropsychiatry: Journal of Treatment Evaluation*, 2(4), 212–222.

W

Wahyuni, S., Sarlita, E., & Sufiawati, I. (2022). Relationship between Cortisol, Cytokines and Stress on Training: A Scoping Review. *Journal of International Dental and Medical Research*, *15*(3), 1340–1347.

Wang, P., Wang, H. P., Xu, M. Y., Liang, Y. J., Sun, Y. J., Yang, L., Li, L., Li, W., & Wu, Y. J. (2014). Combined subchronic toxicity of dichlorvos with malathion or pirimicarb in mice liver and serum: A metabonomic study. In *Food and Chemical Toxicology,* 70, 222–230. https://doi.org/10.1016/j.fct.2014.05.027

Webster, J. I., Tonelli, L., & Sternberg, E. M. (2002). Neuroendocrine regulation of immunity. *Annual Review of Immunology*, *20*, 125–163. https://doi.org/10.1146/annurev.immunol.20.082401.104914

Y

Yakubu, M. T., Akanji, M. A., & Oladiji, A. T. (2005). Aphrodisiac potentials of the aqueous extract of Fadogia agrestis (Schweinf. Ex Hiern) stem in male albino rats. *Asian Journal of Andrology*, *7*(4), 399–404. https://doi.org/10.1111/j.1745-7262.2005.00052.x

Yaseen, H. S., Asif, M., Saadullah, M., Mahrukh, Asghar, S., Shams, M. U., Bazmi, R. R., Saleem, M., Yousaf, H. M., & Yaseen, M. (2020). Methanolic extract of Ephedra ciliata promotes wound healing and arrests inflammatory cascade in vivo through downregulation of TNF-α. *Inflammopharmacology*, 28 (6), 1691–1704. https://doi.org/10.1007/s10787-020-00713-7

Yeager, M. P., Guyre, C. A., Sites, B. D., Collins, J. E., Pioli, P. A., & Guyre, P. M. (2018). The stress hormone cortisol enhances interferon-ϒ–mediated proinflammatory responses of human immune cells. *Anesthesia and Analgesia*, *127*(2), 556–563. https://doi.org/10.1213/ANE.0000000000003481

Z

Zengin, G., Mahomoodally, M. F., Rocchetti, G., Lucini, L., Sieniawska, E., Świa¸tek, Ł., Rajtar, B., Polz-Dacewicz, M., Senkardes, I., Aktümsek, A., Picot-Allain, M. C. N., & Montesano, D. (2020). Chemical characterization and bioactive properties of different extracts from fibigia clypeata, an unexplored plant food. *Foods*, *9*(6). https://doi.org/10.3390/foods9060705

Zerargui, F., Saffidine, K., Guemmaz, T., Laroui, H., Arrar, L., & Baghiani, A. (2022).

Antithrombotic , Antihemolitic and Analgesic Activities of Ephedra Alata Alanda Extracts. *Annals of the Romanian Society for Cell Biology*, *26*(01), 3767-3783.

Zhang, B. M., Wang, Z. Bin, Xin, P., Wang, Q. H., BU, H., & Kuang, H. X. (2018). Phytochemistry and pharmacology of genus Ephedra. *Chinese Journal of Natural Medicines*, *16*(11), 811–828. https://doi.org/10.1016/S1875-5364(18)30123-7

Zhou, K., Kerr, N., Armour, M. A., Rigakis, K., & Crown, B. (1996). Loss of pirimicarb residues from contaminated fabrics. *Bulletin of Environmental Contamination and Toxicology*, *57*(1), 29–33. https://doi.org/10.1007/s001289900151

Ziani, B. E. C., Heleno, S. A., Bachari, K., Dias, M. I., Alves, M. J., Barros, L., & Ferreira, I. C. F. R. (2019). Phenolic compounds characterization by LC-DAD- ESI/MSn and bioactive properties of Thymus algeriensis Boiss. & Reut. and Ephedra alata Decne. *Food Research International*, *116*(8), 312–319. https://doi.org/ 10.1016/ j.foodres.2018.08.041

Appendices

Standards compounds (purity ≥ 95%)**:**

Chlorogenic acid, quercetin-3-O-glucoside, kaempferol-3-O-glucoside, kaempferide, luteolin-7-O-glucoside, apigenin, 2,6-di-tert-butyl-4- hydroxytoluene (BHA), dibutylhydroxytoluene (BHT), α-Tocopherol, ascorbic acid, quercetin, gallic acid, tannic acid and trolox were purchased from Sigma Aldrich (St. Louis, MO, USA). Syringic acid was sourced from Fluka Chemika (Buchs, Switzerland). Stock solutions were prepared at 1 mg/mL in methanol and properly diluted before analysis.

Diclofenac (diclamid 25mg/ml) FRATER-RAZES, was purchased from pharmacy.

Solvents used for extraction, LCMS/MS analysis and histologic sections preparation

Were of analytical and HPLC-MS grades, respectively. Methanol, acetonitrile, and formic acid, they were obtained from Fisher Chemicals (Thermo Fisher, Waltham, MA, USA). Ultrapure water was obtained by a Milli-Q system (Millipore, Bedford, MA, USA).

Ethanol 96% , xylene and formaldehyde 37–38% were obtained from PanReac AppliChem;

Certistain was obtained from Merck, Mayer' hematoxylin was obtained from Specilab, and

neoxylene was obtained from Eukitt.

Dimethylsulfoxid (DMSO) was obtained from sigma.

Others chemicals and reagents

- 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′azinobis-3- ethylbenzothiazoline-6-sulfonic acid (ABTS), potassium persulfate ($K_2S_2O_8$), sodium hydroxide (NaOH), monosodium phosphate (NaH_2PO_4,$2H_2O$), disodium phosphate (Na_2HPO_4,$2H_2O$), potassium phosphate monobasic (KH_2PO4), nitroblue tetrazolium (NBT), linoleic acid, β-carotene, potassium ferricyanide (K_3[Fe(CN)6]), trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), tween 40, chloroform, hydrogen peroxide (H_2O_2) 30%, neocuproine, folin ciocalteu, sodium carbonate (Na_2CO_3), copper (II) chloride ($CuCl_2$), ammonium acetate (CH_3COONH_4), potassium acetate (CH_3COOK) aluminum nitrate ($Al(NO_3)_3$), thiobarbituric acid (TBA), silver nitrate (AgNO3), ferrous sulfate ($FeSO_4$), o-Phenanthroline, trisodium citrate ($Na_3C_6H_5O_7$), 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), sodium chloride (NaCl), bradford reagent, bovine serum albumin (BSA), Trizma (tris), hydrogen chloride (HCl),

ethylenediaminetetraacetic acid (EDTA), pyrogallol, magnesium sulfate ($MgSO_4$),activated charcoal and formazan were all obtained from Sigma Aldrich.

- Pirimor 50 DG “pirimicarb” was obtained from Syngenta.
- Dulbecco’s Modified Eagle Medium (DMEM) and fetal calf serum were purchased from gibco -Thermo Fisher Scientific-
- MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was provided by Invitrogen.
- Ficoll-Paque was obtained from GE Healthcare
- Hanks’ balanced salt solution 10 x (HBSS), trypan blue, dextran were delivered from Leuconostoc Mesenteroides.

République Algérienne Démocratique et Populaire
Ministère de l'enseignement supérieur et de la recherche
Comité Consultatif d'Ethique
Du
Centre de Recherche en Biotechnologie

CERTIFICAT D'APPROBATION D'ÉTHIQUE

Le 18/01/2023

À Madame Khattabi Latifa ;
Doctorante Immunologie Moléculaire et cellulaire
Université des Frères Mentouri Constantine (UFMC1)
latifa.khattabi@umc.edu.dz

Numéro/Réf : **Projet N07KH-2021/2023/CCE**

Madame,

J'ai le plaisir de vous informer que les membres du Comité Consultatif d'Éthique (CCE) du Centre de Recherche en Biotechnologie ont examiné votre projet de recherche intitulé «**Impact D'un Pesticide Sur La Sensibilité Du Système Neuro- Immuno Endocrinien Et L'effet Préventif D'un Extrait D'une Plante Médicinale Chez Les Rats Wistar**» soumis pour des considérations d'éthique et ont émis un avis favorable. L'approbation de votre projet de recherche s'est basée sur des documents et des précisions que vous avez fait parvenir au CCE le **22/07/2021.**

Par ailleurs, le Projet a été finalisé Le **15-12-2022** ; par conséquence le présent document est le certificat final délivré pour le projet, et toute demande de renouvellement pour le même projet ne peut être prise en charge par Le Comité.

Je vous souhaite plein succès dans la poursuite de vos travaux de recherche.

Le président

Président du Comité
Consultatif d'Ethique

Pr. Abdelhamid ABERKANE

Kit components

Micro ELISA Plate (Dismountable), Reference Standard, Concentrated, Biotinylated Detection Ab (100×), Concentrated HRP Conjugate (100×), Reference Standard & Sample Diluent, Biotinylated Detection Ab Diluent, HRP Conjugate Diluent, Concentrated Wash Buffer (25×), Substrate Reagent, Stop Solution, Plate Sealer, Product Description 1 copy, Certificate of Analysis

Test principle

Rat IL-1β ELISA Kit of Elabscience, uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Rat IL-1β. Samples (or Standards) are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Rat IL-1β and Avidin Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Rat IL-1β, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Rat IL-1β. You can calculate the concentration of Rat IL-1β in the samples by comparing the OD of the samples to the standard curve.

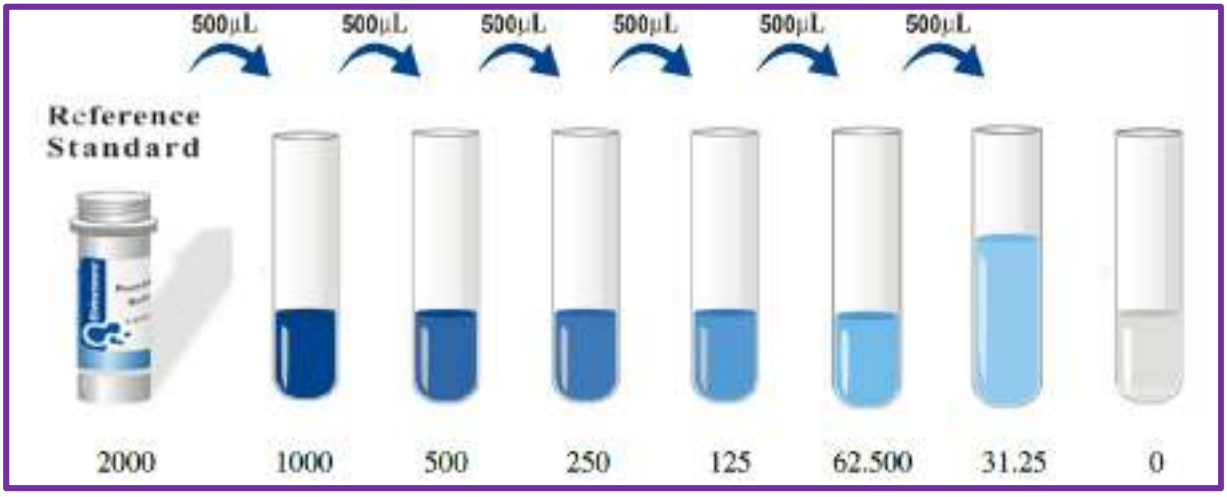
Sample collection

Plasma: Collect plasma using EDTA-Na2 as an anticoagulant. Centrifuge samples for 15 min at 1000 × g at 2-8℃ within 30 min of collection. Collect the supernatant to carry out the assay.
Tissue homogenates: It is recommended to get detailed references from the literature before analyzing different tissue types. For general information, hemolyzed blood may affect the

results, so the tissues should be minced into small pieces and rinsed in ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then homogenized in PBS (tissue weight (g): PBS (mL) volume=1:9) with a glass homogenizer on ice. To further break down the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5-10 min at 5000×g at 2-8℃ to get the supernatant.

Reagent preparation

- Bring all reagents to room temperature (18-25℃) before use
- Wash Buffer: Dilute 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer.
- Standard working solution: Centrifuge the standard at 10,000×g for 1 min. Add 1.0 mL of Reference Standard &Sample Diluent, let it stand for 10 min and invert it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 2000 pg/mL. Then make serial dilutions asneeded. The recommended dilution gradient is as follows : 2000, 1000, 500, 250, 125, 62.500, 31.25, 0 pg/mL.



Dilution procedure using the Reference Standard & Sample Diluent

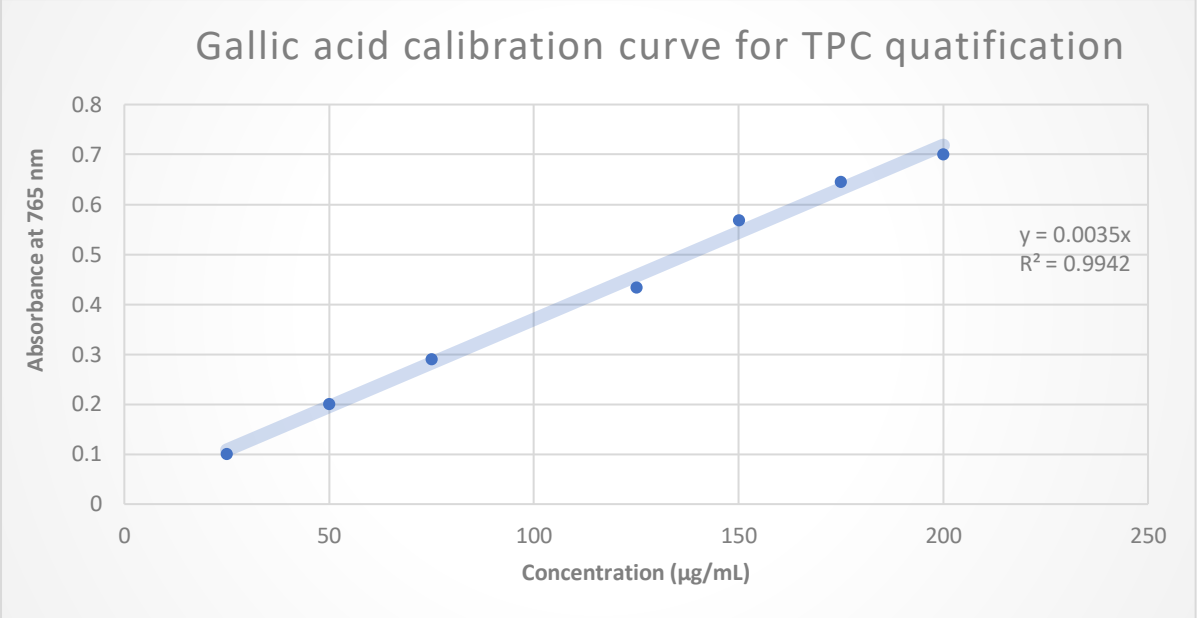
- Biotinylated Detection Ab working solution: Calculate the required amount before the experiment (100 µL/well). Ab at 800×g for 1 min, then dilute the 100× Concentrated Biotinylated Detection Ab to 1× working solution with Biotinylated Detection Ab Diluent(Concentrated Biotinylated Detection Ab: Biotinylated Detection Ab Diluent= 1: 99).
- Concentrated HRP Conjugate working solution: HRP Conjugate is HRP conjugated avidin. Calculate the required amount before the experiment (100 µL/well) Centrifuge the Concentrated HRP Conjugate at 800×g for 1 min, then dilute the 100× Concentrated HRP Conjugate to 1× working solution with HRP Conjugate Diluent(Concentrated HRP Conjugate: HRP Conjugate Diluent= 1: 99).

Assay procedure

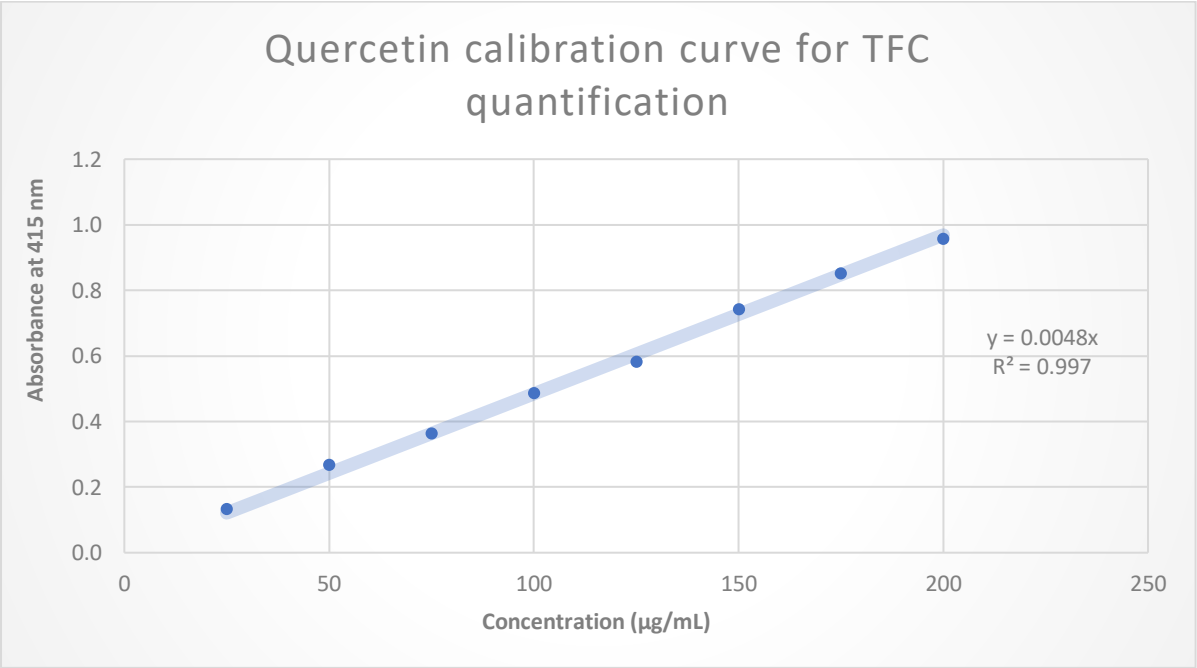
1. Determine wells for diluted standard, blank and sample. Add 100 µL each dilution of standard, blank and sample into the appropriate wells (It is recommended that all samples and standards be assayed in duplicate. Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37℃.
2. Decant the liquid from each well, do not wash. Immediately add 100 µL of Biotinylated Detection Ab working solution to each well. Cover the plate with a new sealer. Incubate for 1 hour at 37°C.
3. Decant the solution from each well, add 350 µL of wash buffer to each well. Soak for 1 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times.
4. Add 100 µL of HRP Conjugate working solution to each well. Cover the plate with a new sealer. Incubate for 30 min at 37°C.
5. Decant the solution from each well, repeat the wash process for 5 times as conducted in step

6. Add 90 µL of Substrate Reagent to each well. Cover the plate with a new sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Preheat the Microplate Reader for about 15 min before OD measurement.
7. Add 50 µL of Stop Solution to each well. Note: adding the stop solution should be done in the same order as the substrate solution.
8. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

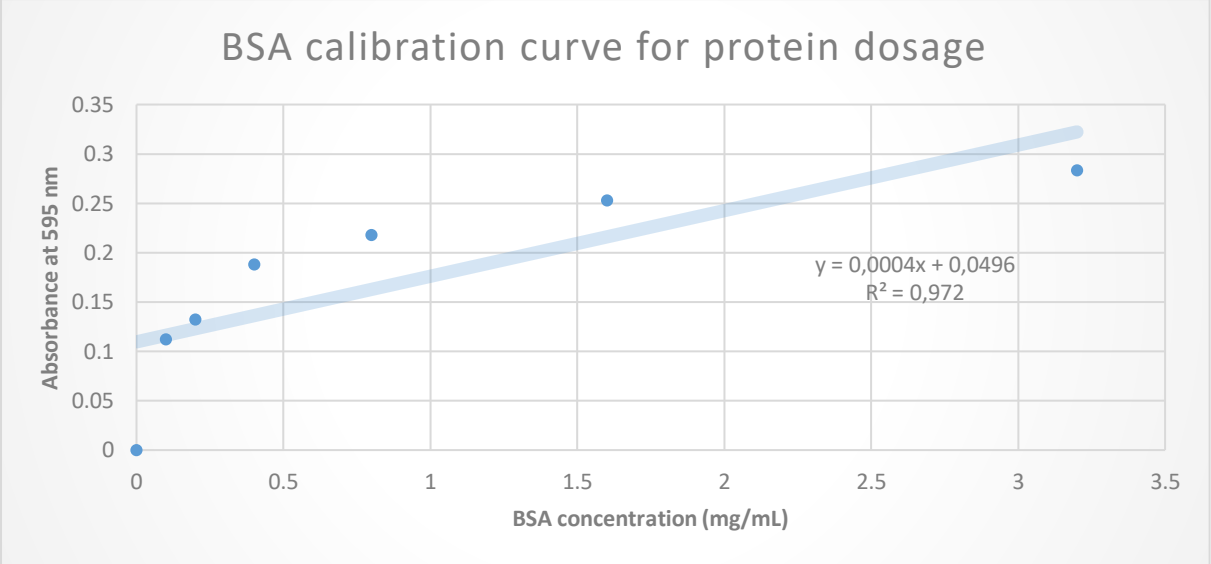
Gallic acid calibration curve



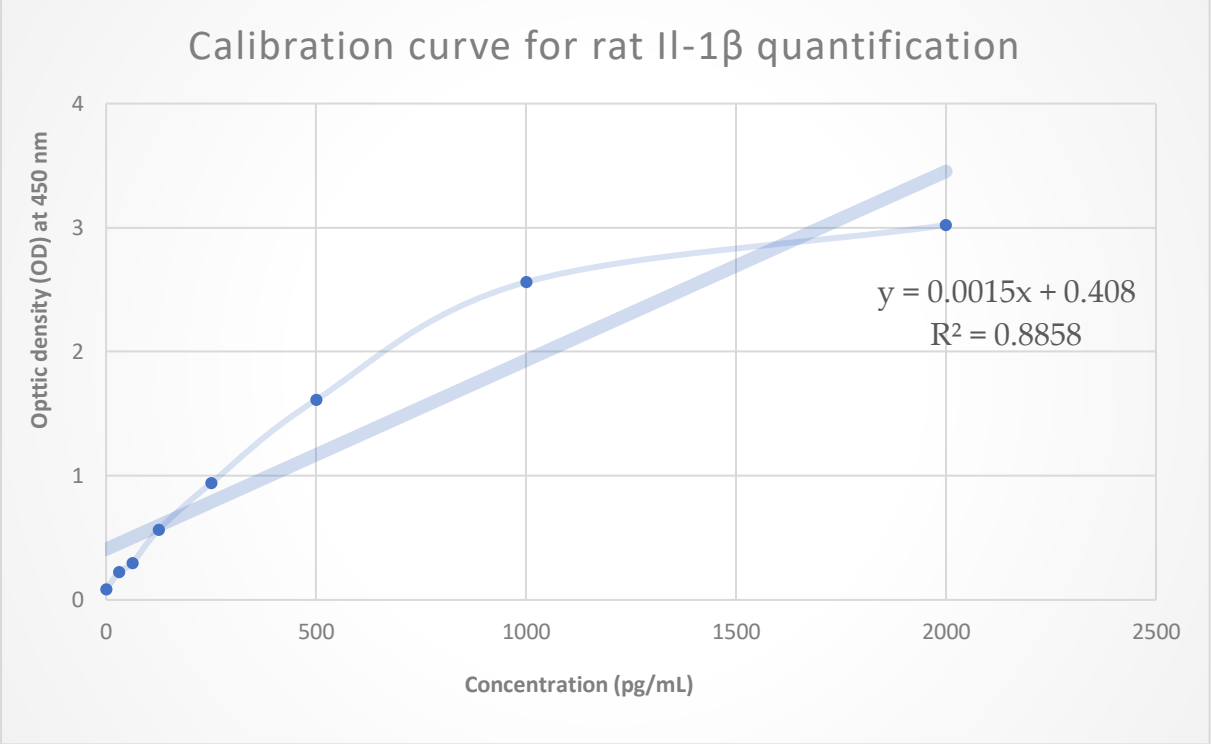
Quercetin calibration curve



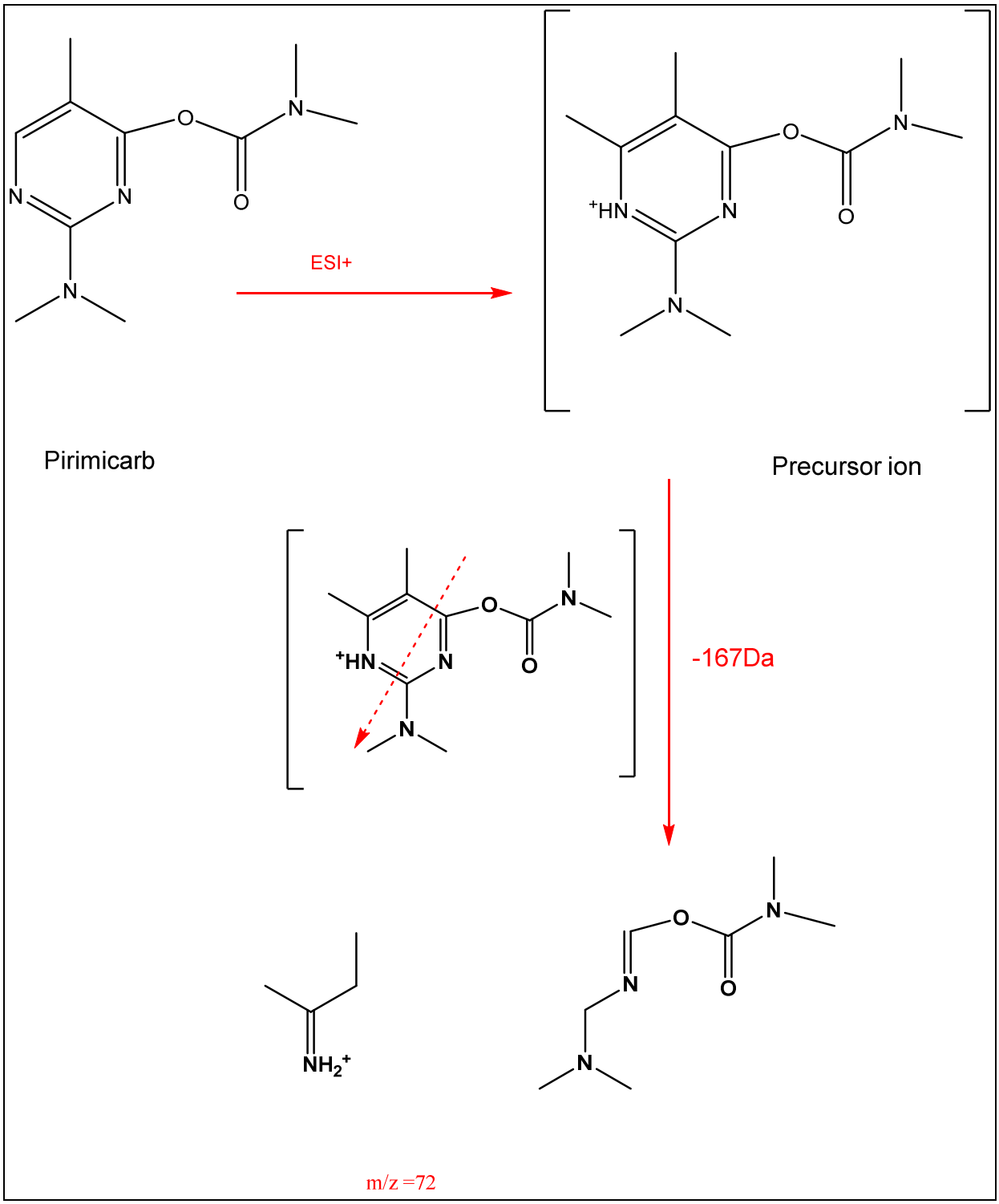
BSA calibration curve



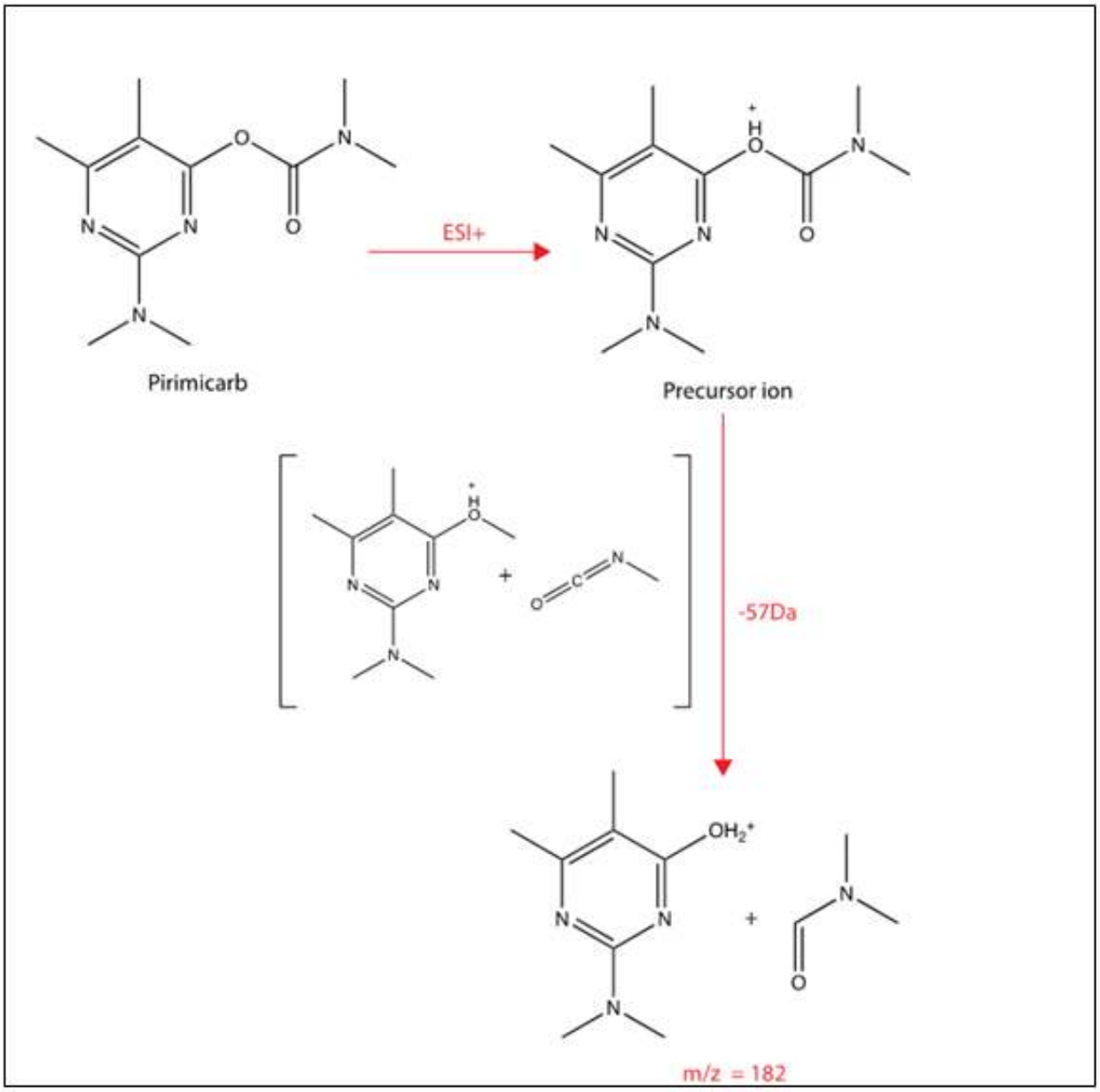
Rat IL-1β calibration curve



Structure of 72-ion fragment



Structure of 182-ion fragment



Abstracts

ABSTRACT

Pesticide users and manufacturers should anticipate exposure to toxicological risks, withal; the general public is not exempted. The use of carbamate insecticides poses a threat to human health, and pirimicarb is the most used carbamate. This research project sought to determine the impact of pirimicarb on the neuroimmune endocrine axis and to evaluate the eventual preventive ability of *Ephedra alata* monjauzeana. Preliminary *in vitro* and *in vivo* investigations were performed to assess the antioxidant, anti-inflammatory and the non-toxic effects of the prepared crude extract from the plant of interest. In addition, LCMS/MS qualitative analysis was conducted for the characterization of the chemical profile. Twenty-one flavonoids and phenolic acids were identified in the EamCE, this latter exhibited a remarkable antioxidant activity and an interesting anti-inflammatory potential. The tests of toxicity and cytotoxicity indicated our plant as non-toxic and relatively harmless. Moreover, the pirimicarb noxiousness was determined by inducing a subacute toxicity on male wistar rats after a period of 28 days of pirimicarb daily gavage (1/10 of LD50 = 145 mg/kg). The exploration of the noxious effects was realized by various analysis. Behavioral tests were used to evaluate the mood and behavior changes and to estimate the physical performance. Oxidative stress was determined by analyzing some parameters, namely: MDA, GSH SOD and CAT. The measurement of inflammatory and hormonal biomarkers such as blood cells count, IL-1 β level, cortisol and testosterone serum titers were also carried. At the histologic scale, the significance of lesions was specifically examined in the brain and testis. In other respects, traces of pirimicarb in extracts of the same organs were searched using LC-MS/MS MRM. Consequently, a considerable status of anxiety and depression was revealed, with a remarkable rise in cortisol, monocytes, IL-1 β (peripheral and cerebral) titers. Likewise, a significant decline in oxidative enzymes and testosterone rate as well as a status of lymphopenia and agranulocytosis were recorded. Additionally, important histological lesions were shown in cerebral cortex and seminiferous tubules. The chromatographic analysis of extracts samples from brain and testis of rats force-fed with pirimicarb provided accurately chromatograms that demonstrated the detection of pirimicarb and approved its accumulation in tissues. The EamCE showed exceptional promising results, as a preventive therapeutic agent, which is characterized by restoring mental and physical performances, enhancing mood, fertility, antioxidant and anti-inflammatory activities, and maintaining tissue integrity. In further, we carried another test to confirm the cytotoxic effect of pirimicarb on immune cells where we used human blood cells from healthy donors, to separate neutrophils and reacting them with different concentrations and mixtures of pirimicarb and EamCE. Therefore, pirimicarb induced the reduction of neutrophils viability percentage, in contrast to the enhanced effect of EamCE. Overall, we believe that is pertinent to mention that EamCE possesses euphoric and preventive properties towards pirimicarb negative impacts that are manifested by disrupting the neuro-immune endocrine axis.

Keywords:

Pirimicarb, *Ephedra alata* monjauseana, neuro-immune endocrine axis, depression, oxidative stress, tissue injuries, infertility, antioxidant, anti-inflammatory, euphoric.

RESUME

Les utilisateurs et les fabricants des pesticides doivent anticiper l'exposition aux risques toxicologiques, en outre, le grand public n'est pas épargné. L'utilisation des insecticides à base de carbamate constitue une menace pour la santé humaine, le pirimicarbe représente le carbamate le plus utilisé. Ce projet de recherche a pour objectif de déterminer l'impact du pirimicarbe sur l'axe neuro-immuno endocrinien et approuver l'éventuelle capacité préventive d'*ephedra alata* monjauzeana. Des investigations préliminaires *in vitro* et *in vivo* ont été menées pour estimer les effets antioxydants, anti-inflammatoires et non toxiques de l'extrait brut préparé à partir de la plante d'intérêt (EamCE). De plus, une analyse qualitative LCMS/MS a été effectuée pour la caractérisation du profil chimique. Vingt et un flavonoïdes et acides phénoliques ont été identifiés dans l'EamCE, ce dernier a montré une activité anti-oxydante remarquable et un potentiel anti-inflammatoire intéressant. Les tests de toxicité et de cytotoxicité ont désigné notre plante comme non toxique et relativement inoffensive. Par ailleurs, la nocivité du pirimicarbe a été déterminée en induisant une toxicité subaiguë chez les rats wistar mâles après une période de 28 jours de gavage quotidien du pirimicarbe (1/10 de la DL50 = 145 mg/kg). L'exploration des effets nocifs a été réalisée par diverses analyses. Les épreuves comportementales ont été utilisées pour évaluer les changements d'humeur, de comportement et pour estimer les performances physiques. Le stress oxydatif a été déterminé en évaluant quelques paramètres, à savoir : MDA, GSH SOD et CAT. L'estimation des bio-marqueurs inflammatoires et hormonaux tels que le nombre de cellules sanguines, le taux d'IL-1 β, les titres sériques de cortisol et de testostérone a également été réalisée. Au niveau tissulaire, les lésions significatives ont été spécifiquement examinées sur les coupes histologiques du cerveau et des testicules. En outres, les traces du pirimicarbe ont été recherchées dans les extraits des mêmes organes par LC-MS/MS MRM. En conséquence, un statut d'anxiété et de dépression considérable a été bien installé, avec une augmentation évidente du cortisol, des monocytes, d'IL-1 β (périphérique et cérébral). De même, il a été décelé, une baisse significative des enzymes anti-oxydantes et du taux de testostérone ainsi qu'un statut de lymphopénie et d'agranulocytose. De plus, des lésions histologiquement importantes ont été révélées dans le cortex cérébral et les tubules séminifères. L'analyse chromatographique des extraits du cerveau et de testicules de rats gavés avec du pirimicarbe, a fourni avec précision des chromatogrammes qui démontrent la détection du pirimicarbe et approuvent son accumulation dans les tissus. L'EamCE a montré des résultats prometteurs et exceptionnels en tant qu'agent thérapeutique préventif, qui se caractérise par la restauration des performances mentales et physiques, l'amélioration de l'humeur, la fertilité et de l'activité anti-oxydante et anti-inflammatoire ainsi que le maintien de l'intégrité tissulaire. Une investigation ultérieure a été additionnée pour confirmer l'effet cytotoxique du pirimicarbe sur les cellules immunitaires, nous avons utilisé des cellules sanguines humaines provenant de donneurs sains, pour séparer les neutrophiles et les faire réagir avec différentes concentrations et mélanges de pirimicarbe et d'EamCE. Dès lors, le pirimicarb a induit une réduction du pourcentage de viabilité des neutrophiles, contrairement à l'effet améliorateur de l'EamCE. En dernier recours, nous pensons qu'il est pertinent de conclure que l'EamCE possède des propriétés euphorisantes et préventives à l'égard des impacts négatifs du pirimicarbe, qui se manifestent par la perturbation de l'axe neuro-immuno endocrinien.

Mots clés:

Pirimicarbe, *ephedra alata* monjauseana, axe neuro-immuno endocrinien, dépression, stress oxydatif, lésions tissulaire, infertilité, antioxydant, anti-inflammatoire, euphorisant.

ملخص

التعرض لمخاطر التسمم هو امر يجب توقعه لدى مستخدمي ومصنعي مبيدات الآفات. كما ان عامة الناس ليسوا مستثنين. يشكل استخدام المبيدات الحشرية الكرباماتية تهديدًا لصحة الإنسان، ويعد البيريميكارب هو أكثر المبيدات الكرباماتية استخدامًا. يهدف هذا المشروع البحثي إلى تحديد تأثير البيريميكارب على مستوى المحور العصبي المناعي والغدي الصماوي وللتأكد من القدرة الوقائية المحتملة لنبتة الإيفيدرا ألاتا مونجوزينا. تم إجراء اختبارات أولية في الوسط الحيوي وفي المختبر (في الأنبوب) لتقييم التأثيرات المضادة للأكسدة و للالتهابات وغير السامة للمستخلص الخام المحضر من النبتة المعنية (EamCE).بالإضافة إلى ذلك ، تم إجراء تحليل الاستشراب السائل المزود بمقياس طيف الكتلة لتحديد المكونات الكيميائية. تم التعرف على واحد وعشرين مركب من الفلافونويدات والأحماض الفينولية في EamCE، هذا الأخير اظهر نشاطًا ملحوظًا مضادًا للأكسدة و مثيرا للاهتمام كمضاد للالتهابات. بينت اختبارات السمية والسمية الخلوية على ان نبتتنا غير سامة وغير ضارة نسبيًا. من جهة أخرى، تم تحديد اضرار البيريميكارب عن طريق إحداث سمية تحت حادة في ذكور جرذان المعمل بعد فترة 28 يومًا من الاطعام القسري اليومي بالبيريميكارب(10/1 من الجرعة المميتة الوسطية =145 ملغ/ كغ). و اجري كشف الآثار المترتبة من خلال القيام بتحليلات مختلفة. استخدمت الاختبارات السلوكية لتقييم التغيرات المحدثة في المزاج والسلوك ومن اجل تقويم التأهيل البدني. حدد الإجهاد التأكسدي من خلال فحص بعض المعلمات مثل MDA، GSH، SODو CAT. تم أيضًا تحديد المؤشرات الحيوية الالتهابية والهرمونية مثل قياس عدد خلايا الدم ومستوى الانترلوكين1 بيتا، الكورتيزول وهرمون التستوستيرون. على مستوى الأنسجة، تم فحص تواجد آفات مهمة في شرائح نسيجية للدماغ والخصيتين على وجه التحديد. علاوة على ذلك، تم البحث عن آثار البيريميكارب في المستخلصات المحضرة لنفس الأعضاء بواسطة الاستشراب السائل المزود بمقياس طيف الكتلة. كحو لة للنتائج، تم إثبات حالة الارق والاكتئاب الحادين، مع زيادة واضحة في الكورتيزول و الانترلوكين1 بيتا (المصلي والدماغي). سجل ايضا انخفاض كبير في الخلايا الليمفاوية، الخلايا المحببة، الإنزيمات المضادة للأكسدة ومستويات التستوستيرون. علاوة على ذلك، تم الكشف عن آفات نسيجية في غاية الجدية على مستوى القشرة الدماغية والأنابيب المنوية. قدم التحليل الكروماتوغرافي لمستخلصات الدماغ والخصيتين التي تخص الفئران التي تم تغذيتها بالبيريميكارب مخططات كروماتوجرافية دقيقة أظهرت استبانة البيريميكارب واثبتت إمكانية تراكمه في الأنسجة. أبدى EamCE نتائج واعدة وممتازة كعامل علاجي وقائي ، والذي يتميز باستعادة المؤهلات العقلية والبدنية، تحسين المزاج، الخصوبة والنشاط المضاد للأكسدة و للالتهاب وكذلك الحفاظ على سلامة الأنسجة. اجري فحص إضافي للتأكد من التأثير السام للبيريميكارب على الخلايا المناعية، استخدمنا خلايا دم بشرية متحصل عليها من متبرعين أ حاء، من اجل التمكن من فصل العَدِلِيَات واخضاعها للتفاعل مع تراكيز وخلائط مختلفة من البيريميكارب و EamCE. تسبب البيريميكارب في انخفاض النسبة المئوية لحيوية العَدِلِيَات، على عكس التأثيرالمحسن لل EamCE. بناءا على ما سبق يمكننا أن نستنتج أن EamCE يمتلك خصائص مبهجة ووقائية ضد الآثار السلبية للبيريميكارب و التي تتمثل في تعطيل المحور الرابط للجهاز العصبي المناعي والغدي الصماوي.

الكلمات الدالة:

البيريميكارب، الإيفيدرا ألاتا مونجوزينا، المحور العصبي المناعي والغدي الصماوي، الاكتئاب، الإجهاد التأكسدي، الآفات النسيجية، اللاخصوبة ، مضاد للأكسدة، مضاد للالتهاب، مبهج.

Academic year : 2022/2023	**Presented by :** Latifa KHATTABI

Thesis presented for the fulfillment of the Doctorate degree, 3rd cycle
Entitled:
Impact of Pirimicarb on neuro-immune-endocrine system sensitivity and the preventive effect of *Ephedra alata* monjauzeana

Abstract:

Pesticide users and manufacturers should anticipate exposure to toxicological risks, withal; the general public is not exempted. The use of carbamate insecticides poses a threat to human health, and pirimicarb is the most used carbamate. This research project sought to determine the impact of pirimicarb on the neuroimmune endocrine axis and to evaluate the eventual preventive ability of Ephedra alata monjauzeana. Preliminary in vitro and in vivo investigations were performed to assess the antioxidant, anti-inflammatory and the non-toxic effects of the prepared crude extract from the plant of interest. In addition, LCMS/MS qualitative analysis was conducted for the characterization of the chemical profile. Twenty-one flavonoids and phenolic acids were identified in the EamCE, this latter exhibited a remarkable antioxidant activity and an interesting anti-inflammatory potential. The tests of toxicity and cytotoxicity indicated our plant as non-toxic and relatively harmless. Moreover, the pirimicarb noxiousness was determined by inducing a subacute toxicity on male wistar rats after a period of 28 days of pirimicarb daily gavage (1/10 of LD50 = 145 mg/kg). The exploration of the noxious effects was realized by various analysis. Behavioral tests were used to evaluate the mood and behavior changes and to estimate the physical performance. Oxidative stress was determined by analyzing some parameters, namely: MDA, GSH SOD and CAT. The measurement of inflammatory and hormonal biomarkers such as blood cells count, IL-1 β level, cortisol and testosterone serum titers were also carried. At the histologic scale, the significance of lesions was specifically examined in the brain and testis. In other respects, traces of pirimicarb in extracts of the same organs were searched using LC-MS/MS MRM. Consequently, a considerable status of anxiety and depression was revealed, with a remarkable rise in cortisol, monocytes, IL-1 β (peripheral and cerebral) titers. Likewise, a significant decline in oxidative enzymes and testosterone rate as well as a status of lymphopenia and agranulocytosis were recorded. Additionally, important histological lesions were shown in cerebral cortex and seminiferous tubules. The chromatographic analysis of extracts samples from brain and testis of rats force-fed with pirimicarb provided accurately chromatograms that demonstrated the detection of pirimicarb and approved its accumulation in tissues. The EamCE showed exceptional promising results, as a preventive therapeutic agent, which is characterized by restoring mental and physical performances, enhancing mood, fertility, antioxidant and anti-inflammatory activities, and maintaining tissue integrity. In further, we carried another test to confirm the cytotoxic effect of pirimicarb on immune cells where we used human blood cells from healthy donors, to separate neutrophils and reacting them with different concentrations and mixtures of pirimicarb and EamCE. Therefore, pirimicarb induced the reduction of neutrophils viability percentage, in contrast to the enhanced effect of EamCE. Overall, we believe that is pertinent to mention that EamCE possesses euphoric and preventive properties towards pirimicarb negative impacts that are manifested by disrupting the neuro-immune endocrine axis.

Keywords:
Pirimicarb, *ephedra alata* monjauseana, neuro-immune endocrine axis, depression, oxidative stress, leucopenia, tissue injuries, infertility, antioxidant, anti-inflammatory, euphoric.