PEOPLE'S DEMOCRATIC REPUBLIC OF ALGERIA

Ministry of Higher Education and Scientific Research

Echahid Echahid Cheikh Larbi Tebessi University

Faculty of Exact Sciences and Natural and Life Sciences

Department of Applied Biology

MASTER GRADUATE

Field: Natural and life sciences

Major: Biological Sciences

Speciality: Applied biochemistry

Title:

Effect of *Withania somnifera* (Ashwagandha) on biochemical and neurobehavioral disturbances induced by chronic restraint stress in an animal model

Presented by:

Mr. GUEHAIRIA Mohamed ***Mr. TALEB Houssam Eddine***

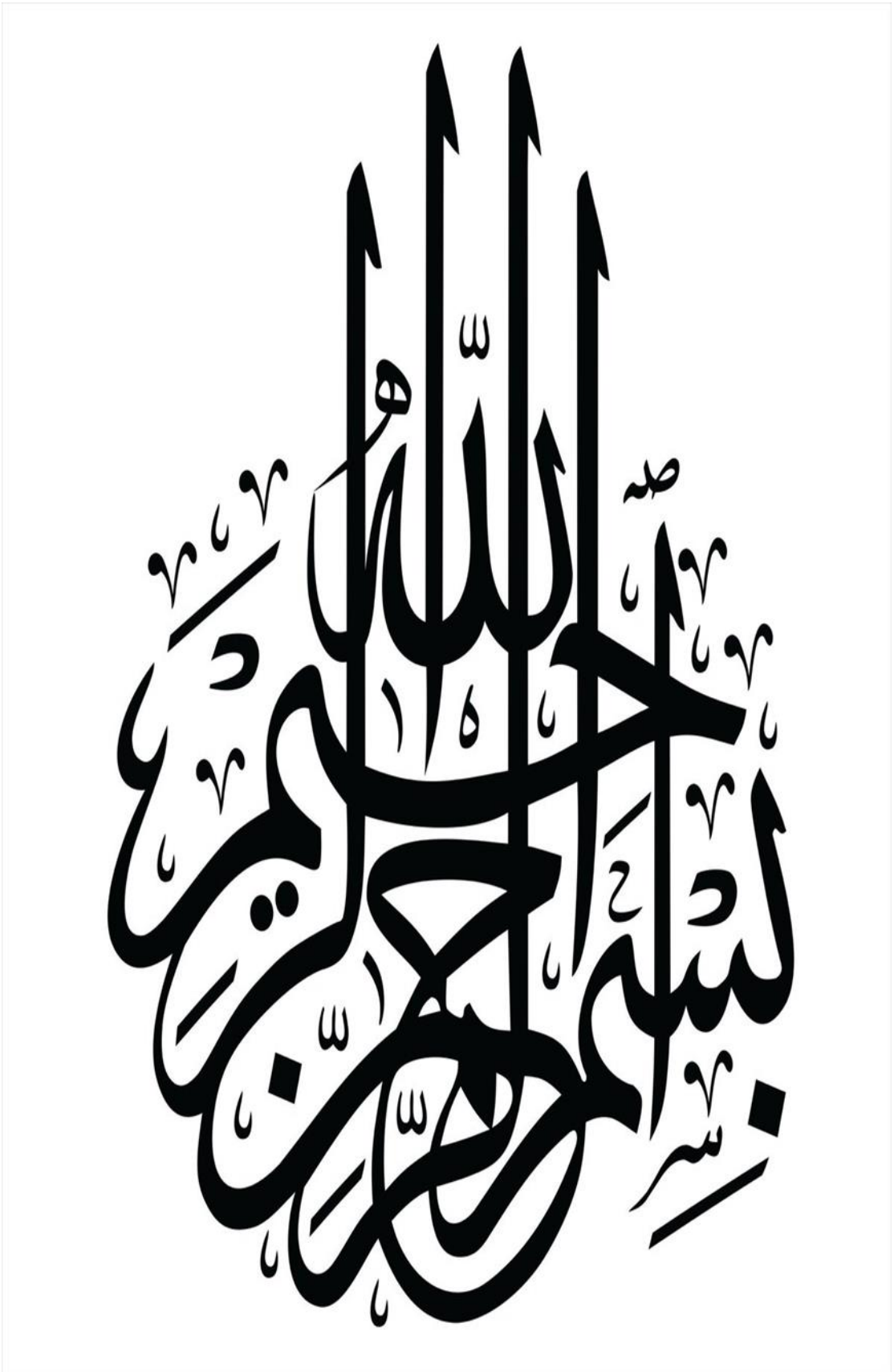
In Front of The jury:

Mm. MESSAADIA Amira	MCB	Examiner	University of Tebessa
Mr. GASMI Salim	MCA	President	University of Tebessa
Mm. GUEDRI Kamilia	MCA	Promoter	University of Tebessa

Defense date : 07 Juin 2023

Note : …… ***Mention :*** ……

بسم الله



Thanks

At the end of this work, we would like to thank Almighty God for giving us the courage, the will and the patience to complete this work.

I have the honor and the pleasure to present my deep gratitude and my sincere thanks to Our supervisor ***Mme. GUEDRI Kamilia****, for his invaluable help, these orientations and the time he granted me for my supervision.*

We would also like to thank the members of the jury, President ***M. GASMI Salim*** *and Examiner* ***Mme. MESSAADIA Amira*** *for having done us the honor of judging our work and attending the defense.*

We extend our sincere thanks to the entire laboratory team, biologists and laboratory technicians for the trust and advice they were able to give me during this study.

Finally, we would like to thank all the people who participated directly or indirectly in the realization of this thesis.

Dedicate

I dedicate this work: To my father **AbdElBaki** and my mother **Salima** who supported me throughout my studies. Also, I dedicate this work: **my brother** & **sister my grandma.**

To all my friends one by one with whom I spent wonderful moments and with who I shared misfortune and happiness in particular: **Houssem berrais,ramzi,yacine,abdelhak,mahdi, both aymen** and **Mr imed eddine benkolli**

Special thanks to **Donia SOLTANIi** my manager without her I couldn't finish this work now.

To all my colleagues: **Houssam, sabah, dhia,ameen, manar, maroua, zineb, sirin, ahlem, sorour, khadidja From Chicago** who helped me through my studies. I wish a future full of joy, success and happiness for them.

To **my Instagram community** and every single one who believed in me.

To all those who have participated directly or indirectly in the development of this project.

Last but not least, I wanna thank me for believing in me

I wanna thank me for doing this hard work.

I wanna thank me for never quitting.

I wanna thank me for always being a giver and trying to give more than I receive.

I wanna thank me for just being me at all time.

GUEHAIRIA Mohamed

Dedicate

A dedication To those whose morals were the Qur'an our Prophet, our beloved, and our leader, Muhammad, may God bless him and grant him peace

To my grandmother and my second mother who left us and left a great place with her loss, may God have mercy on her soul

To the one who took my hand and provided me with the path of learning, **my honorable father**.

To **my dear mother** who gave me kindness and care - **may God preserve her-**

To all the honorable family and the family with all its members. to my colleagues and companions on the path.

To my friend Muhammad Guehairia, who was patient and gave me his time and effort, thank you very very much

To Dr Mme.GUEDRI Kamilia, who gave us her time and helped us accomplish this humble work

To those whom I learned from, and to those who provided me with their advice and guidance, my teachers.

To all who helped me. I dedicate the fruit of this humble effort.

I wanna thank me for doing this hard work.

I wanna thank me for never quitting.

I wanna thank me for always being a giver and trying to give more than I receive.

I wanna thank me for just being me at all time

TALEB Houssam Eddin

Abstract

Stress is a very important current problem for both men and women. in the animal. Each individual, human and animal, is confronted in his daily life with stressful situations, chronic exposure to stress is responsible for the appearance of several pathologies, forced immobilization is one of the best explored stress models in rats. Due to several side effects of chemical medications, many specialists and patients prefer herbal therapy like adaptogen plant as Ashwagandha extracts to cure diseases and disorders associated with chronic stress.

In this study, we will focus on evaluating the possible preventive effects of *Withania somnifera* root extract on oxidation, biochemical and neurobehavioral disturbances induced by chronic restraint stress for 21 days in female Wistar rats.

The application of chronic restraint stress for 3h/d for 21 consecutive days, causes anxiety like depression responses detected by the EPM and FST tests, a deterioration of exploratory and locomotor activity in the OF test associated with a memory deficiency revealed by the TRO test and cerebral oxidative stress revealed by the decrease in the activity of GST and increase in the MDA. and on the other hand, hematological (Leukocytosis) and biochemical (Hyperglycemia, hypercholesterolemia and increased cerebral ACTH disturbances.

In addition, the treatment of stressed rats with the root of organic Ashwagandha attenuated cognitive disorders, decreased anxiety like depression effects and improved cerebral redox status.

In conclusion, Ashwagandha supplementation may improve the biochemical, cognitive, and behavior effects of restraint stress.

Key words: Chronic restraint stress, *Withania somnifera*, Behaviour, Oxidative stress, Rat

Résumé

Le stress est un problème actuel très important pour les hommes et les femmes. Chaque individu, humain et animal, est confronté dans sa vie quotidienne à des situations stressantes, l'exposition chronique au stress est responsable de l'apparition de plusieurs pathologies, l'immobilisation forcée est l'un des modèles de stress les mieux explorés chez le rat. En raison de plusieurs effets secondaires des médicaments chimiques, de nombreux spécialistes et patients préfèrent la phytothérapie comme la plante adaptogène comme les extraits d'Ashwagandha pour guérir les maladies et les troubles associés au stress chronique.

Dans cette étude, nous nous concentrerons sur l'évaluation des effets préventifs possibles de l'extrait de racine de *Withania somnifera* sur l'oxydation, les perturbations biochimiques et neurocomportementales induites par le stress de contention chronique pendant 21 jours chez des rats Wistar femelles.

L'application d'un stress de contention chronique pendant 3h/j pendant 21 jours consécutifs, provoque des réponses anxiogènes et dépressif détectées par les tests EPM et FST, une détérioration de l'activité exploratoire et locomotrice au test OF associée à un déficit de mémoire révélé par le test TRO et stress oxydatif cérébral révélé par la diminution de l'activité de la GST et l'augmentation de la MDA. Et d'autre part hématologique (Leucocytose) et biochimique (Hyperglycémie, hypercholestérolémie et augmentation des troubles cérébraux de l'ACTH.

De plus, le traitement de rats stressés avec la racine d'Ashwagandha organique a atténué les troubles cognitifs, diminué l'anxiété comme les effets de la dépression et amélioré le statut redox cérébral.

En conclusion, la supplémentation en Ashwagandha peut améliorer les effets biochimiques, cognitifs et comportementaux du stress de contention.

Mots clés : Stress de contention chronique, *Withania somnifera*, Comportement, Stress oxydatif, Rat

ملخص

التوتر مشكلة حالية مهمة للغاية عند الرجال والنساء. يواجه كل فرد ، بشريًا وحيوانيًا ، في حياته اليومية مواقف مرهقة، والتعرض المزمن و المستمر للتوتر مسؤول عن ظهور العديد من الأمراض ، ويعد التثبيت القسري أحد أفضل نماذج الإجهاد التي تم اكتشافها في الفئران. بسبب الآثار الجانبية العديدة للأدوية الكيميائية ، يفضل العديد من المتخصصين والمرضى العلاج بالأعشاب مثل نبات التاقلم مثل مستخلص الأشواغاندا لعلاج الأمراض والاضطرابات المرتبطة بالإجهاد المزمن.

في هذه الدراسة، سنركز على تقييم الآثار الوقائية المحتملة لمستخلص جذر الاشوجندا على الأجهاد التاكسدي،والاضطرابات الكيميائية الحيوية والسلوكية العصبية الناجمة عن إلضغط المزمن لمدة 21 يومًا في إناث فئران ويستار.

يؤدي تطبيق إجهاد تثبيت الحركة المزمن لمدة 3 ساعات / يوم لمدة 21 يومًا متتاليًا إلى استجابات الاكتئاب و القلق التي تم الكشف عنها بواسطة اختبارات الصليب المرتفع و السباحة القهرية و ، وتدهور النشاط الاستكشافي والحركي في اختبارالحقل الواسع المرتبط بنقص الذاكرة الذي كشف عنه اختبار التعرف على الاشياء و تم الكشف عن الإجهاد التأكسدي الدماغي من خلال انخفاض نشاط الغلتاثيوت تراتسفيراز و زيادة اكسدة الدهون. ومن ناحية أخرى أمراض الدم (زيادة عدد الكريات البيضاء) والكيمياء الحيوية (ارتفاع السكر في الدم ، فرط كوليسترول الدم واضطرابات ACTH الدماغية.

بالإضافة إلى ذلك، فإن علاج الفئران المجهدة بجذر أشواغاندا العضوية يخفف الاضطرابات السلوكية ، ويقلل من القلق مثل آثار الاكتئاب ويحسن حالة الأكسدة الدماغية و الذاكرة

في الختام، قد تحسن مكملات الاشوجندا من الاضطرابات البيوكيميائية والمعرفية والسلوكية لضغط الاجهاد المزمن.

الكلمات الأساسية: التوتر المزمن، الاشوجندا, السلوك ، الإجهاد التأكسدي ، الجرذ

List of Tables

Table 1 : Classification of Cronquis (L.) 16
Table 2: Variation in weight gain in the control, stressed and treated batch.....39
Table 3: Variation in the absolute weight of the adrenal glands and the brain in the control, stressed and treated batch 40
Tableau 4: Variation in glycaemia and cholesterol in the control, stressed and treated group .41
Table 5: Variations in hematological parameters in control, stressed and treated rats. 42
Table 6: Variation in malondialdehyde levels and glutathione-S-transferase (GST) activity in the brain in the control, stressed and treated group.....44
Table 7: Variation of ACTH in Control, Stressed and Treated Rats.....45
Tableau 8: Variation of OF parameters in the control, stressed and treated batch.....46
Table 9: Variation of FST parameters in the control, stressed and treated batch. 48
Tableau 10: Variation of EPM parameters in the control, stressed and treated batch. 49
Table 11: Variation of TRO parameters in the control, stressed and treated batch. 51

List of Figure

Figure 1: Activation des axes de stress.....9
Figure 2: Withania somnifera plant : (A) plant ; (B) roots and root powder ; (C) flowers ; (D) leaves ; and (E) and fruits**.** 14
Figure 3: Principal biochemical constituents of Withania somnifera.....17
Figure 4: Experimental animals 23
Figure 5: Withania Somnifera root powder 24
Figure 6:schematic illustration of the experiment.....25
Figure 7: Restraint stress procedure 26
Figure 8: The FST test.....27
Figure 9: Elevated plus maze test.....28
Figure 10: The Open Field Test.....29
Figure 11: TRO test 30
Figure 12: The retro-orbital vein bleeding.....31
Figure 13: Dissection and Organs removal.....31
Figure 14: Abacus 380 biochemical automaton with 19 parameters 32
Figure 15:MDA dosage 35
Figure 16:GST dosage 36
Figure 17: calibration curve for bovine serum albumin 37
Figure 18:Protein dosage 37
Figure 19 : Variation in weight gain in the control, stressed and treated batch 39
Figure 20: Variation in the absolute weight of the adrenal glands and the brain in the control, stressed and treated batch 40
Figure 21: Variation in glycaemia and cholesterol in the control, stressed and treated group .41
Figure 22: Variations in hematological parameters in control, stressed and treated rats 43
Figure 23: Variation in malondialdehyde levels and glutathione-S-transferase (GST) activity in the brain in the control, stressed and treated group.....44
Figure 24:Variation of ACTH in Control, Stressed and Treated Rats 45
Figure 25: Variation of OF parameters in the control, stressed and treated batch. 47
Figure 26: Variation of FST parameters in the control, stressed and treated batch.....48
Figure 27:Variation of EPM parameters in the control, stressed and treated batch. 50
Figure 28: Variation of TRO parameters in the control, stressed and treated batch 51

List of Abbreviation

ACTH: adrenocorticotropic hormone

ALT: Alanine Aminotransaminase

AST: aspartate aminotransferase

AVP: arginine vasopressin

AW: Absolut weight

CAM: complementary and alternative medicine

CRH: corticotropin-releasing hormone

CRF: corticotropin-releasing factor

DTNB: 5,5-dithio-bis-2-nitrobenzoic acid

EDTA: Ethylenediaminetetraacetic acid

EPM: the elevated plus maze

FST: the forced swimming test

GAS: General Adaptation Syndrome

GST: Glutathione S Transferase Activity

HPA: hypothalamo- pituitary-adrenal)

LC : locus coeruleus

MDA : Malone-dialdehyde

NaCl: sodium chloride

NOR: Novel object recognition test

OF: Open Field

PK: Pharmacokinetics

PVN: Paraventricular Nucleus

TBA: Thiobarbituric acid

TCA: trichloroacetic acid

TMB: Tetramethylbenzidine

TRO: Object Recognition Test

WS : *Withania somnifera*

Summary

Thanks

Dedicate

Abstract

Résumé

ملخص

List of Table

List of Figure

Liste of Abbrviation

Introduction 1

Chapter I: General Concept on Stress

1 Generality 4

2 Definition of stress 4

3 Types of stress 5

3.1 Acute stress. 5

3.2 Chronic stress 5

4 Physiology of stress 5

5 General adaptation syndrome 6

5.1 The three phases of GAS are: 6

6 The mechanisms of stress 7

7 Effects of acute and chronic stress 9

7.1 Effects of short-term stress (acute stress): 9

7.2 Effects of long-term stress (Chronic stress): 10

8 Restraint stress (Immobilization stress): 11

9 Relationship of Stress to Health Outcomes and Behavior 11

Chapter 2 : Medicinal plant Withania somnifera "Ashwagandha"

1 Description 14

2 Taxonomy 15

3 Chemical composition and pharmacology of Ashwagandha 16

3.1 Chemical composition 16

3.2 Pharmacology 17

4 Bioavailability of WS Compounds 18

5 Biological effects of Ashwagandha 19

5.1 Effects of Ashwagandha on stress and anxiety 19

5.2 Anti-inflammatory 19
6 Safety and toxicity 20
Material and methods 22

Material and methods

1 Material 23
1.1 Animal material 23
1.1.1 Breeding conditions 23
1.1.2 Experimental design 24
1.2 Plant material 24
1.2.1 Preparation of aqueous extract by decoction 24
1.3 Protocol 25
2 Methods 26
2.1 Restraint stress procedure 26
2.2 Behavioral study 26
2.2.1 Forced swimming test (FST) 26
2.2.2 Elevated plus maze test (EPM) 27
2.2.3 Open field test (OF) 28
2.2.4 Novel Object Recognition Test (TRO) 29
2.3 Specimens 30
2.3.1 Blood sample 30
2.3.2 Sacrifice and organs removal 31
2.4 Assay of hematological parameters 32
2.4.1 Study of hematological parameters 32
2.4.2 Study of biochemical parameters 32
2.4.3 Dosage of oxidative stress parameters 34
3 Results 39
3.1 Effect of restraint stress and Ashwagandha treatment on weight change 39
3.2 Effect of restraint stress and Ashwagandha treatment on absolute organ weights 39
3.3 The effect of restraint stress and Ashwagandha on biochemical parameters. ...40
3.3.1 Cholesterol 40
3.3.2 Glycemia 40
3.4 The effect of restraint stress and Ashwagandha on hematological parameters .41
3.5 Effect of restraint stress and Ashwagandha on cerebral redox status 44

3.6 Effect of restraint stress and Ashwagandha on the level of cerebral ACTH: 45

3.7 Behavioral study 45

3.7.1 Effect of restraint stress and Ashwagandha on open field test parameters .45

3.7.2 Effect of restraint stress and Ashwagandha on the parameters of the forced swimming test 48

3.7.3 Effect of Restraint Stress and Ashwagandha on the Parameters of the Elevated Plus-Maze Test.....49

3.7.4 Effect of restraint stress and Ashwagandha on the parameters of the new object recognition test.....51

Discussion.....53

Conclusion and perspectives 62

REFERENCES BIBLIOGRAPHIC 63

INTRODUCTION

Introduction

Stress is a condition arising from external physical or mental overload. It can make a human and animal, feel embattled, nervous, anxious or otherwise less capable of full and normal response to environmental demands. Prolonged exposure to stress can unbalance the mental and physiological state, thereby leading to other illnesses like depression, high blood pressure, cardiac diseases and metabolic disorders. Such conditions, rooted in mental or emotional factors, are rapidly increasing in prevalence and emerging as major global diseases. It is not surprising, therefore, that an increasing fraction of the population is seeking medical help to overcome stress nowadays **(Chandrasekhar et al., 2012);** in response to stressors, a series of behavioral changes occur, neurochemical and immunological that should serve in an adaptive capacity. The hypothalamic-pituitary-adrenal (HPA) axis is a key endocrine adapter against stress factors and plays an important role in the pathophysiology of diseases stress-related psychiatric disorders, such as depression and anxiety disorders **(De Kloet et al., 2005).**

Chronic stress induces hyper activation of the Corticotropic axis (hypothalamo- pituitary-adrenal) and the sympathetic axis, which induces an important secretion of catecholamine and glucocorticoids at the central and peripheral level which is due to the deficienc in the negative feedback exerted by glucocorticoids on the nerve centers and different tissues of the body which would be at the base of the appearance of many physiological and psychological changes, these pathological changes are revealed in the days, weeks, or even years following stressful events through a dysregulation of the stress axis, changes in gene regulations, desensitization of nuclear receptors MRS and GRS. That lead to trouble cardiac, digestive, tumor, hypertension .... etc.

Restraint stress is the most intense type of stress in rodent models and that it has a comparative effect in humans; this type of stress was used in the present study. Exposure to chronic restraint stress in rats has been shown to alter cognitive functions such as learning and memory, as well as performance altered in the Morris water maze test **(Kazushige et al., 2000; Veneroet al., 2002),** as well as certain behavioral parameters in mice, such as the disorders anxious. Furthermore, some studies have shown that reproductive functions are suppressed under various stressful conditions

Naturopathic medicine (also known as naturopathy) is a school of medical philosophy and practice that seeks to improve health and treat disease chiefly by assisting the body's innate

capacity to recover from illness and injury. This alternative medical system of care employs the use of many CAM therapies including acupuncture, herbal medicine, osteopathy, nutrition, homeopathy, and lifestyle counseling **(Smith et al., 2002).** in a combined manner to address the underlying cause of disease. No previous studies have investigated the impact of a naturopathic approach for anxiety.

Adaptogens are herbs that improve an individual's ability to cope with stress. These herbs in times of increased stress, normalize the physiological process of the body and help the body adapt to changes **(Provino et al., 2010)**. A recent definition of an adaptogen is a class of metabolic regulators which increase the ability of an organism to adapt to environmental factors and avoid damage from such factors." Ideally, an adaptogen should: a) decrease stress-induced damage, b) be safe and produce a beneficial effect even if the number of administrations is more than required, c) be devoid of any negative effects such as withdrawal syndromes and d) not influence the normal body functions more than necessary **(Panossian et al., 2009)** *Ashwagandha* is one adaptogen that possesses all of the characteristics listed above.

Withania somnifera (Ashwagandha), has been an important herb in use with in the Ayurvedic and indigenous medical systems for over 3000 years. Clinical trials and animal research support the use of *WS* for anxiety, inflammation, Parkinson's disease, cognitive and neurological disorders and as a useful adjunct for patients undergoing radiation and chemotherapy. *WS* is also used therapeutically as an adaptogen for patients with nervous exhaustion, insomnia, debility due to stress, and as an immune stimulant in patients with low white blood cell counts **(Mishra et al., 2000)**.

The major biochemical constituents of *WS* root are steroidal alkaloids and steroidal lactones in a class of constituents called withanolides **(Mishra et al., 2000)**. At present, 12 alkaloids, 35 withanolides, and several sitoindosides from this plant have been isolated and studied. A sitoindoside is a withanolide containing a glucose molecule at carbon 27. Much of Withania's pharmacological activity has been attributed to two main withanolides, withaferin A and withanolide D **(Mishra et al., 2000).**

The present investigation focuses on the possible ameliorative effects of Ashwagandha organic root extract on biochemical, hematological and behavioral disturbances induced by chronic restraint stress in female Wistar rats.

Chapter I: General Concept on Stress

1 Generality

Nowadays, everyone seems to be talking about stress. You hear about this topic not only in daily conversation but also on television, via radio, in the newspapers, in conferences, centers, and university courses devoted to stress. Yet remarkably few people define the concept in the same way or even bother to attempt a clear-cut definition. The business person thinks of stress as frustration or emotional tension; the air traffic controller, as a problem in concentration; the biochemist and endocrinologist, as a purely chemical event; and the athlete, as muscular tension. This list could be extended to almost every human experience or activity, and, somewhat surprisingly, most people be they chartered accountants, short-order cooks, or surgeons—consider their own occupation the most stressful. Similarly, most commentators believe that ours is the "age of stress," forgetting that the caveman's fear of attack by wild animals or of death from hunger, cold, or exhaustion must have been just as stressful as our fear of a world war, the crash of the stock exchange, or overpopulation. Ironically, there is a grain of truth in every formulation of stress because all demands upon our adaptability do evoke the stress phenomenon. But we tend to forget that there would be no reason to use the **(Selye, 1991).**

Stress affects not only humans, but also the animal world and represents an ethical and economic problem in farm animals. A problematic importance in animal husbandry is that of animal welfare. Many concepts can define the welfare, but it mainly refers to the subjective psychological state of an animal in relationship with its internal and external environment **(Rushen, 2003; Fraser, 2005).**

2 Definition of stress

Stress may be defined as a real or interpreted threat to the physiological or psychological integrity of an individual that results in physiological and/or behavioral responses. In biomedicine, stress often refers to situations in which the adrenal glucocorticoids and catecholamines are elevated because of an experience. Stress is also a subjective experience that may or may not correspond to physiological responses, and the word stress is widely used in many languages as part of daily discourse. There is good stress ' and ' bad stress, and people talk about bad stress as being stressed out **(Fink., 2010).**

Stress involves a stressor and a stress response. A stressor may be a physical insult, such as trauma or injury , or physical exertion , particularly when the body is being forced to operate beyond its capacity Other physical stressors include noise , overcrowding , excessive heat or cold Stressors also include primarily psychological experiences such as time-ressured tasks,

interpersonal conflict , unexpected events , frustration , isolation , and traumatic life events , and all of these types of stressors may produce behavioral responses and evoke physiological consequences such as increased blood pressure , elevated heart rate , increased cortisol levels , impaired cognitive function , and altered metabolism The physiological stress responses include primarily the activation of the autonomic nervous system and the hypothalamo - pituitary - adrenal (HPA) axis, leading to increased blood and tissue levels of catecholamines and glucocorticoids . It is these physiological responses that have both protective and damaging effects **(Fink., 2010).**

3 Types of stress

3.1 Acute stress: In both cases, the body's response is at the physical, psychological level. and biological. It is responsible for disturbances of the different metabolisms and biochemical balance **(Boudarene et al., 1997)**.

3.2 Chronic stress: corresponds to violent or moderate but repeated attacks and close in time. It causes psychological and glandular exhaustion responsible for so-called adaptation diseases. The repetition of stress phenomena requires frequent adaptation and constitutes in the long run an excessive total dose exceeding the resistance threshold of the individual **(Boudarene et al., 1997).** chronic stress models are the most widely used animal models of depression. chronic stressors enhance levels of stress-related hormones by disrupting the hypothalamicpituitary-adrenal (HPA) axis and suppress the production of new neurons in the hippocampus **(Ye Mao et al., 2022)**

4 Physiology of stress

Selye was the first to propose a real theory concerning stress and its effects on the organism. He noticed that different disturbances caused a number of similar responses in animals. According to him, these answers formed the basis of this which he called the General Adaptation Syndrome (GAS) **(Selye., 1956).** The GAS includes three steps. An alarm phase or initial response phase, followed by a resistance phase during which the organism tries to adapt to the disturbance and restore homeostasis. If the body fails to restore balance, it enters a phase exhaustion, which can lead to the appearance of various pathologies or death **(Selye., 1974).**

5 General adaptation syndrome

Physiologists define stress as how the body reacts to a stressor, a stimulus, real or imagined. Acute stressors affect an organism in the short term; chronic stressors over the longer term. The general adaptation syndrome (GAS), developed by Hans Selye, is a profile of how organisms respond to stress; GAS is characterized by three phases: a nonspecific mobilization phase, which promotes sympathetic nervous system activity; a resistance phase, during which the organism makes efforts to cope with the threat; and an exhaustion phase, which occurs if the organism fails to overcome the threat and depletes its physiological resources **(Taylor et Sirois., 2012)**

5.1 The three phases of GAS are:

A. Alarm: Fight or flight, is the immediate response of the body to perceived stress. Physiologically, this starts at the brain's hypothalamus, which acts as the central computer chip of the body regulating such functions as heart rate, blood pressure, respiration, body temperature, digestion, hunger, thirst, and libido. Surrounding the hypothalamus is the limbic system, one of the oldest parts of the brain, which houses the emotions. Powerful feelings such as fear and rage trigger the hypothalamus, which sends messages to the autonomic nervous system by the hypothalamic–pituitary–adrenal (HPA) axis and separately through the autonomic nervous system. The nerves of the autonomic nervous system are split into either parasympathetic or sympathetic branches, the latter of which causes an immediate stress reaction by releasing catecholamine's (adrenaline-like chemicals). These chemicals released from the adrenal gland increase heart rate, respiration, and blood pressure.

The activated hypothalamus also secretes its own hormones and stimulates the pituitary gland to secrete hormones, which produce some of the same effects as those of the catecholamine's but which last some 10 times longer and have a far wider reach. One of these hormones, corticotropin-releasing hormone (CRH), is sent to the pituitary to trigger the release of adrenocorticotropin hormone (ACTH). ACTH travels through the bloodstream to the adrenal glands, on top of the kidneys, to produce glucocorticoids (such as cortisol), which start a cascade of events, including increased blood glucose concentrations, elevated blood pressure, and slowed digestion. Specifically, insulin's ability to facilitate glucose uptake by the cells is reduced, while gluconeogenesis, the synthesis of new glucose (from glycerol and amino acids) is increased. Blood pressure rises as the kidneys are signaled to retain more sodium, which raises water volume in the

blood vessels. Also, digestion slows as hormonal changes cause muscles to become engorged with oxygen and glucose-rich blood that is shunted away from the digestive tract. The body in this conditional response to stressors is ready for fight or flight.

B. **Resistance (adaptation):** the second stage of the stress response, is to achieve optimal adaptation in resisting the stressor. Everyday stressors are beneficial in maintaining the psychophysiological balance that results when the stressor is successfully removed, adapted, or coped with by the person. Stress is actually a necessary component in life because it contributes to survival and, ultimately, growth. Optimal stress fuels maximum performance, but excess stress results when the demands on a person exceed or fall far below their capabilities. Hans Selye said the only time an individual is free from stress is death the ultimate flight. However, too much stress, and failure to adapt and reach a healthy homeostasis, can also result in illness or death.

C. **Exhaustion:** the last stage of the stress response, a continued, chronic response to stress, can be a risk factor for many multifactorial disorders. These in turn may lead to a downward spiral of more stress, exhaustion, and possibly extinction. Eustress becomes distress if not adequately handled by the body and mind. Physical and psychological well-being becomes, decreasing the quality of life, if not its very presence **(Fink., 2017).**

6 The mechanisms of stress

During stress, two axes are mobilized to enable the stress response: the axis corticotrope and sympathetic axis. The control of stress is located in the hypothalamus and the brain. The main players in this central control are the neurons secreting CRF and vasopressin (AVP) located in the paraventricular nucleus of the hypothalamus (PVN) and the locus coeruleus (LC) associated with other groups of brainstem norepinephrine neurons forming the LC/NA system of the sympathetic nervous system **(Chrousos and Gold, 1992; Tsigos and Chrousos, 2002).** The HPA axis and efferents from the sympathetic system to the adrenal medulla represent the 2 main peripheral effector branches allowing the brain to control the different organs during exposure to the stressor **(Tsigos and Chrousos, 2002).**

A. HPA axis

CRF is the first hypothalamic regulator of the HPA axis. He's free at the level of the median eminence in the pituitary portal system by endings nerves coming from the parvocellular zone of the PVN of the hypothalamus following a stimulation of the PVN by other structures. It acts on the corticotropic cells of the anterior pituitary gland to stimulate the release of ACTH into the general circulation. Following when released by the pituitary gland, ACTH stimulates

the adrenal cortex allowing the release of glucocorticoids. Glucocorticoids are the end effectors of the HPA axis and play a major role in the adaptive stress response **(Sapolsky et al., 2000).** They are also of fundamental importance in stopping the stress response because they exert a negative feedback on the hypothalamus and the pituitary allowing to inhibit the release of CRF and ACTH. The activity of the HPA axis is important both in non-stressful conditions only under stressful conditions. Thus, under non-stressful conditions, CRF and AVP are secreted in a pulsatile and synchronized manner into the pituitary portal system following a circadian rhythm **(Charmandari et al., 2005).** This rhythm is controlled by the nuclei suprachiasmatics of the anterior hypothalamus which represent the seat of the regulation of rhythms of many behaviors and physiological processes **(Saper et al., 2005).**

The increase in the amplitude and frequency of pulsatile secretions during early hours of the activity phase (in the morning for diurnal species such as humans, beginning of the night for nocturnal species such as the rat) leads to an increase in the secretion of ACTH and glucocorticoids **(Gudmundsson and Carnes, 1997; Lightman et al., 2008).** Thus, glucocorticoids are secreted according to a precise rhythm during the day time. This rhythm makes it possible to coordinate circadian events such as the phases of glucocorticoids are secreted according to a precise rhythm during the day.

B. Sympathetic axis (Activation of the LC/NA system):

The locus coeruleus (LC), located in the bridge of the brain, is the most important noradrenergic nucleus (A6) of the central nervous system. With noradrenergic nuclei A1 and A2 respectively located in the ventrolateral reticular formation of the medulla oblongata, the LC constitutes the LC/NA system **(Carrasco and Van de Kar, 2003; Itoi, 2008).** Neurons of the LC/NA system have projections between them and towards the limbic system, the hypothalamus, the brainstem and the spinal cord in particular **(Itoi and Sugimoto, 2010).** He seems that this system contributes the majority of the norepinephrine present in the brain because plasma norepinephrine is water soluble and cannot cross the barrier hematoencephalic **(Habib et al., 2001**). During stress, emotional or physical information is integrated by the Relevant structures (amygdala and hippocampus or parabrachial nucleus of the brainstem by example) and can directly regulate the LC/NA system, causing a release massive and very rapid norepinephrine at the central level. The LC/NA system receives fibers nerves of CRF neurons from the PVN of the hypothalamus and reciprocally innervates the PVN with noradrenergic nerve fibers. The activation of PVN by stress then leads to stimulation of the LC/NA system by binding CRF to receptors type 1.

This stimulation is reciprocal because the release of norepinephrine activates the PVN via α1-noradrenergic receptors **(Kiss and Aguilera, 2000; Charmandari et al., 2005; Dunn and Swiergiel, 2008).** As with CRF neurons, noradrenergic neurons are influenced by other central systems. GABAergic and opioid systems inhibit it while the serotonergic and cholinergic systems stimulate it **(Szafarczyk et al., 1993; Kyrou and Tsigos, 2009**).

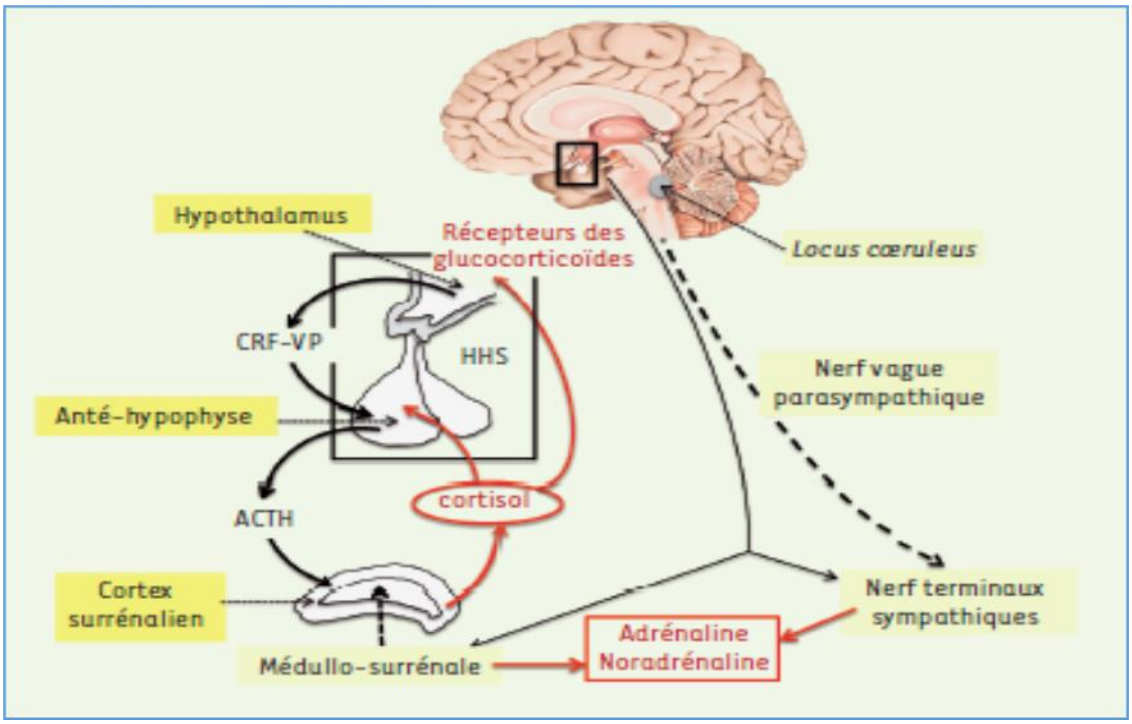


Figure 1: Activation des axes de stress (Moisan, 2012).

7 Effects of acute and chronic stress

In humans as in animals, stress has a beneficial role in the short term, but long-term exposure to adverse consequences on the body.

7.1 Effects of short-term stress (acute stress):

a beneficial adaptive response Acute activation of stress systems leads to a set of changes behavioral and physical defined as the stress syndrome **(Chrousos and Gold, 1992).** These changes are adaptive and normally time-limited as they have aim of optimizing an individual's chances of survival by mobilizing their physical and mental. They are modulated by the stress mediators that are neuropeptides and hormones regulating homeostasis secreted by the hypothalamic-pituitary-adrenal axis (which we will call Corticotropic axis) and the sympathetic nervous system associated with the glands adrenal medulla: corticosteroid-liberin (CRF), adrenocorticotropic hormone (ACTH), glucocorticoids and catecholamines.

The main behavioral adaptations include a state of wakefulness, alertness, and increased cognitive abilities combined with focused attention. Increased intermediate metabolism (glycolysis, gluconeogenesis, lipolysis) promotes the availability of vital substrates. As a corollary, the functions consuming energy such as digestion, growth, immunity is temporarily suppressed allowing optimal use of energy. The success of these adaptations depends not only to the speed with which they are set up, but also to the ability to the body to contain the stress response in quantity and time. Thus, all elements of the stress response are counter-regulated and detoxification functions at cellular level are activated to eliminate from the organism the metabolites produced during the adaptive response. Although adaptive responses to stressors are beneficial in the short term, they can lead to unpleasant manifestations **(Chrousos., 2009).** Indeed, it is common for these responses to lead to allergic manifestations, migraines, episodes of high blood pressure, abdominal pain, indigestion, diarrhea or constipation. Despite these potentially embarrassing manifestations, the response of the organism to a stressor is positive in the short term because it maximizes the survival of the organism. However, the ability to quantitatively and temporally limit these responses adaptation is also crucial for the survival of the individual. If the regulatory processes of the stress response fail or if exposure to stressors is repeated too frequently the adaptive changes may become chronically excessive and the catabolic, anti-reproductive, anti-growth and immunosuppressive effects contribute to the development of adverse effects and pathologies.

7.2 Effects of long-term stress (Chronic stress):

Exposure to chronic stress leads to impaired structures and functions regions of the brain involved in the control of the HPA axis and the system autonomic nervous system. Chronic stress induces structural changes in hippocampus and prefrontal cortex **(Magarinos and Mcewen, 1995; Radley et al., 2008**). At the level of the PVN, an increase in the expression of CRF and AVP and a decrease in expression of GRs are highlighted **(Herman et al. 1995; Makino et al., 1995)** as well as an alteration of many other receptors **(Cullinan, 2000; Ziegler et al., 2005**). Of the neurochemical changes are also demonstrated in many pathways of signaling projecting to the PVN, including increased levels of GABA neurotransmitters in the hypothalamus and the bed nucleus of the stria terminalis **(Bowers et al., 1998).** These changes are at the origin of the deleterious effects of stress chronic. To understand the mechanisms involved in the adaptive response to stress, many stress models have been studied. Indeed, one of the objectives of the study of stress is to know the

mechanisms underlying the activation of the various systems involved and the effects of stress on these mechanisms in different metabolic situations or conditions environmental. Knowing precisely these mechanisms and their modulation makes it possible to put in place programs to combat the negative impact that stress currently has on our society.

8 Restraint stress (Immobilization stress):

Restraint stress is a method used to induce physiological responses in an animal by restricting its free movement. An attractive feature of restraint stress is that it is primarily a psychological stressor, i.e., the physical discomfort induced by restraint is intentionally secondary to the cognitive appraisal of the organism's inability to gain free movement. The popularity of this stressor technique is attributable to its relatively low cost and technical requirements. Restraint stress is also amenable to parametric manipulation, such as duration of restraint, number of restraint sessions, time between restraint sessions, and food intake or deprivation prior to restraint. Moreover, different methodologies have been tailored to meet the needs of researchers from varied disciplines such as neuroscience, endocrinology, physiology, pharmacology, psychology, and gastroenterology **(Servatius et al., 2007)**.

9 Relationship of Stress to Health Outcomes and Behavior

Psychological stress has a deleterious effect on a wide range of physical and mental health outcomes with accumulating evidence that health practices/maladaptive behaviors may mediate these relationships **(McEwen BSet al.,1998)**. Stress has been strongly implicated in the pathogenesis of coronary heart disease and the incidence of acute myocardial infarctions. Those under high stress are less likely to survive cardiac events. Alterations in the immune system by stress are well-established, and those who report high levels of stress are more likely to become infected **(Cohen S et al.,1991; Rozanski A et al.,1999; Kivimaki M et al.,2002; Rosengren A et al.,2004; Segerstrom SC et al.,2004)**. The nervous system is also compromised during times of undue stress **(Woolley CS et al.,1990; Sapolsky RM et al.,1999)**. Stress is associated with a host of mental symptoms as well, including cognitive dysfunction, dementia and excessive fatigue.While stress may have a direct effect on health (e.g., dysregulation of hormonal axes), indirect routes toward mal adaptation also likely exist **(Sandi C et al.,2004; Hasler G et al.,2005; Theorell-Haglow J et al.,2006; Cho HJ et al.,2012; Gerber M et al.,2009; Hamer M et al.,2012)**. For instance, stress is related to declining physical function over time and obesity**,** which contributes to

cardiovascular disease. Another likely factor is impaired health/lifestyle practices and maladaptive behaviors, such as decreased exercise and PA and increased sedentarianism **(Cheng et al., 2000; Hamer et al., 2012; Ogden, et al.,2012; Siervo et al.,2009)**. Furthermore, delays in recovery from exercise and dampened muscular and neural adaptations are observed with chronic stress. It is of no wonder that individuals under high stress are much more likely to incur greater healthcare costs **(Tucker et al., 2002; Stranahan et al.,2006; Bartholomew et al.,2008; Stults-Kolehmainen et al., 2012).**

Chapter 2 : Medicinal plant Withania somnifera “Ashwagandha”

1 Description

Ashwagandha (Withania somnifera, fam. Solanaceae) is commonly known as “Indian Winter cherry” or “Indian Ginseng”. It is one of the most important herbs of Ayurveda (the traditional system of medicine in India) used for millennia as a Rasayana for its wide-ranging health benefits. Rasayana is described as an herbal or metallic preparation that promotes a youthful state of physical and mental health and expands happiness. These types of remedies are given to small children as tonics, and are also taken by the middle-aged and elderly to increase longevity. Among the ayurvedic Rasayana herbs, Ashwagandha holds the most prominent place. It is known as “Sattvic Kapha Rasayana” Herb **(Changhadi, 1938).**

Withania somnifera has small, greenish-yellow flowers and smooth, round fruits with numerous seeds. This plant can be found in tropical and subtropical areas, ranging from South Africa, Middle East India and China **(Dar et al., 2015).**

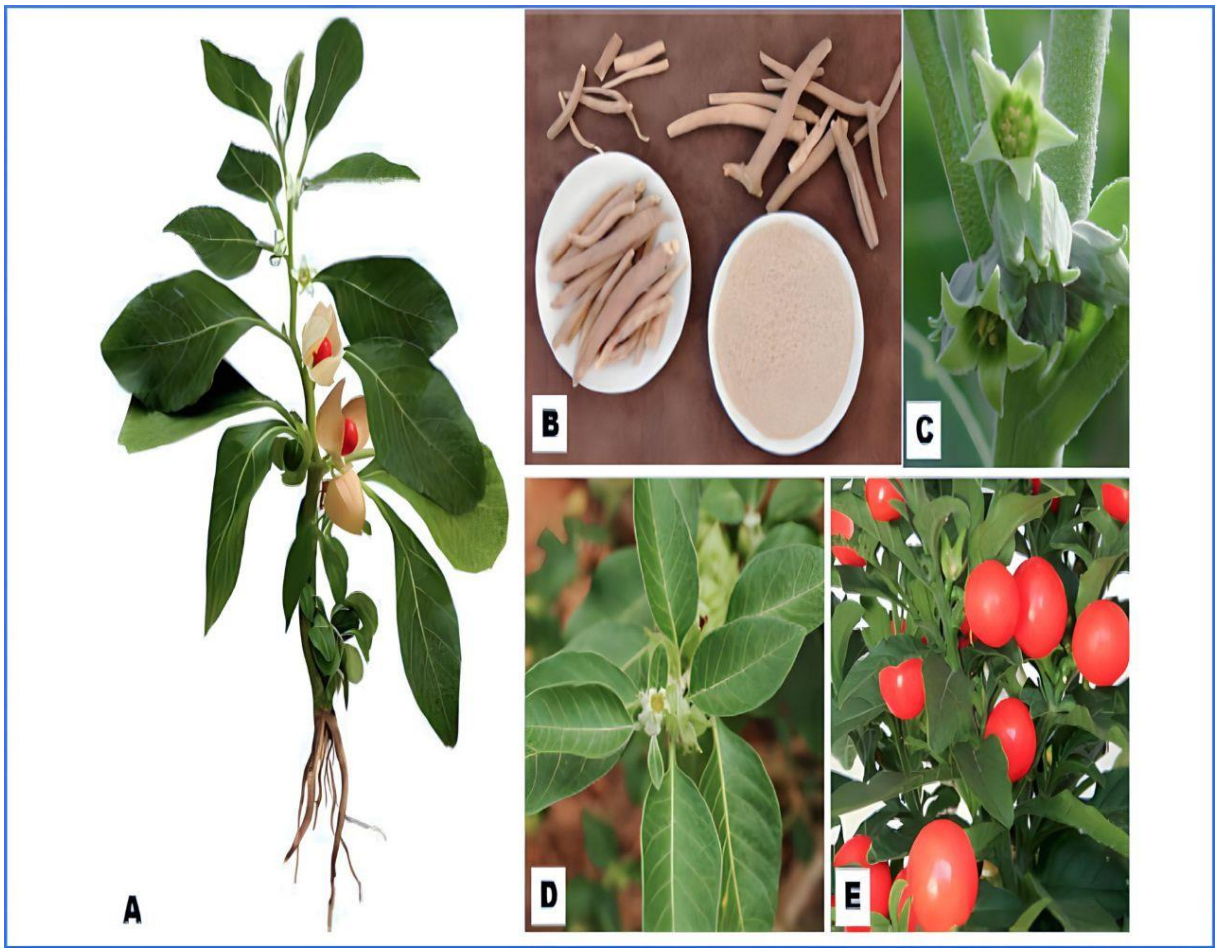


Figure 2: *Withania somnifera* plant : (A) plant ; (B) roots and root powder ; (C) flowers ; (D) leaves ; and (E) and fruits **(Pallav., 2017).**

Withania somnifera is grown as a medicinal crop, and either the whole plant or different parts are used for its medicinal properties. Since antiquity, and to this day, the root of *Withania somnifera* is used as an adaptogen, diuretic, sedative, antioxidant, and aphrodisiac **(Narinderpal et al., 2013).** Other parts of the plant, such as the leaves and fruits, have been used as a pain reliever, memory enhancer **(Choudhary et al., 2017)**, anti-tumor **(Hassannia et al.,2019)**, anti-microbial agent **(Tripathi et al., 2018**), and anti-inflammatory agent **(Narinderpal et al., 2013),** anti-stress Kaur et al., (2001), cardioprotective **(Kaur et al., 2015),** or neuroprotective **(Yenisetti et al., 2016).** It also displays enhanced endothelial function (Dar **et al., 2015)**, reduces reactive oxygen species (**Sun et al., 2016),** regulates apoptosis (**Ahmed et al., 2018)**, and modulates mitochondrial function **(Dar et al., 2015),** showing to be effective to treat aging effects **(Pradhan et al., 2017),** skin diseases (**Li et al., 2016)** and thyroid function **(Sharma et al., 2018).**

Ashwagandha has the antioxidant property that can reduce free radicals induced oxidative stress **(Singh et al., 2002; Mishra ,2009; Bhattacharya at al., 2010).** Thus, some of the useful effects of dietary intake Ashwagandha roots on triglyceride level are attributable to the reduction of stress oxidative and lipid peroxidation.

2 Taxonomy

Ashwagandha, botanically known as Withania somnifera Dunal, is a member of the Solanaceae family. It is commonly known as Indian Ginseng or Winter Cherry. The literal meaning of the word "Ashwagandha" is "smell of horse". The herb is so named for two reasons. One reason is that the fresh roots of the herb emit the smell of horse. The second reason is that there is a commonly held belief that a person consuming extracts of the herb may develop the strength and vitality similar to that of a horse **(Dr. Shastry JLN., 2001)** This herb has a central and prominent place in Ayurvedic medicine. Ashwagandha is referred to as a "royal herb" because of its multifarious rejuvenative effects on the human body. It is a multipurpose herb that acts on various systems of the human body: the neurological system, the immune system, the energy production system, the endocrinal system and the reproductive system.

Table 1 : Classification of Cronquis (L.) **(Dunal, 1852)**

Reign	Plantae
Sub- Reign	*Tracheobionta*
Division	*Magnoliophyta*
Classe	*Magnoliopsida*
Sub-classe	*Asteridae*
Order	*Solanales*
Family	*Solanaceae*
Gender	*Withania*
Species	*Withania somnifera*

3 Chemical composition and pharmacology of Ashwagandha

3.1 Chemical composition

Ashwagandha, also known as Withania somnifera, is an herb commonly used in Ayurvedic medicine. It has a variety of chemical compounds that are responsible for its pharmacological effects.

Several phytochemical studies have been conducted to determine the chemical constituents of the various parts of *Withania somnifera* **(Kuboyama et al., 2014; Rajasankar et al., 2009).** The principal bioactive compounds of *Withania somnifera* are withanolides, which are triterpene lactones (with C28 ergosterone-based skeleton). More than 40 different withanolides, 12 alkaloids and several sitoindosides have been isolated **(Mishra et al., 2000).** The major biochemical constituents of Withania somnifera are withaferin-A, withanolide-D and withanone **(Dar et al., 2016; Mirjalili et al., 2009)** (**Figure 2**). **Lavie et al. (1965)** were the first to isolate withaferin-A from Withania somnifera. In addition to the bioactive compounds, several metabolites have also been identified, which include iron, alanine, aspartate, fructose, lactate, glutamine and many more **(Chatterjee et al., 2010).** Extensive toxicological studies from clinical research on Withania somnifera have shown that the plant is non-toxic in a wide range of practical doses, as well as has no reported herb-herb or herb-drug interactions **(Kulkarni and Dhir, 2008; Patel et al., 2016; Prabu et al., 2013).** These withanolides follow the route of intestinal epithelium absorption when administered orally. Withaferin-A is reported to have highest bioavailability among withanolides in vivo **(Devkar et al., 2015).**

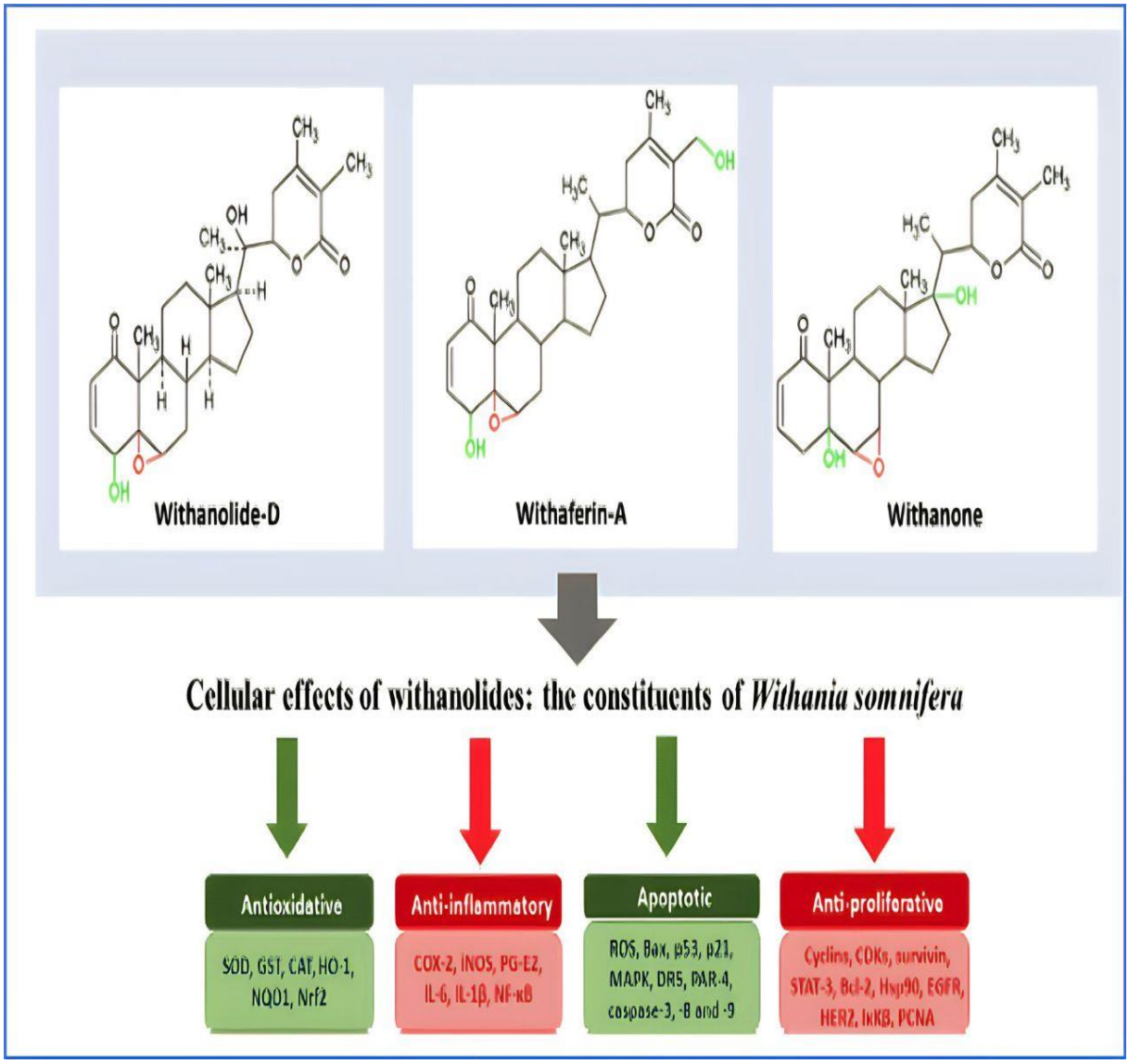


Figure 3: Principal biochemical constituents of *Withania somnifera* **(Pallav., 2017)**

3.2 Pharmacology

The extracts from this plant revealed a new withanolide; these are 14α,15α,17,20β-tetrahydroxy-1-oxo-(22R)-witha-2,5,24-trienolide and the withanolides F and J already identified. The extracts, the semi-purified fractions and the pure compounds showed a significant cytotoxic effect against the human cancer lines tested in a dose-dependent manner. The morphological characteristics of the Hep2 cells treated with the new withanolide and the DNA fragmentation observed are in favor of the induction of apoptosis which is at the origin of the cytotoxicity of these cells. Therefore, the results obtained suggest that the withanolides from *W. adpressa Coss*, can be considered as potential antiproliferative agents **(Abdeljebbar et al 2006).**

Ashwagandha has been extensively studied for its pharmacological effects. Here are some of its notable effects:

- **Adaptogenic**: Ashwagandha is considered an adaptogen, which means it can help the body adapt to stress. It has been shown to reduce the stress hormone cortisol and improve symptoms of stress and anxiety.
- **Anti-inflammatory:** Ashwagandha has anti-inflammatory properties that may help reduce inflammation throughout the body.
- **Immune-boosting:** Ashwagandha has been shown to boost immune function and increase the production of white blood cells.
- **Anti-cancer**: Some studies suggest that ashwagandha may have anti-cancer properties due to its ability to reduce inflammation and boost immune function.
- **Neuroprotective**: Ashwagandha has been shown to protect the brain from damage and improve cognitive function.

4 Bioavailability of WS Compounds

It is desirable to evaluate the in vivo oral bioavailability, Plasma Pharmacokinetics (PK) and tissue distribution (particularly to target organs) of putative active compounds, which are often identified through in vitro bioassays or parenteral administration in vivo. There have been relatively few studies on the PK and brain bioavailability of WS compounds. Plasma bioavailability and PK of withaferin A and withanolide A were recorded after intragastric administration of a WS root aqueous extract in mice **(Patil et al., 2013)**. Plasma bioavailability of withaferin A was also shown in rats after oral administration of a polyherbal formulation containing WS root **(Muhasaparur Ganesan et al., 2021).** Plasma PK of withanolide A was established in rats following oral administration of the compound, which also showed wide tissue distribution; notably, brain levels were considerably lower than in lung, kidney, liver, heart, and spleen **(Singh et al., 2018)**. Four major withanamides were detected in the mouse brain following the intraperitoneal administration of an extract of WS **(Vareed et al., 2014)**. Oral bioavailability of a root extract of WS standardized to 4.5% withaferin A (AshwaMAX®) was assessed in 13 patients with advanced-stage high-grade osteosarcoma **(Pires et al., 2020)**. Patients consumed 1600 mg AshwaMAX®, corresponding to 72 mg of withaferin A. Withaferin A was not detected in plasma samples collected over the following 24 hours **(Pires et al., 2020)**. No other bioavailability studies of WS compounds in humans were found **(Speerset al., 2021)**.

5 Biological effects of Ashwagandha

5.1 Effects of Ashwagandha on stress and anxiety

Ashwagandha is an herb commonly used in Ayurvedic medicine that has been studied for its effects on stress and anxiety. Here are some of the key findings from research studies. Reduced stress and cortisol levels: Ashwagandha has been shown to reduce cortisol levels, a hormone associated with stress. In a double-blind, randomized controlled trial, participants who took ashwagandha for 60 days had significantly lower cortisol levels compared to the placebo group **(Chandrasekhar et al., 2012).**

Reduced anxiety: Several studies have found that ashwagandha can reduce anxiety symptoms. In a randomized controlled trial, participants who took ashwagandha had significantly lower anxiety scores compared to the placebo group (Cooley et al., 2009). Another study found that ashwagandha was as effective as lorazepam, a prescription medication used to treat anxiety **(Andrade et al., 2000).**

Improved mood: Ashwagandha has also been shown to improve mood. In a randomized controlled trial, participants who took ashwagandha had significantly lower scores on depression and anxiety scales compared to the placebo group **(Kumar et al., 2016).**

Enhanced cognitive function: Ashwagandha has been studied for its effects on cognitive function, particularly in individuals with chronic stress. One study found that participants who took ashwagandha had significantly improved reaction time and task performance compared to the placebo group **(Pingali et al., 2014).** Overall, research suggests that ashwagandha may be an effective natural remedy for reducing stress and anxiety symptoms. However, it is important to note that more research is needed to fully understand its effects and potential side effects. It is always recommended to consult with a healthcare professional before taking any herbal supplement **(Chandrasekhar et al., 2012).**

5.2 Anti-inflammatory

Properties The effectiveness of ashwagandha in a variety of rheumatologic conditions may be due in part to its anti-inflammatory properties, which have been studied by several authors. Few studies have been conducted on the mechanism of action for the anti-inflammatory properties of WS. In one study, rats injected with 3.5-percent formaline in the hind leg footpad showed a decrease in absorption of 14C-glucose in rat jejunum.5 Glucose absorption was maintained at the normal level by both WS and the cyclooxygenase inhibitor oxyphenbutazone.

Both drugs produced anti-inflammatory effects. Similar results were obtained in parallel experiments using 14C-leucine absorption from the jejunum these studies suggest cyclooxygenase inhibition may be involved in the mechanism of action of WS.

6 Safety and toxicity

Though numerous plants and their pharmacologically active constituents have been analyzed but still there is scarcity of effective drugs. The reason behind this is the safety and toxicity of the studied plants. Therefore, the discovery of new drug is still continuing. The plant should be selected in such a way that it exerts less toxic effect with higher efficacy. On the basis of literature, we observed that W. somnifera does not possess any significant toxicity **(Padmavathi et al. 2005).**

The widespread use of WS as a traditional medicine and dietary supplement, as well as current scientific literature, supports its general safety. In a review of clinical trials that used root preparations of WS for a wide variety of conditions **(Tandon and Yadav ,2020)** noted that reasonable safety outcomes were seen with no serious adverse events or changes in vital signs, hematological and biochemical parameters **(Tandon et al., 2020)**. Mild to moderate transient adverse events, including somnolence, giddiness, vertigo, and drowsiness, was reported in some studies, while other studies involving adults and children did not report any adverse events **(Tandon et al., 2020).** Similarly, an earlier review by reported that WS has been used in all age groups and both sexes, even during pregnancy, without any reported side effects **(Alam et al., 2012).** The safety of KSM-66® Ashwagandha root extract (Ixoreal Biomed, Inc.) was assessed in a randomized, placebo-controlled **(Verma et al., 2020).** Eighty healthy participants (40 of each sex), aged 18-45 years, were randomized 1:1 to receive 300 mg KSM-66® or a matching placebo twice per day for 8 weeks. To assess safety, researchers assessed vital signs and various hematological and biochemical markers (hemoglobin, neutrophil percentage, platelet count, alkaline phosphatase, Aspartate Aminotransferase (AST), Alanine Aminotransaminase (ALT), thyroid hormone panel). No significant changes were observed in any of the safety parameters compared to placebo, and no adverse events were reported. No serious adverse effects were reported in any of the clinical studies reviewed here. One study **(Kelgane et al., 2020)** formally measured the tolerability of a WS root extract using the Patient's Global Assessment of Tolerability to Therapy and found it to have a high tolerability score. WS extracts have also been shown to be safe in animal toxicity studies. Acute and sub-acute toxicity of a hydroalcoholic root extract of WS was investigated in female Wistar rats **(Prabu et al., 2013).**

No behavioral signs of toxicity or gross pathological changes were observed with acute doses up to 2000 mg/kg. Similarly, no signs of subacute toxicity were observed in rats given 500, 1000, or 2000 mg/kg of the extract daily for 28 days. In a separate study, a hydroalcoholic root extract of WS was used to assess prenatal developmental toxicity in rats at doses of 500, 1000, and 2000 mg/kg daily for 28 days **(Prabu et al., 2015)**. No signs of toxicity, gross pathology changes, or mortality were observed in the pregnant rats or fetuses. Further studies are required to examine the safety of WS extracts prepared using other extraction methods and plant parts.

While these studies suggest that WS does not cause significant toxicity, the possibility of adverse events arising from herb-drug interactions must be considered **(Borse et al., 2019).** Pharmacodynamic interactions between WS and some groups of drugs may exist, as evidenced by additive effects seen in rodent studies between WS and the drugs imipramine **(Jayanthi et al., 2007, Shah et al., 2006)**. diazepam **(Gupta et al., 2007),** and fluoxetine **(Shah et al., 2006)**. The GABA-mimetic and serotonergic activities seen with some WS extracts in rodents would suggest caution when co-administering WS with drugs that work by similar mechanisms. Pharmacokinetic interactions may also occur when botanicals alter the activity of drug transporters or drug-metabolizing enzymes **(Feltrin et al., 2019)**.It has been suggested that an IC50 of less than 100 µg/mL for extracts or 100 µM for active constituents should be classified as potent inhibition that could lead to undesirable herb-drug interactions **(Pan et al., 2011, Obach et al., 2000)**.Various root extracts of WS had IC50 values greater than 100 µg/mL for the cytochrome P450 isoenzymes CYP3A4, CYP2D6, CYP1A2, and CYP2C9 in human liver microsomes (HLM). At the same time, withaferin A and withanolide A did not inhibit these enzymes at doses up to 50 µM **(Savai et al., 2014, Savai et al., 2015).** An aqueous extract of WS did not inhibit human recombinant CYP3A4 at doses as high as 1000 µg/mL [160]. Methanol and ethylacetate extracts of WS root had IC50 values of 79 and 58 µg/mL, respectively, for CYP2B6 in HLM, whereas aqueous and ethanolic extracts were not inhibitory at up to 200µg/mL **(Kumar et al., 2020)**. None of the extracts inhibited β-esterase-dependent rifampicin metabolism in HLM or induced mRNA of CYP2B6 or CYP3A4 in HepG2 cells. In a detailed biopharmaceutical study of withanone, the compound was found to have IC50 values >100 µM for CYP2C9/11 in rat and human liver microsomes and IC50 values between 28.5 and 80 µM for other CYP isoenzymes (the lowest being 28.5 µM for CYP3A4 in human liver microsomes **(Singh et al., 2021).**

Material and methods

Material and methods

1 Material

1.1 Animal material

The basic biological material in our study is the female white rat Rattus from the strain Wistar in the number of 20 rats, obtained from El-Oued- Algeria, aged from (06- 08) weeks weighing (100g ±10). These rodents are nocturnal mammals, it has a broad head small ear, protruding red eyes and a long tail, widely used in various fields of experimental research.

The rats were kept in plastic cages at a steady temperature (23+1°C) with a 12 h/12 h light/dark cycle to adapt them to the natural photoperiod standards; environments. The cages have been cleaned and the bedding changed once every two days. Rats had full access to conventional rodent food and water

1.1.1 Breeding conditions

The rats are raised in polyethylene cages. The litter, made up of shavings of wood, is changed every two days. They are acclimatized under standardized conditions of animal facility, with a temperature of 18 to 21°C and a humidity of 50 to 70%. The food brought to animals is made up of corn, carrots, barley. As for the drinking water, it is served in bottles.

Figure 4: Experimental animals **(Photos personnel)**

1.1.2 Experimental design

After an adaptation period of 21 days, the rats were measured and divided into four experimental groups of five rats each:

- **Control groups (T):** rats received distilled water by gavage (1.5 ml/day).
- **Ashwaghanda groups (WS):** rats were given a solution of the decoction of ashwaghanda at dose of 100 mg/Kg/d of body weight for 21 days
- **Stressed group (S):** rats were exposed to chronic restraint stress 3h/d for 21 days
- **Stressed+ Ashwaghanda group (S+WS):** rats exposed to restraint stress and after 30min received by gavage the decoction of 100 mg/kg/bw) of Ashwaghanda for 21 consecutive days.

1.2 Plant material

Organic Ashwagandha (Withania Somnifera) root powder was brought from USA

Figure 5: Withania Somnifera root powder

1.2.1 Preparation of aqueous extract by decoction

For this extraction we used the method of decoction given by the herbalists, and for this 5g of the dry plant is crushed and mixed with 100ml of distilled water, once the water has boiled, the temperature is lowered until the liquid of half (50ml). The preparation is filtered with Wattman paper.

1.3 Protocol

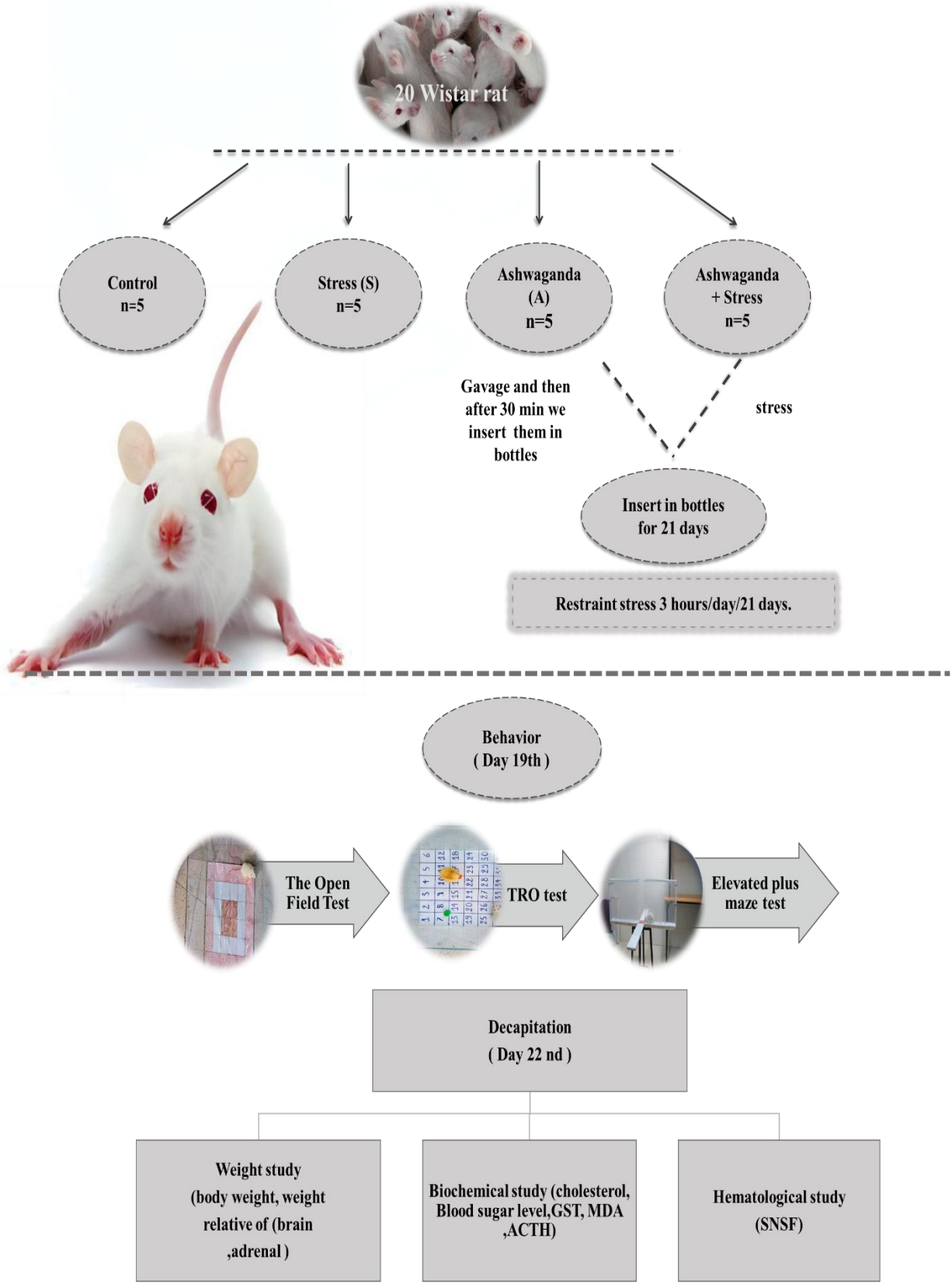


Figure 6: Schematic illustration of the experiment

2 Methods

2.1 Restraint stress procedure

We applied the stress model of **(Bardin et al., 2009)**, as a behavioral evaluation was performed at the end of the stress, female Wistar rats were restrained in a plastic cylinder bottle for 3 hours every day (9 :30 am to 12 :30 am) for 21 days.

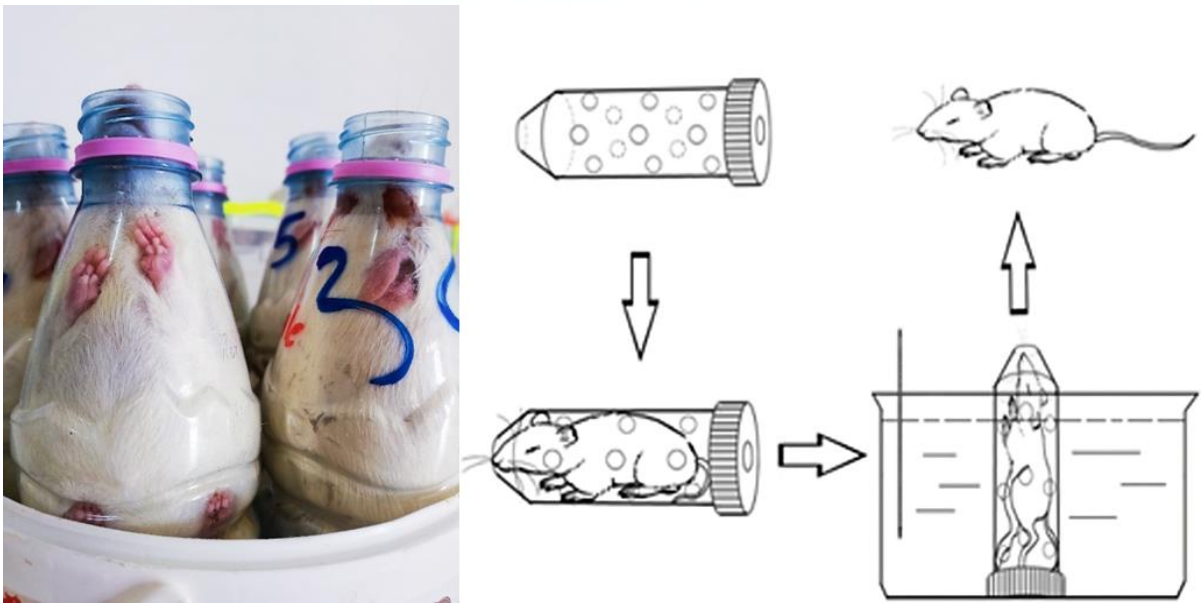


Figure 7: Restraint stress procedure

2.2 Behavioral study

This study is available in our laboratory with the aim of evaluating the stress effect of restraint on locomotor activity, on the state of anxiety, olfaction, the ability to memorization and several tests have been developed such as: the forced swimming test (FST), the elevated plus maze (EPM), the open field (OF) and the TRO. The behavior of the animals was filmed and recorded using video cameras. These tests carried out in the 19th restraint stress day.

2.2.1 Forced swimming test (FST)

The FST or Forced swimming test, is a behavioral model that predicts the effectiveness of antidepressant treatment **(Porsolt et al., 1977)**. This animal model uses both in rats and mice, however, has procedural differences depending on the species used. The test consists in placing the rat individually in an aquarium 40 cm high on 30cm wide. These dimensions ensure that the rat cannot escape by clinging to the edges of the device. The aquarium is filled with water at 25°C. The height of the water reaches 35 cm, to ensure that the rat does not use its lower limbs to keep it on the surface, and therefore force it to swim **(Figure 8).** After a phase of activity vigorous (adaptation time), the control animal stops swimming and freezes, adopting a desperation behavior. The animal is considered to be stationary when it floats in horizontal

position and performs only low amplitude movements, sufficient to keep his head above water. The FST takes place in rats in two phases, the pre-test (FST1) and the test (FST2), separated by a 24-hour interval during which treatment is administered. During the pre-test, the rat is placed for 15 minutes in the aquarium filled with water which it cannot escape. At the end of the session, the animal is motionless. The next day, the animal is dives back into the aquarium for 5 minutes, during which time the immobility is saved. Effective antidepressant treatment decreases immobility time only on the day of the test **(Porsolt et al., 1978)**. Recently, an improvement of the test has been validated. This modification proposes in the rat, not only to evaluate the immobility posture, but also the two active behaviors directly involved in reducing of this immobility, namely swimming and climbing. The variables measured are: The immobility time (second), swimming time (second), climbing time (second).

Figure 8: The FST test

2.2.2 Elevated plus maze test (EPM)

EPM is a widely studied test to highlight the properties anxiolytics or anxiogenic pharmacological compounds. The device consists of a raised maze in the shape of a cross with two open arms (50 × 10cm) and two closed arms (50 × 10 × 45 cm). The device is located at a height of 50 cm above the soil **(Patin et al., 2005).** Each rat is placed individually in the center of the EPM directed towards one of the open arms and its behavior in free exploration is recorded and examined for 5 min. A visit was counted when the rat had all four legs in an arm **(figure 9)** The time spent and the number of entries in the open and closed arms are measured. The experiment exploits the conflict, in rodents, between fear of open spaces and the desire to explore a new environment. Closed arms represent security, while open arms offer exploratory value. An anxious animal will have naturally tended to prefer closed and dark spaces over open

ones and enlightened. Thus, behavioral anxiety is measured by the degree of avoidance of spaces open to the labyrinth. At the end of each session, the animal is returned to its cage and the device is wiped with an alcoholic solution.

The variables measured are: time spent in the center (second), time spent in open arms (second), time passed to the distal and proximal part of the open arm (second), number of entries to the open arm, time spent in closed arm (second), time spent at distal and proximal part of closed arm (second), number of entries in closed arm, Redressement number.

Figure 9: Elevated plus maze test

2.2.3 Open field test (OF)

The OF test, originally described by **(Hall 1934)**, was developed with the aim of measure differences in emotional reactivity in rodents. The OF therefore allows to assess locomotor and exploration behavior in rats. Briefly, the OF is a Plexiglas unit (70 cm × 70 cm × 40 cm) whose floor is divided into central and peripheral zones. Each rat is placed individually in the center of the compartment and left for 5 min of exploration **(Sáenz et al., 2006)**. An anxious animal to the dancing rat prefers the area device while avoiding entry into the central area. Each session is filmed. The device is cleaned after each session with an alcoholic solution to bearing the polarizing effects due to the odors left by the previous rat.

Measuring variables:

- Time spent in the central and the peripheral section (second)
- total traveled distance (cm)
- Redressement time (second)
- Number of passages in the device's center

- Immobility time (second)

Figure 10: The Open Field Test

2.2.4 Novel Object Recognition Test (TRO)

The object recognition test (ORT), also known as the novel object recognition test (NOR), is a relatively fast and efficient means for testing different phases of learning and memory in mice. It was originally described by Ennaceur and Delacour in 1988 and used primarily in rats1; however, since then, it has been successfully adapted for use in mice1. **(Akkerman S et al., 2012 ; Antunes M et al., 2012 ; Leger M et al., 2013 ; van Goethem et al., 2012 ; Lindsay M. Lueptow et al., 2017)**. The ORT is fairly simple and can be completed over 3 days: habituation day, training day, and testing day. During training, the mouse is allowed to explore 2 identical objects. On test day, one of the training objects is replaced with a novel object. Because mice have an innate preference for novelty, if the mouse recognizes the familiar object, it will spend most of its time at the novel object. Due to this innate preference, there is no need for positive or negative reinforcement or long training schedules. Additionally, the ORT can also be modified for numerous applications. The retention interval can be shortened to examine short-term memory, or lengthened to probe long-term memory. Pharmacological intervention can be used at various times prior to training, after training, or prior to recall to investigate different phases of learning (*i.e.*, acquisition, early or late consolidation, or recall). Overall, the ORT is a relatively low-stress, efficient test for memory in mice, and is appropriate for the detection of neuropsychological changes following pharmacological, biological, or genetic manipulations **(Lueptow 2017).**

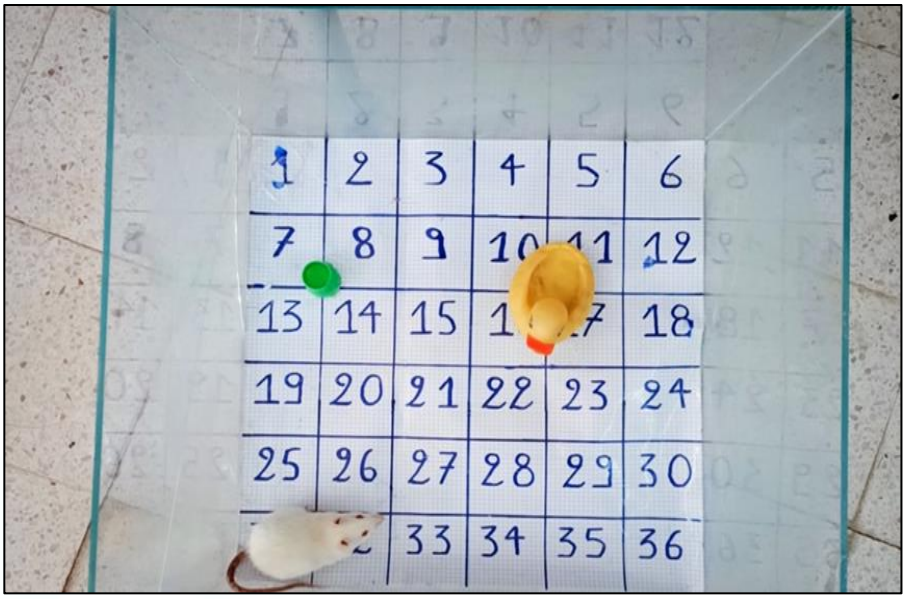


Figure 11: TRO test

2.3 Specimens

2.3.1 Blood sample

Blood samples are taken by retro orbital puncture using tubes hematocrits on the 22th day of experimentation for the four batches. The blood is immediately collected in two labeled tubes, one EDTA and the other containing heparin as anticoagulant.

- EDTA tubes will be used for blood count formula determination (SNSF).
- -The heparin tubes are centrifuged at 5000 rpm for 15 minutes, the sera are collected in the eppendorf tubes will be used to determine the biochemical parameters (Glycemia, Cholesterol, ACTH).

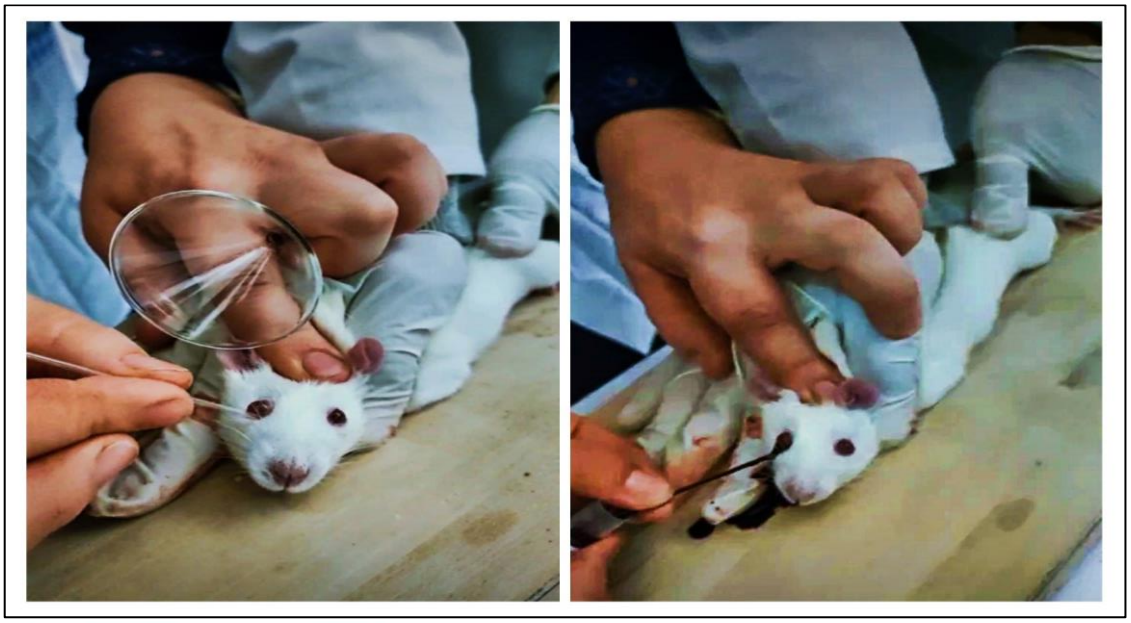


Figure 12: The retro-orbital vein bleeding

2.3.2 Sacrifice and organs removal

At the end of the experimental period, the animals are sacrificed by decapitation of the brain and adrenals were removed quickly and rinsed in cold wash buffer (NaCl 9%), then dried at low temperature (4°C) with semi-absorbent paper and weighed using a precision balance (SCALTEC SBC 51).

- The relative weight of the organs is calculated according to the formula:

Relative weight (g /100gPV) = (Organ weight / Individual body weight) x100

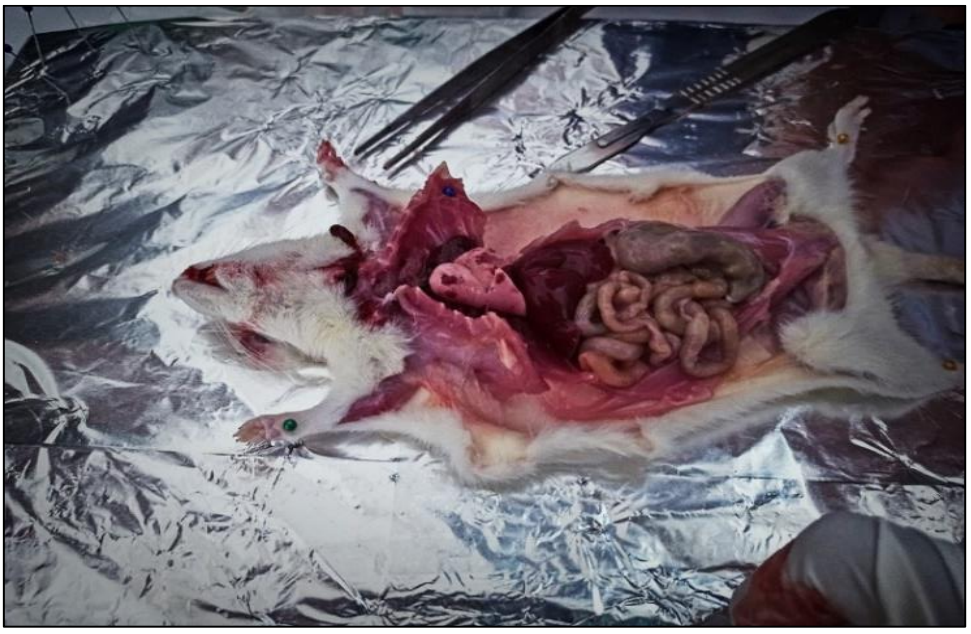


Figure 13: Dissection and Organs removal

2.4 Assay of hematological parameters

2.4.1 Study of hematological parameters

The blood numbering was done by a type counter automaton (of Abacus 380) with 19 parameters. To estimate the figured elements of the blood (number of red blood cells, the number of white blood cells and the rate of hemoglobins, lymphocytes, monocytes, granulocyte, this analysis is carried out on blood stored in tubes with EDTA or heparin have been determined at the end of the experiment, counting was carried out at the Elite laboratory (Tebessa).

Figure 14: Abacus 380 biochemical automaton with 19 parameters

2.4.2 Study of biochemical parameters

2.4.2.1 Blood glucose measurement

- **Principe:**

the measure of blood glucose was carried out by a glucometer which uses test strips. These are intended for in-diagnosis use in vitro for blood glucose testing. They are designed to measure glucose in the capillary whole blood. The test strip contains glucose oxidase, an enzyme that oxidizes glucose in the blood and produces D-gluconic acid and hydrogen peroxide.

- **Operating mode**
  - The reader starts automatically by simply inserting the strip Reactivates Accu-Chek (in the direction of the arrows and up to the stop).
  - The symbol of a drop flashes.

- Place the drop of blood on the orange drop zone of the strip. The result appears in 5 seconds. Blood glucose is given in g/dl.

2.4.2.2 Cholesterol measurement

- **Principe:**

The Dosage is done according to the Biomaghreb technical sheet **(Fasce, 1982; Richmond, 1973; Trinder, 1969)**. The cholesterol present in the sample forms a colored complex according to the reactions below:

$$\textbf{Cholestérol ester} + H_2O \xrightarrow{\text{CHE}} \textbf{Cholestérol} + \textbf{Acides gras}$$

$$\textbf{Cholestérol} + O_2 \xrightarrow{\text{CHOD}} \textbf{4-Cholesténone} + H_2O_2$$

$$2\,H_2O_2 + \textbf{Phénol} + \textbf{4-Aminophénazone} \xrightarrow{\text{POD}} \textbf{Quinonimine} + 4H_2O$$

An already prepared kit (Quinicaclinicaapplicada) was used. The content included cholesterol oxidase, cholesterol esterase, peroxidase, non-reactive stabilizers, phosphate buffer, phenol, 2,5-dichlorophenol and 4-aminoantipyrone. Storage was at 28°C, protected from light. Three test tubes 1, 2, 3 were used and the content of the test specimens of text produced was

The contents of each mixture were mixed well and incubated for 15 min at 37°C and read at a wavelength of 546 nm. Cholesterol content (mg/dl) was calculated as follows:

$$\frac{\textbf{OD sample}}{\textbf{OD standard}} \times 200 = mg\ of\ \frac{cholesterol}{dl\ serum}$$

Units in mg/dl can be converted to mmol/liter by multiplying by 0.0259.

Dosing procedure

DOSAGE

1. Place a sufficient number of streptavidin-coated strips in a holder to test all six (6) ACTH standards, ACTH standards A through F (the exact concentration is indicated on the vial label), the quality control plasma and the samples of patients.

2. Pipette 200 µl of sample into the appropriate well. Freeze (at -20°C) the standards and remaining controls as soon as possible after use.

3. Add or administer 25 µl of Reagent 1 (biotinylated antibody) to each well containing the sample.

4. Add or administer 25 µL of Reagent 2 (enzyme-labeled antibody) to each of the same wells. Cover the plate or plates with aluminum foil or with a tray to avoid exposure to light. Place it (or them) on an orbital shaker or rotator set at 170 ± 10 rpm for 4

Hours± 30 minutes at room temperature (22°C-28°C).

5. Aspirate all fluid, then wash or aspirate each well five (5) times with active wash (prepared with Reagent A) in an automatic microplate washer. He is recommended to limit the volume of wash solution to be poured into each well to 0.35 ml.

6. Add or dispense 150 µl of ELISA Reagent B (TMB Substrate) into each Wells.

7. After covering the plate or plates to avoid exposure to light, the place on an orbital shaker set at 170 ± 10 rpm for 30 ± 5 minutes at room temperature (22°C-28°C).

8. Add or administer 100 µl of blocking solution to each of the wells. To mix together delicately.

9. Read the absorbance of the solution in the wells after 10 minutes with a reader of microplates set at 450 nm against 250 µl of distilled or deionized water.

- The results are measured using a Magellan TECAN ELISA reader fitted with a computer software that automatically calculates the standard range and directly gives the value of testosterone to the desired unit.

2.4.3 Dosage of oxidative stress parameters

2.4.3.1 Measurement of malone-dialdehyde (MDA)

➢ Principle

MDA is a product of lipid peroxidation reactions which is formed upon attack polyunsaturated lipids by reactive oxygen species generated by certain contaminants. In our study, brain MDA levels were assessed using the method from Ohkawa et al (1979). The dosage is based on formation in an acidic and hot medium (100°C) between MDA and Thiobarbituric acid (TBA) of a color pigment absorbing at 530 nm, extractable by organic solvents such as butanol.

Preparation of the homogenate: 500 mg of the brain of the two batches are ground cold using an ultrasonic homogenizer in the presence of 5 ml of a phosphate buffer solution (0.1M, pH 7.4) to obtain a homogenate.

➢ Operating mode

- Take 0.5 ml of the homogenate.
- Add 0.5 ml of 20% trichloroacetic acid (TCA).

- Add 1 ml of Thiobarbituric acid (TBA) 0.67%.
- Mix and incubate in a water bath at a temperature of 100°C for 15 min.
- Cool and add 4 ml of n-butanol.
- Centrifuge for 15 minutes at 3000 rpm.
- Recover the supernatant, and read the optical density at 530 nm against the blank.

Calculation of the MDA concentration: the quantity of MDA in the sample is expressed in nmol/gram of tissue (liver or kidney). It is obtained thanks to a standard curve carried out with 1,1',3,3'-tetraethoxypropane made under the same conditions.

Figure 15:MDA dosage

2.4.3.2 Measurement of reduced Glutathione S Transferase Activity (GST)

➢ **Principe**

Glutathione-S-transferases belong to a family of essentially cytosolic multifunctional enzymes, involved in intracellular transport and biosynthesis. They catalyze conjugation reactions between an endogenous peptide, in the presence of a cofactor glutathione, and reactive molecules comprising electrophilic sites. Conjugation leads to the formation of a new molecule; 1-S-Glutathionyl 2-4 Dinitrobenzene to measure GST activity. The technique we used to measure GST activity is that of Habig et al (1974). It measures the kinetics of formation between a model substrate, chlorodinitrobenzene (CDNB) and glutathione, which absorbs light at 340 nm

➢ **Operating mode**

The samples are homogenized in 1 ml of phosphate buffer (0.1M, pH6). The homogenate is centrifuged at 1400 rpm for 30 min and the recovered supernatant will serve as

a source of enzymes. The assay consists of reacting 200 µl of the supernatant with 1.2 ml of the CDNB mixture. The reading of the absorbances is carried out every minute for 5 minutes at a wavelength of 340nm in a UV/visible spectrophotometer against a blank containing 200 µl of distilled water replacing the quantity of the supernatant. GST activity expressed in nanomoles of C-DNB per minute per milligram of protein (nmol C-DNB/min/prot) according to the following formula:

$$\textbf{GST (nmol C-DNB/min/mg prot)} = \frac{\Delta \text{ DO échantillon} - \Delta \text{ DO Blanc}}{\varepsilon \times L \times \text{mg prot}}$$

▪Δ Sample OD – Δ Blank OD: average OD of samples per minute – average OD of Blanks per minute

▪ε: Molecular extinction coefficient of C-DNB, εCDNB = 9.6 mM-1.cm-1

▪L: Optical path of the tank = 1cm

Figure 16:GST dosage

2.4.3.3 Protein dosage

➢ **Principle**

The protein concentration is determined according to the Bradford method (1976) which uses Coomassie blue (G 250) as a reagent. The amine groups (-NH2) of proteins react with a reagent based on ortho phosphoric acid, ethanol and Coomassie blue to form a blue color complex. The appearance of this color reflects the degree of ionization of the acid medium and the intensity establishes the concentration of proteins in the sample.

➢ **Operating mode**

- Take 0.1 ml of the homogenate.
- Add 5 ml of Bradford's reagent.
- Shake and leave to stand for 5 minutes to stabilize the color.
- Read the optical density at 595 nm, against white.
- The optical density obtained is reported on a calibration curve Previously drawn.

The protein concentration is determined by comparison with a standard range

Bovine serum albumin (BSA) (1 mg/ml) produced under the same conditions

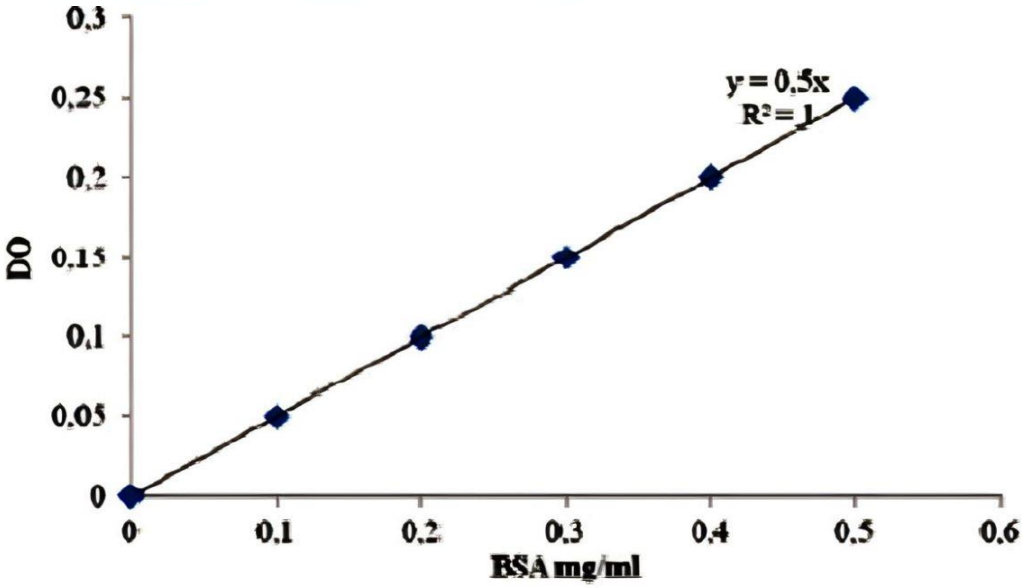


Figure 17: calibration curve for bovine serum albumin

Figure 18:Protein dosage

RESULTS

3 Results

3.1 Effect of restraint stress and Ashwagandha treatment on weight change

According to our results, body weight gain decreased in the stressed batch treated with Ashwagandha and the batch undergoing restraint stress and treatment.

Table 2: Variation in weight gain in the control, stressed and treated batch

Weight/groups	C	S	A	S+A
Initial weight (g)	209,4 ± 8,56	207 ±10,41	199 ± 6.16	203.9 ± 14.70
Final weight (g)	224 ± 6.67	194 ± 4,79	194.9 ± 13.12	195.2 ± 10.61
Weight gain (%)	7.17%	-8,57%	-2.51%	-3.84%

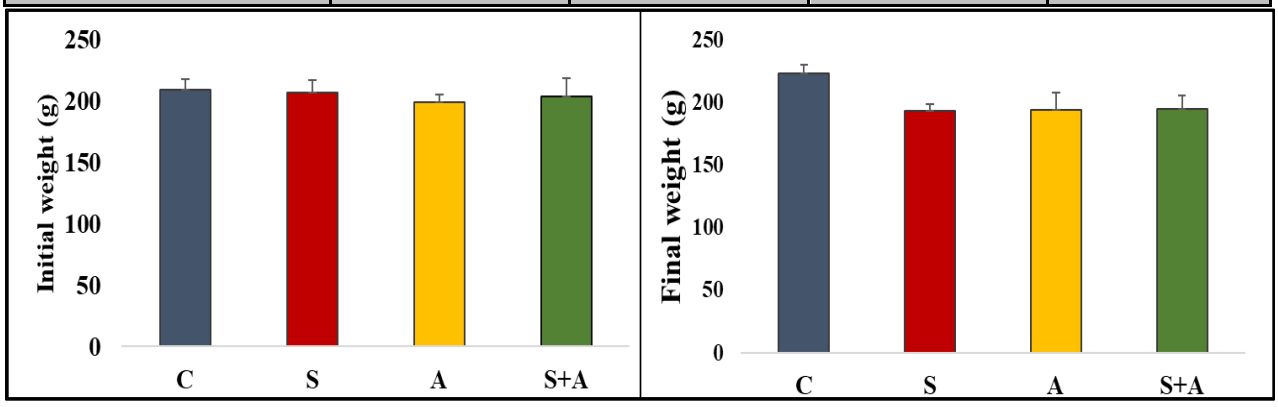


Figure 19 : Variation in weight gain in the control, stressed and treated batch

3.2 Effect of restraint stress and Ashwagandha treatment on absolute organ weights

Our results highlight the evolution of the absolute weights (AW) of the brain and the adrenal glands in control, stressed and treated rats. Concerning the AW of the adrenals, the results obtained show that there was a significant increase ($p \leq 0.05$) in the rats experience restraint stress compared to the control group. On the other hand, a non-significant decrease was noticed in rats treated with Ashwagandha compared to the control, moreover the treatment of rats stressed by our plant reduced this increase.

Concerning the AW of the brain, our results show a decrease significant ($p \leq 0.05$) in the stressed batch compared to the control batch and not significant in the other batches.

Table 3: Variation in the absolute weight of the adrenal glands and the brain in the control, stressed and treated batch

Weight/groups	C	S	A	S+A
Absolute Adrenal Weight(g)	0,0322 ± 0,0031	0,0376 ± 0,0028 *	0,0298 ± 0,0028 Ns	0 ,0346 ± 0,002 ns
Absolute brain weight (g)	1,166 ± 0,011	1,098 ± 0,029 *	1,184 ±0,047 Ns	1,138 ± 0,032 ns

*p<0.05; **p<0.01; *** p<0.001 ns ; not significant (comparison vs T, n=5).

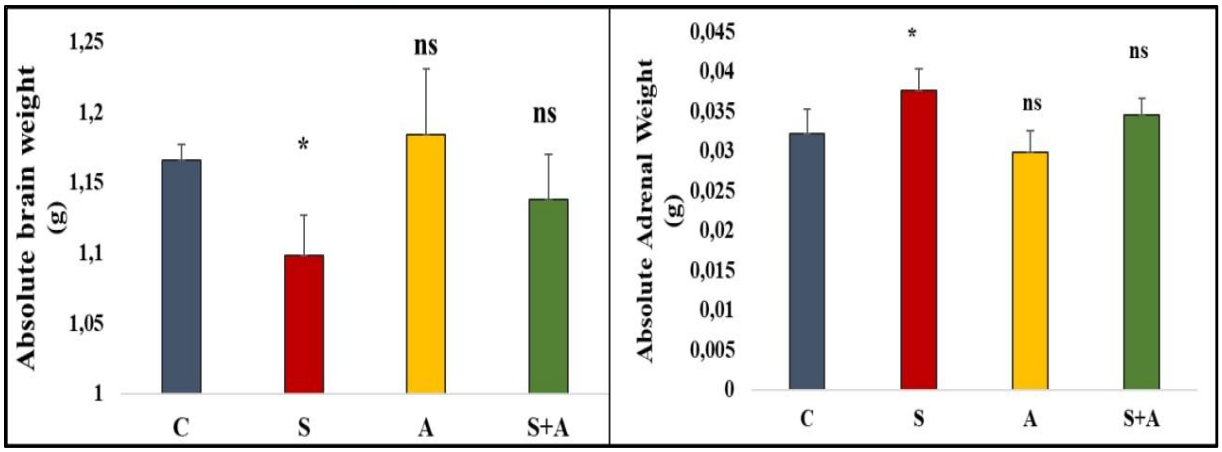


Figure 20: Variation in the absolute weight of the adrenal glands and the brain in the control, stressed and treated batch

3.3 The effect of restraint stress and Ashwagandha on biochemical parameters.

3.3.1 Cholesterol

The results obtained show a significant increase (p≤0.001) in cholesterol in the stressful rats compared to the controls. On the other hand, the treatment of stressed rats with Ashwagandha decreases this increase.

3.3.2 Glycemia

Our results show a significant increase in blood sugar (p≤0.01) and cholesterol (p≤0.001) levels in rats subjected to restraint stress compared to control rats. Treatment of stressed rats with Ashwagandha attenuated this increase somewhat.

Tableau 4: Variation in glycaemia and cholesterol in the control, stressed and treated group

Parameters	C	S	A	S+A
Blood glucose (mg/dl)	1,098 ± 0,0363	1,264 ± 0,068 **	1,130 ± 0,412 ns	1,170 ± 0,220 ns
Cholesterol (g/l)	0,750 ± 0,033	1,204 ± 0,047 ***	0,860 ± 0,119 Ns	0,794 ± 0,279 Ns

*p<0.05; **p<0.01; *** p<0.001 ns ; not significant (comparison vs T, n=5).

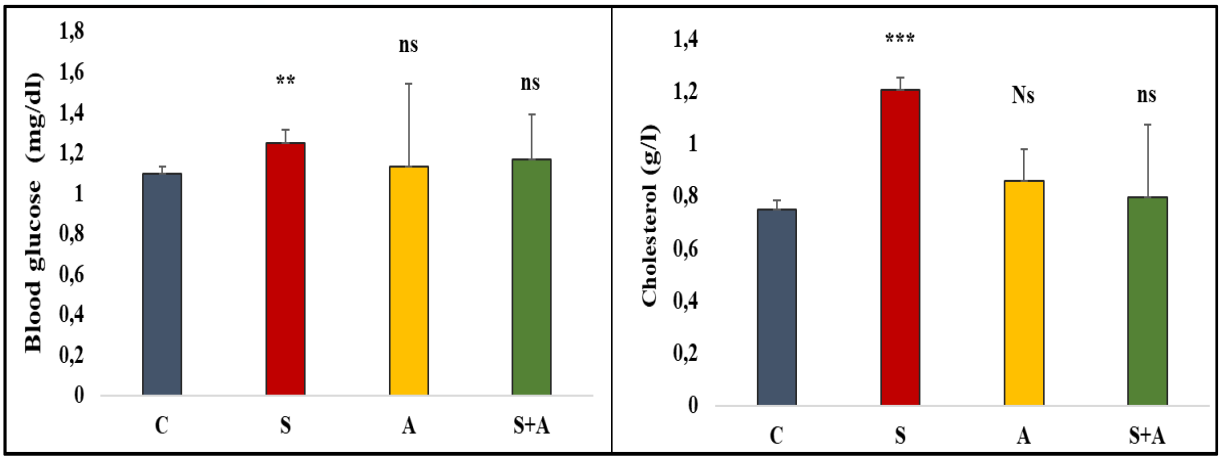


Figure 21: Variation in glycaemia and cholesterol in the control, stressed and treated group

3.4 The effect of restraint stress and Ashwagandha on hematological parameters

The results displayed in the table show a significant increase (P≤0.01) in total leukocytes, Lymphocytes and Eosinophils and a significant increase (p≤0.001) in Monocyte and Neutrophils and a significant increase (P≤0.05) in GR in stressful rats compared to controls. On the other hand, the treatment of stressed rats with Ashwagandha modulated this increase.

Table 5: Variations in hematological parameters in control, stressed and treated rats.

Parameters/groups	C	S	A	S+A
WBC (10^3/µl)	9,540 ± 0,677	10,980 ± 0,936 **	9,254 ± 2,286 ns	9,474 ± 1,700 ns
Lymphocytes (10^3/µl)	8,160 ± 0,673	9,698 ± 0,839 **	8,224 ± 0,528 ns	8,756 ± 1,092 ns
Monocyte (10^3/µ±l)	0,272 ± 0,052	0,568 ± 0,162 ***	0,288 ± 0,080 ns	0,471 ± 0,159 *
Neutrophiles (10^3/µl)	1.795 ± 0.061	1,874 ± 0,175 ***	1,532 ± 0,263 ns	1,558 ± 0,411 ns
Eosinophiles (10^3/µl)	0,210 ± 0,086	0,490 ± 0,185 **	0,202 ± 0,089 ns	0,320 ± 0,176 ns
GR (10^6/µl)	8,556 ± 0,317	9,020 ± 0,207 *	8,166 ± 0,422 ns	8,690 ± 0,466 ns

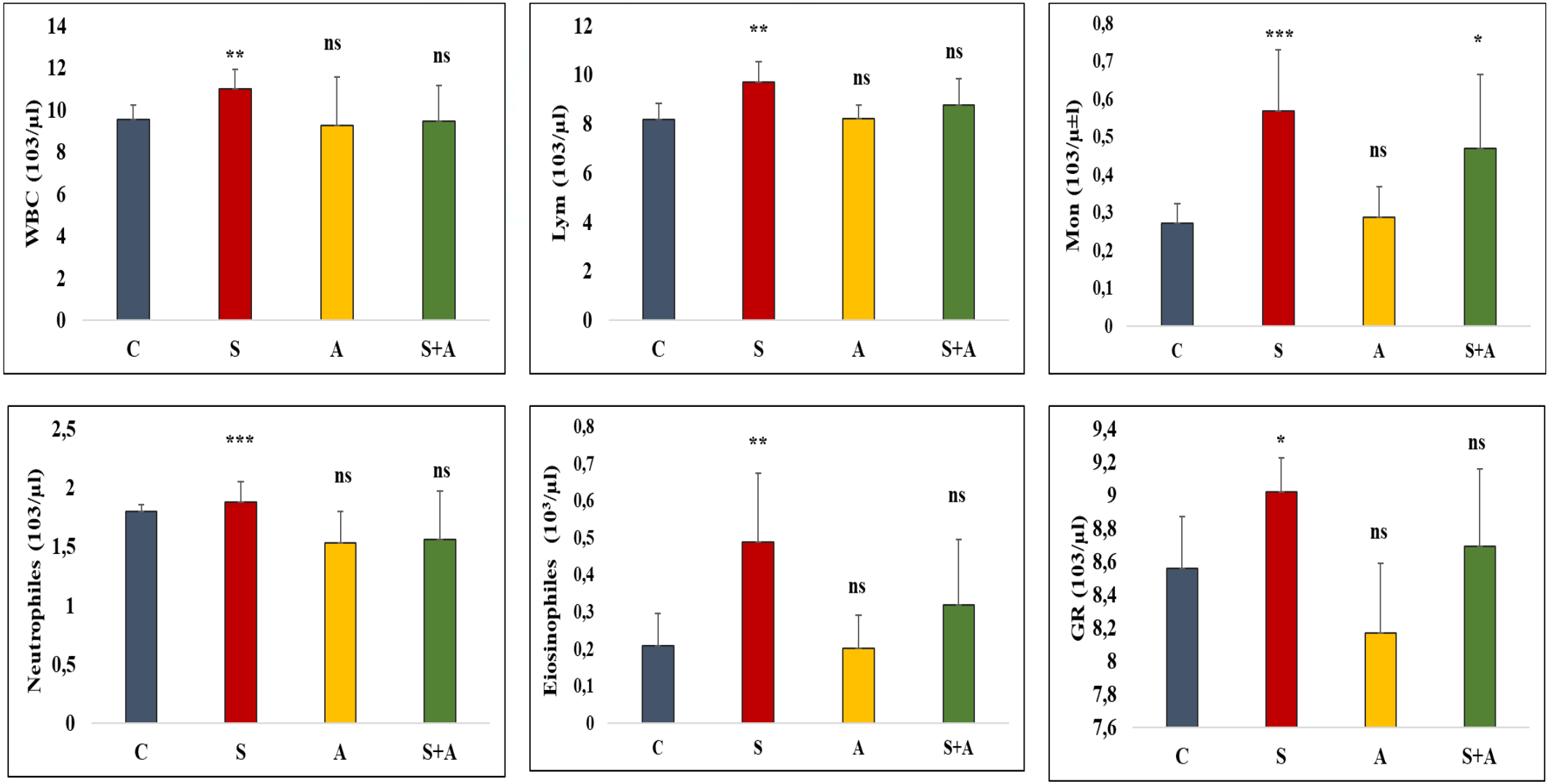


Figure 22: Variations in hematological parameters in control, stressed and treated rats

3.5 Effect of restraint stress and Ashwagandha on cerebral redox status

a. Malondialdehyde (MDA) levels

We found a significant elevation of cerebral MDA ($p \leq 0.01$) in rats undergoing restraint stress compared to the control group, on the other hand, treatment with Ashwagandha significantly attenuated this elevation compared with the stressed group ($p \leq 0.01$).

b. Glutathione S Transferase Activity

Statistical analysis of our results show a significant decrease ($p<0.01$) in glutathione-s-transferase (GST) in the stressed batch, and a significant increase in the batch treated with Ashwagandha ($p<0.05$) compared to the witnesses.

Interestingly, the treatment batch combined S+A modulated significantly ($p<0.05$) this decrease in GST compared to the stressed batch.

Table 6: Variation in malondialdehyde levels and glutathione-S-transferase (GST) activity in the brain in the control, stressed and treated group

Parameters/batches	C	S	A	S+A
MDA (nmol/mg prot)	0,250 ± 0,038	0,372 ± 0,083 ****	0,241 ± 0,024 ns	0,271 ± 0,02 **a^{ns} ; b***
GST(nmol/min/mg port)	0,220 ± 0,020	0,178 ± 0,019 ****	0,262 ± 0,039 *	0,20 1± 0,01 **a^{ns}, b***

(* p<0.05; ** p<0.01; *** p<0.001 ns; not significant (comparison a vs T and b vs S; n=5)

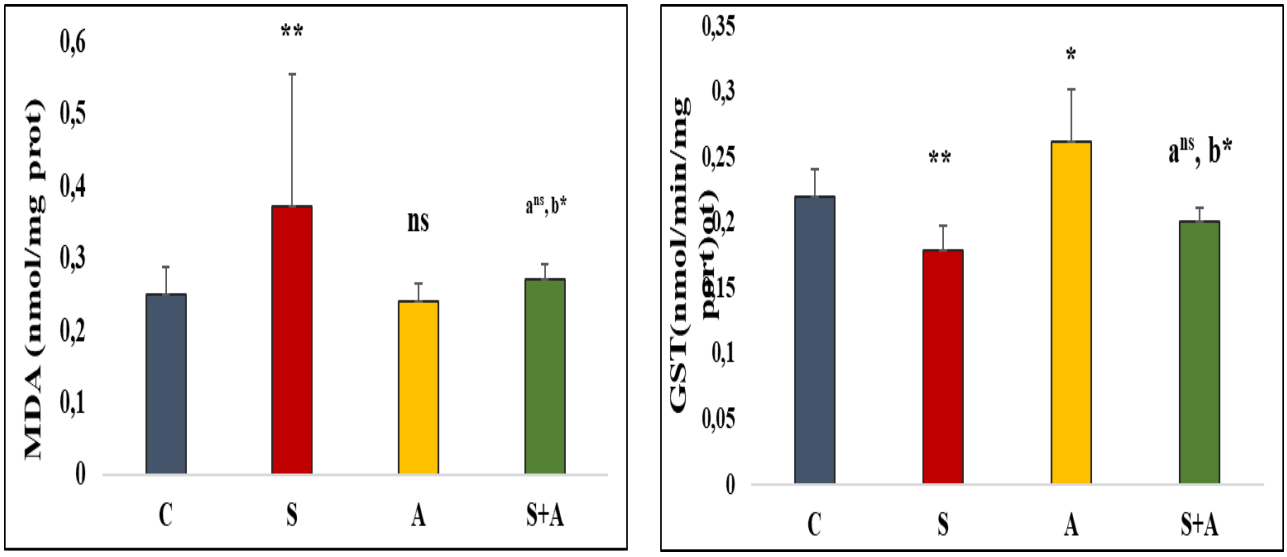


Figure 23: Variation in malondialdehyde levels and glutathione-S-transferase (GST) activity in the brain in the control, stressed and treated group

3.6 Effect of restraint stress and Ashwagandha on the level of cerebral ACTH:

The statistical analysis of our results shows a significant increase ($p \leq 0.001$) in ACTH in stressed rats compared to the control group. On the other hand, treatment with Ashwagandha significantly attenuated this increase compared with the stressed group ($p \leq 0.001$).

Table 7: Variation of ACTH in Control, Stressed and Treated Rats

Parameters/batches	C	S	A	S+A
Brain ACTH (ng/l)	133,20 ± 6,65	599,20 ± 61,85 **a*****	130,80 ± 17,05 **a^{ns}**	215,30 ± 73,01 **a^{*}, b*****

(* p<0.05; ** p<0.01; *** p<0.001 ns; not significant (comparison a vs T and b vs S; n=5)

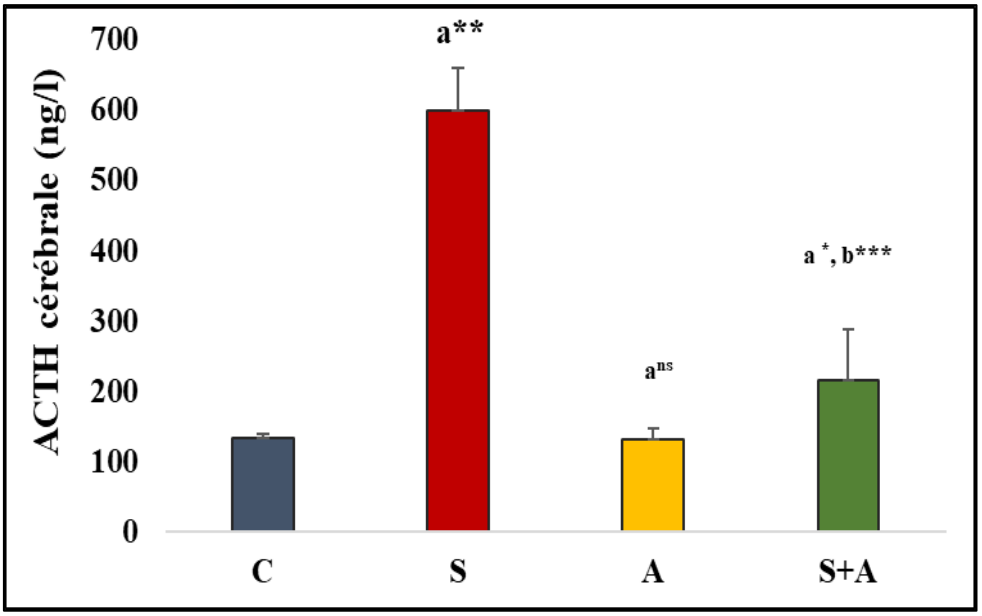


Figure 24: Variation of ACTH in Control, Stressed and Treated Rats

3.7 Behavioral study

3.7.1 Effect of restraint stress and Ashwagandha on open field test parameters

The statistical analysis of the different variables measured in this test revealed a significant difference between the different groups. We noted a significant decrease in the stressed groups compared to the control groups with regard to the total distance traveled ($p<0.001$), time spent at the center ($p<0.001$) and the number of entries to the center ($p<0.01$) and number of rightings ($p<0.05$). However, treatment of stressed rats with Ashwagandha prevented this increase with a significant difference ($p<0.001$) compared to stressed rats. On the other hand, we recorded a significant increase in the time of immobility ($p < 0.001$) and the time spent at the peripheral ($p < 0.001$) compared to the controls.

Interestingly, no significant difference was noted between the Ashwagandha-treated rats and the control rats.

Tableau 8: Variation of OF parameters in the control, stressed and treated batch**.**

Parameters/batches	C	S	A	S+A
Total distance traveled (cm)	564,80 ± 22,31	200,40 ± 7,83 a***	645 ± 01,09 a ^{ns}	413,60 ± 80,08 a**, b***
Immobility time (sec)	168,20 ± 8,29	402,60 ± 13,70 a***	189,40 ± 26,09 a^{ns}	312,20 ± 21,33 a**, b***
Time spent in the periphery (sec)	202,60 ± 10,16	252,60 ± 32,49 a**	219,20 ± 17,24 a^{ns}	241,80 ± 30,67 a**, b***
Time spent in the center (sec)	46,40 ± 13,83	15,00 ± 7,00 a**	29,50 ± 4,82 a*	9,20 ± 5,98 a***,b*
Number of turnarounds	10,800 ± 1,304	2,400 ± 1,673 a***	17,00 ± 1,245 a*	6,00 ± 3,125 A *,b**
Number of entries in the center	3,400 ± 1,342	1,200 ± 0,447 a**	2,100 ± 1,203 a^{ns}	1,200 ± 0,248 a**,b^{ns}

(* p<0.05; ** p<0.01; *** p<0.001 ns; not significant (comparison a vs T and b vs S; n=5)

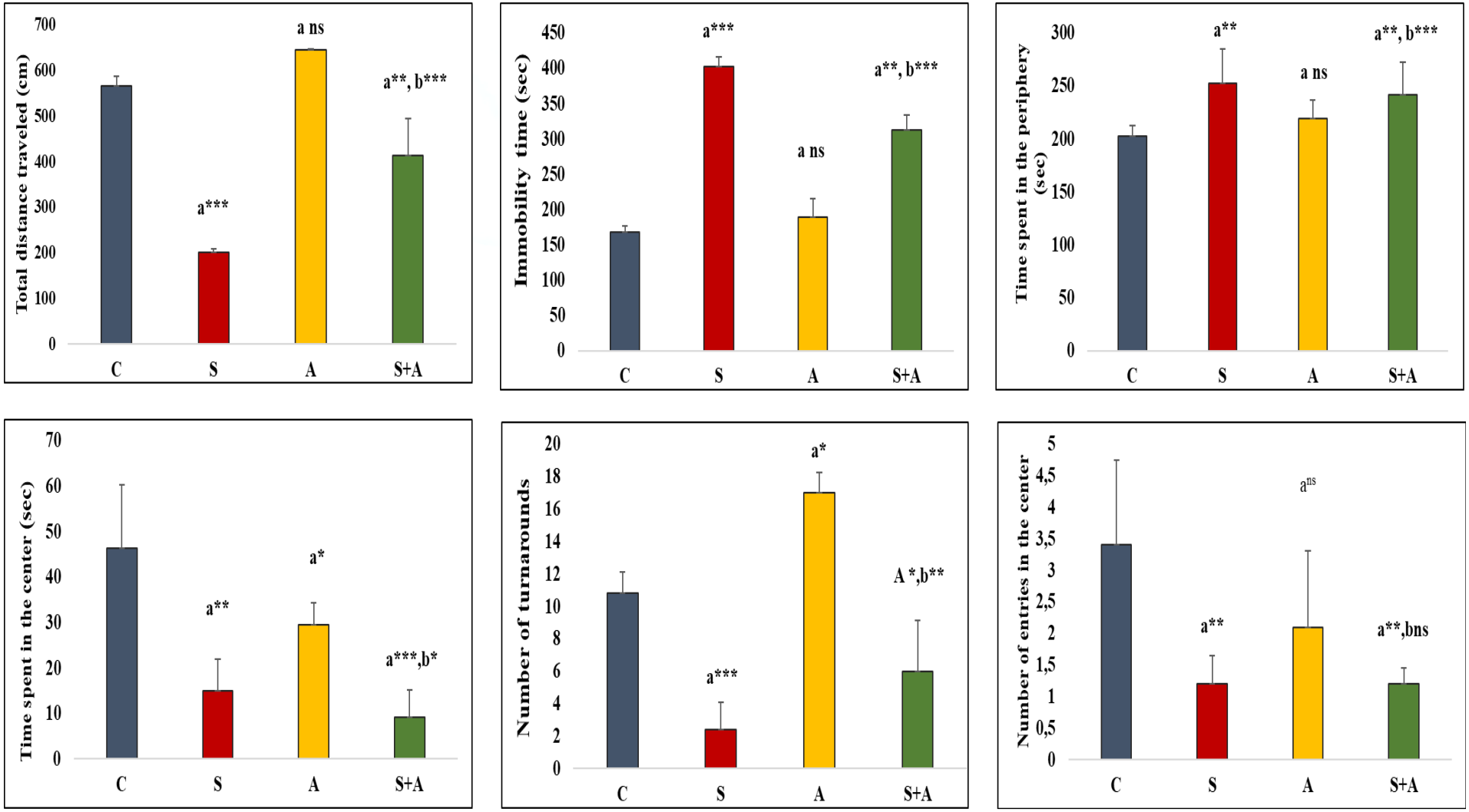


Figure 25: Variation of OF parameters in the control, stressed and treated batch.

3.7.2 Effect of restraint stress and Ashwagandha on the parameters of the forced swimming test

According to our results, the application of restraint stress **induces** a significant increase ($p<0.001$) in the time of immobility compared to the control. Thus, the treatment of this group with Ashwagandha reduced this increase **remarkably** ($p<0.001$) compared to the stressed group.

Regarding swimming and climbing time, stressed rats show a significant decrease ($p<0.001$) in swimming and climbing time ($p<0.001$) compared to controls. Ashwagandha treatment remarkably increased swimming and climbing time significantly compared to the stressed group.

Table 9: Variation of FST parameters in the control, stressed and treated batch.

Parameters/batches	C	S	A	S+A
Swimming time (sec)	140,00 ± 17,45	56,20 ± 8,11 **a*****	147,20 ± 8,42 **ans**	85,80 ± 11,37 **a **, b*****
Immobility time (sec)	69,00 ± 12,90	136,80 ± 18,32 **a*****	80,30 ± 18,94 **a ns**	95,30 ± 14,34 **a *, b****
Climbing time (sec)	82,20 ± 19,94	30,40 ± 8,56 **a*****	80,40 ± 17,06 **a ns**	71,80 ± 13,46 **a ns, b*****

(* $p<0.05$; ** $p<0.01$; *** $p<0.001$ ns; not significant (comparison a vs T and b vs S; n=5)

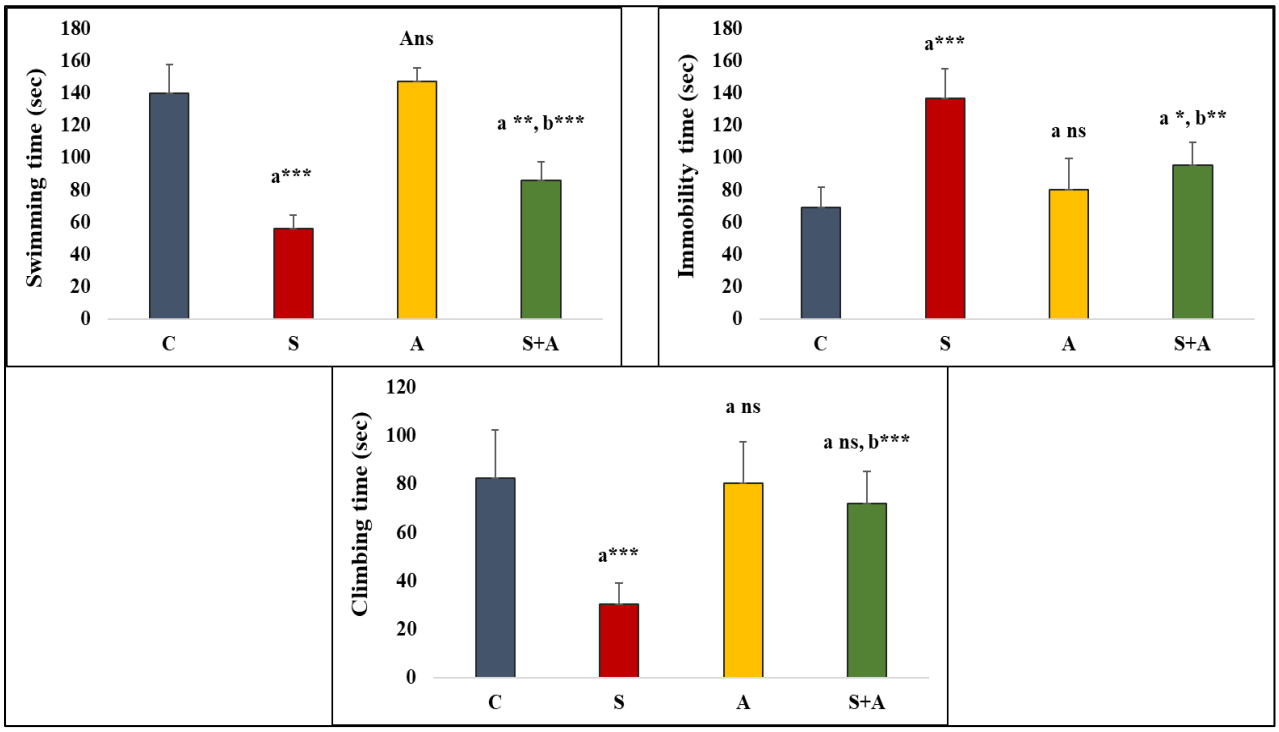


Figure 26: Variation of FST parameters in the control, stressed and treated batch.

3.7.3 Effect of Restraint Stress and Ashwagandha on the Parameters of the Elevated Plus-Maze Test

The results of the stressed rats show a significant increase ($p<0.001$) in the time spent in the closed arms ($p<0.001$) as well as the number of entries into these arms ($p<0.01$) compared to the controls. On the other hand, a significant decrease ($p<0.001$) in the time spent in the open arms ($p<0.001$) as well as the number of entries into these arms ($p<0.01$) compared to the controls. However, treatment of stressed rats with Ashwagandha modulated exploration time in both arms. Regarding the time spent in the center, no significant difference was recorded in all groups.

Concerning the number of rearings, the stressed rats show a significant reduction in the number of rearings ($p<0.001$) compared to the controls.

However, treatment with Ashwagandha increased this decrease significantly ($p<0.05$) compared to stressed rats

Tableau 10: Variation of EPM parameters in the control, stressed and treated batch.

Parameters/batches	C	S	A	S+A
Time spent in the center (sec)	28,800 ± 5,495	35,400 ± 5,413 a^{ns}	32,40 ±12,77 a^{ns}	28,20 ± 7,017 ans, b^{ns}
Time spent in open arms (sec)	173,80 ± 6,53	74,00 ± 19,08 a***	176,20± 29,30 a^{ns}	117,40 ± 7,23 a***, b**
Time spent in the distal part of the open arm (sec)	66,00 ± 2,739	6,200 ± 2,588 a***	56,00 7,30 a*	46400± 6,76 a**, b***
Number of entries at open arms	2,400 ± 1,1402	0,800 ± 0,447 a**	1,600 ± 0,547 a^{ns}	1,00 ± 0,707 a*,b^{*}
Time spent in closed arms (sec)	86,86 ± 4,45	131,80 ± 38,58 a***	85,60± 14,60 a^{ns}	95,00 ± 9,49 a* ; b***
Time spent in the distal part of the closed arm (sec)	77,80 ± 7,85	126,60 ±5,22 a***	72 .80 ± 16,18 a^{ns}	97,60 ± 6,86 a**, b***
Number of closed arm entries	1,800 ± 0,4472	1,200 ± 0,447 a*	1,400 ± 0,547 a^{ns}	1,300 ± 0,5477 a^{ns},b^{ns}
Number of turnarounds	20,200 ± 1,789	11,20 ± 2,387 a***	21,00 ± 1,701 a^{ns}	12,80 ± 2,664 a**, b*

(* $p<0.05$; ** $p<0.01$; *** $p<0.001$ ns; not significant (comparison a vs T and b vs S; n=5

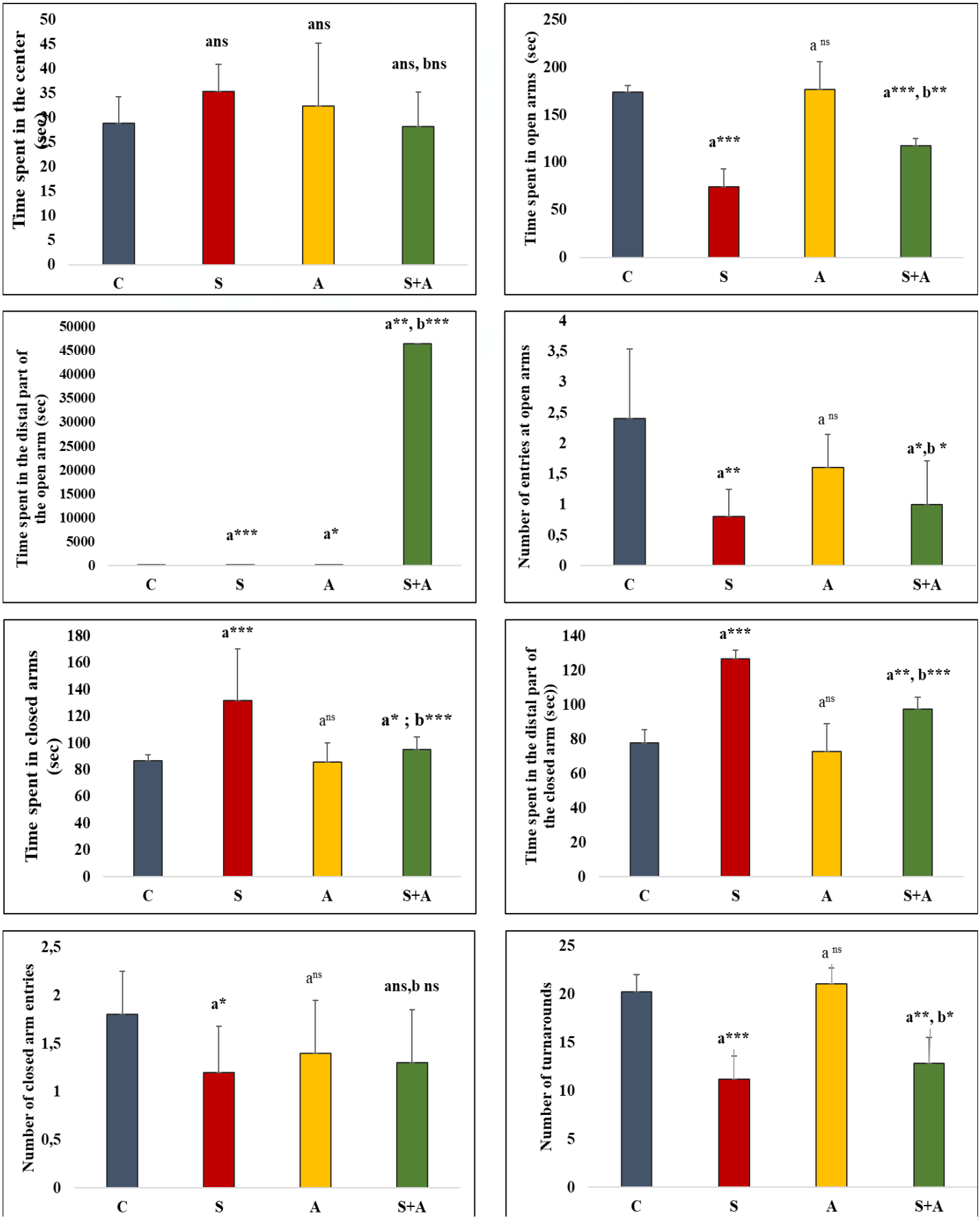


Figure 27:Variation of EPM parameters in the control, stressed and treated batch.

3.7.4 Effect of restraint stress and Ashwagandha on the parameters of the new object recognition test

According to our results, the stressed rats spent less time exploring the new object compared to the family object, unlike the control group, indicating that the latter did not did not remember the family object the statistical study confirms that there is a significant difference ($p \leq 0.01$). On the other hand, the treatment of stressed rats with Ashwagandha improves the rats' memory for the family object so they spent a lot of time exploring the new object.

Table 11: Variation of TRO parameters in the control, stressed and treated batch.

Parameters/batches	C	S	A	S+A
Latency time (sec)	6,200 ± 1,643	15,200 ± 4,65 a**	3,600 ± 1,074 a^{ns}	7,600 ± 2,074 a^{ns}, b**
Family object exploration time (sec)	21,20 ± 7,530	34,80 ± 8,349 a**	28,20 ± 6,155 a^{ns}	25,60 ± 5,215 a^{ns} ;b **
NEW OBJECT EXPLORATION TIME (sec)	49,00 ± 20,36	13,40 ± 4,16 a**	41,80 ± 15.74 a^{ns}	32,60 ± 7,13 a* ; b ***

(* p<0.05; ** p<0.01; *** p<0.001 ns; not significant (comparison a vs T and b vs S; n=5

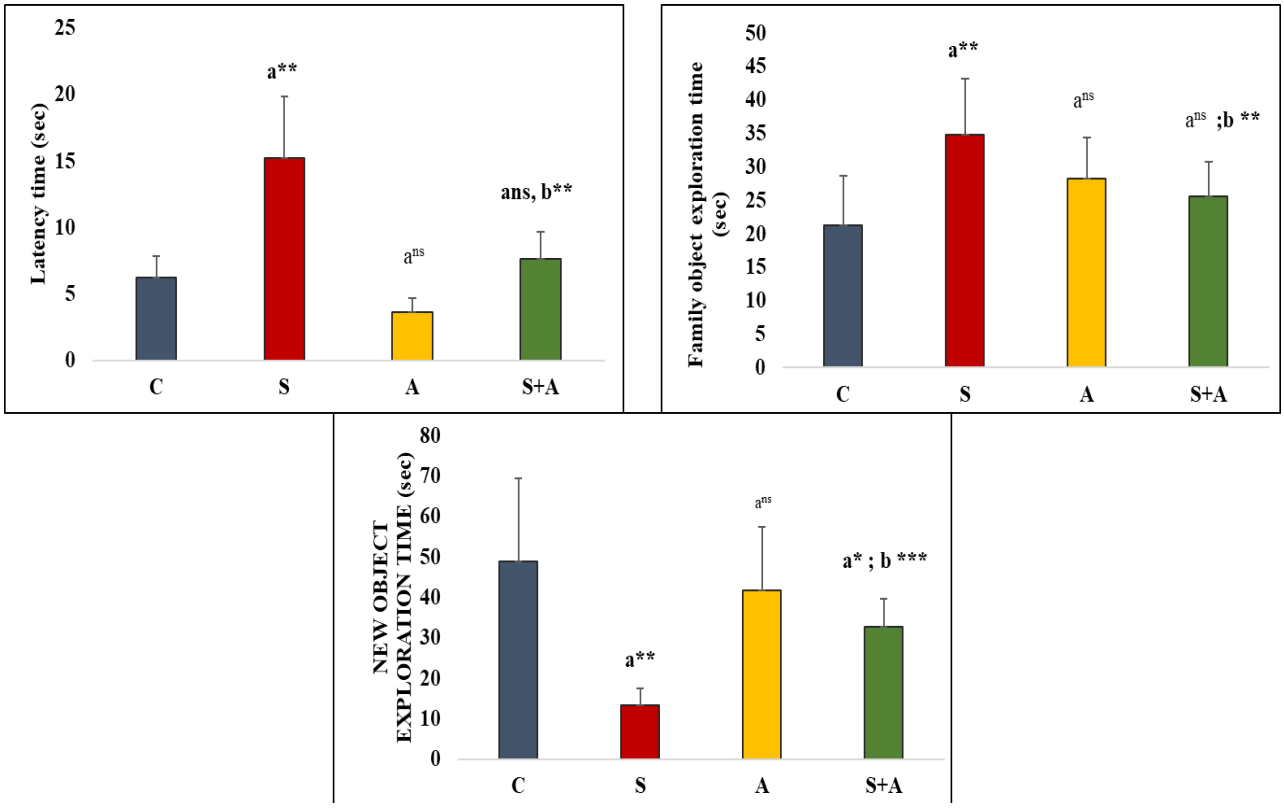


Figure 28: Variation of TRO parameters in the control, stressed and treated batch

DISCUSSION

Discussion

Stress is known to systematically impair the immune, cardiovascular, neuroendocrine and autonomic nervous systems, as well as brain activity. Hypothalamic-pituitary-adrenal (HPA) axis is known to be a major adaptive or maladaptive pathway to psycho-emotional stress (Stratakis and Chrousos, 1995). Thus, Withania somnifera (Ashwagandha) is advertised widely for its apparent "multiple beneficial effects on a number of organs including the central nervous and endocrine systems, as well as the ability to alleviate pain by its anti-inflammatory property and generate calming effects by lowering adrenal steroids". However, similar to many other herbal medicinal products, adverse effects of Ashwagandha on humans have not been well documented (Christopher et al., 2022). It's widely known for its neuroprotective activity and promotion of memory enhancement. These two factors are directly related to its antioxidant properties. Its components play multiple roles in the regulation of oxidative stress, the main mechanism of which is free radical scavenging and indirect inhibition of free radical formation (Remenapp, et al 2022). **Effect of restraint stress and Ashwagandha treatment on body weight gain**

In our study, we recorded a decrease in body weight in stressful rats compared to controls (T: 7.17% vs Stressed: -8.57%) which is due to the decrease in food consumption under the influence of stress and may also have been associated with stress-induced increases in metabolic demands, reduced digestion, and increased adrenal steroid secretion **(Nayanatara et al., 2012).** Our results agree with those of **(Christaki et al., 2013; Katzer and Bradshaw, 2008; Manzoni et al., 2009; Guedri et al., 2017).** On the other hand, a slight decrease of weight gain was noticed in rats treated with Ashwagandha for 21 days, this is due to the anti-obesogenic effect of the plant, it has been shown that WS stimulates the seroteninergic system which induces hypophagia **(Schembre SM et Al .,2011)**

- **Effect of restraint stress and treatment with Ashwagandha on the absolute weight of the adrenal glands**

In this study, we recorded the significant ($P \leq 0.01$) increase in adrenal weight in stressed subjects compared to controls (T: 0.0174 ± 0.001 vs Stressed: 0.0206 ± 0.002) which is explained by the high level of glucocorticoids secreted by the adrenal cortex due to prolonged exposure to chronic stress (**Herman et al., 1995; Ulrich-Lai et al., 2006; Davy et al., 2022**). On the other hand, the treatment of stressed rats with Ashwagandha reduce this increase. Recently, it is possible that the steroidal compounds from ashwagandha, such as

With anolides and alkaloids, may have a direct effect on adrenal function. Adrenal steroidogenesis is complex, involving a pathway of precursor hormones that require specific enzymatic steps (Han et al., 2014). It has been reported that adrenal function was suppressed during the period when the patient was taking Ashwagandha, which was reversed to normal function after discontinuation of this herbal product (Christopher et al., 2022). **Effect of restraint stress and Ashwagandha treatment on absolute brain weight**

In our study, the assessment of absolute brain weight shows a significant decrease ($P \leq 0.05$) in stressful rats compared to controls (T: 1.1880 ± 0.069 vs Stressed 1.0980 ± 0.029), this is due to the action of glucocorticoids which induce atrophy in several brain regions such as the hippocampus and the prefrontal cortex. Chronic Stress Can produce neuronal atrophy and cell death in the hippocampus while leaving other brain regions intact **(Magarinos et al., 1997).**

On the other hand, the treatment of stressed rats with Ashwagandha increased this decrease, this is due to the neuroprotective effect of the root of W somnifera as well as the beneficial actions of the plant against ischemia/reperfusion injury, hypoxia, vascular and cardiovascular diseases **(Hosny et al., 2021; Kuboyama et al., 2014)**

➢ **The effect of restraint stress and Ashwagandha on biochemical parameters**

✓ **Lipid balance**

In our research, we recorded a significant ($p \leq 0.001$) increase in cholesterol in Stressed Rats compared to controls (T: 0.740 ± 0.096 vs Stressed: 1.204 ± 0.047), this increase is due to increased axis activity hypothalamic-pituitary resulting in increased release of catecholamines and corticosteroids **(Lakshmi and Sudhakar, 2009**). It's well known that catecholamines activate adipose tissue lipolysis and increase the flow of free fatty acid to the liver where increased triglyceride synthesis and secretion occurs. Also, the adrenals during chronic stress to continue to produce cortisol will send signals to the liver to Increase its cholesterol production. Our results agree with the work of **(Guedri et al., 2017)**

On the other hand, the treatment of stressed rats with Ashwagandha reduced this increase. Our results agree with that of **(Jain et al., 2000; Shabir Ahmad Rather et al, 2013).** Further interesting was to note the significant reduction in total cholesterol and gradual decreasing trend in triglycerides. Lipid-lowering activity of WS has been reported earlier in hypercholesterolemic subjects **(Raut, A. A et al., 2012)**

✓ **Carbohydrate balance**

In our study, a significant ($P \leq 0.01$) increase in blood glucose level in stress rats compared to controls (T: 1.098 ± 0.0363 vs S: 1.264 ± 0.068). What is translated by the effect of

glucocorticoids have a hyperglycemic action by stimulation of gluconeogenesis and by a reduction in the consumption of glucose by peripheral tissues. Glucose metabolism is a factor resulting from the activation of the metabolic systems body after a stressful situation (**Hargreaves, 1990**). Many studies have showed an increase in glucose concentration both after restraint and after forced swimming in rats **(Rafter, 2001).** Corticosterone is a true initiator and metabolic regulator. This hormone stimulates the increase in blood glucose and which therefore releases energy from the body's reserves.

In our results, WS has decrease the increase level of glycaemia in stressed group, it has been reported that Ashwagandha root powder can be used as a useful ingredient to produce low glycemic index (GI) food product with favorable sensory characteristics and too prove to be positive in the healing and/or hindrance of impair glucose acceptance, insulin resistance and in addition being an effective means of controlling glucose levels. Therefore, it is a vital role for healthy and nutritious products which may be beneficial in avoidance and managing of Type II Diabetes **(Gill., et al 2019).**

✓ **Hormone Balance**

In our study we recorded a significant increase in the level of cerebral ACTH ($p \leq 0.001$) in the stressed rat compared to the control group (T: 133.20 ± 6.65 vs Stressed 599.20 ± 61.85) this increase is reflected by the hyperactivation of the hypothalamic-pituitary-adrenal axis and the deficit of negative feedback exerted by glucocorticoids on the corticotropic cells of the anterior pituitary. On the other hand, treatment with Ashwagandha significantly attenuated this increase compared with the stressed group ($p \leq 0.001$). (Stressed: 599.20 ± 61.85 vs S+G: 209.40 ± 73.73). It has been reported that Ashwagandha also seems to reduce cortisol levels **(Chandrasekhar et al., 2012; Choudhary et al.,2017; Lopresti et al., 2019)**

➢ **Effect of restraint stress and Ashwagandha on hematological parameters**

Several studies have shown that emotional stress could affect the immune response in animals and humans **(Nascimento et al., 2004; Leandro et al., 2006; Ribas et al., 2011**). And induces alterations in hematological parameters.

Our results show a significant increase ($P \leq 0.01$) in total leukocytes, Lymphocytes and Eosinophils. The decrease in white blood cells could be caused by their redistribution in peripheral tissues such as the skin and the lymph nodes or by the destruction of stem cells and the immunosuppression exerted by glucocorticoids and even by catecholamines (Anti-inflammatory and immunomodulatory activity), by inhibiting the proliferation of T lymphocytes, decrease the bactericidal activity of macrophages and suppress the cytotoxic

activity of natural killer (NK) cells. However, glucocorticoids can also exert immunostimulatory properties on B lymphocytes, sometimes playing an immunostimulatory role, sometimes an immunosuppressive one **(Steele, 2002).**

Ashwagandha organic root normalize blood cell perturbation in stressed rats. Recent study showed that the rootextract (concentrations of 25 and 50 mg/kg) exhibits significant anti-stress and apoptogenic activities in stress-induced immunological perturbations of mice (**Bhattacharya et Muruganandam., 2003).**

Effect of restraint stress and Ashwagandha extract on cerebral redox status

Several studies have shown that restraint stress induces oxidative stress in the brain and an impairment of its functions **(Buynitsky and Mostofsky, 2009; Kumar et al., 2012).** Oxidative stress results from an imbalance between oxidative stressors and antioxidant capacity. Mammals have evolved an antioxidant system consisting of non-enzymatic and enzymatic components, including catalase, SOD and GSH **(Storz and Imlay, 1999).** In the present study, the antioxidant capacity of brain tissues was moderately reduced by repeated restraint stress, as shown by a significant decrease ($p \leq 0.01$) in glutathione-s-transferase (GST) in the stressed batch and a significant increase in MDA levels. This due to increased glucocorticoid levels has been associated with inhibition of Nrf2 gene expression leading to reduced expression and activity of antioxidant enzymes **(Ki et al., 2000**). Recent studies have shown that the exogenous administration of glucocorticoids and the application of chronic restraint stress have also been shown to induce a decrease in enzymatic and non-enzymatic factors, including SOD, CAT, GST and reduced glutathione in rat brain samples. Moreover, the increased generation of free radicals induced by glucocorticoids was significantly attenuated by treatment with exogenous antioxidants **(Manikadan, et al., 2006, McIntosh et al., 1998**).

In addition, reactive oxygen species (ROS) have also been shown to cause stimulation of adrenocorticotrophin (ACTH) hormone production by the pituitary, which increases HPA axis activity via a reduction of negative feedback, which may further exacerbate glucocorticoid-induced oxidative stress **(Lacoste et al., 2001, Amber et al., 2018**).

Interestingly, the treatment batch combined S+G modulated significantly ($P \leq 0.05$) this decrease in GST and the increase in MDA compared to the stressed batch (stressed: 0.178 ± 0.019 vs S+G: 0.210 ± 0.015). In our study, Ashwagandha reversed the increase levels of MDA, and decreased GST activities in the brain²s of rats exposed to immobilization stress, confirming that Ashwagandha as a potential agent in the protection of neurons suffering from oxidative stress. Lipid peroxidation activity of aqueous suspension of organic ashwagandha roots was

investigated by administration to mice and rabbits at a dose of 100 mg/kg after 6hr intervals. The concentration of lipid peroxide was decreased in K. pneumoniae and S. aureus which advocated the prophylactic activity against stress induce lipid peroxidation **(Dhuley., 1998).** It was suggested that the anti-oxidant potential of withanolides might be due to the hydroxylated long chain of the carbon-bearing acyl group. Other compounds such as sitoindosides VII-X and withaferin A were investigated as potent initiators for free-radical scavenging enzymes, catalase, glutathione peroxidase, and superoxide dismutase in the striatum and frontal cortex of rat's brain **(Bhattacharya et al., 1997).** It was also noticed that withanoside V displayed prominent free radical scavenging activity at 10 µg/ml concentration **(Sood et al., 2018).** Aqueous extract of the roots was tested for the anti-oxidant effect in male albino rats against cypermethrin induced oxidation. Extract, when administered at a dose of 5 ml (10% root's extract) for 60 days to male albino rats, showed the complete restoration of all biochemical and hematological parameters **(Maheswari et Manisha., 2015).**

➢ **Effect of restraint stress on behavior**

✓ **Effect of restraint stress and Ashwagandha on the parameters of the elevated plus-maze test:**

Exploratory behavior is one of the preliminaries of learning. The latter can be defined as a process of acquiring new knowledge or new skills. This acquisition is at the origin of a new representation of the environment and of specific and persistent behavioral changes **(Vann and Albasser, 2011).**

The results of the stressed rats show a significant increase ($p \leq 0.001$) in the time spent in the closed arms ($p \leq 0.001$) as well as the number of entries into these arms ($p \leq 0.01$) compared to the controls. On the other hand, a significant reduction ($p \leq 0.001$) in the time spent in the open arms ($p \leq 0.001$) as well as the number of entries into these arms ($p \leq 0.01$) compared to the controls. These results show the anxiogenic effect of restraint stress. This is due to hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis which leads to increased plasma glucocorticoids in the brain which lead to neuronal activation of the dopaminergic and cholinergic system which results in a reduction in the concentration of serotonin. Thus, the hyperactivation of the sympathetic axis stimulates the immune system which increases the cerebral pro-inflammatory cytokines which in turn react on these neuronal receptors and alter the metabolism of tryptophan which induces the reduction of serotonin. Various studies have in fact shown that chronic restraint stress induces an increase in the level of anxiety assessed by the elevated plus-maze test (place maze test) **(Guedri et al., 2017; Huynh et al., 2011; Cliona**

et al., al., 2011; Shuichi et al., 2012; Sansri et al., 2014). Chronic restraint stress induces anxiety like behavior in rodents **(Huynh et al., 2011; Cliona et al., 2011; Shuichi et al., 2012)** and a decrease in the number of open arm entrances indicates a decrease general motor activity **(Brummelte et al., 2012**), which agrees with our results.

However, treatment of stressed rats with Ashwagandha modulated the exploration time in both arms in stressed rats, which shows the anxiolytic effect of the plant. The anxiolytic type effects. In interesting investigation, *W. somnifera* revealed significant improvement against anxiety disorder in patients. The primary impact observed was on the "anxious mood" in participants receiving treatment with *W. somnifera* **(Anbalgan et Sadique., 1981).** Ashwagandha (*Withania somnifera*) is an adaptogen herb that is purported to prevent and treat the effects of stress. Research reveals that Ashwagandha de-creases cortisol levels, perceived stress, anti-anxiety, and blood pressure in people under chronic stress **(Chandrasekhar et al., 2012; Choudhary et al., 2017; Pratte et al., 2014)**

- ✓ **Effect of restraint stress and Ashwagandha on open field test parameters**

The statistical analysis of the different variables measured in this test revealed a significant difference between the different groups. We noted a significant decrease in the stressed groups compared to the control groups with regard to the total distance traveled ($p \leq 0.001$), time spent at the center ($p \leq 0.001$) and the number of entries to the center ($p \leq 0.01$) and the number of rectification ($P \leq 0.05$). Indeed, restraint stress applied acutely or repeatedly is usually considered very intense and leads to an increase in plasma corticosterone concentration (**Bhatnagar et al., 2006; Marini et al., 2006; Barnum et al., 2007)** and an increase in behaviors indicating a high level of anxiety within the OF, **(Regenthal et al., 2009)** and therefore a reduction in the number of boxes crossed in the OF, indicates a decrease in motor activity characteristic of a higher level of anxiety in these animals **(Prut and Belzung, 2003; Meerlo et al., 1996; Marini et al., 2006; Kin et al., 2008; Kalvez, 2010 ; Carli et al., 1989; Kasar et al., 2009; Sansri et al., 2014 ; Guedri et al., 2017).**

However, treatment of stressed rats with Ashwagandha prevented this increase with a significant difference ($p \leq 0.001$) compared to stressed rats. Aswagandha is richness in flavonoid **(Ahmeda and El-Darier 2023).** The effect of flavonoids on brain and cell function includes neuroprotection, reduction of neuro-inflammation, and enhancement of memory, learning, and cognitive function **(Lu et al, 2007).** More, flavonoids contribute to permeability to nutrition, oxygen uptake and ATP synthesis **(Mahadevan and Park, 2008).**

- ✓ **Effect of restraint stress and Ashwagandha on the parameters of the novel object recognition test (TRO)**

Chronic restraint stress models are the most popular for studying the mechanisms of cognitive impairments or disturbances **(Chen et al., 2010; Yi et al., 2013).** The main physiological responses of chronic stress include the hypothalamic-hypophysio-adrenal (HPA) axis and the sympatico-adrenal medulla system, through which the levels of corticosterone and catecholamines could be modified **(Cohenet Hamrick, 2003; Yi et al., 2013). This** results in a modification of cognitive functions including learning and spatial memory **(Yi et al., 2013).** Disorders: spatial learning/memory are to be linked to the specific alterations of the hippocampus on which these behavioral tasks largely depend **(Vann and Albasser, 2011**).

The object recognition test (ORT) is a commonly used behavioral test for studying various aspects of learning and memory in mice. The ORT is quite simple and can be completed over a period of 3 days: habituation day, training day and test day. During training, the rats is allowed to explore 2 identical objects. On the day of the test, one of the training objects is replaced by a new object. Because rats have an innate preference for novelty, according to our results, stressed rats spent less time exploring the new object compared to the family object unlike the control group indicating that the latter did not remember the family object the statistical study effectively confirms that there is a significant difference ($p \leq 0.01$). So chronic restraint stress impairs recognition memory. Which is due to the increase in endogenous glucorticoids, which induce an anatomical and functional alteration of the hippocampus which induce atrophy. This hippocampal atrophy would be linked in particular to the neuronal effects of stress, which induces an increase in the release of glutamate. The hippocampus also appears to be an anatomical site correlated with depression **(Zhang et al., 2007; Nasuti et al., 2013).**

On the other hand, the treatment of stressed rats with Ashwagandha improves the rats' memory for the family object so they spent a lot of time exploring the new object. Our results agree with that of **(Khalil et al., 2021).** It has been reported that Ashwagandha improves cognitive function in adults with mild cognitive impairment (**Choudhary et al., 2017; Remenapp et al., 2022).**

- ✓ **Effect of restraint stress and Ashwagandha on the parameters of the forced swimming test (FST):**

The FST is an antidepressant efficacy test, represents an aversive and stressful where the rat cannot escape and produces immobility, hopeless behavior (**Porsolt et al., 1977; Kirby and**

Lucki, 1997). In animals, immobility is interpreted as a lack of will to survive and considered a sign of depression in the mice and rats **(Porsolt et al., 1977; Petit-Demouliere et al., 2005)**

According to our results, the application of restraint stress induces a significant increase ($p \leq 0.001$) in immobility time and a significant decrease in immobility time and escalation time compared to the control. this shows the depressogenic effect of restraint stress. Recent studies have shown that prolonged exposure to stress may increase neuronal and astrocytic death and lead to hippocampal atrophy and a decrease in glutamate and glutamine, which in turn impair neuronal.

In our study, Ashwagandha exerted un antidepressant effect by the reduction of immobility time and the increase of swim and escalade time in stressed rats. Our results are in accord with that of (**Gupta et al., 2018; Haque et al., 2021; Kuboyama et al., 2006; Kaurav et al., 2012; Kanjilal S et al., 2021; Davis et al., 2000).**

CONCLUSION & PERSPECTIVES

Conclusion and perspectives

We can conclude that the repeated exposure of female Wistar rats for 21 consecutive days to chronic restraint stress at a rate of 3h/day induced anxiety and depression like behavior responses associated with cognitive disorders.

These neurobehavioral disturbances are associated with the development of cerebral oxidative stress and alteration of biochemical parameters (glycaemia, cholesterol and ACTH).

In addition, the treatment of stressed rats with the organic root of Withania somnifera (Ashwagandha) attenuated behavior and cognitive disorders and reduce oxidative stress.

From these results, it would be interesting to draw the following perspectives:

- ✓ Investigate memory performance in the Morris aquatic Maze test
- ✓ Phytochemical screening of the plant to qualitatively and quantitatively the major families of metabolites
- ✓ Histological study to allow the search for possible pathological tissue lesions
- ✓ Dosage of interleukin and pro-inflammatory cytokines: IL-1b and IL-6
- ✓ Prolong the duration of restraint stress for 2 months

REFERENCES BIBLIOGRAPHIC

Ahmeda HA and El-Darier SM. (2023). Phytochemistry, allelopathy and anticancer potentiality ofWithania somnifera (L.) Dunal (Solanaceae Brazilian Journal of Biology, 84

Anbalagan K, Sadique J. (1981). Influence of an Indian medicine (Ashwagandha) on acute–phase reactants in inflammation. Ind J Exp Bio.19:245-249.

A. Remenapp, K. Coyle, T. Orange, T. Lynch, D. Hooper, S. Hooper, K. Conway, H.A. (2022). Efficacy of Withania somnifera supplementation on adult's cognition and mood Hausenblas Journal of Ayurveda and Integrative Medicine 13:100510

Balkaya M, Prinz V, Custodis F, Gertz K, Kronenberg G, Kroeber J, Fink K, Plehm R, Gass P, Laufs U, Endres M.(2011). Stress worsens endothelial function and ischemic stroke via glucocorticoids. Stroke.42(11):3258-64.

Barden, J., Maddux, W.W., Petty, R.E., & Brewer, M.B. (2004). Contextual Moderation of Racial Bias: The Impact of Social Roles on Controlled and Automatically Activated Attitudes. Journal of Personality and Social Psychology, 87(1), 5-22.

Bardin, L., Malfetes, N., Newman-Tancredi, A., Depoortère, R. (2009). Chronic restraint stress induces mechanical and cold allodynia, and enhances inflammatory pain in rat: Relevance to human stress-associated painful pathologies.Behavioural Brain Research, 205, 360–366.

Barnum CJ, Blandino P Jr, Deak T. (2007). Adaptation in the cortico-sterone and hyperthermic responses to stress following repeated stressor exposure. J Neuroendocrinol 19(8):632–642.

Benchikh Nora, L.H. (2016). Effet d'un traitement à base d'extrait d'algue marine peyssonnelia sp, associé au BCG, sur deux modeles de stress chez le rat Wistar: CMS et inflammation chronique par ligature du nerf sciatique (Doctoral dissertation)

Bhattacharya SK, Muruganandam AV. (2003). Adaptogenic activity of Withania somnifera: an experimental study using a rat model of chronic stress. Pharma Biochem Behav; 75:547-555

Bhattacharya SK, Satyan KS, Chakrabarti A. (1997). Effect of Transina (TR), an ayurvedic herbal formulation, on pancreatic islet superoxide dismutase (SOD) activity in hyperglycaemic rats. Ind J Exp Bio 1997; 3:297-299.

Bhatnagar et al., (2006). Bhatnagar J, Shinyama H, Morton N, Mullins J, Seckl J, Flier J., A transgenic model of visceral obesity and the metabolic syndrome. Science.; 294:pp.2166-70;;

Borgerson K (2005). Evidence-based alternative medicine? Perspect Biol Med 48: 502–515.

Breier, A., Albus, M., Pickar, D., Zahn, T.P., Wolkowitz, O.M., & Paul, S.M (1987). Controllable and uncontrollable stress in humans:Alterations in mood and neuroendocrineand psychophysiologicalfunc-tion.American Journal of Psychiatry,144, 1419-1425.

Brown RP, Gerbarg PL (2001). Herbs and nutrients in the treatment of depression, anxiety, insomnia, migraine, and obesity. J Psychiatr Pract 7: 75–91.

Brummelte S,. Lieblich SE, Galea LA.M (2012). Gestational and postpartum corticosterone exposure to the dam affects behavioral and endocrine outcome of the offspring in a sexuallydimorphic manner. Neuropharmacology ; 62, 406e 418

Buynitsky T& Mostofsky DI (2009).Restraint stress in biobehavioral research: Recent developments. Neurosci Biobehav Rev; 33, 1089-1098.

Calvez, J. (2010). Stress et prise alimentaire-Application à l'étude de l'effet anti-stress d'un extrait de levure chez le rat (Doctoral dissertation, Agro Paris Tech)

Carli M, Prontera C, Samanin R., (1989). Effect of 5-HT1A agonists on stress-induced deficit in open fieldlocomotor activity of rats: evidence that this model identifies anxiolyticlike activity. Neuropharmacology . 28:471-476

Chandrasekhar, K., Kapoor, J., & Anishetty, S. (2012). A prospective, randomized double-blind,placebo-controlled study of safety and efficacy of a high-concentration full-spectrum extract of Ashwagandha root in reducing stress and anxiety in adults. Indian Journal of Psychological Medicine, 34(3), 255-262.

Chen Y, Mao Y, Zhou D, Hu X, Wang J, Ma Y. (2010). Environmental enrichment and chronic restraint stress in ICR mice: effects on prepulse inhibition of startle and Y-maze spatial recognition memory. Behav Brain Res. : 212, pp.49–56.

Choudhary, D., Bhattacharyya, S., Joshi, K. (2017). Body weight management in adults under chronic stress through treatment with ashwagandha root extract: a double-blind, randomized, placebo-controlled trial. Journal of evidence-based complementary & alternative medicine, 22(1):96-106

Choudhary D, Bhattacharyya S, Bose S (2017). Efficacy and safety of Ashwagandha (withania somnifera (L.) dunal) root extract in improving memory and cognitive functions. J Diet Suppl.14:599e612

Christaki E, Kokkinos A, Costarelli V, Alexopoulos EC, Chrousos GP., Darviri C (2013). Stress management can facilitate weight loss in Greek overweight and obese women: a pilot study. J Hum Nutr Diet 26: 132 .

Christopher H. David F· Thang S (2022). Adrenal hypofunction associated with ashwagandha (Withania somnifera) supplementation: a case report. Toxicology and Environmental Health Sciences14:141–145

Cliona, M. et al., (2011). Strain differences in the neurochemical response to chronic restraint stress in the rat: relevance to depression. Pharmacol. Biochem. Behav. 97: 690-699 .

Conrad DF, Pinto D, Redon R, Feuk L, Gokcumen O, Zhang Y, (2010). Origins and functional impact of copy number variation in the human genome. Nature. 464(7289):704– 712.

Datson, N.A., Morsink, M.C., Meijer, O.C., De Kloet, E.R. (2008). Central corticosteroid actions: Search for genetargets , Eur J Pharmacol, 583(2-3):272-89

De Kloet, E. R., Joëls, M. and Holsboer, F (2005). Stress and the brain: from adaptation to disease. Nat. Rev. Neuro. Sci. 6: 463-475 .

Dhuley JN. (1998). Effect of ashwagandha on lipid peroxidation in stress–induced animals. J Ethnopharma 1998; 60:173-178.

Shastry JLN. (2001). Ayurvedokta oushadha niruktamala, Chaukhambha Orientalia, Ist ed.

Varanasi, India.10.

Elder C, Aickin M, Bell IR, Fonnebo V, Lewith GT, et al. (2006) Methodological challenges in whole systems research. J Altern Complement Med 12: 843–850.

Fakhri S., Piri S., Moradi S.Z., Khan H. (1998). Phytochemicals Targeting Oxidative Stress, Interconnected Neuroinflammatory, and Neuroapoptotic Pathways Following Radiation. Curr. Neuropharmacol)

Fink, G. (2010). Stress: Definition and history. Stress science: neuroendocrinology, 3(9): 3-14.

Gill, M. K., Kumar, S., Sharma, M., Singh, T. P., Kumar, K., & Kaur, R. (2019). Role of Ashwagandha Incorporated Functional Foods for Betterment of Human Health: A Review. Journal of Agricultural Engineering and Food Technology p-ISSN, 2350-0085.

Finsterwald C, Alberini CM (2014). Stress and glucocorticoid receptor-dependent mechanisms in long- term memory: from adaptive responses to psychopathologies. Neurobiol Learn Mem. ;112C:17–29.

Furukawa T, Watanabe N, Churchill R, (2007). Combined psychotherapy plus antidepressants for panic disorder with or without agoraphobia. Cochrane Database Syst Rev. pp CD004364.

Goldstone, B. (1952). The general practitioner and the general adaptation syndrome. South African medical journal, 26(5), 99-92.

Gregus A, Wintink AJ, Davis AC, Kalunchuk L E., et al., (2005). Effect of repeated corticosterone injections and restraint stress on anxiety and depression-like behavior in male rats. Behav Brain Res.156:105-14.

Han TS, Walker BR, Arlt W, Ross RJ. (2014). Treatment and health outcomes in adults with congenital adrenal hyperplasia. Nat Rev Endocrinol 10:115–124

Hargreaves KM,(1990). Neuroendocrine markers of stress. AnesthProg ; 37:99-105

Harikrishnan VS et al. (2018). A comparison of various methods of blood sampling in mice and rats : Effects on animal welfare. Laboratory Animals.

Herman, J.P .; Adams, D.et Prewitt, C. (1995). Regulatory changes in neuroendocrine stressintégrative circuitry produced by a variable stress paradigm. Neuroendocrinology 61(2): 180– 190. PMID: 775333

Hosny EN , El-Gizawy M , Sawie G, Abdel-Wahhab G, Khadrawy K. (2021). Neuroprotective Effect of Ashwagandha Extract against the Neurochemical Changes Induced in Rat Model of Hypothyroidism. J Diet Suppl;18(1):72-91.

Hotopf M, Churchill R, Lewis G. (1999). Pragmatic randomised controlled trials in psychiatry. Br J Psychiatry 175: 217–223.

Huynh, T. N., Krigbaum, A. M., Hanna, J.J., Conrad, C.D.(2011). Sex differences and phase of light cycle modify chronic stress effects on anxiety and depressive-like behavior. Behav. Brain. Res. 222: 212-222 .

Jackson, M., Ramsden, E., & Cantor, D. (2014). The invention of the stressed animal and the development of a science of animal welfare, 1947-86.

Jo EJ et al. (2021). Comparison of murine retroorbital plexus and facial vein blood collection to mitigate animal ethics issues. Laboratory Animal

Kalvez J. (2010), Stress et prise alimentaire Application à l'étude de l'effet anti-stress d'un extrait de levure chez le rat.Paris,ABIES. 259

Guedri K, Frih H, Chettoum A., Rouabhi R1.(2017). Chronic Restraint Stress Induced Neurobehavioral Alterations and Histological Changes in Rat .Toxicol. Environ. Health. Sci. 9(2), 123-129

Katzer L, Bradshaw AJ, Horwath CC, Gray AR, O&39 ; Brien S, Joyce J.(2008). Evaluation of a stress reduction program for overweight women: a randomized trial. Am J Health Promot. 22(4):264-74.

Kazushige, M. et al.,(2000). Chronic Stress Induces Impairment of Spatial Working Memory Because of Prefrontal Dopaminergic Dysfunction. J. Neurosci. 20: 1568 -1574 .

K Thakur, A., Dey, A., S Chatterjee, S., & Kumar, V. (2015). Reverse Ayurvedic pharmacology of ashwagandha as an adaptogenic anti-diabetic plant: A pilot study. Current Traditional Medicine, 1(1): 51-61

Khalil, H. M., Eliwa, H. A., El-Shiekh, R. A., Al-Mokaddem, A. K., Hassan, M., Tawfek, A. M., & El- Maadawy, W. H. (2021). Ashwagandha (Withania somnifera) root extract attenuates hepatic and cognitive deficits in thioacetamide-induced rat model of hepatic encephalopathy via induction of Nrf2/HO-1 and mitigation of NF-κB/MAPK signaling pathways. Journal of Ethnopharmacology, 277:114-141

Krisanaprakornkit T, Krisanaprakornkit W, Piyavhatkul N, Laopaiboon M.(2006). Meditation therapy for anxiety disorders. Cochrane Database Syst Rev. CD004998.

Kuboyama T, Tohda C, and Komatsu K. (2014). Effects of Ashwagandha (Roots of Withania somnifera) on Neurodegenerative. Diseases Biol. Pharm. Bull. 37(6) 892–897

Kumar D, Puja B1, Sangeeta R1 and Vinod K.(2012). Persistence of Circannual Rhythms under Constant Periodic and Aperiodic Light 1 Conditions: Sex Differences and Relationship with the External Environment. Journal of Expérimental Biology. 10.1242/jeb.065581.

Lakshmi BVS et Sudhakar M.(2009). Screening of Psidium guajava Leaf Extracts for Antistress Activity in Different Experimental Animal Models Pharmacognocy Res. 1 (6): 359-366.

Leder S., Siwiak-Kobayashi M., Nerwice W.B.A.(2016) Psychiatria, Podręcznik dla Studentów Medycyny. Wydawnictwo Lekarskie PZWL; Warszawa, Poland.

Lopresti AL, et al. (2019). An investigation into the stress-relieving and pharmacological actions of an ashwagandha (Withania somnifera) extract: A randomized, double-blind, placebo-controlled study Medicine (Baltimore)

Lu, L., Wu, C., Lu, B., Xie, D., Wang, Z., Bahaji Azami, N.L., An, Y., Wang, H., Ye, G., Sun, M., (2020). BabaoDan cures hepatic encephalopathy by decreasing ammonia levels and alleviating inflammation in rats. J. Ethnopharmacol. 249, 112301.

Lupien SJ, McEwen BS, Gunnar MR, Heim C. (2009) . Effects ofstress throughout the lifespan on the brain, behaviour andcognition. Nat Rev Neurosci 10:434 – 445

Maccari S. Korte, Koolhaas JM, Wingfield JC, and McEwen., BS (2005). The Darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease, Neurosci. Biobehav. Rev. 29 (2005) 3-38.

Maheswari R, Manisha P. Withania Somnifera L (2015). Root extract ameliorates toxin induced cytotoxicity. Int J Pharma Sci Res; 6:848-855.

Mao, Y., Xu, Y., & Yuan, X. (2022). Validity of chronic restraint stress for modeling anhedonic-like behavior in rodents: a systematic review and meta-analysis. Journal of International Medical Research, 50(2);

Marini F, Pozzato C, Andreetta V, Jansson B, Arban R, Domenici E, Carboni L. (2006) .Single exposure tosocial defeat increases corticotropin-releasing factor and glucocorticoid receptor mRNA expression in rathippocampus. Brain Res ; 1067:25-35.

McEwen, B. S., Sapolsky, R. M.(2001). Stress and cognitive function. Curr. Opin. Neurobiol. 5, 205-216.

Meerlo P, De Boer S, Koolhaas Koolhaas J, Daan S , Hoofdakker R.(1996). Changes in daily rhythms ofbody temperature and activity after a single social defeat in rats. PhysiolBehav ; 59, pp.735-739.

Mikulska, P., Malinowska, M., Ignacyk, M., Szustowski, P., Nowak, J., Pesta, K., Cielecka-Piontek, J. (2023). Ashwagandha (Withania somnifera)—Current Research on the Health-Promoting Activities: A Narrative Review. Pharmaceutics, 15(4): 1057

Mishra LC, Singh BB, Dagenais S (2000). Scientific basis for the therapeutic use of Withania somnifera (ashwagandha): a review. Altern Med Rev 5: 334–346.

Nasuti C, Carloni M, Fedeli D et al. (2013). Effects of early life permethrin exposure on spatial working memory and on monoamine levels in different brain areas of presenescent rats. Toxicology 303: 162- 168

Nayanatara AK, Tripathi Y, Nagaraja HS, Jeganathan PS, Ramaswamy C, Ganaraja B, Sheila R., Asha K. (2012). Effect of Chronic Immobilization Stress on some selected Physiological, Biochemical and Lipid Parameters in Wistar Albino Rats. Research Journal of Pharmaceutical, Biological and Chemical Sciences 3(1):34-42

Panossian A, Wikman G. (2009). Evidence-based efficacy of adaptogens in fatigue, and molecular mechanisms related to their stress-protective activity. Curr Clin Pharmacol;4:198-219.

Pilkington K, Kirkwood G, Rampes H, Fisher P, Richardson J (2006). Homeopathy for anxiety and anxiety disorders: a systematic review of the research. Homeopathy 95: 151–162.

Pittler MH, Ernst E (2003). Kava extract for treating anxiety. Cochrane Database Syst Rev. Pp CD003383.

Provino R. (2010). The role of adaptogens in stress management. Aust J Med Herbal;22:41-9.

Prut L, Belzung C (2003). The open field as a paradigm to measure the effects of drugs on anxiety- likebehaviors: a review. Eur J Pharmacol ;463:3-33.

Rafferty AP, McGee HB, Miller CE, Reyes M (2002). Prevalence of complementary and alternative medicine use: state-specific estimates from the 2001 Behavioral Risk Factor Surveillance System. Am J Public Health 92: 1598–1600

Rafter. Psychiatry Res (2001). 103:93-96

Raut, A. A., Rege, N. N., Tadvi, F. M., Solanki, P. V., Kene, K. R., Shirolkar, S. G., ... & Vaidya, A. B. (2012). Exploratory study to evaluate tolerability, safety, and activity of Ashwagandha (Withania somnifera) in healthy volunteers. Journal of Ayurveda and integrative medicine, 3(3), 111.

Regenthal R, Koch H, Köhler C, Preiss R, Krügel U.(2009). Depression-like deficits in rats improved by subchronic modafinil. Psychopharmacology ; 204:627–39

Remenapp A, Coyle K, Orange T, Lynch T, Hooper D, Hooper S, Conway K,Hausenblas HA. (2022). Efficacy of Withania somnifera supplementation on adult's cognition and mood Journal of Ayurveda and Integrative Medicine 13:2

Rojo L, Conesa L, Bermudez O, Livianos L., (2006). Influence of stress in the onset of eating disorders: data from a two-stage epidemiologic controlled study. Psychosom. Med ; 68:628–635.

Roy-Byrne PP, Bystritsky A, Russo J, Craske MG, Sherbourne CD, et al. (2005). Use of herbal medicine in primary care patients with mood and anxiety disorders. Psychosomatics 46: 117–122.

Salima, B. (2019). Etude de l'effet du stress chronique de contention sur les paramètres biologiques et biochimiques chez les Hamsters syriens (Master memory, Universite laarbi tebessi tebessa)

Schembre SM (2011). Weight-related eating behavior questionnaires:applying theory to measurement. In: Preedy VR, Watson RR,Martin CR, eds. Handbook of Behavior, Food and Nutrition. NewYork, NY: Springer. 3487-3506.

Selye, H (1991). History and Present Status of the Stress Concept. In Stress and coping: An anthology (pp. 21-35). Columbia University Press.

Selye H (1976). The stress of life, 2d edition ed: Papermark Edition.

Sharma A et al., (2014). Safety and blood sample volume and quality of a refined retro-orbital bleeding technique in rats using a lateral approach. Lab Animal

Shuichi C, Tadahiro N, Midori N, Misty C R, Chisato W, Hiroshi K., (2012). Chronic restraint stress causes anxiety- and depression-like behaviors, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic

factor in the prefrontal cortex. Progress in NeuroPsychopharmacology and Biological Psychiatry ; 39, pp.112-119.

Smith MJ, Logan AC (2002). Naturopathy. Med Clin North Am 86: 173–184

Sood A, Mehrotra A, Dhawan DK, Sandhir R. (2018). Indian ginsing (Withania somnifera)

supplementations ameliorates oxidative stress and mitochondrial dysfunction in experimental model of stroke. Meta Brain Dis. 1-14.

Steele TA. (2002). Chemotherapy-iduced immunossupression and reconstitution of immune function.Leukemia Res ; 26: 411-4.

Storz, G., Imlay, J.A. (1999). Oxidative stress. Curr. Opin. Microbial., 2: 188-194

Stratakis, C.A. and Chrousos, G.P (1995). Neuroendocrinology and Pathophysiology of the Stress System. Annals of the New York Academy of Sciences, 771: 1-18

Stults-Kolehmainen, M. A., Sinha, R. (2014). The effects of stress on physical activity and exercise. Sports medicine, 44, 81-121

Swaab DF, van Pelt J, Hofman MA. Introduction to the sixteenth C.U. AriÃ«ns Kappers lecture. Progress in Brain Research. 147: 39-41

Swiergiel A, zhou Dunn A.(2007). effects of chronic footshok,restraint and corticotropin releasing factor on freezing,ultrasonic vocalization and forced swim behavior in rats. Behav Brain Res ; 183:178- 87.

Tobach, E. (1966). Manipulation effects, open-field experience and digestive transit time in wistar male and female rats. Psychological Reports, 19(2), 375-378

Timmermans S, Berg M. (2003). The gold standard: The challenge of evidence based medicine and standardization in health care. Philadelphia: Temple University Press.

Ulrich-Lai, Y.M.; Figueiredo, H.F.; Ostrander, M.M(20 06). Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner. American Journal of Physiology. Endocrinology and Metabolism 291(5):e965–e973

Vann S, Albasser M (2011). Hippocampus and neocortex: recognition and spatial memory.

CurrOpin Neurobiol. ; 21(3) :440-5.

Van Herck H et al. (1992). Histological changes in the orbital region of rats after orbital puncture. Lab Animal

Van Herck H et al. (1998). Orbital sinus blood sampling in rats as performed by different animal technicians: the influence of technique and expertise. Lab Animal

Venero, C. et al. (2002). Chronic stress induces opposite changes in the mRNA expression of the cell adhesion molecules NCAM and L1. Neuroscience 115, 1211-1219 .

Verhoef MJ, Lewith G, Ritenbaugh C, Boon H, Fleishman S, et al. (2005). Complementary and alternative medicine whole systems research: beyond identification of inadequacies of the RCT. Complement Ther Med 13: 206–212.

Walter Heinz et al. (1998). Refinement of blood sampling from the sublingual vein of rats. Laboratory Animals

Xu Y, Lin D, Li S, Shyamala S, Barish P, Vernon M., (2009). Curcumin reverses impaired cognition and neuronal plasticity induced by chronic stress. Neuropharmacology. 57:463–71

Yenisetti S.C., Manjunath M.J., Muralidhara C.(2016). Neuropharmacological properties of withania somnifera - indian ginseng: an overview on experimental evidence with emphasis on clinical trials and patents. Recent Patents CNS Drug Discov. 10(2):204–215.

Yi L, Xuemei Z, Lingshan G, Xin L, Xia T, Ling L, Youguang Z, Ling Z, Xiaoxing Y.(2013). Protective effects of nizofenone administration on the cognitive impairments induced by 118 chronic restraint stress in mice. Pharmacology, Biochemistry and Behavior ; 103:474–480.

Zhang HT, Huang Y, Massoud A, Stolinski LR, Li YF, Zhang L, Dlaboga D, Jin SLC, Conti M, O'donnell JM .(2007). Anxiogenic-like behavioral phenotype of mice deficient in phosphodiesterase 4B. Neuropsycho-pharmacology 33: 1611-1