INVESTIGATION OF THE PHARMACOLOGICAL EFFECTS OF *MORINGA OLEIFERA* AQUEOUS EXTRACT ON OXIDATIVE DAMAGES AND REPRODUCTIVE PERFORMANCES INDUCED BY HEAT STRESS IN FEMALE RABBIT

A thesis submitted in fulfillment of requirements for Doctor of Philosophy degree of the University of Nairobi (Pharmacology and Toxicology)

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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DEDICATION

I dedicate this work to my parents MUTWEDU MUZIKWA Valeric and METRE NTAKWINJA Véronique. A special gratitude to my loving wife, AKONKWA CIRIMWAMI Dulce for the daily encouragement. My son, ANCHESA MUTWEDU Kenan-Jophiel who has never left my side and is very special in my life.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ALAT :   Alanine Aminotransferase

ASAT :   Aspartate Aminotransferase

CAT :   Catalase

DPPH :   2,2-diphenyl-1-picrylhydrazyl

DRC :   Democratic Republic of the Congo

ELISA: Enzyme-Linked Immunosorbent Assay

ER :   Effect Rate

F.I. :   Feed Intake

FSH :   Follicle Stimulating Hormone

GSH :   Reduced Glutathione

GTA :   Graduate Teaching Assistantship

H/E :   Hematoxylin/Eosin

Hb :   Hemoglobin

LH :   Luteinizing Hormone

LYM :   Lymphocytes

MCH :   Mean Corpuscular Hemoglobin

MCHC: Mean Corpuscular Hemoglobin Concentration

MCV :   Mean Corpuscular Volume

MDA :   Malondialdehyde

MO :   *Moringa oleifera*

MOASE: *Moringa Oleifera* Aqueous Seed Extract

NRC :   National Research Council

PCV :   Packed Cell Volume

PLT :   Platelet Count

RBC **:** Red Blood Cell

ROS **:** Reactive Oxygen Species

RUFORUM: Regional Universities Forum for Capacity Building in Agriculture

SENTINEL: Social and Environmental Trade-offs in African Agriculture

SOD **:** Superoxide Dismutase

UEA **:** Université Evangélique en Afrique

W.C **:** Water Consumption

W.G. **:** Weight Gain

WBC **:** White Blood Cell

ABSTRACT

Heat stress is considered as one of the factors altering animal productivity and reproductive performances by impairing animal physiology due to the imbalance between heat accumulation and heat loss. *Moringa oleifera* (MO) is one of the best antioxidant plants because of its numerous bioactive compounds. It is reported to alleviate damages caused by oxidative stress and improve animal productivity and reproductive performances. The general objective of this study was to investigate the pharmacology of MO extract in the management of heat stress disturbances on reproductive performances and oxidative stress in rabbit does. Fresh MO leaves, seeds and barks were collected from 2 to 3 years old MO trees of Bukavu city of South Kivu province in Democratic Republic of Congo and Masii village of Machackos County in Kenya and 300 g of each dried powder was mixed with 700 ml of distilled water. Qualitative and quantitative assessment of bioactive compounds including flavonoids, tannins, alkaloids, phenols, glycosides, saponins and terpenoids were performed following standard methods while the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to evaluate the antioxidant activity. Thereafter, twenty-eight rabbit does were randomly distributed to 4 treatments groups (with 7 animals per group) and assigned for 80 days to different room temperatures, relative humidity, temperature humidity index (THI) and of *Moringa oleifera* aqueous seed extract (MOASE) as follows: group 1 (T0): ambient temperature (18–24°C), 59 ±0.48%, 23.6 ±1.52; group 2 (T1): 35–36°C, 64 ±0.6%, 32.5 ±0.7, 100 mg of MOASE; group 3 (T2): 35–36°C, 64 ±0.6%, 32.5 ±0.7, 200 mg of MOASE; group 4 (T3): 35–36°C, 64 ±0.6%, 32.5 ±0.7. In each rabbit cage, electrical heaters were used from 08:00 h to 16:00 h following with the exposition to the room air temperature as in the control group. The selected range of temperature, relative humidity and THI were chosen according to results observed in the pilot study. During the experiment period, food consumption, body weight and body weight gain as well as rectal and skin temperature were measured daily. Reproductive functions including mating method, number of services per conception, self'fur removal, gestation length, litter size, kit body weight, the weight of the litter, milk intake and milk efficiency were recorded or calculated. All animals were sacrificed after 80 days and data referring to blood parameters, oxidative stress biomarkers, weight of organs, hormones, kidney and ovary histology were collected. Results

indicate the presence of glycosides in all the plant parts but the absence of terpenoids in seeds from DRC, phenols and flavonoids in barks from both countries. The highest extraction yield was in leaves of MO from DR Congo and seeds from Kenya. The DPPH showed that MO leaf and seed extracts have appreciable and concentration-dependent increase in scavenging effect. A significant decrease ($P<0.05$) was observed in the skin and rectal temperatures, weight of lung, liver, kidney, hematological parameters such as mean cell volume, white blood cells, lymphocytes, biochemical parameters such as creatinine, urea, alanine aminotransferase, aspartate amino-transferase as well as oxidative stress biomarkers like kidney lipid peroxidation in rabbits from T1 and T2 compared to those of T3. However, a significant increase ($P<0.05$) in ovary weight, hemoglobin, red blood cells, glucose, total protein as well as kidney protein, catalase, superoxidase dismutase, reduced glutathione and lipid peroxidation was observed in animals of T1 and T2 compared to those of T3. Regarding reproductive parameters, a significative increase ($P<0.05$) body weight and body weight gain of both pregnant and lactating female rabbit's, fur removal and litter weight, litter size, kit body weight and kit weight gain, weekly milk production and daily milk efficiency and estradiol, luteinizing hormone, follicular stimulating hormone and cortisol contents in rabbits from T1 and T2 compared to those of T3. A significant decrease ($P<0.05$) in the number of services per conception, milk intake and progesterone content in animals of T1 and T2 compared to those of T3 was noted. Kidney histology of animals of T1 and T3 indicated cortical renal parenchyma with fibroblast proliferation in the interstitial tissues and a tubular epithelial cell swelling while ovary tissue of T1, T2 and T3 animals showed structural alterations. It was concluded that *Moringa oleifera* seeds possess antioxidant activities which could be attributed to some phytochemical components observed in Moringa. These bioactive compounds, when *Moringa oleifera* aqueous seed extract is administered at 200mg/kg b.w., minimize the adverse effects of heat stress inducing physiological and reproductive impairment in female rabbits. Other parts of *Moringa oleifera* (pods, stembark) on heat stress disturbances in rabbits should be studied using organic solvents while the potential toxicity of *Moringa oleifera* aqueous seed extract should be checked.

Keywords: Female rabbits, *Moringa oleifera*, Physiological changes, Reproduction, Thermal stress

CHAPTER ONE: GENERAL INTRODUCTION

1.1. BACKGROUND INFORMATION

Animal reproduction is an essential function of animal production (Ruth, 2002). It is linked to the improvement of production and animal productivity as well as the preservation of the species and productivity (Ruth, 2002; Nse-Abasi *et al*., 2013). It is greatly affected by changes in environmental and xenobiotic factors as well as management (Mutwedu *et al.*, 2019; Mutwedu *et al.*, 2020; Mutwedu *et al.*, 2021).

In Africa, extreme temperature variations are reported between -23.98°C and 57.88°C (Collins, 2011) due essentially to variability of climatic zones and climate change (IPCC, 2007 Collins, 2011). Rabbits, whose ideal environmental temperature ranges between 16 and 21ºC, are subject to heat stress because they do not have enough sweat glands to dissipate excess body heat (Rafel *et al.*, 2012).

The term *'stress'* describes a series of physiological and behavioral reactions to a stressful situation (Merlot, 2004). Stress is caused by external stimuli that disrupt homeostasis, according to physiologists. Animals are affected by various forms of stress which include nutritional, chemical, psychological, physical and thermal (Nardone *et al.*, 2010; Ngoula *et al.,* 2017 b). The latter occurs when the animal's thermoneutrality zone, also known as "thermal comfort zone" is exceeded, limiting the animal's reproductive performances (Kumar *et al.*, 2011). Oxidative stress is an upshot of a disparity between oxidants (free radicals) and antioxidants (Lukaszewicz-Hussain, 2010), but in favor of oxidants. Increased oxidative stress raises the risk of spontaneous abortion (Toboa-Wróbel *et al.*, 2020) as well as other aspects such as litter performances, animal well-being and health status, including reduced milk

production, reproductive performance and longevity (Agarwal and Said, 2003; Fan *et al.*, 2015; Mutwedu *et al.*, 2021).

Several studies have reported heat stress disturbances on physiological and reproductive performances. For instance, exposure to 41°C lead to decrease of 18% of RBC, 20% of hemoglobin and 22% of blood platelet; 24% of albumin, 11.2% of protein, 21% of globulin in New Zealand White rabbits (Sabah and Dalal., 2016). When exposed respectively to 35°C and 45 °C, male guinea cavies' (*Cavia porcellus*) superoxide dismutase activity decreased, level of malondialdehyde increased while concentration in nitric oxide decreased, and the number of damaged and altered sperm DNA increased when compared to animals exposed to ambient temperature (Ngoula *et al.*, 2017b).

During pregnancy and lactation, rabbits kept at temperatures between 25 and 36°C had smaller litters (9.7 vs 11.4), litter weight (503.0 vs 630.5g), and kit weight at delivery (56.6 vs 61.4g), as well as a higher stillbirth rate (25.4 vs 9.9%) compared to those kept at temperatures between 14 and 20°C (Marco-Jiménez *et al.*, 2017). Oogenesis is affected by high temperatures, particularly during telophase I and metaphase II of meiosis, and hence poor quality of quality of oocytes are produced (Hamam *et al.*, 2001). Al-Shimaa *et al.* (2013) reported that in Buffalo, ovarian samples collected in the warm months versus those collected in the spring, were characterized by a significant rate of degenerated oocytes (19.43 vs 10.28), lower number of good quality oocytes (72.77 vs 81.69) and lower maturation rate (63.40 vs 71.49).

In other studies, female rabbits exposed to 41°C significantly increased the progesterone and estradiol release (Sabah and Dalal, 2016). Further, studies in rabbits showed that heat stress also affects gene expression. In pre-implantation embryo, the level of expression of OCT-4, VEGF and IFN-ɣ were reported to be higher for heat-stressed female rabbits compared to control (Marco-Jime´nez *et al.*, 2012).

In order to address these issues, use of plant extracts are a more promising way because of their availability and their low toxicity (Molla *et al.*, 2012) compared to synthesized antioxidants that have been shown to be carcinogenic (butylate hydroxyanisole, tert-butylate hydroxyquinone and butylate hydroxytoluene) (Vijayakumar *et al.*, 2012; Ikpeme *et al.*, 2014). Their phytochemical properties such as antioxidant properties, minerals and vitamins (Machebe *et al.*, 2013) act synergistically and protect against the harmful actions of free radicals. These protective properties are attributed to the existence of bioactive compounds like iridoid, phenylpropanoid glycosides, phenols, flavonoids, alkaloids and labdane diterpenoids (Indumathi *et al.*, 2015).

Moringa oleifera, the most widely cultivated species of the *Moringaceae* family (Mahmood *et al.*, 2010), is widely distributed all over the world including in India, Himalayan tracts, Pakistan, and Africa and could be found even in the hardest and driest soils (Luqman *et al.*, 2012). It possesses 46 antioxidant compounds such as flavonoids, phenolic compounds, carotenoids, and ascorbic acid (Anwar *et al.*, 2007; Adedapo *et al.*, 2009; El-Alfy *et al.*, 2011). In fact, all parts of *Moringa oleifera* such as leaves, gum, root, bark, flowers, fruit (pods), seeds and seed oil are rich in proteins, vitamins, minerals, antioxidants, β-carotene amino acids, and different phenolic compounds, making them highly nutritious (Anwar *et al.,* 2007). The leaves of *Moringa oleifera* are edible in both humans and animals (Makkar *et al.*, 2007) and exhibit anti-inflammatory, anti-ulcer, anti-atherosclerotic, anti-tumor, antispasmodic, hypotensive, anticonvulsant as well as oxidative stress activities in humans (Dahiru *et al.*, 2006; Chumark *et al.*, 2008; Dehshahri *et al.*, 2012; Fayazuddin *et al.*, 2013). Seeds possess several interesting biological properties such as anticancer, anti-inflammatory, antibacterial, antioxidant and anti-asthmatic proprieties antioxidant, antibacterial, anticancer, anti-inflammatory, anti-asthmatic properties as well as hepatoprotective and hypotensive effects (Mehta *et al.*, 2003; Mahajan *et*

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al., 2007; Hamza, 2010). Roots are used as stimulant and diuretic while barks possess analgesic, anti-inflammatory and antiviral potential (Siddhuraju and Becker, 2003).

Considerable data on the protective effects of *Moringa oleifera* against oxidative stress and some reproductive performances in mammalian species are documented (Musa *et al.*, 2014; Ojo and Abdurahman, 2017; Omodanisi, 2017). For instance, Mansour *et al.* (2018) indicated that administration of 300 mg of *Moringa oleifera* tea to mice exposed to 0.05 mg/kg b.w. of cypermethrin improved some biochemical parameters including Aspartate Amino Transferase (ASAT), Alanine Amino Transferase (ALAT), Alkaline Phosphatase (ALP), urea, creatinine, the histological status of liver and brain, and antioxidant enzymes such as Superoxide Dismutase (SOD), Total Antioxidant Capacity (TAC) and Lipid Peroxidation (MDA). Obembe and Raji (2018) reported that, in male rats exposed to cadmium, the administration of aqueous extract of seeds of *Moringa oleifera* at dose of 100 mg/kg b.w. improved their relative organ weight (liver, kidney, heart, spleen, lung, epididymis, prostate, testis, seminal vesicle), sperm quality (motility, morphology, volume and viability), oxidative stress biomarkers (catalase, SOD, MDA) and reproductive hormones (testosterone and Luteinizing Hormone) compared to those exposed exclusively to cadmium. Administration of 0.37% of *Moringa oleifera* ethanolic leaf extracts to female rabbits increased their feed consumption, body weight gain, milk production, alveoli number and size as well as the number of the epithelial cells compared to the control (Setiasih *et al.*, 2019). However, information related to its role on heat stress is scanty.

In South Kivu, region of East of Democratic Republic of Congo (DRC), which has great temperature variabilities, no study has been reported on how the heat stress affects animal oxidative stress, reproductive performances as well as the effects of plant extracts mitigation in the context of climate change. The present study was therefore designed to investigate ways

of mitigating against challenges associated with climate change in animal production and reproduction using female New Zealand White rabbits as experimental animal.

1.2. JUSTIFICATION OF THE STUDY

Heat stress has caused massive losses in Congolese rabbit farming communities. The status of heat stress in these communities is not well understood since there are very limited available studies that have been conducted to elucidate the situation of the heat stress in the region. In South Kivu province of the Eastern part of Democratic Republic of Congo (DRC), *Moringa oleifera* is widely cultivated but no study has been conducted on its role in the management of heat stress damages in animals. This study will help to develop strategies to address heat stress at rabbit farm level by using *Moringa oleifera* extracts that are not toxic and locally available. Once the heat stress problem has been controlled, animal prolificacy will be increased, the mortality reduced and production and productivity will be enhanced, resulting in increased household incomes, reduced food insecurity and poverty.

New Zealand White rabbit was chosen for this study because it is readily adaptable to laboratory conditions, is a good model for studies on reproduction and offers easier way to collect blood samples for hormonal analysis.

1.3. OBJECTIVES

1.3.1. General objective

To investigate the pharmacology of *Moringa oleifera* extract in the management of heat stress disturbances on oxidative stress and reproductive performances in rabbit does.

1.3.2. Specific objectives

1. To screen for bioactive compounds in leaves, seeds and barks of *Moringa oleifera* species from Bukavu city (DRC) and Masii sub-county (Kenya).

2. To assess the efficacy of *Moringa oleifera* extract on oxidative stress biomarkers, blood parameters and biochemical parameters involved in management of heat stress using rabbits does.

3. To evaluate the pharmacological effect of Moringa oleifera extract on some reproductive performances, hormonal profile and ovarian histology of rabbit does due to heat stress.

1.4. HYPOTHESIS

1. The phytochemical composition and antioxidant activity of *Moringa oleifera* do not vary with geographical zones.

2. *Moringa oleifera* aqueous extract have no efficacy on the heat stress damage on blood parameters, biochemical parameters and oxidative stress biomarkers of rabbit does.

3. *Moringa oleifera* aqueous extract have no efficacy on the heat stress damage leading to impaired reproductive performances, altered ovarian histology and reproductive hormonal profile of rabbit does.

CHAPTER TWO: LITERATURE REVIEW

2.1. Anatomy and physiology of the female rabbit reproductive system

The genital tract of the female rabbit consists of a vagina, a double uterus composed of two uterine horns separated over almost their entire length, two oviducts and two ovaries (Percy and Barthold, 2007).
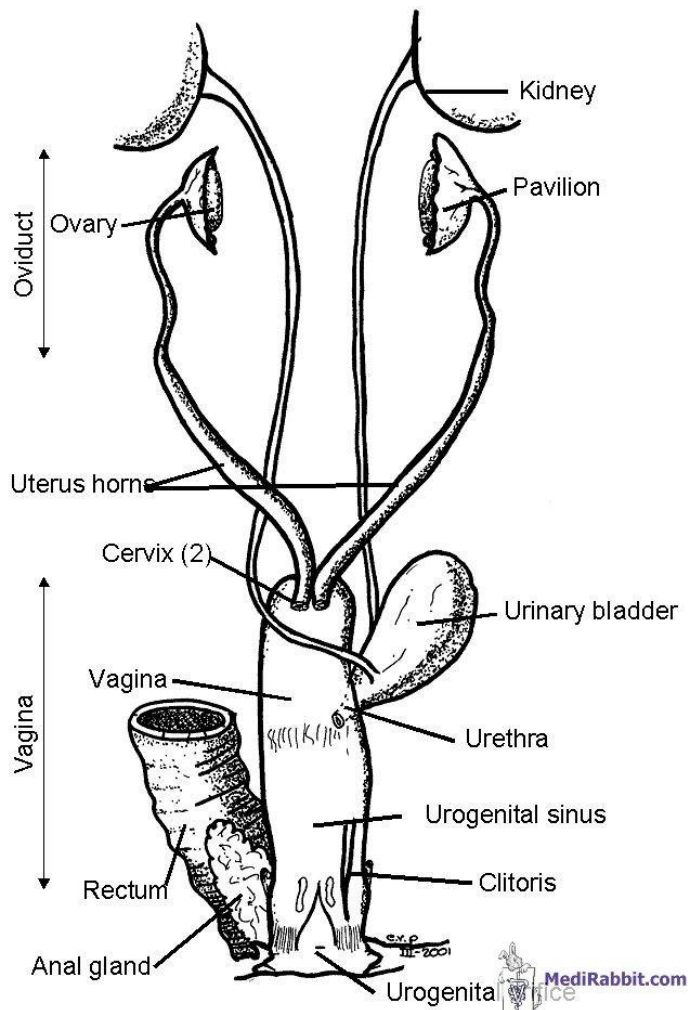
Figure 2.1 : Female rabbit reproductive system

2.1.1. Ovaries

Ovaries are two nodular glands located one on either side of the uterus. Their shape, size and location variation depend on the species (Percy and Barthold, 2007).

They have a layer of simple cuboidal epithelium termed germinal (ovarian) epithelium on the outside and a dense connective tissue capsule called the tunica albuginea underneath (Guyton and Hall, 2006). The ovaries' content is separated into two parts: an exterior cortex and an interior medulla. The presence of many saclike entities known as ovarian follicles at various stages of development makes the cortex look denser and more granular histologically. The medulla is a loose connective tissue that is densely packed with blood arteries, lymphatic vessels, and nerve fibers (Fortune, 2003).
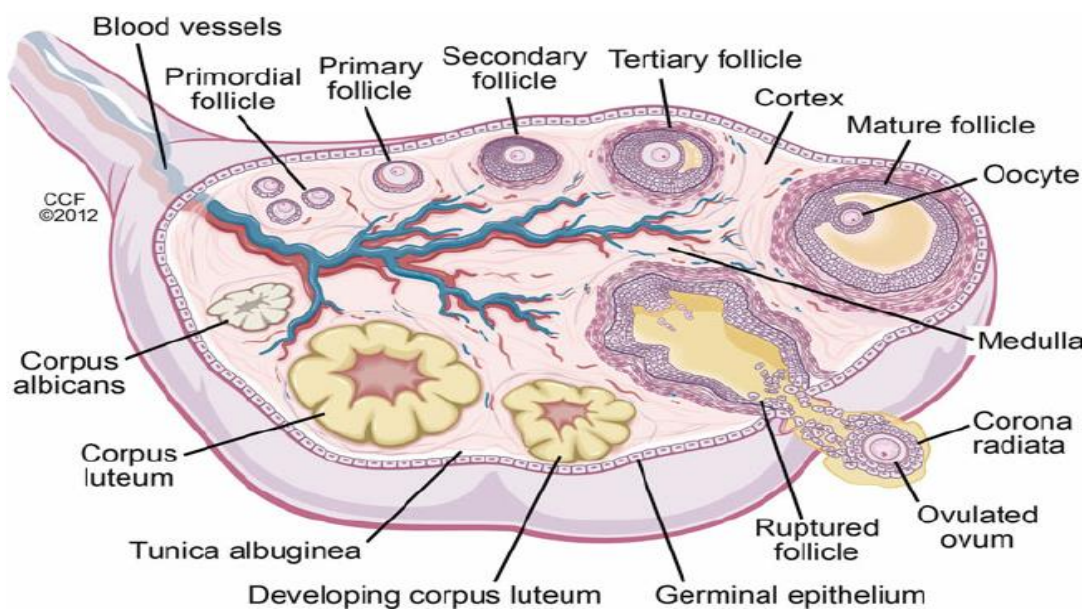
Figure 2.2: Structure of ovary vascularization, folliculogenesis and ovulation (Gayrard, 2007)

2.1.1.1. Function of the ovaries

Ovaries have two main functions; the exocrine function which results in the periodic production of follicles and the endocrine function concerned with hormone production.

2.1.2. Female genital tract

2.1.2.1. Oviducts

There are two of them in the female rabbit reproductive system (also known as fallopian tubes or uterine tubes); the length varying from species to species. The ovary is enclosed by the

lateral end of a fallopian tube, whereas the medial end is connected to the uterus (Johnson, 2000). The fallopian tube's structure guarantees that the conceptus continues to move toward the uterus. The zygote is propelled by the smooth muscle layer of the tube, which contracts in peristaltic waves. The lining mucosa is made up of ciliated epithelial cells and is heavily folded (Gayrard, 2007). The cilia sweeps the zygote toward the uterus. Even if the zygote does not enter in the uterus, it will continue to develop outside of it. This is known as an ectopic pregnancy; referings to a pregnancy that occurs in an unusual location (Percy and Barthold, 2007).

2.1.2.2. The uterus

The uterus (womb) is a hollow organ in which the fetus develops. This is a pear-shaped, thick-walled, muscular organ in the centre of the pelvis, behind the bladder, and in front of the rectum. The fundus is the higher section where the uterine horns connect the uterus, while the body is the major, central portion of the uterus in terms of functionality (Percy and Barthold, 2007). The cervix is a thin section of the uterus that connects to the vaginal canal. Peritoneum (serosa), myometrium (muscle), and endometrium are the three layers of the uterus (Johnson and Everitt, 2002).

2.1.3. The mammary gland

The rabbit mammary gland is structurally similar to those of other mammals, containing tubulo-alveolar glands. This secretory structure is drained by a hierarchical grid of canaliculi and lobular, lobar and mammary drains. These open outside separately at the nipple level of the lagomorphs (Gayrard, 2007). Their development and secretion depend on reproductive hormones such as estrogen, progesterone and prolactin (Théau-Clément, 2005).

The number of functional teats is variable in rabbits. Depending on rabbit breed, two rows of 4, 5 or 6 udders can be available. Thus, the total number of functional udders can be even (8 or

10 teats) or impair (9 or more rarely 11 teats). Each teat has 5 to 6 evacuating drains and is independent of others (Lebas, 2010). Rabbits does with 12 teats were also reported (Coisne, 2000, Lebas, 2010). Whatever their number, there is always a pair of axillary teats located between the front legs, at the 7th and 8th ribs and a pair of inguinal teats located between the thighs. The variations thus concern the pairs of ventral teats, to which the young rabbits have the easiest access (Coisne, 2000).

2.1.4. Puberty

The reproductive organs are fully in place at birth but only become functional at puberty (Salissard, 2013) and the animal becomes able to reproduce, thus perpetuating its species (Gayrard, 2007). Onset of puberty is always earlier in females than in males and it occurs later in animals living in the natural state than in domestic animals (Percy and Barthold, 2007).

Puberty in female rabbits is usually reached when they are 70-75% of their adult weight (4.5-5 months for small breeds and 6-8 months for large breeds). However, it is often preferable to wait until it reaches 80% of this adult weight before mating (Salissard, 2013). These relative weights should not be considered as imperative levels, but as valid limits for the population average. Indeed, if the percentage of female rabbits able to ovulate increases with the average live weight between 14 and 20 weeks, at a given age there is no difference in live weight between ovulating and non-ovulating female rabbits (Lebas, 2011).

2.1.5. Sexual behavior: sexual receptivity of the female rabbit

Female rabbits do not have a clear estrous cycle: heats are not observed at regular time intervals; ovulation is induced by nervous and hormonal stimuli following copulation. They are, therefore, considered to be in quasi-permanent estrus, i.e., sexually receptive on a near-permanent basis (Donnelly, 2004). However, there is some variation in sexual receptivity in rabbits. In fact, there are short phases where the rabbit refuses to mate: this is called the diestrus

phase where the rabbit is "non-receptive" (Salissard, 2013).

Salissard (2013) further stated that a receptive female rabbit behavior will be characterized according to observations of:

- acceptance of the male and the mating,

- lying down position with the rump raised, especially when the hindquarters are stimulated, is one of the most reliable indicators,

- change in the appearance of the vulva which becomes red and wet (90% of female rabbits with this characteristic accept the male and ovulate, against only 10% of females with a pale and pink vulva),

- some hyperactivity and overlapping between conspecifics.

On the other hand, an unreceptive female presented to the male for mating will tend to huddle in a corner of the cage or try to escape, or even be aggressive. It is difficult to predict the time interval between each diestrus. A study by Moret and Barette (1980) revealed a great individual variability between female rabbits.

2.1.6. Sexual cycle, mating and ovulation

2.1.6.1. Sexual cycle

Ovulation occurs at regular intervals during the heat phase or estrus, in most domestic mammals. The period of time between two estrus phases is the length of the estrous cycle (21 days in sows and cows, 4 days in rats, 17 days in sheep) (Gayrard, 2007). The rabbit, unlike most mammals, does not have a regular estrous cycle. It is in heat more or less permanently. Its "induced ovulation" is induced by mating (Johnson and Everitt, 2002; Lebas, 2011).

2.1.6.2. Mating

When the female rabbit is on heat, mating occurs quickly when it stops after a short chase. It then lifts its rump to facilitate copulation. If the female rabbit is not receptive, it will stick to

the cage walls or stick her tail to the floor to avoid mating (Johnson and Everitt, 2002). Mating is generally quick, 10 to 15 seconds, after putting the male and the female together, it will take around 3 seconds and can resume in the following minutes with 20 mattings in 30 minutes if animals are left free (Lebas, 2011). During this mating, various stimuli are transmitted to the cerebral cortex via the nerve pathway following the excitation of the female's erogenous zones. The cerebral cortex also considers other hormonal (steroid levels) and external cues such as pheromones and various sensory stimuli to activate ovulation through an electrical message transmitted to the hypothalamus. The latter produces gonadotropin-releasing hormone (GnRH), which in turn causes the synthesis of LH, the hormone responsible for ovulation, and FSH, which plays an important role in the maturation of the follicles and reinforces the action of the LH (Johnson, 2000). Sometimes ovulation is not followed by fecundation, for example when females overlap, when males are sterile, or when using too young male rabbits or have poor quality semen. In these cases, a corpus luteum is established for a period of 15 to 19 days and produces progesterone preventing any new ovulation a process called pseudopregnancy (Johnson, 2000).

2.1.6.3. Ovulation

Usually, ovulation is induced by stimuli associated with copulation and occurs 10 to 12 hours after mating. Within one minute of mating, oxytocin levels increase while prolactin levels decrease (Donnelly, 2004; O'Malley, 2005; Lebas, 2011). This discharge of oxytocin appears to have the function of allowing sperm to cross the cervix and begin to progress into the uterus (Lebas, 2011). At the same time, the hypothalamus sends a discharge of GnRH which reaches the pituitary almost immediately through the hypothalamic-pituitary "gate" system. Only a very small fraction of this GnRH discharge is introduced into the general blood stream, with the result indicating that circulating levels in the peripheral blood bear no relation to "effective"

physiological levels (Lebas, 2011). Figure 2.5 shows the evolution of oxytocin and prolactin few minutes following rabbit mating.
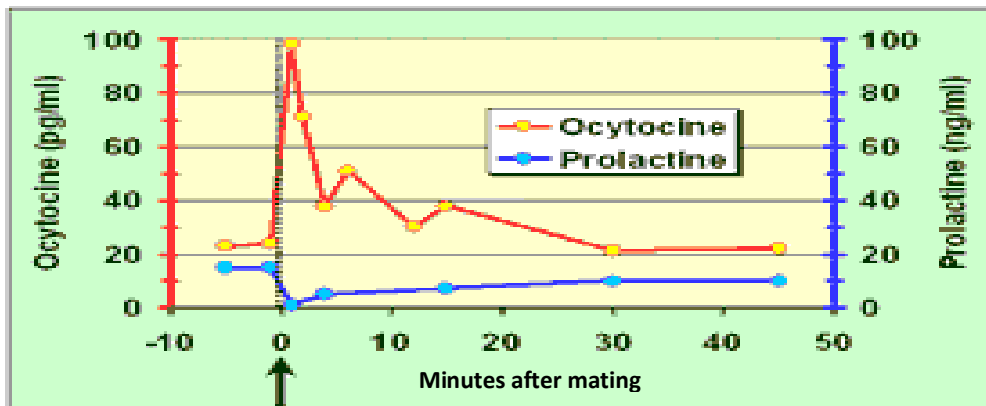
Figure 2.3: Changes in blood levels of oxytocin and prolactin in rabbits within 45 minutes after mating (Sherwood, 2006)

In response to the arrival of GnRH, there is a "discharge" of LH from the anterior pituitary. The maximum concentration is observed 90 minutes after copulation (Figure 2.6). A slight rise in FSH blood levels is observed with a maximum of 30 minutes later. This discharge of LH allows for the final evolution of large antrum follicles (diameter greater than 0.8 mm), which then transform into a De Graaf follicle in approximately 10 hours and release one oocyte each (Lebas, 2011). Figure 2.6 shows the concentration of FSH and LH in few hours after mating.
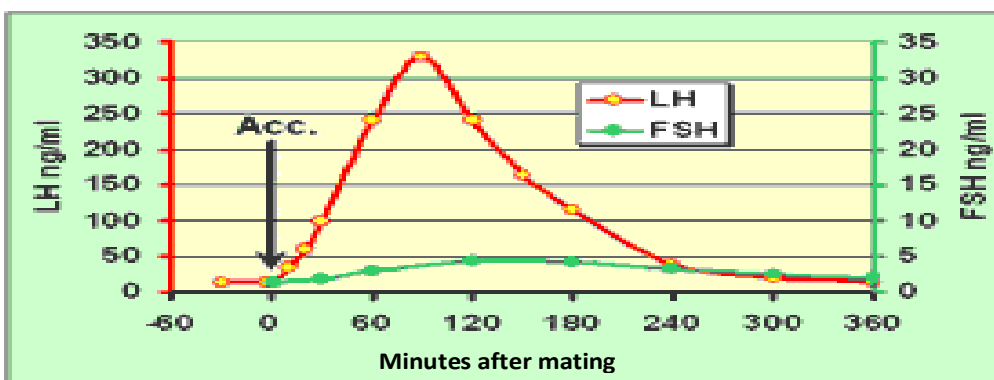
Figure 2.4: Evolution of serum LH and FSH concentration within 6 hours following mating (Sherwood, 2006)

In the rabbit, ovulation can also be artificially induced by intervening at different body sites.

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Mechanical stimulation of the vagina by action on the brain or injections of GnRH, hCG hormones can induce ovulation (Lebas, 2011).

2.1.7. Fertilization and Implantation

When there is ovulation after mating, the oviduct pavilion covers the ovary. As soon as they are released, the oocytes are sucked up by the oviduct's pavilion and are able to be fertilized 1 hour and 30 min after their emission. The sperm is deposited by the male or the artificial insemination device in the upper part of the vagina, at the entrance of the 2 cervices (Percy and Barthold, 2007).

The spermatozoa ascend very rapidly once in female rabbit reproductive tract and reach the fertilization site 30 minutes after ejaculation. During their ascent, the spermatozoa undergo a maturation process that makes them suitable for fertilizing the oocytes. Of the 150 to 200 million ejaculated spermatozoa, only 2 million (1%) will be present in the uterus as they encounter obstacles, mainly in their ascent at the cervix level and the utero-tubal junction (Lebas, 2011). At the time of fertilization, only about twenty spermatozoa are present on each oocyte, but only one crosses the oocyte membrane and ensures fertilization (Lebas, 2010).

Seventy-two hours after ovulation, the fertilized egg reaches the uterus. The egg divides as it goes through the oviduct. The uterine wall differentiates, but the uterine lace will only appear between 5 and 8 days after mating under the progesterone action. The implantation of the fertilized egg is achieved by the synchronization of all these events. The actual implantation takes place at the blastocyst stage, 7 days after mating. Each horn has an equal number of blastocysts, but it is almost never the case that blastocysts change uterine horns under normal physiological conditions (Lebas, 2010; Lebas, 2011).

From day 3 to day 12 after mating, progesterone levels increase steadily, then remain relatively stable and decline rapidly in the few days before parturition (Donnelly, 2004; O'Malley, 2005;

Lebas, 2011). Figure 2.7 shows the evolution of progesterone levels during gestation. However, estrogen levels undergo slightest changes.

Progesterone rate evolution in blood plasma during pregnancy

PROGESTERONE

ng / ml

Days after mating
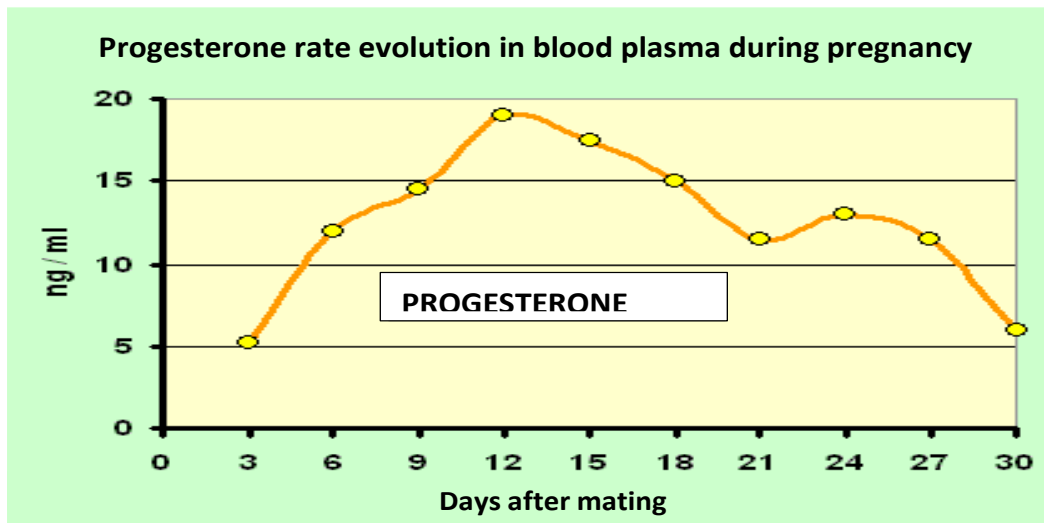
Figure 2.5: Changing in progesterone levels in female rabbit blood plasma during gestation (Sherwood, 2006)

2.1.8. Gestation

In rabbit, gestation period takes 31 to 32 days with variations observed depending on genetic and environmental factors, ranging from 29 to 35 days (30 to 32 days on average) (Lebas, 2011). Below 29 days of gestation, the kids are generally not viable and above 33 days, when the litter size is small (<4 kids), they are almost stillborn (Coisne, 2000).

All embryos are present in the isthmus 24 hours after mating. They will then grow and develop through successive mitotic multiplications, but also migrate along the oviduct to reach their implantation site, in the uterine horn, 72 hours after fertilization (Lebas, 2011). The survival of the embryo depends on the two extracellular layers that surround it:

1) The zona pellucida, which is the internal layer. It is generated during folliculogenesis in the ovaries, with a thick layer comprising between 11 and 30 μm, and is very essential for the oocyte nutrition during follicular growth and gamete recognition during fertilization.

2) The mucosa layer (trophectoderm), which is the external layer is generated during the transition of the embryo through the oviduct. It then increases from 10 μm at 24 hours post-mating in the isthmus to 100 μm at 72 hours post-mating, thus increasing its thickness in just 48 hours. It plays an important role during embryo implantation at the uterine wall (Gayrard, 2007).

Contrary to the migration, the uterine wall begins to differentiate in order to host the embryos. However, the uterine lace necessary for their implantation does not appear until 5 to 8 days after mating, under the action of progesterone secreted by the growing corpora lutea (Donnelly, 2004; Gayrard, 2007). Therefore, there is a close synchronization between this occurrence and the implantation of 5 mm diameter embryos at the blastocyst stage.

During gestation period, changes in hormonal profiles occur. Progesterone levels increase steadily between the 3rd and 12th day after mating, then remain relatively stable and decrease rapidly in the days before delivery. This secretion is mainly carried out by the ovarian corpora lutea, which remain throughout gestation to ensure its maintenance (Salissard, 2013). In fact, even if a little quantity is produced by the placenta from mid-gestation, female rabbits are considered as "corpus luteum dependent" animals: an oophorectomy will systematically result in abortion of the female rabbit independently of the gestation stage; contrary to most other species where the progesterone secretions are significantly produced by the placenta, after a given gestation stage (O'Malley, 2005).

Hemochorionic placentation allows exchanges between the mother and the fetus from the 10th day (Gayrard, 2007). Estrogen levels undergo smaller changes. In fact, a part (17-ß estradiol and estrone) continues to be produced in the ovaries by the new follicular wave while estriol is secreted by the placenta (O'Malley, 2005; Stanczyk, 2009).

At the end of gestation, the activity of prolactin secreted by the pituitary gland occasioned by

the interaction between progesterone and estrogens, will indirectly stimulate the development of the mammary gland, as well as the nest building behavior (Stanczyk, 2009).

2.1.8.1. Uterine and fetal growth

Major uterus and fetuses' growth begin around day 15 of gestation, allowing then a diagnosis of pregnancy by abdominal palpation (Lebas, 2011). The growth of the rabbits is exponential, starting on day 12 of conception. Between days 24 and 31, the weight of the young rabbit increases 6-fold, going approximately from 10 to 60 g on the day of birth. At birth, individual kid weights are very variable mainly due to the position of the fetuses along the uterine horns (Donnelly, 2004). Thus, the first rabbit kid on the ovarian side is almost always the heaviest because of a better blood supply to this part of the reproductive tract. On the other hand, the last rabbit kids on the vaginal side have a much lower weight. The magnitude of the variation increases with the number of kids per horn (Johnson and Everitt, 2002; Lebas, 2011).

2.1.8.2. Diagnosis of pregnancy

Pregnancy diagnosis is done using different methods depending on the stage of gestation:

1. From the 10th gestational day or from 12-14 days otherwise, pregnancy diagnosis can be done using abdominal palpation method. For this purpose, using one hand, the female rabbit is held by the neck skin and the other hand is placed under the belly, between the hind legs, slightly in front of the pelvis in order to feel the uterine horns: the fetuses can then be felt as small soft and slippery balls. This method is not recommended after 20 to 25 days of gestation because of the high risk of abortion.

2. From 11th gestational day, an X-ray can be used: the uterus appears enlarged and filled with a liquid substance. Thereafter, the skeleton calcification process starts. It starts with the clavicles and the mandible around the 15th or 16th day of gestation and the cranial bones are apparent from the 18th to 22nd day of gestation (Lebas, 2010; Lebas, 2011).

3. From day 7 of gestation, an experienced farmer can visualize fetal bulbs by abdominal ultrasound and even count the litter by day 9 if there are less than 6 fetuses (Donnely, 2004).

4. The ELISA test, performed 17 or 18 days after mating, has also been used to measure the concentration of progesterone in the rabbit serum in order to differentiate a pregnant female from a pseudo gestating one (Salissard, 2013).

2.1.9.   Embryonic mortality, farrowing, litter size, viability and body weights of rabbit kids

2.1.9.1. Embryonic mortality

Most embryonic mortality occurs between 0 and 15 days of gestation. This mortality is influenced by several factors such as:

(i) The physiological viability of the embryo;

(ii) The embryo position in the uterine horns: rabbits on the ovarian side are heavier and less fragile because of a better blood supply to this part of the reproductive tract (Donnelly, 2004).

(iii)Environmental factors: embryonic mortality increases when the ambient temperature exceeds 30-33°C (Behrman *et al.*, 2001) and the nutritional status of the mother.

(iv)The female rabbit physiological status such as infections, pharmaceutical agent that interfere with placental integrity, stress or lactation. In a female rabbit that is both lactating and postpartum pregnant, i.e. mated within 24 hours of giving birth, late embryonic mortality is increased compared to a female rabbit that is only pregnant and reared under the same conditions (Coisne, 2000).

2.1.9.2. Parturition

During the last week of gestation, behavioral changes appear and constitute the indicators in the parturition diagnosis. These changes vary greatly from one animal to another and can range from 7 days to a few hours before delivery (Lebas, 2011). The female rabbit becomes more

nervous, feeds less, her mammary glands develop rapidly and it starts to build the nest by gathering large quantities of hay and straw in the mouth and pulling out hair in large clumps on the belly, dewlap and flanks. If possible, the female rabbit can also seek and retrieve fibers and tissues. In addition, the fur removal from the ventral side of the body allows the mammary glands to be freed and facilitates access to the young rabbits at birth (Donnelly, 2004; O'Malley, 2005; Lebas, 2011).

Parturition usually occurs early in the morning: 68% of births occur between 5:00 a.m. and 13:00 p.m., while only 8% occur between 9:00 p.m. and 5:00 a.m. (Salissard, 2013). Normally, the birth process is very rapid, taking no more than 30 minutes, regardless of the size of the litter. All presentations to the expulsion of the pups are possible: by the anterior, by the posterior or by the seat. As a result, dystocia is uncommon and mostly due to overly large rabbits (O'Malley, 2005; Percy and Barthold, 2007).

Sometimes giving birth can be spread in two stages: the second phase of the litter is born within a few hours or even one or two days of the first phase of birth. This is not considered abnormal. However, in case of any delay, it is more than 2 days; the fetuses are most often stillborn and should be removed as soon as possible in order not to compromise the health of the female rabbit and its reproductive potential. To avoid this situation, when the delay exceeds 1 or 2 days or when the gestation period exceeds 33 days, it is possible to induce the expulsion of the fetuses with an injection of oxytocin (1-2 IU/kg) (Donnelly, 2004).

Immediately after giving birth, the female quickly takes care of each young rabbit. They are naked, blind and rather fat that allows them to regulate their temperature and to have energy reserves. The hairs appear within three days while eyes open after 10 days (Lebas, 2011). Sometimes the female rabbit eats her young, especially during the first litter. This behavior is usually due to stress, lack of water, a cage that is too small or young rabbits that are touched

earlier (Percy and Barthold, 2007). The female rabbit cuts the umbilical cord, licks and cleans the residues of fetal envelopes that remain on young rabbit bodies. Then they crawl into the nest and begin to suckle. The rabbit also consumes the placentas within minutes of giving birth. Therefore, the observation of placentas in the nest box more than one hour later may be considered as a normal situation (Donnelly, 2004).

After parturition, the uterus volume and weight decrease very rapidly and loses more than half of its weight in less than 48 hours. However, results will not be satisfactory for fertilizations obtained within one week after the young rabbit birth (O'Malley, 2005; Sherwood, 2006; Lebas, 2011).

2.1.9.3. Litter size, young rabbits' viability and weight

The litter size depends on the breed, parity, age of the rabbit and the season. Small breeds tend to have litters of 4 to 5 kids while large breeds can have an average of 8 to 12 kids per farrowing. For parity, primiparous breeds generally have the smallest litters (Donnelly, 2004; Lebas, 2011).

Kids weigh approximately 50 to 55 g at birth, with wide variation by litter size and breed. The largest litters are observed in the 2nd and 3rd gestation, then their size decreases in subsequent gestations. For the season, the smallest litters are observed during the warmer months (Donnelly, 2004; Percy and Barthold, 2007; Lebas, 2011).

Raji *et al.* (2012) observed a high rate of live-born young rabbits in animals given 200 and 400 mg/ kg body weight of aqueous garlic (*Allium sativum*) extract compared to the control group. The highest mortality rates are observed from birth to weaning with proportions up to 60% (Belhadi *et al.*, 2002). These mortalities have various causes and reduce litter size at weaning. Litter size at birth and viability of rabbits from birth to weaning are very important criteria in the characterization of any rabbit population. These criteria vary from a genetic type to another

and for the same breed, depending on temporary or permanent environmental factors (Belhadi *et al.*, 2002; Zerrouki *et al.*, 2005). According to Coisne (2000) and Zerrouki *et al.* (2005), mortality of mother rabbits depends on maternal qualities, litter size and birth weight.

Mortality also varies from one farm to another, even if the feeding and health monitoring of the young rabbits are identical (Baba, 2004). In a study by Zerrouki *et al.* (2005) on rabbits in Algeria, a birth mortality rate of $16.2 \pm 23.1\%$ and a birth-weaning mortality rate of $13.3 \pm 20.1\%$ were observed. In female rabbits, mortality during lactation is almost twice high in the first litter compared to upcoming litters and is much lower in the heaviest animals (Ouyed *et al.*, 2007).

Rabbits weigh 25-80 g at birth depending on litter size (Berchiche *et al.*, 2012; Salissard, 2013). The evolution of the weight of the young rabbits from birth to weaning depends on their own growth potential and the mother's ability to breastfeed (Garreau and Rochambeau, 2003). Suckling a large litter of 10 young rabbits induces a decrease in the litter weight at weaning (Coisne, 2000; Percy and Barthold, 2007).

Salissard (2013) indicated that in rabbits, the average weight of kids at weaning is negatively related to litter size at birth. However, Ainehchi and Zahedi (2014) showed a significant increase in litter weight in females given 200 and 400 mg/Kg of *Artemisia lanata*. Belhadi *et al.* (2002) reported that in rabbits, growth between birth and weaning increases rapidly and between the 2nd and 3rd week postnatal, growth rate decreases as the mother's milk production becomes limited.

2.1.9.4. Reproductive female rabbit live weight

According to Coisne (2000), the increase in the female rabbits' nutritional needs during lactation (1 to 19 days) leads to an increase in daily intake and a decrease in body protein reserves. The quantity of milk produced seems to be one of the major factors of energy

mobilization during the reproductive cycle (Percy and Barthold, 2007). Females inseminated 25 days after parturition have a higher live weight (+3.7%) at the time of the second parturition compared to those inseminated 11 days after parturition (Feugier *et al.*, 2005).

According to Moumen *et al.* (2009), age at weaning affects the evolution of reproductive female rabbit's body condition because females whose litter is weaned at 23 days of age have greater fat reserves at the second parturition than those whose young are weaned at 35 days of age. This is due to a reduction in the energy deficit of females as the duration of lactation decreases (Coisne, 2005; Lebas, 2011).

2.1.10. Lactation

Milk synthesis and secretion begins during gestation and is uninterrupted throughout lactation period (Houdebine, 2007; Salissard, 2013). At parturition, the mammary gland becomes fully functional under the action of prolactin and decreases after weaning (Coisne, 2000). However, the number of alveoli (secretory cells) increases with the gestation number (Senger, 2005) until the 5th lactation (Houdebine, 2007).

During gestation period, estrogen and progesterone exert a negative feedback control on prolactin secretion. The rabbit produces a lot of milk, which is the only food of the young during the first 17 days following parturition (Percy and Barthold, 2007; Boucher *et al.*, 2007). During this period, this milk quantity is sufficient for the harmonious growth of the rabbit. In addition, lactation allows the establishment of a special connection between the mother and her litter (Gayrard, 2007).

The composition of milk varies with the species, in order to cover the growth needs of the young (Moumen *et al.*, 2009). Rabbit milk is more in nutrients except for lactose compared to cow, goat or sheep milk (Table 2.1) (Zerrouki *et al.*, 2005). The milk composition affects the health status of the young rabbits. Indeed, a delay in growth and/or the risk of developing

disease could be due to the consumption of milk without all the required nutritional or health characteristics (Boucher *et al.*, 2007).

Between the 22nd and 28th day of gestation, Belhadi *et al.* (2002) observed that the milk of pregnant female rabbits has high energy (15 Mjoule/kg) and protein (30 g/kg) contents, compared to that of non-pregnant females (10 Mjoule/kg and 19 g/kg respectively). However, lactation depresses reproductive performance, through competition between maintenance and production needs (Lebas, 2011). According to Gayrard (2007), in lagomorphs, rodents and horses, there is no lactation anestrus. However, the duration of postpartum anestrus is dependent on genetic factors and diet, just as the sow can only ovulate after weaning.

Table 2.1: Comparative composition of cow, goat, sheep and rabbit milk

Components (g/kg of milk)	Cow	Goat	Ewe	Rabbit
Dry matter	129	114	184	**284**
Lactose	48	43	44	6
Fat	40	33	73	**133**
Proteins	33,5	29	58	**153**
Ash	7.5	8	9	**24**
Calcium	1.25	1.30	1.90	**5.60**
Phosphorous	0.95	0.90	1.50	**3.38**
Magnesium	0.12	0.12	0.16	**0.37**
Potassium	1.50	2.00	1.25	**2.00**
Sodium	0.50	0.40	0.45	**1.02**
Coisne, 2000 ; Johnson, 2000 ; Gayrard, 2007				

Rabbits generally suckle once a day, the rate of suckling depending mostly on the mother coming to stand over the litter to give the rabbits access to the teats. The suckling takes only 3 to 4 minutes. Young begin to consume the food and little water at 3 weeks of age, when animals become mobile (Lebas, 2010). The rabbit can stay 48 hours without suckling. They have no intestinal flora at birth (Lebas, 2010; Lebas, 2011). Lactation period can take about 30 days,

but can easily go up to 2 months (Kenfack *et al.*, 2015).

Milk production can be measured by weighing either the mother or the litter, before and after breastfeeding (Lebas and Zerrouki, 2011). It can also be estimated by the growth of the young rabbits (Kenfack *et al.*, 2015). In fact, up to 3 weeks, the weight of the young rabbits reflects the quantity of milk they have consumed (Lebas, 2011). Lebas and Zerrouki (2011) reported a positive correlation between the growth of suckled rabbits and milk production, especially the first three weeks. Thereby, Kenfack *et al.* (2015) reported a correlation coefficient r = 0.999 in the local Cameroonian rabbit. The mammary gland of the rabbit involutes rapidly beyond day 20-25 of lactation (Coisne, 2000; Gayrard, 2007). Kenfack *et al.* (2015) reported that milk production increases until day 20 of lactation and then drops. Feugier *et al.* (2005) reported that in simultaneously pregnant and lactating females, milk production drops on day 16 ,19 or 20 of lactation respectively in some breeds.

From day 22 of lactation period, the milk quantity decreases slightly in non-pregnant females while it drops greatly in pregnant rabbits up to zero by day 28 of gestation (Garreau and Rochambeau, 2003). Salissard (2013) noted that the quantity of the produced milk highly increased until the top quantity (250g/day) around the third week of lactation and decreases rapidly depending on the stimulation of the rabbits. Boucher *et al.* (2007) reported that each suckling female can produce about 250 ml of milk per lactation day at peak lactation with 8 to 9 suckling young rabbits. Suckling stimuli and the mother's willingness to suckle induce the oxytocin secretion which stimulates the action of prolactin for milk release (Lebas and Zerrouki, 2011). However, the milk ejection reflex is inhibited by stress (O'Malley, 2005).

2.1.11. Weaning

Lebas (2011) indicated that weaning can be early (before 26 days of age) or late. The classic weaning of young animals is done at 35 days of age (Djago *et al.*, 2007). These young animals

can be transferred to fattening cages, known as weaning by removal of the mother, or remain in the mother cage, known as weaning by removal of the mother.

Early weaning improves the body condition of female rabbits by reducing the lactation period (Feugier *et al.*, 2005; Percy and Barthold, 2007). The age of the young rabbits at weaning does not impact the reproductive performance of females. However, it limits the lipid reserve mobilization between the 1st and 2nd farrowing (-40.5% vs. -56.5% in the weaned groups at 23 and 35 days respectively) (Feugier *et al.*, 2005).

2.1.12. Blood parameters in the rabbit

The adult rabbit heart weighs approximately 2.7 to 2.8 g per kg live weight (Solau, 2010). The total blood volume is quite stable and represents 55 to 57 ml per kg of body weight, regardless of the age of the animal. It increases during gestation and lactation in rabbits, but remains stable with respect to the animal's body weight.

A female adult weighing 3.5-4.0 kg has a cardiac output of 500-600 ml per minute at 220-240 heartbeats per minute while the heart rate increases by 20-40% in the pregnant female (Lebas, 2010). Tables 2.2 and 2.3 summarize the values of the rabbit hematological and biochemical blood parameters respectively as reported by Chilson (2016). Most of the data are provided from rabbit's laboratory, kept under conditions that differ from domestic rabbits. Other factors that influence blood biochemistry are food, environment, age, sex, health and metabolic activity (Solau, 2010).

Table 2.2: Hematological parameters in female rabbits

Parameters	Abbreviations	Values	Units
Red Blood Cells	RBC	$3.8 – 7.9 \times 10^6$	/mm3
Hematocrit	PCV	$33 – 50$	%
Mean Corpuscular Volume	MCV	$50 – 75$	mm3
Hemoglobin	Hb	$9.4 – 17.4$	g/dl
Mean Globular Volume	MGV	$18 – 24$	pg/cell
Corpuscular Hemoglobin Mean Concentration	C HMC	$27 – 34$	%
White Blood Cells		$5 – 13 \times 10^9$	/l
Basophils		$<0.5 \times 10^9$	/l
Eosinophils		$< 1.0 \times 10^9$	/l
Lymphocytes		$3 – 9 \times 10^9$	/l
Monocytes		$< 0.5 \times 10^9$	/l
Neutrophils		$34 - 70$	%
Platelets		290×10^3	/mm3
		$200 – 650 \times 10^9$	/l

Adopted from Chilson (2016)

Table 2.3: Biochemical parameters in female rabbits

Parameters	Abbreviation	Values	Units
Albumin		**25 - 40**	**g/l**
Total Bilirubin		**0 – 0.75**	**mg/dl**
Globulin		**25-40**	**g/l**
Total Protein		**5.4 – 7.5**	**g/dl**
Acid Phosphatase	**AP**	**0.3 – 2.7**	**IU/l**
Alanine aminotransferase	**ALAT**	**55 - 260**	**IU/l**
Alkaline Phosphatase	**ALP**	**10 - 96**	**IU/l**
Amylase		**200 - 500**	**IU/l**
Aspartate aminotransferase	**ASAT**	**10 - 98**	**IU/l**
Phosphokinase Creatinine	**CK - CPK**	**140 – 372**	**IU/l**
Dehydrogenase Lactate		**132 - 252**	**IU/l**
Cholesterol		**10 - 80**	**mg/dl**
Creatinine		**0.5 – 2.6**	**mg/dl**
Glucose	**Glc**	**75 – 140**	**mg/dl**
Triglycerides		**1.4 – 1.76**	**mmol/l**

Adopted from Chilson (2016)

2.2. Thermal stress

Stress is a cumulative response of the body, following the interaction with its environment experienced by the sensory system. It is, therefore, a phenomenon that disrupts homeostasis and gives rise to new adaptations that can be beneficial or not to the animal (Merlot, 2004). Reactions to stress threaten animal welfare, but also animal production and health, because they consume energy and weaken the body at several levels (Breteau, 2010; Baumgard and Rhoads, 2012). Animals are subjected to several forms of stress including psychological, physical, chemical, nutritional and thermal stress. The latter is most prevalent when referring to climate change. Thus, animal production in tropical and subtropical regions is seriously affected by temperature increase (Marai *et al.*, 2007a; Nardone *et al.*, 2010). In animals, the heat stress is caused by an imbalance between heat production and heat loss. It is therefore, one of the factors responsible for oxidative stress (Kumar *et al*., 2011). In females, heat stress reduces fertility, conception and fetal viability (Edwards and Hansen, 1997), reduces the production of some steroids, and weakens the development of follicles and oocytes while a decrease in the concentration of protein in heat-stressed goats has also been reported (Zeron *et al*., 2001). In addition, Helal *et al.* (2010) reported a decrease in total plasma protein, albumin and globulins in goats. Heat stress affects practically all body systems; it reduces the libido, the level of testosterone and spermatogenesis. In addition, it also impairs sperm motility and the increase of abnormal spermatozoa (Kumar *et al*., 2011).

2.2.1. Thermo-neutrality and thermal comfort zone in homeotherms

The thermoneutrality zone is defined as a temperature range where the animal's energetic consumption is low, static and does not depend on the ambient temperature. In this zone, for a given level of ingestion, the energy available for production needs is maximal (Marai *et al.*, 2007a). It is limited by the minimal and the maximal critical temperature. The superior critical

temperature is the ambient temperature above which the animal must, in the short term, increase its insensible thermolysis to overcome the increase in its body temperature. The lower critical temperature, on the other hand, is the temperature below which the animal must rise its heat production in order to cope with the chilly environment (Capdeville and Veissier, 2001). In a cold as a hot environment, the body temperature of warm-blooded animals is kept relatively constant, because of a careful regulation of the heat production and loss balance (Turpenny *et al.*, 2000).

2.2.2. Thermoregulation mechanisms in homeotherms

Warm-blooded animals have a thermal regulation function that allows them to adapt to production or elimination of the heat according to the needs of the body and maintain their temperature relatively constant (Hansen, 2009). The animal generally gains heat in three different ways: metabolic heat from the oxidation of food, the heat from solar radiation and objects that surround the animal, and heat by conduction and convection from the ground (Guerouali *et al.*, 2003).

The core body temperature reflects the average level of thermal energy of the body. It is close to the rectal temperature which varies depending on the races, species (Table 2.4), heartbeat, and level of production. In a normal thermal environment, the rectal temperature is constant; a variation, even if minimal (less than 1°C) reflects significant physiological changes that cause the decline in animal production performances (Silanikove, 2000; Ahmed *et al.*, 2015). Homeothermic animals therefore require that the heat produced or stored is equivalent to the heat lost to the environment (Silanokove, 2000).

Table 2.4: Normal rectal temperature of some domestic animals

Species	Average (°C)	Limits of variation (°C)
Cow	38.5	37.5-39.5
Sheep	39.3	38.8-39.8
Goat	39.5	38.5-40.5
Pig	39	38-40
Rabbit	**39**	**38.5-39.5**
Cavy	38.5	37.5-39.5
Duck	42	41-43
Goose	40.5	40-41
Fowl	41	40.5-42

Source: Hansen, 2009

2.2.3. Heat production

Thermogenesis is the result of the metabolic activity of different tissues or organs that participate more or less importantly in the thermal balance. Therefore, even representing 4-6% of body mass, the digestive system components such as the liver, stomach, small intestine or mesentery contribute 40% to basic thermogenesis. In contrast, skeletal muscles, which represent about 50% of body mass, produce only 20-30% of basal thermogenesis (Govern and Bruce, 2000). Thermogenesis is the heat production associated with the use of energy consumed for maintenance and production needs. Heat gain in animals has two origins: exogenous and endogenous.

- The exogenous thermogenesis is derived from the solar radiation that the animal receives (Hansen, 2009).

- Endogenous thermogenesis corresponds to the heat produced in the animal body as a result of the various metabolic activities such as muscular activity, breathing and cardiac functioning (Hansen, 2009).

2.2.4. Heat loss or thermolysis

The elimination of heat by the body is an essential process to compensate for the continual production of heat in the tissues (Govern and Bruce, 2000). Heat can be exchanged between the animal and its surroundings in two ways: the sensitive path (non-evaporative) and the latent path (evaporative).

- **Sensitive heat losses**

They follow three mechanisms (Govern and Bruce 2000, Turpenny *et al*., 2000):

- Conduction thermolysis, which is the transfer of heat between two different temperature environments, is the simple thermal conductivity between the body area and the other elements in contact with it.

- The convection thermolysis correspond to the renewal of the heated air in contact with the animal.

- Radiation thermolysis which results in the energy release by the animal body area like infrared radiation.

- **Insensitive heat losses**

According to Govern and Bruce (2000) and Turpenny *et al*. (2000), insensitive heat losses are under two main modalities:

- Sweating allows thermolysis by water evaporation on the skin surface.

- Thermal polypnea through water evaporation is achieved by using the superior respiratory tract. It consists of a sudden respiratory rate acceleration during exposure to heat.

2.2.5. Physiological mechanisms of thermal control

In homeotherms, thermogenesis is very important, but has a higher energy consumption (Silva, 2006). The body temperature is the result of a thermal homeostasis (balance between thermogenesis and thermolysis) ensured mainly by the hypothalamus which receive

information by thermoreceptors; this body thermostat has the ability to integrate physical and emotional signals from all body areas, and therefore release appropriate physiological and behavioral responses (Turpenny *et al.*, 2000). There are two types of thermoreceptors:

- Peripheral thermoreceptors located under the skin, with a sensitive part to low temperatures and another sensitive part to high temperatures (cutaneous receptors of hot and cold).

- Central thermoreceptors located in the hypothalamus and abdomen.

However, an excitation of the cold receptors located under the skin or a lowering of the blood temperature via the thermoregulatory centers on the one hand induce a decrease in the skin blood supply by vasoconstriction and on the other hand enhances muscle tone and steroidal stimulation to increase oxidation processes (Silva, 2006). Hence, the body fights against cooling by an increase of the thermogenesis and a decrease of the caloric loss. However, an excitation of the hot cutaneous receptors and a rise in the blood temperature induce cutaneous capillary vasodilatation which increases the skin temperature and thus the caloric loss by radiation conduction. This reaction is responsible for the reduction of basal metabolism and internal thermogenesis (Turpenny *et al.*, 2000; Silva, 2006).

2.2.6. Homeothermy bearing and consequences of thermoregulation on reproduction

Thermal comfort zone in mammals as well as endotherms is approximately 35-39°C (Singh *et al.*, 2011). These extreme temperatures, which are almost always higher than the ambient temperature, are reached through the combustion of organic matter to increase thermogenesis. However, body temperature is tightly regulated by a pairing between thermogenesis and thermolysis through convection, evaporation, conduction and radiation (Heldmaier *et al.*, 2004). It can cause disturbances in the reproduction process through two main mechanisms; first, the reproduction function could be damaged by the the ability to regulate body temperature (example: the dispensing of blood flow from the center to the periphery which

increases the sensitive thermolysis), and secondly, the reduction in food consumption (Wolfenson *et al.*, 2000). This reduction in food intake lowers metabolic energy generation, but it can also lead to changes in energy balance and the availability of nutrients, both of which have a significant impact on the cycle, pregnancy establishment, and fetal development. The second mechanism is the loss of reproductive regulatory systems, because increasing body temperature may compromise germ cell function, embryonic development, as well as other cells involved in reproduction (Hansen, 2009).

2.2.7. Heat stress and effects on female reproduction

Animals' reproductive functions are very sensitive to environmental heat increase, some species being more affected than others. Heat stress was reported among the major factors leading to infertility and reproductive inefficiency in animals, resulting in profound economic losses (Singh *et al.*, 2011). At various stages of pubertal development, conception, and embryonic death, elevated ambient temperatures harm the female reproductive system. Stress reduces animal reproduction by stimulating the hypothalamic-pituitary-adrenal axis, which activates the pituitary gland and therefore induces the adrenocorticotropic hormone release.

2.2.7.1. Heat stress and effects on female reproductive hormones

A substantial negative association between environmental temperature and reproductive hormone concentration has been reported in several studies and can therefore compromise animal reproductive capacity (Naqvi, 2012).

On day 12 of the estrous cycle, exposure of cattle to 40°C during 16 h induced a significant decrease in GnRH, while FSH secretion was increased eventually due to reduced inhibition of negative feedback from small follicles (Ozawa *et al.*, 2005). Heat stress decreases LH concentration in cattle with low estradiol. Therefore, the maturation and ovulation of the dominant follicles are delayed and damaged by heat stress, while in cyclic cows, low tonic LH

levels also impede luteal development by limiting follicular expansion and turnover (Wolfenson *et al.*, 2000). Moreover, during heat stress, a decrease in pre-ovulatory LH release lowered the manifestation of estrus clinical symptoms and delayed ovulation (Ozawa *et al.*, 2005). Following a change in ovulation, goats exposed to high ambient temperatures had lower follicular fluid and plasma estrogen concentrations, as well as lower LH receptor levels (Ozawa *et al.*, 2005). Estradiol secretion in the follicle is lowered under heat stress environments due to a number of factors including reduced theca cell androstenedione production coupled with low 17-hydroxylase expression, as well as reduced granulosa cell aromatase activity and survival. Furthermore, decreased progesterone secretion has a significant impact on endometrial function and subsequent embryo development. During heat stress, there is observed suspension of estrous cycles and infertility mainly due to the increased level of prolactin (Bridges *et al.*, 2005; Alamer, 2011; Singh *et al.*, 2013).

2.2.7.2. Heat stress and effects on follicular growth and development

When the body temperature exceeds 40°C, heat stress affects the developing follicles (Roth *et al.*, 2000). It also impairs oocyte development by lowering steroid hormone release which inhibits oocyte growth, decrease the dominant follicular growth while enhancing that of subordinate follicles. In lactating Holstein cows, heat conditions reduced the follicular diameter at 14.5 mm instead of 16.4 mm recorded in non-stressed cows, and also reduced follicular fluid volume of 1.1 ml in heat-stressed cows compared to 1.9 ml in non-heat-stressed animals (Badinga *et al.*, 1993). Moreover, thermal stress has been associated with a decrease in follicular dominance by promoting the formation of several follicles with diameter higher than 10 mm with ovulatory follicle dominance maintained for a long time. (Hansen, 2009). Therefore, normal follicle dominance and selection could be disrupted by a high availability of tonic FSH (Wolfenson *et al.*, 2000). During the summer, low LH and a negative animal energy

balance inhibit dominant follicle growth and ovulation (Wakayo *et al.*, 2015). The extended follicular dominance interferes with oocyte normal maturation and reduce their developmental competence. Therefore, during the high temperatures the small dominant follicle development induces an infertile oocyst ovulation or sub functional corpora lutea. The substantial decrease in the ovulation rate is a consequence of the premature dominant follicle regression before reaching a large size (Al-Katanani *et al.*, 2002; Ozawa *et al.*, 2005; Wakayo *et al.*, 2015).

2.2.7.3. Heat stress and its effects on estrus incidences

Photoperiod is considered as the main factor controlling seasonal reproductive cycle and it was found to be systematically affected by climate change (Marai *et al.*, 2004). Negative effects on duration and incidence of estrus and, therefore, on the detection of estrus in females have been previously reported (Dash *et al.*, 2016). Estrus duration and intensity have been reported to be inversely associated with the environmental temperatures, the elevation of temperatures activating and enhancing the anestrus prevalence and silent heat in farm animals (Kadokawa *et al.*, 2012; Singh *et al.*, 2013).

In Japanese black cattle, a significant decrease in the inter estrous interval of 21.5 days in summer compared to 23.5 days in winter have been observed (Sakatani *et al.*, 2012). Estrus occurrence and delay have been observed in Bharat Merino ewes exposed to elevated temperatures and were associated with weak synthesis of estrogen and/or abnormal LH pulsatility when submitting animals to heat stress (Maurya *et al.*, 2005). In addition, Bulbul and Ataman (2009) noticed a decrease in estrus occurrences in cattle reared in regions where ambient temperature is more than 20.5°C. When consecutively submitted to several stresses including heat stress, Malpura ewes showed a decrease in estrous of 41.7% and estrus duration of 14.4 h compared to control group (66.67% and 32 h respectively) (Sejian *et al.*, 2011). Also, when the ambient temperature is low and the temperature- humidity index is below 70, it has

been noticed that cattle are not able to exhibit estrus, compared to buffalos (Vale, 2007; Singh *et al.*, 2011).

2.2.7.4. Effects of heat stress on sexual behavior

Female sexual behavior has been reported to be varying following several stresses such as heat stress (Alejandro *et al.*, 2014). During the warmest times, it is reduced in female animals. For insistence, Marai *et al.* (2004) detected a reduction in the voluntary mating and fur plucking in female rabbits submitted to heat stress under subtropical conditions of Egypt. Wilson *et al.* (1998) indicated that during the pre-ovulatory period of proestrus, elevated temperatures suppress the follicular growth thus decreasing estrus intensity indicators by reducing blood estradiol content. In heat stressed cows, low estrus detection has been reported during summer than winter generally due to a reduction in the time to walk during estrus (Kadokawa *et al.*, 2012). Therefore, animals exhibit estrus at night times because of the drop in temperature as compared to day time when temperature is high (Marai *et al.*, 2007a). During summer season, Indian buffaloes express low estrus activities due to the poor level of blood estradiol during the day, inducing a low estrus detection (Vale, 2007; Upadhyay *et al.*, 2009). In addition, poor locomotor activity in estrus period was detected in Japanese Black cattle when submitted to hot temperatures and was attributed to a reduced 17β estradiol production (Sakatani *et al.*, 2012).

2.2.7.5. Effects of heat stress on oocyte competence

In heat stressed animals, the developmental competence of oocyte can be decreased when heat impairs their growth and maturation by promoting the oxidative damage and apoptosis, as well as generating irreversible cytoskeleton and meiotic spindle alterations (Hansen, 2009). The temperature elevation can have a negative effect on the growth of oocyte, the synthesis of protein and the transcripts creation and development needed for subsequent embryonic

development (Edwards and Hansen, 1997). The transport of protein as well as the transduction of the signals are greatly impaired following the reduction in mRNA content and storage proteins for early embryonic development as well as the alteration of membrane integrity during heat conditions. Hence, premature meiosis and aging oocytes with bad developmental prospects are consequences of the prolonged follicular overview. In vitro, when submitting oocytes to 38.5, 40 and 41°C, results indicated a disrupted maturation such as a low number of mature oocytes collected when cultured at 40-41°C with respect to the number collected when cultured at 38.5°C (Wilson *et al.,* 1998). It has been reported that, when submitted to 41°C, oocyte development is stopped at metaphase I stage (Roth and Hansen, 2005). When environmental temperature is high, several experiments carried out *in vitro* indicated a lower protein synthesis, an interfering architecture of microtubule and microfilament, an elevated rate of cell death caused by the apoptosis as well as a meiotic spindle disturbance (Edwards and Hansen, 1997). The sperm penetration in the oocyte is impaired due to the alteration of the cytoplasm of the oocyte and the zona pellucida following a rise in free radicals produced and the protein impairment in heat stressed animals. Thus, the oocyte competence reduction and heat stress result in an oocyte lesion during the early stages of follicular growth hence low level of fertility (Wolfenson *et al.*, 2000).

2.2.7.6. Effects of heat stress on fertility

Marai *et al.* (2001) reported a decline in the conception rate in rabbit does exposed to a Temperature-Humidity Index of 28.9 (indicating a severe heat stress) during summer in Egypt. It has been also reported a decrease in conception rate of female rabbits following the color of vulva at mating or number of services per conception (Marai *et al.,* 2004). The findings of Zeweil and El-Gindy (2016) indicated a decrease in kindling rate in female rabbits exposed to ambient temperature varying from 27.5-33.5°C and relative humidity of 64-76%. It has been

also reported a significant decrease in the conception rate in female rabbits reared in dry periods compared to rainy seasons (Bassuny, 1999). Marai *et al.* (2004) observed a reduction in the conception rate when the ambient temperature increased female rabbit rectal temperature by 1 to 2°C. It has been noticed a decrease in kindling intervals and conception rate in female rabbits exposed to the very severe heat stress in summer (Marai *et al.*, 2006). Because of their enhanced metabolism, which creates greater internal heat, cows with high milk production yield are more susceptible to heat stress than heifers, and therefore decreases their fertility quality in summer and fall with respect to winter seasons (Ahmed *et al.*, 2015). A decrease in fertility rate was observed in heat stressed sheep and cows (Al-Katanani *et al.*, 2002). When submitted to a temperature humidity index above 75 in subtropical climatic condition, cattle manifested an increase in fertility rate compared to buffaloes because of their low resistance to heat conditions (Dash *et al.*, 2016). A lower fertilization rate has been associated with a rise of 0.5°C in cow uterus during hot periods (Alejandro *et al.*, 2014) as almost all of conceptus damages take place between estrus and day 7 of gestation (Ealy *et al.*, 1993). Infertility induced by heat stress is very common in animal production. A decrease in the conception rate during hot months when compared to coolers seasons has been observed in cattle (Upadhyay *et al.*, 2009). However, during artificial insemination under hot temperatures, a decrease in the conception rate has been observed (Nabenishi *et al.*, 2011; Schuller *et al.*, 2014). On the other hand, in livestock, thermal stress has been associated with conception troubles during reproduction period, 42 days before or 40 days after insemination (Schuller *et al.*, 2014). When submitted to winter, high yielding Israeli cows show a conception rate of 20% compared with 45% observed in winter (Wolfenson *et al.*, 2000; Amundson *et al.*, 2006). A decrease of 20-27% in conception rate as well as a decrease in 90-day non-return rate to the first service in lactating cows during heat conditions has been noted (Chebel *et al.*, 2004). When there is an increase of a unit of

temperature humidity index above 70, the conception rate level was decreased to 4.6% in milking cows, and practically, conception rate was almost reduced below 10% during summer periods (Paula-Lopes *et al.*, 2012).

2.2.7.7. Effects of heat stress on embryonic growth and development

In animals, embryonic loss is one of the most components affecting reproduction and fertility since they are sensitive to thermal stress during the first days after fertilization (Wakayo *et al.*, 2015; Ahmed *et al.*, 2015) probably due to the harmful effects of the elevated body temperature on the growing zygotes and then embryos. It was reported by Amundson *et al.* (2006) that development and viability of growing embryos were reduced during oocytes growth, ovulation or during the first 3 to 7 gestational days in animals submitted to hot temperatures. Even embryo pre-fixation state is affected by thermal stress, the impact decreases to allow the embryo development. Thermal stress induces oxidative cellular impairment with decreasing gestation recognition and expression of genes related to stress hence impairment of protein sysnthesis and consequently apoptosis (Marco-Jime´nez *et al.*, 2012). Suckling cows exposed to heat stress one day after estrus reduce embryos development till the blastocyst stage after day 8 of estrus (Kadokawa *et al.*, 2012), negative effects of thermal stress on embryos being more important at the first stages of their development. In cows, a decrease in pregnancy rate until day 30 and an increase in embryos rate of loss at day 42 of the gestational period were observed. These reductions are results of elevated temperatures until day 7 (blastocyst stage) when in vitro and in vivo embryos are submitted to heat conditions (Demetrio *et al.*, 2007). Embryos at days 3 to 7 are less sensitive to thermal stress than those at 1 day. Moreover, it has been noticed that embryos submitted to heat stress at post implantation period are characterized by a fetal malnutrition and other related teratologic situations, which mainly lead to embryonic death (Kadokawa *et al.*, 2012).

2.2.7.8. Effects of heat stress on pregnancy

Heat stress is known to affect the ability to reproduce in female. It impairs the pregnancy process through several mechanisms altering fertilization, early embryonic and follicular development. According to Demetrio *et al.* (2007) a raise of 38.5 to 40°C in rectal temperature 72 h following artificial insemination in beef cattle may decrease the pregnancy rate for up to 50%. Similar observations were reported by Amundson *et al.* (2006) who indicated that a significant decrease in the pregnancy rate for up 62% may occur in cattle in dry seasons when the temperature-humidity index was above 72.9. Previous studies also showed that, during pregnancy, heat stress may additionally slow down the growth of the fetus. The mechanism underlying this is a decrease in uterine blood supply (Savasani *et al.*, 2015), which disrupts the supply of nutrients and hormones to the conceptus (Amundson *et al.*, 2006). As a consequence, the growth of the embryos will be slow leading in a failure to indicate pregnancy to the maternal system in a timely manner. Besides, during heat stress, endometria prostaglandin F_2 alpha ($PGF_2\alpha$) keeps rising, causing the luteolysis of the graafian follicles, thereby threatening the maintenance of pregnancy (Upadhyay *et al.*, 2009). Ahmed *et al.* (2015) demonstrated that each single rise of approximately 1.05 unit in the temperature, may induce pregnancy loss during the peri–implantation period, or the period between 21-30 up to 90 days, when the humidity index is over 72. Furthermore, heat stress causes the reduction of the placental weight, decrease in hormone secretion, that along with vascular resistance, further contributes to reducing the perfusion of nutrients to the fetus (Sakatani *et al.*, 2012).

2.3. Oxidative stress

2.3.1. Definition

Oxidative stress is known as the imbalance between oxidants (free radicals) and antioxidant defense mechanisms of the organism (Terry and Jeffrey, 2008), in favor of the former, with many impacts on cellular functioning causing chronic diseases (decreased fertility) (Amin and Hashem, 2012). Stress reactions negatively impact on welfare by impairing animal production and health status, as it is energy consuming and weaken the body at multiple levels (Breteau, 2010; Baumgard and Rhoads, 2012).

2.3.2. Free radicals

A free radical is a molecule or an atom with several unpaired electrons (Shkolnik *et al.*, 2011). Therefore, free radicals are molecules with an imbalanced electronic structure that gives them a high level of reactivity toward organic constituents and cellular structures. Thus, due to their particular structure, they tend to attract electrons from other atoms and molecules to gain stability (Pasqualotto *et al.*, 2001). They are inevitably formed in parallel with energy metabolism and by a multitude of other ways. Free radicals are both positive and negative for the reproductive function. Under normal physiological conditions, they are generated in low quantities and act as secondary messengers capable of regulating the apoptosis and fertilization process (Haleng *et al.*, 2007). When produced in large quantities, they become pathological by activating the expression of genes encoding pro-inflammatory cytokines or adhesion proteins (Haleng *et al.*, 2007). The generation of reactive oxygen species is caused by these free radicals. These free radicals are in charge of the formation of reactive oxygen species (ROS) such as: the hydroxyl free radical (OH^-), the peroxide radical (H_2O_2) and the superoxide ion (O_{2^-}) which are highly toxic and originate from the normal metabolic activity of the mitochondria. These mitochondria metabolic activity use a large part of the inspired oxygen for energy production

and, on the other hand, the reactive non-oxygenated free species (peroxidized fatty acids) which are the products of the reactions of certain molecules with the reactive species derived from nitrogen (ROS). They can in turn react with other molecules and be the cause of the multiplication of oxidation reactions and spread of the oxidative damage (Pasqualotto *et al.*, 2001).

2.3.2.1 Sources of free radical production

The first source of free radicals is quite normal and natural. It is produced by the internal activity of animal cells to provide them with energy. Every time when cells use oxygen, free radicals are formed (Haleng *et al.*, 2007). The second source of free radicals is external. They appear during exposure to pollutants, during the ingestion of food treated with pesticides, etc. (Pasqualotto *et al.*, 2001).

Exogenous sources: The environment and lifestyle are at the origin of an increase in oxidative stress in the organism. Free radicals are produced under unfavorable environmental conditions such as xenobiotics and temperature variations (Sikka, 2001). All these elements strengthen the production of pro-oxidant elements and promote the defense overflow, then the occurrence of oxidative stress (Haleng *et al.*, 2007).

Endogenous sources: In this category inflammations, infections (Halliwell and Gutteridge, 2007; Sharma *et al.*, 2012), immature and abnormal oocyte and spermatozoa (Agarwal and Said 2003) may be involved. Oocytes and spermatozoa are very rich in mitochondria that require a significant amount of energy for their mobility hence pose high chance of generating free radicals through these metabolic processes. Mitochondria malformation has been observed in immature and abnormal spermatozoa implying an increased consumption of oxygen leading to a high production of activated oxygen species (AOS) (Sikka, 2001; Kartikeya *et al.*, 2009).

2.3.2.2 Mode of action of free radicals

Activated oxygen species (AOS) (e.g., OH^-, H_2O_2, O_2-, etc) are key targets of all biological components, including lipids, proteins, carbohydrates, and nucleic acids (Haleng *et al.*, 2007; Kartikeya *et al.*, 2009). These AOS are the sources of reproduction impairment both in males and females' animals (Kartikeya *et al.*, 2009; Raghuveer *et al.*, 2010).

Lipids

Lipids are considered as the major constituents of the cell's membrane, also known as polyunsaturated fatty acids (Kartikeya *et al.*, 2009). In a stressful situation, the hydroxyl radicals (OH^-) once formed attach themselves to the carbons located between two double bonds of the polyunsaturated fatty acids and pull out a hydrogen atom (Haleng *et al.*, 2007): this is the initiation phase. A peroxyl radical (ROO^-) is formed when a lipidic radical combines with an oxygen molecule; this peroxyl radical is sufficiently reactive to pull an H^+ proton from a neighboring polyunsaturated fatty acid, thus propagating the reaction (Aitken *et al.*, 2007). This results in an alteration of membrane fluidity, which in turn results impaired cell function hence decreased reproductive performances (Agarwal and Said, 2003; Raghuveer *et al.*, 2010). Through lipid peroxidation, (OH^-) can also alter the process of protein phosphorylation in the germinal cells (Aitken, 2007) resulting in the loss of the ability of the axonal membrane to retain ATP, the only form of energy used by the spermatozoa and oocytes for their motility. Furthermore, hydrogen peroxide (H_2O_2) once formed has the ability to cross the cell membrane and inhibit glucose-6-phosphate dehydrogenase (G6PD) activity (Raghuveer *et al.*, 2010). Via the hexose monophosphate pathway, the enzyme glucose-6-phosphate dehydrogenase regulates glucose flux (Kartikeya *et al.*, 2009). Glucose is an excellent substrate allowing oocyte to obtain the necessary energy for its movement, and the blockage of its passage by the AOS would therefore be at the origin of some impairments (Haleng *et al.*, 2007).

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Proteins

The activated oxygenated species binding to amino acids induce their oxidation, resulting in the appearance of carbonyl groups, division of peptide chains and intra- and inter-chain bi-tyrosine bridges. This damage leads to important modifications (non-recognition of a receptor by a ligand, loss of enzymatic activity) (Haleng *et al.*, 2007). All this contributes to the decrease of hormone levels and even immunoglobulins and enzymes of protein oigin in organisms under stress.

Deoxyribonucleic Acid (DNA)

DNA being the vector of genetic information, is the organelle responsible for the good structure and functioning of all cells. It constitutes a privileged target for AOS. The hydroxyl radical (OH^-) formed during the oxidation process acts with guanine, for example, to form 8-hydroxy-2-deoxyguanosine (8-OH-dG) which, instead of pairing with cytosine, will associate with adenine, causing mutations in the DNA and leading to alterations in the genetic message (Sharma *et al.*, 2012; Haleng *et al.*, 2007). This results in increased sperm abnormalities (Agarwal and Said, 2003; Raghuveer *et al.*, 2010), followed by a decrease in fertility parameters in both males and females. Agarwal and Said (2003) indicated that the decrease in fertility rate and the increase in embryonic mortality is due to significant alterations in DNA. Moreover, Aitken (2007) showed that the alteration of the portion of DNA that codes for the formation of the Y chromosome induces the infertility. Oxidative stress is demonstrated by determination of the oxidative stress markers.

2.3.3. Oxidative stress biomarkers

A biomarker is an observable and/or measurable alteration at the molecular, biochemical, cellular, physiological, or behavioral levels that discloses an individual's current or previous exposure to at least one pollutant chemical (Badiou-Bénéteau *et al.*, 2012). Biomarkers are

indicators that respond early and sensitively to dysfunction and their use takes into account the bioavailability of pollutants and their effects on organisms and populations (McCarthy and Shugart, 1990).

A biomarker measured at the individual level is only meaningful if it can describe, explain and sometimes even predict the effects of pollutants on populations living in this contaminated environment (Bourbia-Ait, 2013). Thus, in organisms exposed to pollutants, measured biomarkers usually highlight:

- genotoxic responses (micronucleus tests);

- neurotoxic responses (cholinesterase activity);

- the capacity of individuals to mobilize defense systems in the presence of toxic substances: biotransformation, metabolism or enzymatic detoxification (Glutathione-S-transferase); reaction of the antioxidant system (Malondialdehyde, superoxide dismutase, catalase).

2.3.4. Oxidative stress and female reproduction

Excessive free radical production has been linked to a disruption in the body's natural antioxidant defense system, resulting in an environment unsuited for typical female physiological reactions (Al-Gubory *et al.*, 2010). Endometriosis, polycystic ovarian syndrome, and unexplained infertility are just a few of the reproductive problems that can result from this. It can also lead to pregnancy difficulties such as spontaneous abortion and recurrent pregnancy loss, etc (Webster *et al.*, 2008). In female, oxidative stress is reported to impair oocyte growth and development in the ovary (Behrman *et al.*, 2001), impair and/or delay ovulation (Shkolnik *et al.*, 2011), induce endometriosis (Giudice and Kao, 2004), which can impair uterus structure and therefore embryo implantation (Carvalho *et al.*, 2011). Polycystic ovary syndrome, characterized by hyperandrogenism, ovulatory dysfunction and polycystic ovaries can be the consequence of oxidative stress (Fauser, 2004). Moreover, it is associated with pregnancy

complications such as placenta dysfunction (Jauniaux *et al.*, 2000; Myatt and Cui,2004), spontaneous abortion (Jauniaux *et al.*, 2000; Gupta *et al.*, 2007), repeated pregnancy loss (Gupta *et al.*, 2007, 2006). Furthermore, it has been associated with intra uterine growth restriction (Biri *et al.*, 2007; Scifres and Nelson, 2009) and preeclampsia characterized by maternal and fetal morbidity and mortality worldwide (Gupta *et al.*, 2009; Reslan and Khalil, 2010). Oxidative stress also increases or decreases excessively the pregnant female body weight, which can complicate both maternal and fetal health (Rotterdam, 2004; Herrero-Mercado *et al.*, 2011).

2.4. Antioxidants

Antioxidants are substances that are capable of suppressing, delaying or preventing oxidation processes at the initiation or propagation state (Klein and Kurilich, 2000; Pszczola, 2001). They are also present in food where they affect the physiological functions of animals by significantly reducing the adverse effects of reactive oxygen species, nitrogenous oxygen species, or both (Adedapo *et al.*, 2009). Antioxidants, an important source of maintaining the body balance, can be classified according to their cellular location and origin.

2.4.1. The different cellular locations of antioxidants

Antioxidants can also be classified into fat-soluble or water-soluble molecules. Depending on their physicochemical characteristics, they have a preferential cell localization: cell membranes for fat-soluble substances and cytosol and/or extracellular location for water-soluble substances. Inadequate antioxidants grade or the antioxidant enzymes inhibition is the cause of oxidative stress induction, which is confirmed to produce cell harm and even cell death (Bartholomew *et al.*, 2013).

2.4.2. Origin of antioxidants

Antioxidants are classified into two categories, namely: non-enzymatic or exogenous antioxidants and enzymatic or endogenous antioxidants (Siddhuraju and Becker, 2003). These antioxidants have very different mechanisms of action.

2.4.2.1. Enzymatic antioxidants

They are produced by the body and are represented by three enzymatic systems: glutathione catalase (CAT), superoxide dismutase (SOD) and peroxidase (GPO). These antioxidants require food-based cofactors like manganese, copper, zinc, magnesium and selenium as well as some vitamins (C, E, A) or molecules such as glutathione (Kevers *et al.*, 2007).

The damages' severity to the cell membrane is determined by measuring the by-products of 4-hydroxynonemal (HNE) and lipid peroxidation: malondialdehyde (MDA) (Hassanpour et *al.*, 2011). However, the organism also possesses enzymatic defenses such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) that are used as predictors of oxidative stress damages (Saxena and Raja, 2014).

Superoxide dismutase

It provides the first organism's line of defense against oxidative stress. Superoxide dismutase fights against both intra and extracellular superoxide anion and thus prevents cell membrane against lipid peroxidation (Pham-Huy *et al.*, 2008). SOD needs trace elements like copper and zinc (Cu-ZnSOD) present in the cytosol or manganese (MnSOD) present in the mitochondria to perform properly. There is also extracellular SOD. Low concentrations of these trace elements can explain low SOD scores. SOD will act in two separate ways to mitigate the oxidative stress. First, the organism will react to moderate oxidative stress by overexpressing SOD (Levine and Kidd, 1996). SOD will be depleted, and its concentration declined if the stress continues and produces massive amounts of free radicals. Paradoxally, a too high

quantity of SOD can be harmful because it is the source of excessive hydrogen peroxide production in this context (Saxena and Raja, 2014).

Glutathione peroxidase

It is a family of enzymes whose activity is prevention of lipid peroxidation. To operate efficiently, glutathione and selenium are required. Its primary function is to remove lipid peroxides produced by oxidative stress on polyunsaturated fatty acids (Saxena and Raja, 2014). Like SOD, selenium-dependent glutathione peroxidase (GPx) will operate in two different ways to fight oxidative stress: the enzyme is first overexpressed, then destruction if oxidative stress remains permanently available. A decrease in GPx activity can also result from a dietary intake of little amount of selenium (Raghuveer et *al*., 2010).

Catalase

Catalase (CAT) detoxifies hydrogen peroxide exclusively and does not require electron donors. Although CAT is well known as an antioxidant enzyme and is involved in the protection of the body against hydrogen peroxides, its localization is limited to peroxisomes. It plays an important role in the liver (Kartikeya et *al*., 2009).

2.4.2.2. Non-enzymatic antioxidants

Compounds having phenolic structures are called non-enzymatic antioxidants. with varying degrees of substitution of alkyl groups. They are generally used to limit oxidation like butyl hydroxyanisole (BHA) and butyl hydroxytoluene (BHT) (Kortenska *et al.*, 2002). However, these synthetic antioxidants are less used these days because of their carcinogenic effect (Ikpeme *et al.*, 2014) with natural antioxidants being preferred (Molla *et al.*, 2012). These are represented by vitamins (E, C, A), molecules such as glutathione and plant extracts (polyphenols) (Haleng *et al.*, 2007).

2.4.2.2.1. Vitamin E

This term refers to all the isomers, tocopherols and tocotrienols. They naturally exist as four isomers: α, β, δ and γ where natural or synthetic α-tocopherol remains the most used antioxidant. It acts directly at the biological membranes level where it traps free radicals before they reach their targets (Brigelius-Flohé, 2007). Its hydrophobic character allows it to be inserted into the fatty acids of the cell membrane and lipoproteins where it plays a protective role by reacting with peroxyl radicals (ROO$^-$) to form a tocopheryl radical, thus preventing the propagation of lipid peroxidation (Wills *et al.*, 2007; Yuan *et al.*, 2007). Tocopherol (Vitamin E) reduces cholesterol content and therefore the risk of heart disease, and promotes the healthy development of the fetus. In addition, it protects the cell membranes from free radical damage and serves as a "scavenger" and antioxidant (Cahoon, 2003). This vitamin is available in several forms. Vitamin E can be secondarily regenerated in the presence of vitamin C or other reducing agents (Yihua *et al.*, 2006). Vitamin E can be found in nuts, vegetable oils, almonds, seeds, milk, eggs and green leafy vegetables (Wills *et al.*, 2007).

2.4.2.2.2. Vitamin C

Also called ascorbic acid, Vitamin C is a major water-soluble antioxidant. It also inhibits lipid peroxidation by regenerating vitamin E from the radical form resulting from its reaction with lipidic radicals (Haleng *et al.*, 2007). It is also an important antioxidant involved in chain breakage and thus increases the antioxidant capacity of seminal plasma by more than 65%. It is an excellent scavenger of activated oxygen species (OH$^-$, O$_2^-$) which protects various biological substrates (proteins, fatty acids, DNA) from oxidation (Hull *et al.*, 2000). It is found in fresh fruits and green leafy vegetables. Citrus fruits, kiwi fruit, peppers, broccoli, cabbage, and parsley have particularly high vitamin C concentration. It is frequently added to foods as a preservative (Kristiina and Marika, 2003).

2.4.2.2.3. Plant extracts

Plant extracts are odorant products with various composition obtained from plant material using (aqueous extracts) or organic solvents (organic extracts) (Bartholomew *et al.*, 2013). The organic extracts can be obtained by solvents such as ethanol, methanol, acetone (Mahmoudi *et al.*, 2013). The aqueous extract differs from organic extracts not only in composition but also in concentration of bioactive compounds. Plant extracts are rich in compounds such as: tripenes, saponins, phenols, tannins, carotenoids, fiber, flavonoids, fatty acids, lectins. There are also bioactive compounds such as: quercetin, luteol, rutin (Tabart, 2011).

Water is commonly used for its non-toxicity and is very easy to obtain extracts compared to organic extracts. Moreover, the extracting power of organic extracts is very efficient than that of aqueous extracts, hence the frequently use of organic extracts (Maja *et al.*, 2013). The efficacy of an extract is based on the required bioactive compounds. Thus, ethanol and acetone maceration are the best extraction techniques for polyphenols and flavonoids, whereas aqueous decoction is preferable for the extraction of condensed tannins (Mahmoudi *et al.*, 2013). However, the use of both types of solvents gives better results for the extraction of phenolics due to their polarity (Maja *et al.*, 2013). To better highlight the role of extracts it is necessary to know the mechanism of action of their bioactive compounds.

Plant extracts contribute to improving the organism's performances, minimizing or even correcting the impact of free radicals on tissues (Béguel *et al.*, 2012). Several investigations have been undertaken in this regard to isolate and characterize the ingredients of natural plants (Morello *et al.*, 2002). These, in the normal life of a plant, protect it from oxidative stress and external attacks such as herbivores, ultraviolet radiations and destructive agents. It is in this optics of plant defense that constituents are produced, and therefore become beneficial to

consumers (Ahn *et al.*, 2002).

Polyphenols

Polyphenols are considered to be almost universal compounds in plants. Structurally, they are divided into several classes ranging from compounds with a simple phenolic core (e.g. gallic acid) to complex polymeric compounds like tannins (Mucha *et al.*, 2021). Polyphenols are the active ingredients of many medicinal plants and can be found in a variety of organs in all vascular plants, including, stems, leaves, roots, fruits, flowers and wood.

In vitro, polyphenols show antioxidant, anti-inflammatory, anticancer and antiviral activities (Rice-Evans *et al.*, 1996). These activities are ascribed to the capacity of these compounds to reduce free radicals such as hydroxyl (HO^-) and superoxide (O_2^-) radicals, but also to their affinity for a diverse range of proteins such as certain enzymes and receptors (Ikpeme *et al.*, 2014).

The chemical properties of polyphenols are essentially linked to those of the phenolic nucleus, particularly the electron-withdrawing (-M) and electron-donating (+M) mesmeric substituents (Rice-Evans *et al.*, 1996). Phenolic compounds' structural characteristics confer them the capacity to counteract free radicals and to chelate transition metal ions (Mucha *et al.*, 2021). It was reported that this activity is associated with the quantity of hydroxyl groups present on the benzoic rings, and also to the alkyl groups proximity. Thus, among the different known families of polyphenols, the flavonoids are those which, in particular, combine all these characteristics (Rice-Evans *et al.*, 1996).

An important characteristic of the hydroxyl groups of phenols is their acidity due to the lability of acidic protons, which leads to the phenoxide anions formation stabilized by resonance. This anion has the possibility of losing an electron to form a radical (Sartori-Thiel, 2003) and the electron can be recovered by a free radical. The aromatic structure of the formed phenoxide

radical provides a certain stability, thus a weaker reactivity, due to the delocalization of the radical and can then react with another free radical (Leopoldini *et al.*, 2011).

Polyphenols are categorised into different groups based on the number of constituent atoms on the one hand and on the other hand, on the basic skeleton structure:

- **Phenolic acids**

These compounds are universally found in plants. Manach *et al.* (2004) reported two sub-groups:

- Hydroxybenzoic acids, the most widespread being salicylic acid and gallic acid,

- Hydroxycinnamic acids, the most abundant are caffeic acid and ferulic acid.

- Flavonoids

Flavonoids are a group of natural chemicals that belong to the polyphenol family. Some of them are nearly universal plant pigments. Flavonoids are classified into numerous types of compounds, with flavonols, flavanones, flavones, flavanols, isoflavones, dihydroflavanols, chalcones, anthocyanins, isoflavanones and aurones (Mucha *et al.*, 2021). These different compounds can be present in both free form and as glycosides. In all vascular plants, these compounds are found in diverse organs including fruits, leaves, flowers, wood, roots and stems (Manach *et al.*, 2004; El Gharras, 2009).

- Tannins

Tannins represent a heterogeneous group rather difficult to define in a rigorous and concise way because there is no basic chemical structure. Their chemical structures are changed and gathered in families according to common activities. Therefore, any chemical classification of tannins is necessarily arbitrary. However, distinction between hydrolysable tannins and condensed tannins is possible (Manach *et al.*, 2004; Leopoldini *et al.*, 2011).

Hydrolyzable tannins are made up of a sugar molecule (most often glucose) esterified with gallic acid or one of its derivatives (ellagic, chebulic or valonic acid). They are easily hydrolyzed by chemical or enzymatic ways.

Condensed tannins are products of the polymerization of flavan-3-ols (catechins) and flavan-3,4-diols. They are also known as "catechic tannins" and are only hydrolyzable under strongly acidic conditions (Manach *et al.*, 2004; Leopoldini *et al.*, 2011).

- Lignins

These high molecular weight compounds contribute to form, with cellulose and hemicellulosic derivatives, the plant cell wall. They are three-dimensional polymers resulting from the condensation (copolymerization) of three phenylpropenic alcohols (Manach *et al.*, 2004).

- Carotenoids

Carotenoids are among the micronutrients that participate in the defense of the body against reactive oxygen species and, consequently, contribute to prevention of various pathologies. They are essentially oxygen molecule scavengers, but they can also neutralize free radicals (Mucha *et al.*, 2021). A single molecule of β-carotene can neutralize several hundred unstable oxygen molecules (Rice-Evans *et al.*, 1996). Carotenoids consumption against oxidative stress is also reflected by a decrease in lipid peroxides content in plasma and 8-hydroxydeoxyguanosine in lymphocytes, which are respectively products of lipid oxidation and nucleic acids (Edmond, 2003). The mechanisms by which carotenoids protect biological systems from damage by singlet oxygen consist of a set of physical and chemical reactions between carotenoids and the "excited" oxygen molecule: the excitation energy of singlet oxygen is transferred to the pigment and subsequently dissipated at the level of the conjugated double bonds (Edmond, 2003). Orange or yellow vegetables such as green leafy vegetables and carrots are considered as the most important sources of this essential antioxidant. Taken in

supplement form, it was reported that a mixture of natural carotenoids is preferable to synthetic beta-carotene (Meyers and Latscha, 1997).

- Selenium

Selenium is a trace element, widely distributed at relatively low concentrations in the food chain. It is an essential nutrient since it contributes in the antioxidant defense system. Brazil nuts and wheat products are good sources of this nutrient. The recommended dose of 55 µg/day is easily achieved with a healthy and balanced diet, containing several types of vegetables, grain products and fruits (Kristiina and Marika, 2003).

2.4.3. Plants extracts and reproduction

Various investigations have revealed positive effects of plant extracts on reproductive performances in animals. For insistence, when administrated 10, 25 and 50 mg/kg body weight *Moringa* leaf ethanolic extract to female rabbits for 50 consecutive days, hormones such as progesterone and prolactin increased as well as the the kindling rate, litter weight at birth, litter size at birth and at weaning (El-Desoky *et al.*, 2021). Habeeb *et al.* (2019) observed that supplementing the diet with 250 mg of crushed roots of Curcumin and Ginger increased progesterone levels, as well as conception rate, litter weight during both birthing and weaning, litter size in female rabbits. Qiong *et al.* (2006) reported that the gavage of 50, 100 and 200 mg/kg b.w of *Lycium barbarum* to male rat exposed at the temperature of 43°C reduced testicular hormones, increased testis and reproductive organ weights and sexual hormones as well as sperm quantity and quality.

In rabbit does, Attia *et al.* (2015) reported that bee pollen administrated at 200 mg/kg b.w with propolis significantly increased litter size and weight, alive kids at birth, weight of kits, higher milk yield as well as milk conversion ratio. There was observed increase in fecundity, conception rate, kindling rate, number of liveborn, stillborn and weaned pups per doe and

progesterone level in female rabbits administrated with 5 g/100 kg feed and 20 g/100 kg feed of *Yucca schidigera* extract for 50 consecutive days (Földešiová *et al.*, 2017). Mohammed *et al*. (2019) observed that, supplementation of ginger, black pepper or garlic in female rabbit diet based on *Moringa oleifera* leaf meal increased their young rabbit survival rate, total milk production, doe and kits average daily feed intake from 3 weeks after kindling to weaning, litter weight at kindling, at 3 week lactation and total milk yield. In both the first and second lactation periods, female rabbits diet supplied with molasses, sprouted fenugreek + molasses, fenugreek oil + molasses and fenugreek powdered seeds + molasses had higher protein, lactose, fat, solid nonfat and total solids content in their milk. These treatments also raised female rabbit milk yields, kids' body weights suckling at 1st and 2nd pregnancy and a significant decrease in white blood cells compared to controls (Abdel-Rahman *et al.*, 2016). In female rabbits receiving a diet containing 79.83 percent cassava byproduct, Oliveira *et al.* (2011) observed an increase in body weight of female rabbits at weaning, number, body weight and body weight gain of kids at birth and weaning compared to the control group.

2.5. Moringa oleifera

2.5.1. Botany and description

Moringa oleifera (Figure 6) is native to Northwest India, with its origins in Agra and Oudh, both of which are located south of the Himalayan Mountains (Mishra *et al.*, 2011). This specie is now spread throughout Asia, Africa, Caribbean, and America. *Moringa* is a member of *Moringaceae* family (Sreelatha and Padma, 2009) and recognized by various different names in the areas where it is found (Fahey, 2005). It has been discovered for centuries (back as 2000 BC early) and due to its extensive therapeutic qualities, it is considered as a very valuable plant. The plant is perennial tree growing up to 5 to 12 m of height and 20 to 40 cm in diameter at breast height. The branches develop in an unorganized mode, and the canopy of the tree has

the form of a shape of an umbrella (Mohammed, 2015). The fruits are made up of three lobed

pods that dangle from the tree branches and open into three halves when dry. Each pod contains

anywhere from 12 to 35 seeds. The seeds have a brownish semi-permeable seed shell and are

spherical. Three white wings go from top to bottom on the hull. Each seed weighs about 0.3

gram. The blossoms are aromatic and cream or white in color, with golden flecks at the base

(Meena *et al.*, 2010).

Figure 2.6: *Moringa oleifera* plant and leaves

2.5.2. Phytochemicals in *Moringa oleifera*

Ndong *et al.* (2007) reported that *Moringa oleifera* contains various phenols such as rutin,

kaempferol glycosides, quercetin glucosides and chlorogenic acids, as well as carotenoids such

as xanthin, β -carotene, lutein, luteoxanthin and zeaxanthin (Pullakhandam and Failla, 2007;

Saini *et al.*, 2014). Their leaves have high level of phytochemicals like niazirin and niazirinin

(Goyal *et al.*, 2007). *Moringa oleifera* is rich in iron and other minerals, vitamins A, B, C as

well as some amino acids like valine, aspartic, glutamic, alanine and leucine (Ruttarattanamongkol *et al.*, 2014).

2.5.3. Antioxidant activity of *Moringa oleifera*

Antioxidants are essential for human nutrition and play a significant function in health maintenance and illness prevention. Antioxidant actions are important in preventing stress, suppressing and scavenging free radicals that can be sources of various diseases including hypertension, heart disease, stroke and cancer (Fang *et al.*, 2002). Furthermore, when antioxidants are added to food, they reduce or postpone oxidation caused by free radicals created by their exposure to environmental variables such as temperature, light and air (Ara and Nur, 2009). Natural antioxidants among others flavonoids, ascorbic acid and carotenoids, can be found in this plant. The most often utilized antioxidants are ascorbic acid and [β]-carotene (Mohammed, 2015). *Moringa oleifera* is a plant that contains a variety of natural antioxidant components, including phenolic compounds, vitamins and flavonoids (Siddhuraju and Becker, 2003). Moreover, this plant can withstand a wide range of drought, soil conditions and rainfall (Iqbal and Bhanger, 2006). The content of nutrients and flavonoids in the *Moringa* can be affected by such agro-climatic factors (Siddhuraju and Becker, 2003).

2.5.4. Uses and therapeutic potentials of *Moringa oleifera*

All *Moringa oleifera* composantes (flowers, leaves, bark, root, gum, and seeds) has a wide range of applications (Anwar *et al.*, 2007; Kumar *et al.*, 2010). Young leaves of the plant can be eaten as a vegetable and used as animal feed. It is used in the production of the biomass, washing material (grinding), food for animals, biogas (from leaves), fertilizer (seed-cake), leaves are a good green manure, honey (flower nectar) and powder can be used as gum (from tree trunks), ornamental paintings, bio pesticide and water purification (Fahey, 2005).

Flowers of the plant are cooked and eaten like a vegetable, while seeds can be eaten raw,

powdered or roasted, and the seed powder can be used to purify water (Fahey, 2005). The young pods are consumed in the same way that green beans are (Foidl et al., 2001) while the root bark is eaten as a snack (Villafuerte and Abonal, 2009).

Leaves, seeds, flowers, fruits, roots and bark are utilized to cure a variety of diseases (Anwar *et al.*, 2007), with anticancer, antipyretic cholesterol-lowering, antidiabetic, anti-inflammatory, fungicidal and antioxidant capabilities among the plant's main therapeutic benefits (Mehta *et al.*, 2003). Many parts of *Moringa oleifera* like leaves, seeds, roots, fruit, bark, immature pods and flowers stimulate the cardiovascular and circulatory system, while they also own anti-tumor, anti-ulcer, anti-pyretic, anti-epileptic activities (Farooq *et al.*, 2012). Further, *Moringa oleifera* is used against microbes, fungi, tuberculosis, cancer, diabetes and has some diuretic, analgesic and antihypertensive proprieties (Kumar *et al.*, 2010; Luqman *et al.*, 2012). *Moringa oleifera* is known for traditional uses like rheumatism, hepatotoxicity, cardiac stimulation and venomous bites (Wadhwa, 2013). It has also been used for centuries as a traditional treatment against several microbial and infectious diseases (Chumark *et al.,* 2008).

CHAPTER THREE: PHYTOCHEMICAL PROFILE AND ANTIOXIDANT ACTIVITIES OF AQUEOUS EXTRACT OF *MORINGA OLEIFERA* (LAM) COLLECTED FROM DEMOCRATIC REPUBLIC OF CONGO AND KENYA

3.1. INTRODUCTION

Oxidative stress is defined as a physiological disturbance when the production of potentially destructive reactive oxygen species (ROS) exceeds the body's own natural antioxidant defense (Tremellen, 2008; Nimse and Pal, 2015). It has been associated with several chronic diseases such as diabetes, hypertension, inflammation, cancer, reproductive impairment in both humans and livestock (Nimse and Pal, 2015, Mutwedu *et al.*, 2021) through DNA damage, lipid peroxidation, tissue injury and protein degradation (Unuigbe *et al.*, 2014). Research findings have revealed that humans affected with such ailments use synthetic antioxidants, which quench or trap ROS (Sreelatha and Padma, 2009; Nimse and Pal, 2015). However, due to healthy lifestyles and poverty especially in rural areas, the use of these synthetic antioxidants has increasingly declined in favor of dietary sources of antioxidants (Ibrahim *et al.*, 2013).

In recent years, extensive research on medicine derived from plants have focused on treatment of a wide variety of clinical diseases. Plants are widely preferred due to their availability and low toxicity on the one hand and, on the other hand, are considered as pure and ecologically friendly for treatment of various ailments (Molla *et al.*, 2012; Vijayakumar *et al.*, 2012; Ikpeme *et al.*, 2014). Several studies have reported the beneficial effects of medicinal plants on many diseases including gastrointestinal and respiratory disorders (Ojewole and Amabeoku, 2006; Owolabi *et al.*, 2010), hepatotoxicity (Adeyemi *et al.*, 2002; Ekor *et al.*, 2006), fertility impairment and improvement (Owolabi *et al.*, 2010; Ngoumtsop *et al.*, 2017; Mutwedu *et al.*, 2019).

Moringa oleifera, a tree growing up to 5 to 12 m high with an open umbrella-shaped crown, is

the most widely cultivated species of the *Moringaceae* family (Mahmood *et al.*, 2010). It is widely distributed over the world including in India, Himalayan tracts, Pakistan, and Africa and is found even in the hardest and driest soils (Luqman *et al.*, 2012). Due to its several traditional medicinal properties, industrial and nutritional uses, this plant is considered as one of the most beneficial trees in the world (Anwar *et al.*, 2007; Wadhwa, 2013). In fact, all parts of *Moringa oleifera* such as leaves, gum, root, bark, flowers, fruit (pods), seed and seed oil are highly nutritious and contain important minerals, proteins, vitamins, antioxidants, β-carotene amino acids and various phenolic compounds (Anwar *et al.*, 2007).

The leaves of *Moringa oleifera* are edible in both humans and animals (Makkar *et al.*, 2007) and exhibit anti-tumor, anti-inflammatory, anti-ulcer, anti-atherosclerotic and anticonvulsant activities (Dahiru *et al.*, 2006; Chumark *et al.*, 2008). Seeds possess several interesting biological activities including antioxidant, antimicrobial, anticancer, anti-inflammatory, antiasthmatic activities as well as hepatoprotective and hypotensive effects (Mahajan *et al.*, 2007; Mehta and Agrawal., 2008; Hamza, 2010). Roots are used as stimulant and diuretic while the bark act as analgesic, anti-inflammatory and antiviral (Siddhuraju and Becker, 2003). All these activities are due to *Moringa oleifera* bioactive compounds which has been reported to possess 46 antioxidant compounds including flavonoids, phenolic compounds, carotenoids, and ascorbic acid (Anwar *et al.*, 2007; Adedapo *et al.*, 2009; El-Alfy *et al.*, 2011).

However, intrinsic factors such as age and cultivar of the plant and extrinsic factors including extraction solvent, postharvest treatment, harvesting season, sunlight, soils, region of cultivation have been shown to affect the phytochemical composition and antioxidant activity of plant materials (Tlili *et al.*, 2014; Agamou *et al.*, 2015). Gelain *et al.* (2012) indicated that bioactive concentrations in plants is strongly dependent on the prevalence of growing conditions and its impact on the accumulation of related natural products. Plants belonging to

the same species but occurring in different geographical zones may significantly differ in qualitative and quantitative content of their particular bioactive compounds (Szakiel *et al.*, 2011). Therefore, different preparations from plants harvested from separate locations in the world may produce different results (Tlili *et al.*, 2014). Whether these concentrations increase or decrease in response to differences in growth environmental conditions, is still not clear.

Moringa oleifera phytochemical screening and antioxidant activity have previously been reported but scientific evidences on the comparison in their phytochemical composition as well as antioxidant activity at different geographical locations remains obscure. It was hypothesized that the phytochemical composition and antioxidant activity of *Moringa oleifera* does not vary with geographical zones. On this basis, the present study was designed to study the effect of different harvest sites with regard to the phytochemical composition and antioxidant activity of *Moringa oleifera* leaves, seeds and barks aqueous extracts.

3.2. MATERIALS AND METHODS

3.2.1. Geographical locations

Fresh Moringa leaves, barks and dry seeds were collected from Bukavu city, East of DRC and Masii village of Machakos county in Kenya in July 2019. All laboratory works were done in the laboratory of phytochemistry, Centre for Traditional Medicine and Drug Research of the Kenya Medical Research Institute (KEMRI) in Kenya.

The city of Bukavu, capital of the Province of South Kivu, is located in the East of the DRC between 2°30'55" South latitude and 28°50'42" East longitude precisely in the basin called Eastern Valley Grabben (region of the great lakes). With an altitude between 1,500 m and 2,194 m above sea level Bukavu has a climate similar to that of sub-equatorial or humid tropics (of short duration). Two seasons are available: the rainy season (lasting more or less 8 months from September to mid-May) and the dry season (starts from July to mid-September). The average temperature is around 20°C throughout the temperate coast and due to the presence of Lake Kivu the rainfall varies between 1,000 mm and 2,500 mm with an annual average of 1,320 mm. The soil of Bukavu is much more compact and less impermeable and less porous since at the slightest drought, water runoff is experienced.

Machakos County, on the other hand, is found in Kenya and located between latitude -1°31'0.01" S and longitude 37°16'0.01" E and has very unique physical and topographical features. The County rises from 790 to 1594 m above sea level and has Hills and a small plateau rising to 1800-2100 m above sea level constitute the Central part of the County. The soils are well drained shallow, dark red clay soils particularly in the plains. However, the vegetation across the entire County depends on the altitude of any given area/location. The average rainfall is between 500 mm and 1300 mm. The short rains occur in October and December while the long rains occur in March to May. Temperature varies between 18°C and 29°C throughout the

year with July as the coldest month while October and March are the warmest months of the year.

The maps showing the location of Bukavu city (DRC) and Machakos County (Kenya) are presented in Figure 1.
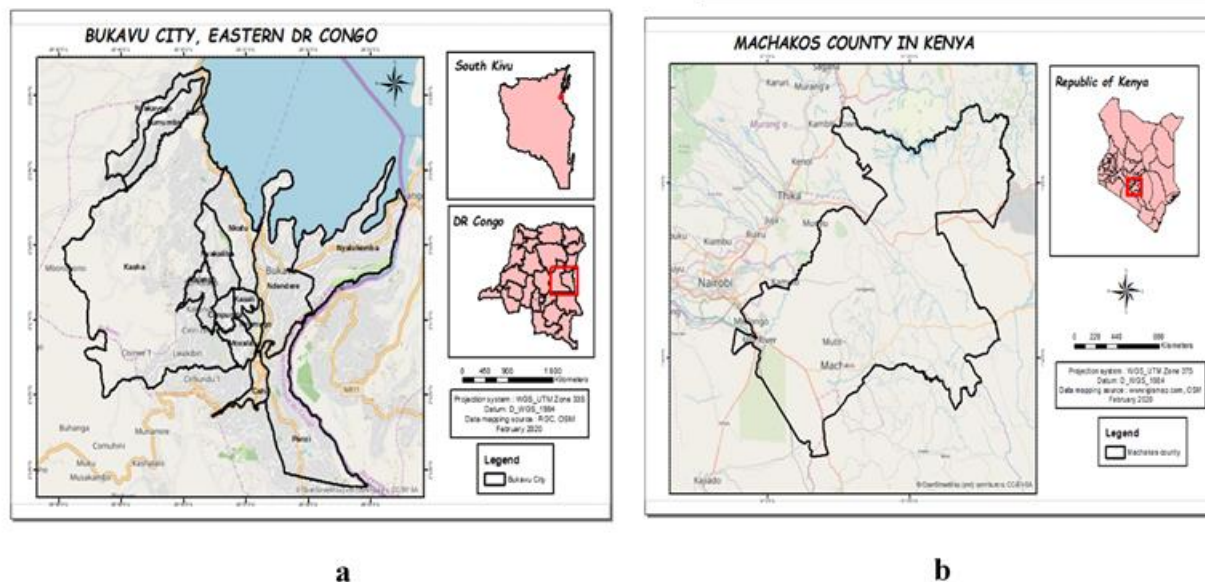
Figure 3.1: Map of Bukavu city in DRC (**a**) and Machakos county in Kenya (**b**)

3.2.2. Plant collection and preparation

The plant materials of *Moringa oleifera (Lam)* aged 2 to 3 years were collected and taxonomically identified at the Department of Biology and Biotechnology, University of Nairobi, Kenya. The leaves, seeds and barks were air dried at 24 to 31°C for 14 days. After spreading, the plant materials were ground using an electric mill and the fine powder was collected and stored in airtight glassware until use.

3.2.3. Extraction of crude powdered sample

A total of 300 grams of dried *Moringa oleifera* powder was mixed with 700 ml of distilled water using a flask wrapped in aluminum foil. The distilled water was gradually added to the powder with gentle shaking until slurry of uniform consistency was formed. Phytochemicals present in the powder were extracted using a magnetic bar and stirrer operating at 200 RPM

for 48 hours. The resultant slurry was centrifuged at 3000 RPM for 5 minutes. The supernatant was then collected into light resistant bottles and freeze-dried.

3.2.4. Qualitative analysis of the phytochemicals of *Moringa oleifera* aqueous extracts samples

To assess the presence of phytochemicals such as alkaloids, saponins, phenols, flavonoids, glycosides, terpenoids and tannins, simple chemical tests were performed according to standard methods developed by Harborne (1998) and Evans and Trease (2009). The resultant color development was reported as presence (+) or nil (-) following the color intensity developed from the reactions.

Test for alkaloids (Dragendorff test)

A total of 50 mg of each extract was added in 5 ml of distilled water until dissolution. Then, 2 M HCL was added until an acidic reaction was observed. The resultant solution was filtered and 2 ml of the filtrate placed in a test tube. One milliliter (1 ml) of Dragendorff reagent was then added along the walls of the test tube. The orange color of the precipitate indicated the presence of alkaloid.

Test for saponins (Foam test)

Five milliliters (5 ml) of the test solution of each of the extracts were added into respective test tubes followed by vigorous shaking to a stable persistent froth for 5 minutes. The formation of emulsion on addition of three drops of olive oil showed positive result.

Test for phenolics (Ferric chloride test)

In order to confirm for presence of phenolics, 1 mg of the sample extract was put in test tube and to 2 ml of distilled water added followed by a few drops of 10% aqueous Ferric chloride solution ($FeCl_3$). The formation of green, red, purple, or blue-black color indicated the presence of phenolics.

Test for flavonoids (alkaline reagent test)

Each sample (0.5 g) weighed into a beaker was extracted with 30 cm^3 of distilled water for 2 hours and filtered with Whatman filter paper number 42 (125 mm). To 10 cm^3 of the aqueous filtrate of each wood extract was added 5 cm^3 of 1.0 M dilute ammonia solution followed by the addition of 5 cm^3 of concentrated tetraoxosulphate (VI) acid. Appearance of yellow coloration which disappeared on standing showed the presence of flavonoids.

Test for glycosides (Keller-Killiani test)

To assess the presence of glycosides, 0.5 g of the extract was diluted with 5 ml of distilled water followed by 2 ml of glacial acetic acid and then add 2 drops of Ferric chloride solution ($FeCl_3$). Then, 1ml of concentrated sulphiric acid (H_2SO_4) was added along one side of the test tube. High precipitate content indicated the presence of glycoside while low content shows the absence of glycoside.

Test for terpenoids (Salkowski's test)

One milliliter (1 ml) of each extract was added to 4 ml of chloroform followed by careful addition of 6 mL of concentrated H_2SO_4 to form a layer. A reddish-brown coloration of the interface indicated the presence of terpenoids.

Test for tannins (Ferric chloride test)

To test for the presence of tannins, each wood powder sample (0.30 g) was weighed into a test tube and boiled for 10 minutes in a water bath containing 30 ml of water. Filtration was carried out after boiling using number 42 (125 mm) Whatman filter paper. To 5 cm^3 of the filtrate 3 drops of 0.1% ferric chloride was added. A brownish green or a blue-black coloration showed positive test.

3.2.5. Quantitative analysis of the phytochemical's constituents of *Moringa oleifera* aqueous extracts samples

Determination of Alkaloids

This was determined according to the method developed by Harborne (1973). A volume of 200 ml of 10% acetic acid in ethanol was added to each powder sample (2.50 g) in a 250 ml beaker and allowed to stand for 4 hours. The extract was concentrated on a water bath to one-quarter of the original volume followed by addition of 15 drops of concentrated ammonium hydroxide dropwise to the extract until the precipitation was complete immediately after filtration. After 3 hours of mixture sedimentation, the supernatant was discarded and the precipitates were washed with 20 cm^3 of 0.1M of ammonium hydroxide and then filtered using Gem filter paper (12.5 cm). The residue was then dried in an oven and the percentage of alkaloid was expressed mathematically as:

$$\% \text{ Alkaloid} = \frac{\text{Weight of alkaloid}}{\text{Weight of sample}} \times 100.$$

Determination of saponins

The quantitative determination of saponin content was carried out using the method reported by Ejikeme *et al.* (2014). Exactly 100 ml of 20% aqueous ethanol was added to 5 g of each sample in a 250 ml conical flask. The mixture was heated over a hot water bath for 4 hours with continuous stirring at a temperature of 55°C. The residue of the mixture was re-extracted with additional 100 ml of 20% aqueous ethanol after filtration and heated for 4 hours at a constant temperature of 55°C with constant stirring. The combined extract was evaporated to 40 ml over water bath at 90°C. Twenty milliliters (20 ml) of diethyl ether was added to the concentrate in a 250 ml separator funnel and vigorously agitated from which the aqueous layer was recovered while the ether layer was discarded. This purification process was repeated

twice. Sixty milliliters (60 ml) of n-butanol was added and extracted twice with 10 ml of 5% sodium chloride. After discarding the sodium chloride layer, the remaining solution was heated in a water bath for 30 minutes, after which the solution was transferred into a crucible and dried in an oven to a constant weight. The saponin content was calculated as a percentage:

$$\% \text{ Saponin} = \frac{\text{Weight of saponin}}{\text{Weight of sample}} \times 100.$$

Determination of total phenols

The determination of phenol contents was carried out according to the spectrophotometric method described by Edeoga *et al.* (2005). The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 min. Five milliliters (5 ml) of the extract was pipetted into a 50 ml flask, then 10 ml of distilled water was added. Two milliliters (2 ml) of ammonium hydroxide solution and 5 ml of concentrated amylalcohol were also added. The samples were made up to mark and left to react for 30 min for color development. This was measured at 505 nm wavelength.

Determination of flavonoid

Flavonoid content determination was assessed using the method reported by Ejikeme *et al.* (2014). For this purpose, 50 ml of 80% aqueous methanol was added to 2.50 g of sample in a 250 ml beaker, covered, and allowed to stand for 24 hours at room temperature. After discarding the supernatant, the residue was reextracted (three times) with the same volume of methanol. Whatman filter paper number 42 (125 mm of pore diameter) was used to filter whole solution of each sample. Each sample filtrate was later transferred into a crucible and evaporated to dryness in a water bath. The content in the crucible was cooled in a desiccator and weighed until constant weight was obtained. The percentage of flavonoid was calculated as:

$$\text{\% Flavonoid} = \frac{\text{Weight of flavonoid}}{\text{Weight of sample}} \times 100.$$

Determination of Glycoside

Cyanogenic glycoside quantitative determination methodology used in this research followed the method described by Amadi *et al.* (2004) and also reported by Ejikeme *et al.* (2014). It was weighed into a 250 ml round bottom flask and about 200 ml of distilled water was added to 1 g of each dry powder sample and allowed to stand for 2 hours for autolysis to occur. Full distillation was carried out in a 250 ml conical flask containing 20 ml of 2.5% NaOH in the sample after adding an antifoaming agent (tannic acid). 100 ml of cyanogenic glycoside, 8 ml of 6 M ammonium hydroxide (NH_4OH), and 2 ml of 5% potassium iodide (KI) were added to the distillate(s), mixed, and titrated with 0.02 M silver nitrate ($AgNO_3$) using a microburette against a black background. Continuous turbidity indicated the end point.

Determination of Terpenoid content

Total terpenoid content in the sample extracts was determined by the method as described by Ferguson (1956). One gram (1g) of the sample powder was put in a conical flask and soaked in ethyl alcohol for one day. Then it was filtered and the filtrate extracted with petroleum ether. The ether extract was taken as the measure of total terpenoid.

$$\text{\% Terpenoid} = \frac{\text{Final weight of the sample- Initial weight of the extract}}{\text{Weight of sample}} \times 100$$

Determination of Tannin content

Tannin determination was done according to Van-Burden and Robinson (1981) method. Five hundred milligrams (500 mg) of the sample were weighed into a 50 ml plastic bottle. Fifty milliliters (50 ml) of distilled water was added and shaken for 1 hour using a Moulinex® shaker.

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This was filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtered solution was pipetted out into a test tube and mixed with 2 ml of 0.1 M $FeCl_3$ in 0.I N HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 min.

3.2.6. Antioxidant activity

This was performed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Siddhuraju *et al.*, 2003; William *et al.*, 2006). The DPPH free radical scavenging activities of the crude extracts from three different parts of *Moringa oleifera* were evaluated according to a previous method of Zhu *et al.* (2015) with some modifications. Firstly, 10 µL of the adequate diluted samples (dissolved in methanol) or the positive control solutions (Ascorbic acid, 31.25–1000 µM) were mixed with 190 µL of methanol solution of DPPH (0.1 mM) in a 96-well plate. After 30 minutes of reaction in darkness at room temperature, the absorbance of the reaction mixture was recorded at 517 nm with a multifunctional microplate reader (Tecan Infinite M200 PRO, TECAN, Männedorf, Switzerland). Methanol was used as a blank control in this assay, and all the extracts and controls were tested in triplicate. The percentage of inhibition of DPPH free radicals was estimated and computed using the following equation:

DPPH-free radical scavenging effect (%) = [(OD_C − OD_S)/OD_C] × 100 (Xu *et al.*, 2019)

Where, OD_C is the absorbance value of the blank control, OD_S is the absorbance value of the tested sample or positive control. The 50% inhibitory concentration (IC_{50}) was expressed as the quantity of the extracts that react with 50% of DPPH radicals.

3.2.7. Statistical analysis

The experimental results were expressed as mean ± standard deviation of three replicates. Statistical comparison was established by one-way ANOVA and P-value < 0.05 was considered significant. All statistical analyses were performed using XL STAT for Windows 10 Software.

3.3. RESULTS

3.3.1. Preliminary phytochemical screening of *Moringa oleifera* leaves, seed and bark extracts

The phytochemical characteristics of leaves, seeds and barks of *Moringa oleifera* from Bukavu city and Machakos County are summarized in Table 3.1. The results revealed the presence of glycosides in all the plant parts with absence of terpenoids in seeds from DRC and phenols and flavonoids in barks from Kenya and DR Congo. Alkaloids were absent in leaf samples while saponins absent in seeds collected from Kenya and DRC while tannins were absent in barks and seeds of samples collected from Kenya.

Table 3.1: Phytochemical composition of aqueous extracts of *Moringa oleifera* leaf, seed and bark collected from Bukavu city and Machakos County.

Chemical groups	Leaves		Seeds		Barks	
	Kenya	DRC	Kenya	DRC	Kenya	DRC
Alkaloids	-	-	+	+	-	+
Saponins	+	+	-	-	+	+
Phenols	+	+	+	+	-	-
Flavonoids	+	+	+	+	-	-
Glycosides	+	+	+	+	+	+
Terpenoids	+	+	+	-	+	+
Tannins	+	+	-	+	-	-

+: presence, -: absence. Note the presence of glycosides in all the plant parts but the absence of terpenoids in seeds from DRC and phenols and flavonoids in barks from both countries and the absence of tannins in seeds and barks of Kenyan samples.

3.3.2. Aqueous extract yield (percentage weight by weight) from leaves, seeds and barks of *Moringa oleifera*

The aqueous solvent produced more extract when using leaves of *Moringa oleifera* from DRC while seeds and barks had the same yield. Samples collected in Kenya had the highest yield in seeds followed by leaves and barks (Figure 3.2).
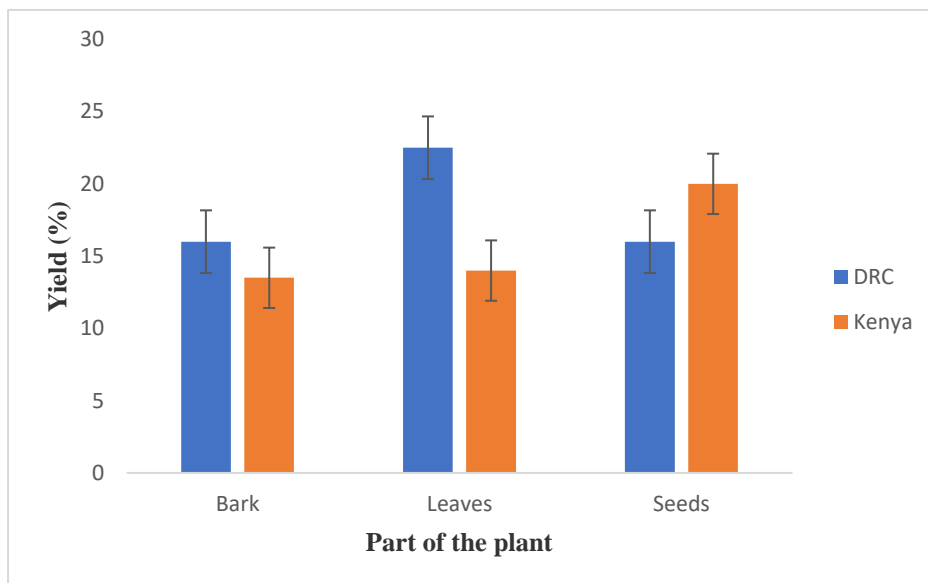
Figure 3.2: Yield of aqueous extracts of *Moringa oleifera* leaf, seed and bark collected from Bukavu city and Machakos county. Note the highest extraction yield in leaves of *Moringa oleifera* from DR Congo (24%) and seeds from Kenya (20%) followed by barks in Kenyan samples (16%).

3.3.3. Quantitative phytochemical analysis of leaves, seeds and barks of *Moringa oleifera* from DRC and Kenya

Glycoside in seeds from DRC had the highest concentration (6.17%) followed by alkaloids in seeds from Kenya (5.56%) and saponin in barks from Kenya (5.33%). Where available, terpenoids and flavonoids had low concentration in all samples compared to other compounds.

Table 3.2: Percentage of crude phytochemicals in aqueous extract of *Moringa oleifera* leaf, seed and bark collected from Bukavu city and Machakos County.

Chemical groups (%)	Leaves		Seeds		Barks	
	Kenya	DRC	Kenya	DRC	Kenya	DRC
Alkaloids	-	-	5.56	3.12	-	2.46
Saponins	1.78	3.78	-	-	5.33	4.92
Phenols	3.47	2.84	3.88	0.97	1.98	-
Flavonoids	0.84	0.71	0.53	0.51	-	-
Glycosides	4.34	3.89	4.18	6.17	4.86	4.61
Terpenoids	0.53	-	0.25	0.57	-	0.46
Tannins	3.73	4.12	-	-	1.65	-

Note a highest concentration in glycoside from seeds of DRC followed by alkaloids in seeds from Kenya and saponin in barks from Kenya and lowest concentration in terpenoids and flavonoids.

3.3.4. Antioxidant activity by DPPH free radical scavenging assay

The result of DPPH free radical scavenging activity showed that *Moringa oleifera* leaf and seed extracts have appreciable and concentration-dependent increase in scavenging effect with the fraction of the leaves being the most active compared to the seeds and barks. At the highest concentration (10 µg/mL), the mean percentage antioxidant inhibition for aqueous extract of leaves, seeds and barks from Kenya and DRC were 88.29±1.12, 80.17±3.59, 30.08±0.80, 33.21±2.62, 37.34±10.26 and 41.86±2.01, respectively whereas the reference standard (ascorbic acid) had a mean percentage inhibition of 92.63±2.76 at 10 µg/mL (Table 3.3).

Table 3.3: DPPH-scavenging activity of aqueous extract of *Moringa oleifera* leaf, seed and bark collected from Bukavu city and Machakos County

Concentration (µg/mL)	Percentage Inhibition						
	Ascorbic acid	Leaves		Seeds		Barks	
		Kenya	DRC	Kenya	DRC	Kenya	DRC
0.5	41.12±1.16	28.02±3.47	22.19±2.53	15.44±6.51	14.63±2.42	17.01±1.13	14.29±1.41
1	64.35±2.28	42.11±4.23	25.81±1.88	15.74±0.42	14.21±3.67	21.38±4.37	25.09±3.53
2	77.49±5.21	44.17±0.65	37.85±3.32	17.40±1.71	22.34±3.29	24.58±4.62	27.38±0.70
5	84.36±1.28	62.83±0.14	51.22±13.03	21.93±2.34	27.14±4.95	30.17±2.66	34.82±0.70
10	92.63±2.76	88.29±1.12	80.17±3.59	30.08±0.80	33.21±2.62	37.34±10.26	41.86±2.01

3.3.5. IC$_{50}$ value in selected aqueous extracts

The IC$_{50}$ value refers the concentration that will scavenge 50% of the initial DPPH radicals. Evaluation of the IC$_{50}$ indicated that the leaves from Kenya (23.59 µg/mL) and DRC (28.67 µg/mL) exhibit the highest antioxidant activities compared to IC50 values of seeds and barks from the two geographical zones and much lower compared to that of the ascorbic acid, which was considered as the reference standard (3.16 µg/mL).

Table 3.4: IC50 of aqueous extract of *Moringa oleifera* leaf, seed and bark collected from Bukavu city and Machakos county.

Sample	Origin	IC$_{50}$
Reference standard	Ascorbic acid	3.16 µg/mL
Leaves	Kenya	23.59 µg/mL
	DRC	28.67 µg/mL
Seeds	Kenya	64.21 µg/mL
	DRC	72.94 µg/mL
Barks	Kenya	84.11 µg/mL
	DRC	75.48 µg/mL

3.4.DISCUSSION

Results on phytochemical screening of the aqueous extract of leaves, seeds and barks of *Moringa oleifera* collected from DRC and Kenya indicated that compounds such as alkaloids, saponins, phenols, flavonoids, glycosides, terpenoids and tannins are found either in leaves, seeds or barks of the same plant either from DRC or Kenya. These results agree with findings of Ferreira *et al.* (2008) in leaves, seeds and stembark of *Moringa oleifera* and Ayirezang *et al.* (2020) in leaves and seeds of *Moringa oleifera*. According to Ifesan *et al* (2013), the presence of these bioactive compounds in these *Moringa* parts is a strong indication that this plant has medicinal potency. Indeed, many studies have shown that the bioactive compounds contained in several plant extracts possess pharmacological proprieties such as hypoglycemic (Tlili *et al.*, 2014) and anti-hypertensive effects (Da Costa *et al.*, 2018; Tsabang *et al.*, 2015).

All these phytochemicals possess good antioxidant activities and have been reported to exhibit multiple biological effects including anti-inflammatory and antitumor activities (Sharma *et al.*, 2011). In fact, alkaloids have diverse pathophysiological effects: antibacterial, antimitotic, anti-inflammatory, analgesic, local anesthetic, hypnotic, psychotropic, and antitumor activity and many others and are still of great interest to organic chemists, biologists, biochemists, pharmacologists, and pharmacists (Chisholm, 2015). Saponins are used as foaming agents in carbonated beverages and cosmetics, as emulsifiers in preparations containing lipophilic colors or flavors, as preservatives, and for removal of dietary cholesterol (Güçlü-Ustündağ and Mazza, 2007). Phenolic compounds can be used as antibiotics and antidiarrheal, antiulcer, and anti-inflammatory agents, as well as for the treatment of diseases such as hypertension, vascular fragility, allergies, hypercholesterolemia (Saito *et al.*, 1998). Flavonoids are well known to protect enzyme systems, cardiovascular diseases, cancers, steroid hormone-dependent cancers, and other age-related diseases (Yao *et al.*, 2004). Glycosides have been shown to suppress or

inhibit growth of one type of cells (microbial, fungal, tumor, genetically altered, etc.) and do not or to a lesser extent affect the growth of host cells (Chisholm, 2015). Many terpenes have biological activities and are used for medical purposes; they have antioxidant, anticonvulsant, antiulcer, anti-inflammatory, antiseptic, antitumor, antiviral, analgesic, antihypertensive, antibacterial, and therapeutic antidiabetic properties (Vuerich *et al.*, 2019). Tannins have shown many health promoting properties like antiviral, anti-inflammatory, immune modulator and antioxidant effects (Manzoor *et al.*, 2020).

Results of the present study showed that alkaloids were absent in leaves from Kenya and DRC. These results agree with the findings of Unuigbe *et al.* (2014) which reported absence of alkaloids in leaves but present in seeds of *Moringa oleifera*. According to Nantongo *et al.* (2018), the concentration of alkaloids can only be found in 20% of the plant species particularly in young, actively growing tissues. Therefore, the production and abundance of alkaloids are mainly related to factors that affect growth of fresh plant tissues such as light, soil nutrients and moisture, temperature and other physicochemical factors (Kirk *et al.*, 2010; Desgagné-Penix, 2017).

It is well known that flavonoids endow a wide range of pharmacological and biochemical properties, such as antimicrobial and anti-inflammatory activities as well as inhibition of platelet aggregation (Kang *et al.*, 2010). In the present study the flavonoids were not detected in bark but were abundant in leaves compared to seeds from Kenya and DRC. Although not the focus of our study, the results of the present study are in agreement with the findings of Tlili *et al.* (2014) in *Rhus tripartitum* which reported variation in flavonoid content from different localities and stage of maturity and with a decrease observed in advanced maturity of the plant (Menichini *et al.*, 2009).

Total phenol and glycoside concentration were higher in leaves, seeds and barks from Kenya

compared to those from DRC. The observed variation in these metabolites have been reported among and within plant species primarily due to several factors such as genetic factors (Adesina, 2006; Sun *et al.*, 2013), environmental effects and their interaction (War *et al.*, 2012). In fact, when growth conditions are not the same, especially nitrogen availability, the abundancy of phenolic compounds in plant tissues can vary from plant to plant within the same species. For instance, nitrogen deficiency or limitation leads to phenolic accumulation in different plant parts (Larbat *et al.*, 2014).

The concentration of terpenoids seems to be very low in each plant part when compared to other compounds in plant samples collected from DRC and Kenya. Generally, in most plants the common order of secondary metabolites with respect to abundance is phenolics > alkaloids > cyanogenic glycosides > tannins > flavonoids and saponins > terpenoids (Nantongo *et al.*, 2018). In addition, concentrations of terpenoids in plant tissues are regulated by the availability of substrate and the activity and type of biosynthetic enzymes. Therefore, emission rates of volatile terpenoids from plant leaves are controlled by their synthesis rates and compound-specific physicochemical characteristics, mainly their solubility, volatility and diffusivity (Desgagné-Penix, 2017). These are affected by physicochemical constraints caused by temperature, stomatal conductance and leaf structure. Rapid changes in the physiological status e.g., stomatal function and allocation of resources (i.e., substrate availability) at high temperatures could have dramatic changes in production of terpenes by plants. This can explain the low terpenoids concentration found in this study as Moringa samples were collected during dry season when sunlight was stinging (Nantongo *et al.*, 2018).

Leaves of *Moringa oleifera* collected from DRC showed a higher extraction yield (22.5%) compared to other plant parts. This result is comparable to findings of Ayirezang *et al.* (2020) in *Moringa oleifera* aqueous leaves extract (24.9%) when compared to seeds (11.45%) but

much higher compared to those reported by Okumu *et al.* (2016) where *Moringa oleifera* aqueous extraction yield was around 14.23%. The variance may be attributed to a large variability of bioactive compounds and their high concentration found in this study and reported by several authors (Kumbhare *et al.*, 2012; Okumu *et al.*, 2016).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is one of the most widely used methods and has become routine in establishing the antioxidant activity of herbal extracts and phytochemicals. DPPH is known to obstruct labile hydrogen and the ability to scavenge the DPPH radical is related to the inhibition of lipid peroxidation (Zheng and Wang, 2001). The result of DPPH radical scavenging activity showed that aqueous extract of *Moringa oleifera* leaves from DRC and Kenya have the highest inhibition percentage depending on the concentration used. These results are comparable to those of Unuigbe *et al.* (2014) in leaves and seeds of *Moringa oleifera*. At the highest concentration (10 µg/mL), the percentage inhibition was 88.29±1.12, 80.17±3.59 for leaves of Kenya and DRC respectively, 30.08±0.80, 33.21±2.62 for seeds of Kenya and DRC respectively and 37.34±10.26, 41.86±2.01 for barks of Kenya and DRC respectively, whereas the reference standard (ascorbic acid) had a percentage inhibition of 92.63 ± 2.76. This result is in accordance with findings of Unuigbe *et al.* (2014), Sharma *et al.* (2011), Tlili *et al.* (2014) who reported that, in most cases, sample concentrations do not exceed the ascorbic acid concentration (considered as the standard). The IC50 value denotes the concentration that will scavenge 50% of the initial DPPH radical. The evaluation of the IC50 in this study indicate that aqueous extract of *Moringa oleifera* leaves from DRC and Kenya exhibit the highest antioxidant activity with IC50 value of 23.59 µg/mL and 28.67 µg/mL respectively. This is the result of the large variability and concentration of bioactive compounds in leaves compared to seeds and barks as reported in this study. These values were very low and different from that of the reference standard (3.16 µg/mL). These

results concur with those of Unuigbe *et al.* (2014) where IC50 of *Moringa oleifera* leaf and seed samples were lower than that of ascorbic acid (standard). However, the IC50 values of *Moringa leaves* were very high compared to Kenyan seeds (64.21 μg/mL) and barks (84.11 μg/mL) and DRC seeds (72.94 μg/mL) and barks (75.48 μg/mL).

3.5. CONCLUSION

In this study, a phytochemical characterization and antioxidant activity comparisons of *Moringa oleifera* leaves, seeds and barks collected from DRC and Kenya were carried out. Results clearly indicate that the composition and concentration of bioactive compounds as well as antioxidant capacity of *Moringa oleifera* vary significantly across geographical regions and the different plant parts used. In this work, we also reported the richness of leaf extracts from DRC and Kenya compared to seeds and barks. Based on this observation, leaves, especially those from Kenya, are recommended as a satisfactory antioxidant. However, despite their low composition in bioactive compounds and antioxidant capacity compared to leaves, seeds and barks also have pharmacological proprieties. Our study has for the first time provided insights into the use of *Moringa oleifera* seeds and barks as important natural source of antioxidant and this can offer reprieve on the over use of leaves hence conservation of ecosystem.

CHAPTER FOUR: EFFECTS OF THERMAL STRESS ON OXIDATIVE STRESS AND PHYSIOLOGICAL CHANGES IN FEMALE RABBITS

4.1. INTRODUCTION

Climate change, a long-term imbalance of customary weather conditions such as temperature, radiation, wind and rainfall characteristics of a particular region, is likely to be one of the main challenges of the present century for mankind (Ganaie *et al.*, 2013). The earth's climate has warmed in the last century (0.74 ±0.18 ∘C) with the 1990s and 2000s being the warmest on instrumental record (Intergovernmental Panel on Climate Change (IPCC, 2019)). Furthermore, the earth's climate has been predicted to change continuously at rates unprecedented in recent human history (IPCC, 2007). The variation in climatic variables like temperature, humidity and radiations have been recognized as the potential hazards in the growth performances and production of all domestic livestock species. High ambient temperature accompanied by high air humidity is reported to cause discomfort and enhance the stress levels which results in depression of the physiological and metabolic activities in animals (Ganaie *et al.*, 2013).

In Africa, rabbits have been promoted as tool for poverty alleviation, food security management, reducing rural-urban migration, entrepreneurial skills, humanitarian services including recovery efforts from natural disasters and gender empowerment (Lukefahr, 2000; Mutwedu *et al.*, 2015). These animals are highly preferred because of their body size, high rate of reproduction, adaptability to inexpensive housing and useful by-products (Mapara *et al.*, 2012). However, African rabbit husbandry is facing several constraints such as lack of reproductive management, predation, uncontrolled cross-breeding practices, inbreeding, negative selection (Mapara *et al.*, 2012; Mutwedu *et al.*, 2015) and environmental stress (Kumar *et al.*, 2011; Rafel *et al.*, 2012; Sabah and Dalal, 2016).

The term 'stress' here refers to a set of physiological and behavioral responses to a hostile

environment (Merlot, 2004). According to physiologists, stress results from external forces that disrupt homeostasis. Several types of stress affecting animals include physical, nutritional, chemical, psychological and thermal factors (Ngoula *et al.*, 2017a, 2017b). The latter arises when the environmental temperature exceeds the thermoneutrality zone of the animal, otherwise referred to as "thermal comfort zone" hence reducing its productive performances (Kumar *et al.*, 2011). Rabbits' ideal environmental temperature ranges between 16°C and 21°C (Marai *et al.*, 1994; Sabah and Dalal, 2016). Environmental temperatures above this range results into heat stress due to few sweat glands that aid in removal of excess body heat (Rafel *et al.*, 2012). Their long exposure to thermal stress leads to an increase in free radicals which may induce oxidative stress (Kumar *et al.*, 2011). Oxidative stress occurs when the production of potentially destructive reactive oxygen species (ROS) exceeds the body's own natural antioxidant defense (Tremellen, 2008). In female, elevated oxidative stress increases not only the risk of spontaneous abortion (Vural *et al.*, 2000) but also affect other factors such as litter performance, the well-being and health status of animals including reduced milk production, reproductive performance and longevity (Agarwal and Gupta, 2003; Jabbour *et al.*, 2009; Zhao *et al.*, 2011).

Female rabbits are very sensitive to heat stress, an important factor influencing their fertility and physiological traits (Rafel *et al.*, 2012; Sabah and Dalal, 2016). Exposure of New Zealand rabbits to 41°C led to 18% decrease in red blood cells count, 20% decrease in hemoglobin content and 22% decrease in blood platelet count as well as 11.2% decrease in total protein, 24% of albumin, 21% of globulin (Sabahand Dalal, 2016). Rabbits exposed to the temperature between 25°C and 36°C compared to those maintained between 14°C and 20°C during pregnancy and lactation, produced lower litter size (9.7 vs 11.4), lower litter weight (503.0 vs 630.5 g) and lower kit weight at birth (56.6 vs 61.4 g) as well as higher stillborn rate (25.4 vs

9.9%) (Marco-Jiménez *et al.*, 2017). Marai *et al.* (2004) reported a decrease in milk yield at day 7 of suckling and milk intake per kit at 7 and 14 days of age in female rabbits reared in hot climate in Egypt. High temperature also affects oogenesis especially during telophase I and metaphase II of meiosis thereby affecting quality of oocytes produced (Hamam *et al.*, 2001). Rabbits, as homoeothermic animals, can regulate the heat input and output of their bodies using physical, morphological, biochemical, and behavioral processes to maintain a fairly normal body temperature (Marai *et al.*, 1994). Apparently, the link between the oxidative stress status, physiological parameters and heat stress in female rabbits under different ranges of temperature is not well documented in literature. This study aimed at evaluating the oxidative stress status and ensuing physiological and behavioral changes in female rabbits exposed to different ranges of temperature.

4.2. MATERIALS AND METHODS

4.2.1. Animals and housing

Twenty-four mature female New Zealand White rabbits, clinically healthy, aged 6 months and weighing between 1953.1 and 2375.4 g were used for the study. They were purchased from a local recognized, licensed breeder and transported to the animal house at the Department of Veterinary Anatomy and Physiology, University of Nairobi, Kenya. Rabbit does were fed the basal commercial pelleted ration containing 18.18% crude protein, 13.43% crude fiber, 2656 MJ/kg diet digestible energy and 2.29% ether extract that met all nutritional requirements of rabbit does according to the National Research Council (NRC, 1977). Fresh water was available to the animals *ad libitum*. The rabbits were housed in wire cages (0.8 ×0.6 ×0.6 m) at room temperature of 22 ± 4°C with animal house relative humidity of 68 ± 5% during the acclimatization period and kept under the same hygienic and managerial conditions. Fecal matter and urine were removed from the cages and floor every morning.

4.2.2. Ethical consideration

The experimental protocols used in this study were approved by the Ethical Committee of the Faculty of Veterinary Medicine, University of Nairobi (REF: FVM BAUEC/2019/244). The experiments were strictly performed in accordance with the internationally accepted standard ethical guidelines for Laboratory Animal Use and Care as described in the European Community guidelines; EEC Directive 86/609/EEC, of November 24, 1986.

4.2.3. Experimental design

Following 2 weeks of acclimatization, all the 24 female rabbits were randomly assigned to 4 groups (T0: ambient temperature (19–26°C), T1: 27–28°C, T2: 31–32°C, T3: 35–36°C) of 6 animals each with comparable weight. The heat was induced in each rabbit cage, using electrical heaters (brand: ARMCO) from 08:00 h to 16:00 h followed by exposure to the normal

air temperature as in the control group from 16:00 h to 08:00 h. During the experimental period, the relative humidity and ambient temperature were recorded twice daily using an automatic thermo-hygrometer. Animals were submitted during 30 consecutive days to the temperature, relative humidity and temperature humidity index (THI) as follows: T0: ambient temperature (19–26°C), 58±0.72%, 22.3±1.84, T1: 27–28°C, 65±0.12%, 26.1±0.6; T2: 31–32°C, 62±0.8%, 29.5±0.6, T3: 35–36°C, 63±0.4%, 32.9±0.6. The THI was calculated following the formula described by Marai *et al.* (2001): THI =db°C-[(0.31–0.31RH) (db°C-14.4) where RH =relative humidity/100, t =ambient temperature. The obtained values of THI for rabbit were classified as follows: <27.8°C =absence of heat stress, 27.8–28.9°C =moderate heat stress, 28.9–30°C =severe heat stress and above 30°C =very severe heat stress (Marai *et al.*, 2001). In the present study, animals of T0 and T1 were exposed to no heat stress and moderate heat stress respectively while those of T2 and T3 were respectively exposed to severe heat stress and very severe heat stress. During the trial period, water consumption (W.C), feed intake (F.I.) and weight gain (W.G.) were measured daily. Daily body weight gain and feed conversion were calculated for each female rabbit according to the equations developed by Sabah and Dalal (2016): Daily body weight gain =final body weight - initial body weight/period (days); Feed conversion ratio =feed intake/body weight gain.

4.2.4. Rectal and skin temperatures

Respective values of rectal and skin temperatures were recorded daily, at 13 h using a digital thermometer brand Kinetik and precision 0.1°C.

4.2.5. Behavioral assessment

Previous studies have shown that stress induces behavioral modifications in animals with the aim of coping with the stressor (Rafel *et al.*, 2012; Nyongesa *et al.*, 2014). It is on this basis that the present study assessed the behavioral changes following induction of heat stress.

Anxiety was scored using behavioral indicators of pacing, scratch and self-directed behaviors; difficulty in standing in upright position, staying alert and moving were indicators of dizziness; aggression was assessed by animals showing ears held flat and turned back, nipping hands, shaking the cage wall, growling and thumping in presence of observer, biting and scratching at the slightest sign of danger; withdrawal was present when the animal appeared isolated to the corner of the cage) and impaired feed intake assessed by lag time in response to introduction of feed and water Shepers *et al.* (2009); Rafel *et al.* (2012); Nyongesa *et al.* (2014). These behaviors were evaluated during the last week of the experimentation.

Three observers, standing at strategic positions in full view of focal subjects, made the behavioral scoring. Following heat-induction, all behaviors were observed for 7 consecutive days before the end of the trial from 10.00 h to 12.00 h of each observation day. Focal subjects in individual cages were observed for their interest with empty toys and/or toys enriched with food. Three different observers that had been habituated to the animals scored individual behavioral scores. Inter-rater reliability used was 90%; it defined number of times behavior was scored divided by the number of times each of the observers scored the behavior. Following the method developed by Ngoula *et al.* (2017a), each behavioral alteration was scored from 0 to +4 (0 =none, +1 =very weak, +2 =weak, +3 =moderately and +4 =severely) depending on the severity and frequency.

4.2.6. Blood and organ collections

At the end of the experimental period (30 days), all animals were fasted for 24 h and humanely sacrificed by euthanizing using ether vapor and then dissected. For hematological and biochemical analysis, 10 ml of the blood was collected directly by cardiac puncture before euthanasia using ether vapor by inhalation. After sacrifice, the ovaries, uterus, lung, heart, liver and kidney were collected by dissection, freed of adipose tissue, washed using saline solution

and blot-dried for weight evaluation. The relative weights of the organs were expressed as percentage of slaughter weights.

4.2.7. Oxidative stress biomarkers

Immediately after weighing, the right kidney was ground in a 0.9% NaCl solution to obtain its 15% homogenates. The resulting homogenate was centrifuged at 4800 rpm for 60 min at 4°C and the aliquots of supernatant kept at -20°C for biochemical estimation of oxidative stress biomarkers. The bovine serum albumin as standard was used to determine the protein content according to the method described by Lowry *et al.* (1951). Enzymatic activities of catalase (CAT) and reduced glutathione (GSH) as well as the levels of superoxide dismutase (SOD) and malondialdehyde (MDA) were assessed in kidney homogenates using a spectrophotometer (GENESYS 20.0) according to the methods described by Habbu *et al.* (2008), Dimo *et al.* (2006), Kodjo *et al.* (2016) and Sajeeth *et al.* (2011) respectively.

4.2.8. Hematological and biochemical analysis

Blood for hematological analysis was collected in a test tube with K3 EDTA anticoagulant and hematological parameters of white blood cell count (WBCs), red blood count (RBCs), hemoglobin content (Hb), lymphocytes (LYM), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), packed cell volume (PCV) and Platelet count (PLT) were analyzed immediately after collection using the Coulter Counter System (Beckman Coulter®, Thermo Fisher, UK) (Sabah and Dalal, 2016; Jensen, 2009). Meanwhile, 5 ml of blood for biochemical analysis collected in tube free from anticoagulant was centrifuged at 3000 rpm for 15 min and supernatant separated as serum and preserved at -20°C for the evaluation of serum content of total cholesterol, albumin, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), urea, creatinine, protein and glucose using commercial assay kits. Analyses were done according to

manufacturer's instructions.

4.2.9. Statistical analysis

All data were submitted to analysis of variance using XL STAT for Windows 10 Software. Results are expressed as mean±SD, and treatment effects among experimental groups alongside controls assessed using one-way ANOVA. The differences in mean values among treatment groups were compared using the Tukey HSD post hoc test at 5% significance level. The Effect Rate (ER) was calculated as the ratio between the value of the control group and the value of the group with the most relevant impact.

4.3. RESULTS

4.3.1. Effect of heat stress on growth performances

There was a significant decrease ($P < 0.05$) in final body weight and body weight gain, by 11% and 62% respectively, in animals exposed to 31–32°C and 35–36°C compared to those of the control group (19–26°C). However, there was apparent opposite trend in feed conversion ratio (64.81%) and water intake (24.19%) while no significant effect ($P > 0.05$) was observed on the average feed intake in all treatment groups compared to controls (Table 4.1).

Table 4.1: Growth performances for female rabbits, as a function of different ranges of temperature

Parameters	T0 (Control)	T1 (27-28°C)	T2 (31-32°C)	T3 (35-36°C)	*p*-value
Initial body weight (g)	2178.30±167.10	2110.1±110.75	2143.5±91.88	2156.30±161.75	0.852
Final body weight (g)	2608.2±163.65[a]	2556.4±129.03[a]	2359.1±65.70[b]	2319.6±75.10[b]	<0.001***
Body weight gain (g)	429.90±194.53[a]	446.28±72.41[a]	215.60±84.44[b]	163.28±107.68[b]	0.0009**
Feed conversion ratio (g)	2.19±0.93[b]	1.81±0.32[b]	3.95±0.49[b]	3.61±0.61[ab]	0.006**
Average feed intake (g)	750.37±130.95	756.42±136.03	781.02±178.62	776.82±116.12	0.8149
Average water intake (ml)	1102.3±149.98[bc]	985±161.43[c]	1196.70±256.69[b]	1368.8±219.1[a]	<0.001***

The superscripts a, b, c: means the mean values are significantly different at P <0.05; T0 control group, T1: 27–28°C, T2: 31–32°C, T3: 35–36°C; ER: Effect Rate. *, **, ***: significant at P <0.05; 0.01 and 0.001, respectively; n=7. Note a decrease in final body weight and body weight gain and an opposite trend in feed conversion ratio and water intake in animals exposed to 31–32°C and 35–36°C compared to those of the control group.

4.3.2. Effect of heat stress on behavioral scores

The behavioral scores recorded on heat stressed female rabbits are presented in Table 4.2. No behavioral changes were observed in animals of the control group (19–26°C) as well as those exposed to 27–28°C. Dizziness was observed in animals exposed to 31–32°C and 35–36°C. All animals exposed to 31–32°C and 35–36°C showed a very weak response to feed introduction (score 1) and severely withdrawn to a corner of the cage (score 4). On the other hand, animals exposed to 19–26°C and 27–28°C had severe response to feed introduction (score 4).

Table 4.2: Behavioral alterations for female rabbits, as a function of different ranges of temperature

Parameters	T0 (Control)	T1 (27-28°C)	T2 (31-32°C)	T3 (35-36°C)
Anxiety	0	0	0	0
Dizziness	0	0	+2	+2
Aggression	0	0	0	+1
Withdrawal	0	+1	+4	+4
Response to feed introduction	+4	+4	+1	+1

T0 control group, T1: 27–28°C, T2: 31–32°C, T3: 35–36°C; 0, +1, +2, +3, +4 represent respectively none, very weak, weak, moderately and severely behavioral scores; n=7. Note presence of dizziness, very weak response to feed introduction and severely withdrawn to a corner of the cage in animals of T2 and T3.

4.3.3. Effect of heat stress on rectal and skin temperature

Results obtained in Figure 4.1 show that severe and very severe heat stress caused a significant increase ($P<0.05$) of both rectal and skin temperatures. Rectal temperature increased significantly by 3.64% with the increase in temperature range while the skin temperature increased by 2.42% in rabbits submitted to the highest temperature compared to controls.
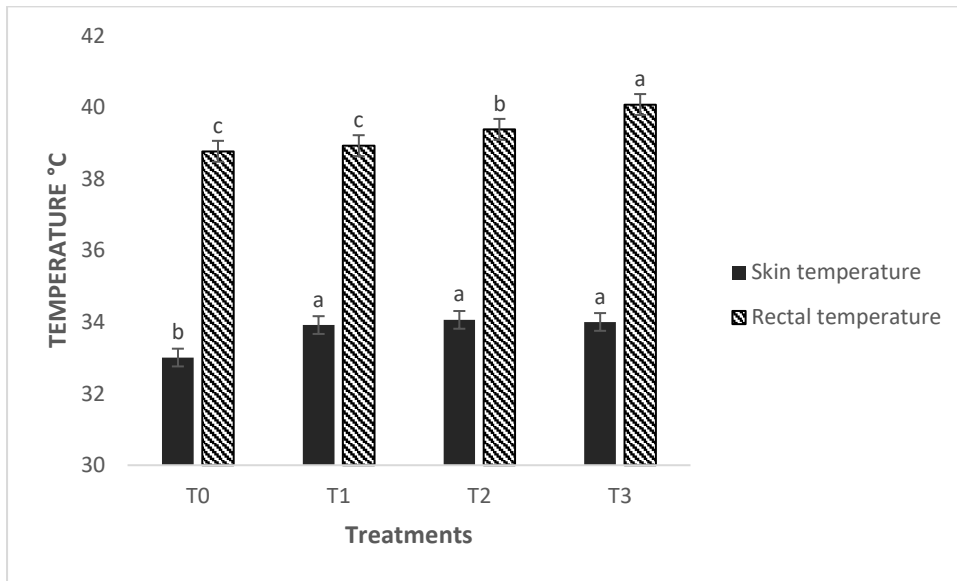
Figure 4.1: Skin and rectal temperatures for female rabbits, as a function of different ranges of temperature.

The superscripts a, b, c: means the mean values are significantly different at P <0.05; T0 control group, T1: 27–28°C, T2: 31–32°C, T3: 35–36°C; n=7. Note a significant increase in rectal and skin temperatures of animals from T2 and T3 compared to those of T0 and T1.

4.3.4. Effect of heat stress on relative weight of some internal organs

The relative weights of heart and liver was not significantly affected ($P>0.05$) by the temperature levels considered in this study (Table 4.3). However, the relative weight of the lung and kidney were significantly increased ($P <0.05$), by 37.72% and 19.5% respectively, in animals exposed to 31–32°C and 35–36°C. The weight of ovaries and uterus decreased significantly ($P<0.05$) by 30.4% and 18.1% respectively at the same temperatures when compared to those submitted to the control group (19–26°C) and 27–28°C.

Table 4.3: Relative organs weight for female rabbits, as a function of different ranges of temperature

Parameters	T0 (Control)	T1 (27-28°C)	T2 (31-32°C)	T3 (35-36°C)	*p*-value
Heart	0.51±0.08	0.54±0.11	0.55±0.06	0.56±0.13	0.101
Lung	1.14±0.32^b	1.19±0.22^b	1.28±0.14^b	1.57±0.06^a	0.027*
Liver	4.18±1.09	4.21±1.52	5.36±1.47	5.59±1.40	0.258
Kidney	0.82±0.04^b	0.81±0.08^b	0.94±0.04^a	0.98±0.07^a	0.023*
Ovaries	0.23±0.02^a	0.22±0.03^a	0.19±0.06^{ab}	0.16±0.02^b	<0.001***
Uterus	0.44±0.01^a	0.46±0.06^a	0.35±0.01^b	0.36±0.02^b	<0.001***

The superscripts a, b, c: means the mean values are significantly different at P <0.05; T0 control group, T1: 27–28°C, T2: 31–32°C, T3: 35–36°C; ER: Effect Rate. *, ***: significant at P<0.05 and 0.001, respectively; n=7. Note a significant increase in the weight of the lung and kidney and a significant decrease in the weight of ovaries and uterus in animals of T2 and T3.

4.3.5. Effect of heat stress on hematological parameters

There was a significant decrease ($P<0.05$) in Hb by 23.64% and 12.73% of RBC contents in animals exposed to 31–32°C and 35–36°C but total WBC, MCV and LYM counts increased in the same animals by 42.37%, 10.73% and 15.53% respectively when compared to control. The PCV decreased only in animals exposed to 35–36°C when compared to control and other treated groups. The other blood parameters; MCH, MCHC and PLT were not significantly affected by the heat stress (Table 4.4).

Table 4.4: Hematological parameters for female rabbits as a function of different ranges of temperature

Parameters	T0 (Control)	T1 (27-28°C)	T2 (31-32°C)	T3 (35-36°C)	*p*-value
Hb (g/dl)	14.17±1.12[a]	14.02±1.26[a]	11.33±1.04[b]	10.82±1.54[b]	0.017*
PCV (%)	41.21±1.76[a]	43.72±2.16[a]	40.10±0.65[a]	36.29±0.76[b]	0.032*
RBC (x10^{12}/l)	5.89±0.22[a]	5.81±0.25[a]	5.36±0.16[b]	5.14±0.21[b]	0.018*
MCV (fl)	69.90±0.81[b]	71.33±2.21[b]	75.33±1.46[a]	77.40±1.83[a]	<0.001***
MCH (pg)	25.73±0.85	26.22±0.13	25.41±0.77	26.85±0.91	0.246
MCHC (g/dl)	33.36±1.82	32.44±2.87	33.06±2.13	34.87±1.48	0.083
WBC (x10^9/l)	11.61±2.73[b]	11.72±1.89[b]	12.62±1.34[b]	16.53±1.12[a]	<0.001***
PLT (×10^3/µl)	185.33±5.60	190.50±6.93	192.67±8.12	188.09±6.74	0.061
LYM (%)	56.57±3.67[b]	58.06±2.51[b]	63.36±1.49[a]	65.36±2.33[a]	0.024*

The superscripts a, b, c: means the mean values are significantly different at *P* <0.05; T0 control group, T1: 27–28°C, T2: 31–32°C, T3: 35–36°C. Hb: hemoglobin; PCV: packed cell volume; RBC: Red blood Cell; MCV: mean cell volume; MCHC: mean corpuscular hemoglobin concentration; MCH: mean corpuscular hemoglobin; WBC: white blood cells; PLT: Platelet count; LYM: lymphocytes; ER: Effect Rate. *, ***: significant at *P* <0.05 and 0.001, respectively; n=7. Note a significant decrease in Hb and RBC followed by an increase in WBC, MCV and LYM in animals of T2 and T3.

4.3.6. Effect of heat stress on biochemical parameters

Creatinine, urea and ASAT were significantly increased ($P < 0.05$) by 28.98%, 53.20% and 23.31% respectively at 31–32°C and 35–36°C while total protein decreased by 12.02% in animals in the same groups compared to those of the control group (19–26°C) and 27–28°C. There was no significant difference on cholesterol, ALAT, glucose and total albumin in treated groups compared to controls (Table 4.5).

Table 4.5: Serum biochemical parameters for female rabbits, as a function of different ranges of temperature

Parameters	T0 (Control)	T1 (27-28°C)	T2 (31-32°C)	T3 (35-36°C)	*p*-value
Cholesterol (mg/dl)	122.12±6.08	123.40±9.13	111.93±9.11	108.42±10.21	0.241
Creatinine (mg/dl)	0.69±0.06[b]	0.69±0.04[b]	0.84±0.05[a]	0.89±0.04[a]	0.004**
Urea (mg/dl)	92.12±11.15[b]	89.30±17.42[b]	136.93±13.81[a]	141.13±12.87[a]	0.003**
ALAT (U/L)	49.22±3.70	46.29±6.02	51.93±5.15	53.01±7.19	0.072
ASAT (U/L)	23.90±1.19[c]	23.64±1.22[c]	25.91±0.08[b]	29.47±1.28[a]	0.036*
Glucose (mmol/L)	7.26±0.08	6.55±0.16	6.61±0.12	5.81±0.39	0.241
Total protein (g/L)	73.87±2.04[a]	72.12±1.41[a]	67.80±1.08[b]	64.99±2.25[b]	0.014*
Total albumin (g/dl)	4.51±0.23	4.60±0.19	4.48±0.31	4.55±0.33	0.131

The superscripts a, b, c: means the mean values are significantly different at $P < 0.05$; T0 control group, T1: 27–28°C, T2: 31–32°C, T3: 35–36°C. ALAT: alanine aminotransferase; ASAT: aspartate amino-transferase, ER: Effect Rate. *, **: significant at P<0.05 and 0.01, respectively; n=7. Note an increase in creatinine, urea and ASAT followed with a decrease in total protein contents in animals from T2 and T3.

4.3.7. Effect of heat stress on oxidative stress biomarkers

In female rabbits exposed to 31–32°C and 35–36°C, the protein levels in the kidney significantly decreased by 3.55% as compared to those exposed to 19–26°C and 27–28°C. The opposite trend was recorded for MDA concentration (74.29%) (Table 4.6). The activities of CAT, SOD and GSH were significantly lower ($P<0.05$) in animals exposed to 35–36°C by 32.82%, 24.28% and 34.34% respectively compared to other temperature ranges.

Table 4.6: Oxidative stress biomarkers for female rabbits, as a function of different ranges of temperature

Parameters	T0 (Control)	T1 (27-28°C)	T2 (31-32°C)	T3 (35-36°C)	*p*-value
Protein (mg/ml)	10.65 ± 0.55^a	10.50 ± 0.67^a	9.3 ± 0.84^b	6.62 ± 0.43^c	<0.001***
MDA (nmol/mg tissues)	19.88 ± 0.99^a	20.63 ± 1.64^b	30.75 ± 1.65^c	34.65 ± 1.29^c	<0.001***
CAT (UI/mg tissues)	9.75 ± 1.03^a	9.65 ± 1.39^a	8.40 ± 0.60^a	6.55 ± 0.84^b	<0.001***
SOD (UI/mg tissues)	6.30 ± 0.52^a	6.73 ± 0.42^a	5.02 ± 0.41^b	4.77 ± 0.69^b	<0.001***
GSH (nmol/mg of tissue wet)	9.55 ± 0.57^a	9.55 ± 0.39^a	6.38 ± 0.60^b	6.27 ± 0.75^b	<0.001***

The superscripts a, b, c: means the mean values are significantly different at $P <0.05$; T0 control group, T1: 27–28°C, T2: 31–32°C, T3: 35–36°C. CAT: catalase, GSH: reduced glutathione, SOD: superoxide dismutase, MDA: lipid peroxidation; ER: Effect Rate. ***: significant at $P < 0.001$; n=7. Note a decrease in the kidney protein levels, CAT, SOD and GSH activities and an increase in MDA concentration in animals of T2 and T3.

4.4. DISCUSSION

Heat stress causes several drastic changes in the biological function which are responsible for damages in both production and reproductive performances in rabbits (Abdel–Samee *et al.*, 2005). Results of the present study showed effect of elevated temperatures on some parameters defining growth performance where final body weight and body weight gain decreased while feed conversion ratio (FCR) increased in animals exposed to 31–32°C and 35–36°C as compared to the controls. Similar observations were reported in earlier studies where New Zealand White rabbits exposed to hot ambient temperature ranges from 33 to 38°C (Sabah and Dalal, 2016) and 36 ±3°C (Ondruska *et al.*, 2011) for one month. The decrease in final body weight and body weight gain observed in the present study may be attributed to the increase in the FCR, which might have led to less protein biosynthesis and less fat deposition (Marai *et al.*, 2001, 2004; Ogunjimi *et al.*, 2008). In fact, increase in FCR with the increasing temperature indicates a poor digestibility of feed and absorption of the resultant nutrients in heat stressed rabbits. Poor digestibility, in this context, has been explained in earlier findings where elevated temperature suppressed enzymatic activities at the brush border and pancreatic secretions on duodenum, which led to a weakened digestion and nutrient assimilation (Jinap and Hajeb, 2010). It has also been associated with increase in the energy loss used in synthesis of macromolecules (Garlick, 2005). Studies have shown that extreme temperatures can disrupt stability of alpha diversity within gut micro-biota while favoring beta diversity (relative abundance of specific bacteria) microbiota within host organisms and consequently influence beneficial and deleterious effects that play a key role on host phenotypes and fitness (Kohl and Yahn, 2016). Each host species displays a distinct microbial response to thermal stress but some gut bacterial taxa (Firmicutes and proteobacteria) show shift with temperatures that appear to be reproducible across host species. It is however, not clear whether changes in gut

microbiota in response to high ambient temperature are responsible for reduced host energy acquisition from diet. Thermal stress also interferes with redistribution of blood from internal organs such as intestines thereby interfering with digestion and absorption. Thermoregulatory mechanisms here favor cutaneous heat dissipation while internal tissues experience hypoxia (Pearce *et al.*, 2013) hence impairment of secretion of digestive enzymes and absorption. In addition, high ambient temperatures have been shown to impact on the composition of animal gut microbiota thus altering their function and consequently influencing host phenotypes and general performance (Sepulveda and Moeller, 2020). The observed increase in FCR in the present study shows the influence of high temperature on food assimilation by gut microbiota. This is supported by earlier studies in salamanders that reported high temperature different from their preferred body temperature that led to specific changes in microbiome composition hence decrease in energy assimilation, feed intake, and digestive efficiency. Energy assimilation, in this case, was associated with the relative abundances of Sphingopyxis, Roseococcus and Stenotrophomonas, which contain lineages capable of digesting cellulose polymers (Dantur *et al.*, 2015). Similar studies in *Bos taurus* reared at 20°C, 28°C and 30°C showed decrease in relative abundance of Firmicutes within gut micro-biota (Tajima *et al.*, 2007). From the foregoing, understanding how changes in ambient temperature impacts on gut microbiota of animals may help predict future responses of animal genotypes and phenotypes to climate change.

The high water consumption in heat stressed rabbits observed in the present study can be explained by the normal homeostatic mechanisms governing thermoregulation. Rabbits are almost dependent on pulmonary ventilation for regulation of body temperature in hot conditions and so the increase in water consumption may help them to increase heat loss through pulmonary ventilation mechanisms (Marai *et al.*, 2001; Badr, 2015). Results of the

present study showed high skin and rectal temperatures in rabbits exposed to heat, with significant effects at 31–32°C and 35–36°C compared to the group exposed to moderate temperatures as well as for controls. These results are similar to the findings of Sabah and Dalal (2016) in New Zealand White rabbits subjected to hot ambient temperature in the range 33°C–38°C. The high skin temperature observed in the present study may be due to the insulating effect of the hair coat (Marai *et al.*, 2008). On the other hand, the increase in rectal temperature in animals at high temperatures may be the result of increasing metabolic rate and ultimately hyperthermia as a result of impaired thermoregulation (Shafie *et al.*, 1982). In separate studies, Marai *et al.* (2004) reported that at temperatures above 30°C, rabbits stretch out in an attempt to lose as much heat as possible by radiation and convection with significant rises in rectal temperature. This finding is linked to the behavioral manifestations reported in animals exposed to 31–32°C and 35–36°C in the present study. Rafel *et al.* (2012) in their study indicated that stress induces reactions in rabbits including behavioral modifications in attempt to cope with the stressor. The appearance of dizziness in heat stressed animals reported in the present study may be due to the inhibition of acetyl cholinesterase enzyme (AchE) activity by the heat thus resulting to accumulation of acetylcholine in cholinergic synapses (Sarkar *et al.*, 2000). The inhibition of this AchE activity induces an accumulation of the neurotransmitter acetylcholine (ACh) in neuronal synapses and neuromuscular junctions, thereby leading to prolonged over-stimulation of cholinergic receptors and resulting cholinergic toxicity (Major *et al.*, 2018). The withdrawal near the window of the cage observed in heat stressed animals indicates an attempt to cooling effect with cooler ambient temperatures outside of the animal house. The heat stressed animals were not excited with feed introduction in cages. McManus *et al.* (2009) reported that heat stressed animals are not very attracted to feed as they need to slow down their basal metabolism thereby causing hypo-function of thyroid gland in order to

prevent the additional metabolic heat production.

Results of this study showed that the relative weight of lung and kidney were significantly increased in heat stressed animals. The increase in weight of these organs has been previously reported in studies by Badawi and El-Aasar (2018) in New-Zealand White and Baladi Black rabbits exposed to Egyptian hot conditions for 35 consecutive days. On the contrary, results of the present study showed a decrease in the weights of uterus and ovaries as compared to the control. The reduction in uterine and ovary size has been previously associated with the fetal growth restraint (IbaÂnÄez et al., 2003). The increased weight of lung in heat stressed rabbits reported in the present study may be due to the larger air volumes required by these animals to dissipate heat to the environment through pulmonary ventilation (Marai et al., 2007b). The increase in weight of the kidney could be due to the intensive activity of detoxification carried out by this organ due probably to the increased insensible loss of body water and salt (Lloyd, 1994). This leads to substantial fluid deficit which, if not replaced, may result in vasoconstriction resulting in renal injury driven by effects of hyper-osmolarity (via activation of the polyol pathway) (Lloyd, 1994; Glaser et al., 2016). Urea and creatinine are biochemical markers usually used in the exploration of the renal function. It was observed from this study that the urea and creatinine concentrations significantly increased in animals at 31–32°C and 35–36°C. This is in agreement with findings of Okab et al. (2008) in New-Zealand White rabbit males submitted to 26.5°C-32.2°C corresponding to summer conditions in Egypt. The level of increase of creatinine and urea in the study is probably indicative of damage to the kidney functional units hence compromised glomerular filtration (Walmsley and White, 1994). Further, the increase in urea and creatinine could also be a result of increase in protein catabolism owing to high stimulation of synthesis of the enzyme arginase by elevated heat, which intervenes in the production of urea (Yanardag and Sacan, 2007). The latter argument is

complimented by results in the same animals where a decrease in serum protein level was noticed in rabbits exposed to 31–32°C and 35–36°C. Similar to earlier reports in New Zealand White rabbits exposed to 33–38°C (Sabah and Dalal, 2016) and 36 ±3°C (Ondruska *et al.*, 2011) for 1 month. The decrease in total protein observed in the present study was probably a consequence of dilution of plasma proteins caused by the increase in water consumption or could have arisen from enhanced protein utilization and amino acid transamination in the rabbits exposed to heat (Ayyat *et al.*, 2002).

Previous reports show that the liver functional transaminase (ASAT and ALAT) enzyme activities are indicators of liver diseases such as infectious hepatitis, alcoholic cirrhosis, biliary obstruction, toxic hepatitis and liver cancer (Arafa *et al.*, 2007). In the present study, ALAT concentration was not significantly affected by heat, which is in contrast to the findings of Okab *et al.* (2008) in New-Zealand White male rabbits exposed to 26.5°C-32.2°C where a significant decrease in liver ALAT levels was recorded. On the contrary, ASAT levels significantly increased in rabbits exposed to 31–32°C and 35–36°C in the present study. This finding points to the fact that ASAT is dependent on the amino acid groups of alanine and glutamine taken up by the liver and reflect the changes in the liver metabolism associated with glucose synthesis (El-Maghawry *et al.*, 2000). The decrease in serum glucose content in the heat stressed rabbits by the rise in glucose utilization during muscular movements was required for high respiratory activity or due to increase in corticosteroid concentration (Habeeb *et al.*, 1997).

The results of this study showed a decrease in Hb, PCV and RBC counts but an increase in MCV, WBC, LYM in female rabbits exposed to 31–32°C and 35–36°C. These results are similar to the findings in New Zealand White male rabbits exposed to summer conditions in Egypt (Okab *et al.*, 2008) and in New Zealand White rabbits exposed to 33–38°C (Sabah and

Dalal, 2016). Hemoglobin of erythrocytes plays a vital role in carrying approximately 98% of oxygen throughout the respiring tissue cells of the animal body system while the PCV is a measure of the proportion of blood that is made up of cells (Jensen, 2009). In the present study, we reported suppression of appetitive behavior in terms of increased latency of feed intake with high temperature exposure to animals. The reduced feed intake may have been a strategy to minimize metabolic heat production, which may explain, in part, the physiological, biochemical and behavioral adaptations that were observed in female does. Studies in piglets showed that heat stress increase skin blood flow circulation that promote heat dissipation (Collin *et al.*, 2001) while most internal organs such as intestinal epithelium may have less blood supply that leads to tissue hypoxia (Pearce *et al.*, 2013). Thermoregulatory mechanisms in this case favor increased erythropoiesis hence more red blood cells to allow for oxygen redistribution to curb hypoxia in internal tissues. The decrease in hemoglobin concentration in the present study is surprising since increased erythropoiesis means increase in hemoglobin content in red blood cells. Nonetheless, it is possible there was increased attack by free radicals on red blood cell membrane, which is rich in lipid content thereby leading to hemolysis. Measurements on the RBC deformability were not considered in the present study. Earlier studies showed involvement of increased insulin concentration during thermal stress (Pearce *et al.*, 2013) in synthesis of nitric oxide by red blood cells, which causes deformability (Grau *et al.*, 2013). The increase in RBC deformability during heat exposure has been shown to slow down peripheral resistance hence accelerating tissue oxygenation and blood supply to heat exchange surfaces (Zhou *et al.*, 1999). Although these studies were mainly done on pigs, the mechanisms of homeothermic thermoregulation favor extrapolation of these results to rabbit physiology among other terrestrial homeotherms. In fact, studies in sheep exposed to high ambient temperatures for 8 h daily for 45 days showed significant effect on increased RBC,

hemoglobin and PCV (Al-Haidary, 2004; Rana *et al.*, 2014). The increase in PCV in their study, was attributed to adaptation mechanism to provide water necessary for evaporative cooling process. The same scenario could hold true for rabbits in the present study since rabbits have few functional sweat glands (Naqvi *et al.*, 1995). Moreover, heat stress is reported to decrease adrenocorticotropic hormone (ACTH) levels, which in turn decreases RBC, Hb and PCV contents (Okab *et al.*, 2008). Lymphocytes and WBC are responsible for both humoral and cellular immunity. The increase in their contents in heat stressed rabbits reported in the present study may be attributed to stress due to pathogens during high temperature exposure, which may increase blood viscosity and produce allergic effects that induce WBC increase (Okab *et al.*, 2008). The increase in MCV is a consequence of the decrease in salt concentration in blood plasma of rabbits exposed to 31–32°C and 35–36°C (Badawi and El-Aasar, 2018). In the present study, the level of oxidative stress biomarkers such as kidney protein, MDA, CAT, SOD, GSH were significantly affected ($P<0.001$) by the increase in temperature. Similar results have been reported in exotic breeds of rabbits during peak of heat stress in Nigeria for 7 consecutive weeks (Jimoh, 2019) and in guinea pigs exposed to heat stressed temperatures for 60 days (Ngoula *et al.*, 2017b). Oxidative stress has been reported to impair both productive and reproductive functions in animals (Spears and Weiss, 2008; Celi, 2011; Ngoula *et al.*, 2017a). An increase in the activity of MDA as well as a decrease in activity of SOD, CAT and GSH reported in the present study may have been caused by their intensive utilization in protection against oxidative tissue damage (Seven *et al.*, 2001). In fact, the increased amount of MDA levels indicates the lipid peroxidation process in tissues wherever the fatty acids in the cell membrane lose hydrogen molecules (Celi, 2011). Serum GSH activity has a major role in the oxidative defense of animal tissues by catalyzing the reduction of hydrogen and lipid peroxides (Halliwell and Chirico, 1993). Superoxide dismutase catalyses the dismutation of

superoxide to hydrogen peroxide (H_2O_2) and this is considered the first line of defense against pro-oxidants while CAT is known for its facile ability to convert hydrogen peroxide into water and oxygen thereby reducing H_2O_2 concentration in animal cells (Halliwell and Chirico, 1993).

4.5. CONCLUSION

Results of this study indicated that exposure of female rabbits to 31–32°C and 35–36°C for 30 consecutive days impaired their growth performances, relative organ weights and caused some behavioral abnormalities. Moreover, heat stress increased rectal and skin temperature, impaired hemato-biochemical parameters, resulted in increase in the level of MDA while reducing the levels of enzymatic antioxidant biomarkers. The deleterious effects of heat stress observed in the present study may be attributed to the oxidative stress. However, further studies are needed for alternative ways of alleviating the effects of heat stress on oxidative stress and physiological damages in animals.

CHAPTER FIVE: ASSESSMENT OF THE EFFICACY OF *MORINGA OLEIFERA* AQUEOUS SEED EXTRACT ON PHYSIOLOGICAL PARAMETERS IN MANAGEMENT OF HEAT STRESS USING FEMALE RABBITS

5.1. INTRODUCTION

Rabbits (*Oryctolagus cuniculus*) are widely present in domestic farming, due to affordability of their feed and the rearing method is very easy to conduct. Rabbits are simple to raise, require little space and are very prolific throughout the year (Lebas, 2008). Moreover, they are easy to house, produces meat with good dietary qualities and provide manure for nourishing the soil (Guindjoumbi, 2007; Schiere and Coustiaensen, 2008). They are also preferred because of their early maturity, fast growth rate, high genetic selection potential, high feed conversion efficiency and economic utilization of space (Hassan *et al.*, 2012). However, in tropical and subtropical regions, rabbit production is affected by several factors including inadequate nutrition due to lack of feed, a range of parasitic diseases (Ola-Fadunsin *et al.*, 2018), low genetic potential, inbreeding (Mutwedu *et al.*, 2015) and environmental stress (Ngoula *et al.*, 2017b; Mutwedu *et al.*, 2020).

Thermal stress is one of the main environmental impediments and occurs when the ambient temperature is under or exceeds the thermoneutral (comfort) zone of the animal (Kumar *et al.*, 2011). Heat stress is one of the most important stressors in animal production, especially in rabbit farming due to the lack of functional sweat glands (Marai *et al.*, 2002). It has been reported to induce many disturbances in rabbit physiological system. For instance, in an earlier study, there was a decrease in standing and walking behavior while red blood cells, white blood cells, hemoglobin, packed cell volume, lymphocytes as well as the respiration rate, rectal temperature were impaired in rabbit bucks submitted to 37±0.5°C (Hassan *et al.*, 2015).

Okab *et al.* (2008) reported a decrease in hematological values such as hemoglobin, packed

cell volume, red blood cell and an increase in biochemical values including globulins, total lipids and cholesterol as well as plasma enzymes such as alanine aminotransaminase and alkaline phosphatase in rabbits submitted to 32.2°C. A decrease was observed in lipid peroxidation, total antioxidant activity, catalase, glutathione peroxidase, and superoxide dismutase in rabbits reared during peak of heat stress in Nigeria (Jimoh, 2019).

To counter the harmful effects of heat stress, the antioxidant capacity of the animal can be improved by supplementation of vitamin C and E, minerals and trace elements such as zinc, copper, sodium, potassium (Kumar *et al.*, 2011). Additionally, substances with antioxidant activity such as green tea extract (Abshenas *et al.*, 2011), powder of *Zingiber officinale* (Habeeb *et al.*, 2019) and *Moringa oleifera* extract (El-Desoky *et al.*, 2021) can be used.

Moringa oleifera is perennial tree with 5 to 12 m of height belonging to the *Moringaceae* family and is considered as one of the best antioxidant plants throughout the world (Wadhwa, 2013). All parts of this plant including leaves, stembarks, pods and seeds, have been reported to be rich in various bioactive compounds such as alkaloids, saponins, phenols, flavonoids, glycosides, terpenoids and tannins (El-Alfy e*t al.*, 2011; Ifesan *et al.*, 2013; Ayirezang *et al.*, 2020) and have been reported to alleviate damages caused by oxidative stress (Sharma *et al.*, 2011; Chisholm, 2015). However, leaves of *Moringa oleifera* have been reported to possess better antioxidant activity (Chumark *et al.*, 2008) and this has led to its overuse compared to other parts of this plant. It is worth exploring the efficacy of other parts of this plant for their role in overcoming oxidative stress in animals. This study was designed to evaluate the efficacy of *Moringa oleifera* aqueous seed extract in attempt to overcome the effects of heat stress on some internal organ weight, hematological and biochemical parameters, oxidative stress biomarkers and kidney histology in female rabbits.

5.2. MATERIAL AND METHODS

5.2.1. Experimental location

The study was conducted from September to December 2019 at the Department of Veterinary Anatomy and Physiology, University of Nairobi, Kenya. The average temperature was 24°C and the relative humidity was generally greater than 58%.

5.2.2. Plant material

The seeds of *Moringa oleifera (Lam)* aged 2 to 3 years were collected from Masii village of Machakos County in Kenya. The plant was taxonomically identified at the Department of Biology and Biotechnology, University of Nairobi, Kenya. The seeds were air dried at 21 to 25°C for 14 days. After spreading, the plant material was ground using an electric mill and the fine powder collected and stored in airtight glassware until aqueous extraction.

5.2.3. Extraction of crude powdered sample

A total of 1000 g of dried *Moringa oleifera* seed powder was aliquoted into a flask wrapped in aluminum foil. Ten (10) liters of distilled water was gradually added to the powder with gentle shaking until slurry of uniform consistency was formed. The resultant slurry was centrifuged at 3000 RPM for 5 minutes. The supernatant was then collected into light resistant bottles and freeze-dried for 24 hours to obtain the aqueous extract.

5.2.4. Phytochemical screening

The bioactive compounds were detected using phytochemical tests according to standard methods developed by Harborne (1998) and Evans and Trease (2009). Thus, phytochemical screening of bioactive compounds contained in seeds of *Moringa oleifera* revealed the presence of alkaloids, phenols, flavonoids, glycosides and tannins.

5.2.5. Experimental animals and their feeding

A total of 28 female rabbits, aged 6 months and weighing between 2015.6 and 2322.7 g, purchased from a local recognized and licensed breeder, were used. Throughout the experimental period, feed and water were provided *ad libitum* to animals. All animals used for the experiment were provided with the basal commercial pelleted ration containing 18.18% crude protein, 13.43% crude fiber, 2656 MJ/kg diet digestible energy and 2.29% ether extract that met all nutritional requirements of rabbit does according to the National Research Council (NRC, 1977). The rabbit cages were routinely cleaned every morning before clinical observations to the animals.

5.2.6. Ethical consideration

It is the same as was described previously in section 4.2.2 of the Chapter 4.

5.2.7. Experimental design

During the acclimatization period, the rabbits were housed in wire cages (0.8 ×0.6 ×0.6 m) at room temperature of 21 ±3°C with animal house relative humidity of 66 ± 3% and kept under the same hygienic and managerial conditions. Before starting the experiment, the animals were weighed and randomly assigned to 4 groups of 7 female rabbits each with comparable body weight and distributed to T0 and T3 (normal and positive control respectively), T1 and T2 (low and high dose of *Moringa oleifera* respectively). Thereafter, during 80 consecutive days, animals were submitted to different room temperatures, relative humidity, temperature humidity index (THI) and of *Moringa oleifera* aqueous seed extract (MOASE) as follows: T0: ambient temperature (18–24°C), 59 ±0.48%, 23.6 ±1.52; T1: 35–36°C, 64 ±0.6%, 32.5 ±0.7, 100 mg of MOASE; T2: 35–36°C, 64 ±0.6%, 32.5 ±0.7, 200 mg of MOASE; T3: 35–36°C, 64 ±0.6%, 32.5 ±0.7. The heat was induced in each rabbit cage, using electrical heaters (brand: ARMCO from India) from 08:00 h to 16:00 h followed by exposure to the normal air

temperature as in the control group from 16.00 h to 08:00 h. During the experimental period, the relative humidity and ambient temperature were recorded twice daily using an automatic thermo-hygrometer (Brand: RC dalys, Size: 48*28.6*15.2mm, temperature precision: $\pm 1°C$, hygrometry precision: ± 5). The selected range of temperature, relative humidity and THI were chosen according to results observed in the pilot study as reported by Mutwedu *et al.* (2020) and was classified as very severe heat stress (Marai *et al.*, 2001). *Moringa oleifera* aqueous seed extracts (MOASE) were administrated *per os* once a day for 80 days using an endogastric cannula while normal and negative control animals (T0 and T3 respectively) orally received 10 ml of distilled water daily.

5.2.8. Rectal and skin temperatures

It is the same as was described previously in section 4.2.4 of the Chapter 4.

5.2.9. Sacrifice of rabbits and organ collections

At the end of the treatment (80th day), all female rabbits were fasted for 24 h.

Animal sacrifice, tissue harvesting and handling are as described previously in section 4.2.6 of the Chapter 4.

5.2.10. Oxidative stress biomarkers

It is the same as was described previously in section 4.2.7 of the Chapter 4.

5.2.11. Hematological and biochemical analysis

Blood sampling and processing for hematological and biochemical analysis followed steps described previously in section 4.2.8 of the Chapter 4.

5.2.12. Kidney histology

Immediately after sacrifice by euthanizing using ether vapor, the left kidney of each female rabbit was removed and prepared as follows:

Fixation

After harvesting, the kidney was pre-fixed in 10% formalin, in a volumetric ratio (organ/formalin) of 1/3. Twenty-four (24) hours before dehydration, the pre-fixed organ was soaked in Bouin's solution.

Dehydration

After its stay in Bouin solution, the biopsies of the organ that had been flushed in distilled water for 15 min were dehydrated in ethyl alcohol containers of increasing temperature (70°C, 95°C, 95°C, 100°C, 100°C, 100°C) for 45 min, 45 min, 60 min, 45 min, 60 min and 90 min respectively. Subsequently, these biopsies were introduced into xylene for 2 hours for brightening.

Inclusion and coating

Immediately after being dehydrated, the organ tissues were embedded in three successive steps in molten paraffin (approximately 60°C) for 60 min, 90 min, and 120 min respectively for soaking. Subsequently, these tissues placed in molds were embedded in paraffin wax and then left on a cooling surface for curing.

Realization of sections

The tissue blocks were placed on the microtome (Brand: Leica RM2155) and cut to a thickness of 5 µm. The sections were then spread on the surface of a gelatin water bath (40°C) for defriping, then mounted on microscope glass slides before being placed in the oven (45°C) to dry for 24 hours.

Dewaxing

After removal from the oven, the slides were dewaxed in three successive xylene containers for 10 min respectively, then rehydrated in decreasing degrees of ethyl alcohol (three times in 100°C alcohol, once in 95°C alcohol and once in 70°C alcohol) (10 min per container), before

being flushed with distilled water for another 10 min.

Hematoxylin-Eosin staining

After rehydration, the slides were stained in Mayer's hematoxylin (nuclear stain) for 10 min and flushed with tap water for 10 min for dark blue staining of the cell nucleus. These slides were then successively soaked for 10 min in two containers of ethyl alcohol (70 and 80%) before being stained for 10 min in a 0.5% alcoholic eosin solution, to which a few drops of acetic acid were added at a rate of 40 µl per 100 ml of solution (for pink staining of the cell cytoplasm and orange-red staining of the red blood cells). After quickly flushing the slides with water to remove excess stain, they were dehydrated by successive passages for 10 min each, in two containers of absolute ethyl alcohol and finally, in xylene to make the parts uniformly translucent before being placed on Eukitt for fast drying.

Observation and photography

After drying the slides at room temperature for 24 h, they were observed using a light microscope (Leica DM 750, X10 and X40) equipped with a DCM35 digital camera (350 Kpixels, USB 2.0), which was connected to the central processing unit of a computer. The images were directly observed on the computer equipped with an image capture program. The captured images were magnified 100 times (100X) for histology and cellular integrity.

5.2.13. Statistical analysis

Data were submitted to one-way analysis of variance at $P<0.05$. Tukey HSD test was used to to measure significant differences in means among different treatment subject groups. Dependent variables including some internal organ weight, hematological and biochemical parameters, oxidative stress biomarkers were compared following *Moringa oleifera* aqueous seed extract doses in heat stressed animals. Results are expressed as mean ± SD and the analyses were performed using XL STAT for Windows 10 Software. The Effect Rate (ER) was

calculated as the ratio between the value of the control group and the value of the group with the most relevant impact. Histological parameters were observed by comparing the changes in different kidney tissues of each treatment with normal kidney tissues.

5.3. RESULTS

5.3.1. Rectal and skin temperature

There was a significant increase ($P<0.05$) of both rectal and skin temperatures (by 10.3% and 10.4% respectively) in animals subjected to heat stress compared to the control group. However, the administration of doses of *Moringa oleifera* aqueous seeds extract (MOASE) tended to moderate these parameters compared to animals solely submitted to heat stress (6.2% and 3.8% for rectal temperature and skin temperatures respectively).
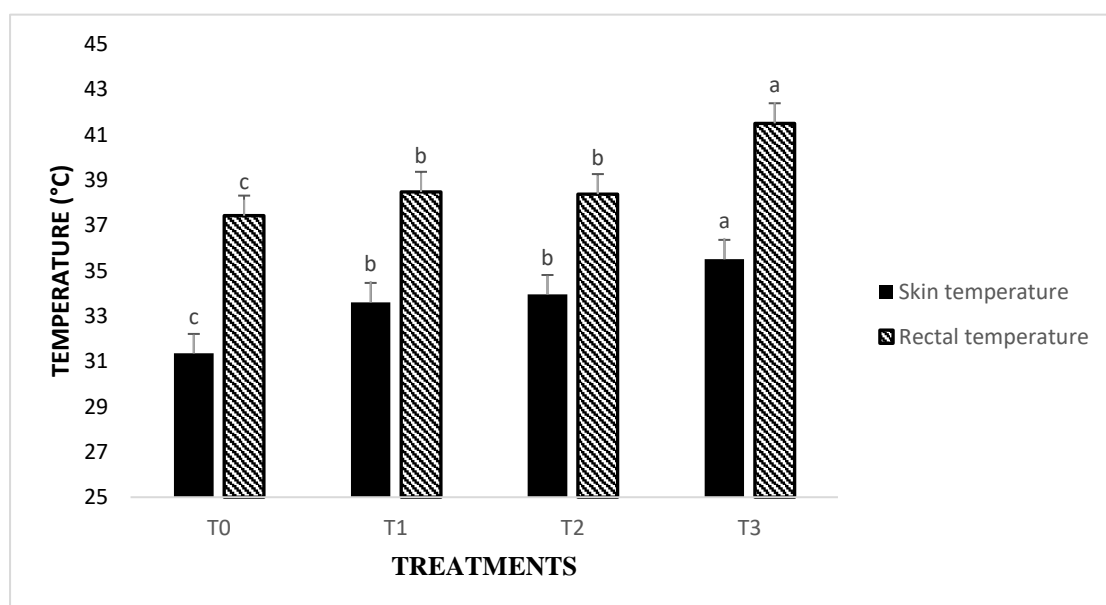
Figure 5.1 : Variation in the rectal and skin temperatures following different doses of the *Moringa oleifera* aqueous seeds extract in female rabbits exposed to heat stress.

The superscripts a, b, c, d: means the mean values are significantly different at $P<0.05$; T0 control group, T1: 35–36°C+100 mg *MOASE*, T2: 35–36°C+200 mg *MOASE*, T3: 35–36°C; n=7. Note a significant decrease in the skin and rectal temperatures in rabbits submitted to heat stress and receiving doses at 100 mg and 200mg/kg b.w. of MOASE compared to dose 0 mg/kg of b.w. of MOASE

5.3.2. Relative weight of some internal organs

Results indicate that relative weights of lung and kidney significantly increased in female rabbits submitted to heat stress compared to those of control group (34% and 30.5% respectively). However, when aqueous extract of *Moringa oleifera* seeds were administered to different treatment groups, these parameters decreased in a dose dependent manner by 19.4% and 7.1% for lung and liver respectively. The relative weight of liver of animals from T0 and T2 was lower than those of animals from T1 and T3 while the relative weight of ovary of animals from T0, T1 and T2 was comparable and significantly higher than those of animals from T3.

Table 5.1: Variation of the relative weight of heart, lung, liver, kidney, ovary and uterus following different doses of the *Moringa oleifera* aqueous seeds extract in female rabbits exposed to heat stress.

Parameters	T0	T1	T2	T3	*p*-value
Heart	0.54±0.12	0.58±0.23	0.56±0.14	0.58±0.27	0.209
Lung	1.28±0.25^d	1.66±0.08^b	1.59±0.16^c	1.94±0.12^a	0.031*
Liver	5.09±1.21^c	7.24±0.55^b	5.48±0.83^c	8.06±0.06^a	0.046*
Kidney	0.66±0.03^d	0.84±0.02^b	0.71±0.06^c	0.95±0.08^a	0.029*
Ovaries	0.30±0.08^a	0.28±0.07^a	0.28±0.05^a	0.19±0.06^b	<0.001***
Uterus	0.53±0.08	0.52±0.09	0.52±0.06	0.52±0.08	0.613

The superscripts a, b, c, d: means that mean values are significantly different at $P < 0.05$; T0 control group, T1: 35–36°C+100 mg *MOASE*, T2: 35–36°C+200 mg *MOASE*, T3: 35–36°C. *, ***: significant at $P < 0.05$ and 0.001, respectively; n=7. Note a significant amelioration in lung, liver, kidney, ovary and uterus in rabbits submitted to heat stress and receiving doses at 100 mg and 200 mg/kg b.w. of MOASE compared to dose 0 mg/kg b.w. of MOASE

5.3.3. Hematological parameters

There was a decrease in Hb and RBC (-62.1% and -22.6% respectively) and an increase in MCV, WBC and LYM contents by 19.3%, 25.1% and 17.5% respectively in female rabbits exposed to heat stress compared to the control group. However, Hb and RBC contents increased by 30.8% and 11.1% respectively in animals co-treated with different doses of *Moringa oleifera* aqueous seed extract compared to those submitted to heat stress alone; the opposite trend was noticed for MCV, WBC and LYM contents (-11.9%, -39.8% and 7.1% respectively).

Table 5.2: Variation in the hematological parameters following different doses of the *Moringa oleifera* aqueous seed extract in female rabbits exposed to heat stress.

Parameters	T0	T1	T2	T3	*p*-value
Hb (g/dl)	15.06 ± 1.12^a	13.11 ± 1.09^b	13.42 ± 1.17^b	9.29 ± 1.88^c	0.035*
PCV (%)	39.37 ± 2.04	41.35 ± 3.44	40.21 ± 2.83	40.06 ± 2.50	0.740
RBC ($\times10^{12}$/l)	6.18 ± 0.41^a	5.37 ± 0.03^c	5.67 ± 0.24^b	5.04 ± 0.16^d	0.026*
MCV (fl)	66.24 ± 1.15^d	77.18 ± 2.27^b	73.26 ± 1.44^c	82.04 ± 1.77^a	0.041*
MCH (pg)	28.15 ± 2.44	24.31 ± 3.92	26.15 ± 2.82	29.03 ± 3.79	0.064
MCHC (g/dl)	35.45 ± 2.18	33.91 ± 3.06	33.27 ± 2.74	36.53 ± 2.11	0.309
WBC ($\times10^9$/l)	14.18 ± 1.05^b	13.04 ± 0.77^b	13.53 ± 1.30^b	18.92 ± 1.88^a	<0.001***
PLT ($\times10^3$/µl)	184.14 ± 3.81	186.77 ± 4.01	186.16 ± 2.29	198.91 ± 5.10	0.63
LYM (%)	57.61 ± 2.88^c	65.18 ± 3.82^b	64.28 ± 2.21^b	69.83 ± 2.89^a	0.047*

The superscripts a, b, c, d: means the mean values are significantly different at *P* <0.05; T0 control group, T1: 35–36°C+100 mg *MOASE*, T2: 35–36°C+200 mg *MOASE*, T3: 35–36°C. Hb: hemoglobin; PCV: packed cell volume; RBC: Red blood Cell; MCV: mean cell volume; MCHC: mean corpuscular hemoglobin concentration; MCH: mean corpuscular hemoglobin; WBC: white blood cells; PLT: Platelet count; LYM: lymphocytes; *, ***: significant at *P* =0.05 and 0.001, respectively; n=7. Note a significant increase in Hb and RBC while a decrease in MCV, WBC and LYM in rabbits submitted to heat stress and receiving doses at 100 mg and 200mg/kg b.w. of MOASE compared to dose 0 mg/kg of b.w. of MOASE

5.3.4. Biochemical parameters

There was a significant increase ($P<0.05$) in creatinine, urea, ALAT, ASAT contents (by 25.6%, 35.4%, 24.7% and 41.4% respectively) and decrease ($P <0.05$) in glucose and protein content (by -76.1% and -28.3%) in animals submitted to heat stress compared to those of the control group. However, in animal submitted to heat stress there was an amelioration of these parameters following *Moringa oleifera* aqueous seed extract dose dependent manner (an increase of 19.4%, 30.2%, 21.3% and 23.1% in creatinine, urea, ALAT, ASAT respectively and an increase of -66.7% and 18.7% in glucose and protein contents respectively).

Table 5.3: Variation in biochemical parameters according to different doses of the *Moringa oleifera* aqueous seed extract in female rabbits exposed to heat stress.

Parameters	T0	T1	T2	T3	*p*-value
Cholesterol (mg/dl)	136.48±5.27	141.12±8.87	139.14±10.65	136.22±8.46	0.241
Creatinine (mg/dl)	0.64±0.04[d]	0.75±0.08[b]	0.72±0.09[c]	0.86±0.07[a]	0.007**
Urea (mg/dl)	94.41±2.15[d]	122.15±6.25[b]	101.92±5.92[c]	146.09±16.22[a]	0.038*
ALAT (U/L)	44.56±2.08[d]	53.21±3.37[b]	48.81±1.08[c]	59.22±2.38[a]	0.035*
ASAT (U/L)	19.54±1.86[d]	27.15±1.93[b]	25.88±1.74[b]	33.37±2.15[a]	0.028*
Glucose (mmol/L)	7.94±0.18[a]	7.12±0.88[a]	7.52±0.71[a]	4.51±0.42[b]	<0.001***
Total protein (g/L)	78.12±2.27[a]	71.03±1.33[c]	74.91±1.25[b]	60.86±3.54[d]	0.014*
Total albumin (g/dl)	6.92±0.48	7.12±0.67	6.56±0.88	6.81±0.77	0.421

The superscripts a, b, c, d: means that mean values are significantly different at $P <0.05$; T0 control group, T1: 35–36°C+100 mg *MOASE*, T2: 35–36°C+200 mg *MOASE*, T3: 35–36°C. ALAT: alanine aminotransferase; ASAT: aspartate amino-transferase, *, **, ***: significant at $P < 0.05$; 0.01 and 0.001, respectively; n=7. Note a significant decrease in creatinine, urea, ALAT and ASAT while an increase in glucose and protein in rabbits submitted to heat stress and receiving doses at 100 mg and 200 mg/kg b.w. of MOASE compared to dose 0 mg/kg b.w. of MOASE

5.3.5. Oxidative stress biomarkers

Results showed that the level of protein significantly decreased (P <0.05) by -169.8% while MDA significantly increased (P <0.05) by 56.3% in animals submitted to heat stress compared those of the control group. However, these parameters improved with the increase of doses of *Moringa oleifera* seeds aqueous extract in these animals submitted to heat stress without reaching the values of the control group (a decrease of -96.8% for proteins and an increase of 40.8% for MDA). In addition, the level of CAT, SOD and GHS significantly increased (P <0.05) in animals of control group and those submitted to different doses of *Moringa oleifera* aqueous seed extract compared to animals submitted only to heat stress (by 44.8%, 29.9% and 32.7% respectively).

Table 5.4: Variation in the kidney levels of oxidative stress biomarkers according to different doses of the *Moringa oleifera* aqueous seed extract in female rabbits exposed to heat stress.

Parameters	T0	T1	T2	T3	*p*-value
Protein (mg/ml)	13.87 ± 0.23^a	8.81 ± 0.37^c	10.12 ± 0.33^b	5.14 ± 0.69^d	0.026∗
MDA (nmol/mg tissues)	16.37 ± 1.21^d	26.04 ± 2.91^b	22.16 ± 2.45^c	37.43 ± 3.84^a	0.029∗
CAT (UI/mg tissues)	11.23 ± 1.27^a	10.77 ± 1.16^a	10.82 ± 1.08^a	5.97 ± 0.941^b	<0.001∗∗∗
SOD (UI/mg tissues)	7.21 ± 0.77^a	7.08 ± 0.25^a	7.25 ± 0.66^a	5.08 ± 0.44^b	<0.001∗∗∗
GSH (nmol/mg of tissue wet)	9.37 ± 0.18^a	8.81 ± 0.69^a	8.93 ± 0.75^a	6.01 ± 0.14^b	<0.001∗∗∗

The superscripts a, b, c, d: means the mean values are significantly different at P <0.05; T0 control group, T1: 35–36°C+100 mg *MOASE*, T2: 35–36°C+200 mg *MOASE*, T3: 35–36°C. CAT: catalase, GSH: reduced glutathione, SOD: superoxide dismutase, MDA: lipid peroxidation; ∗, ∗∗∗: significant at $P <$ 0.05 and 0.001, respectively; n=7. Note a significant increase in kidney protein, CAT, SOD and GSH while a decrease in MDA in rabbits submitted to heat stress and receiving doses at 100 mg and 200 mg/kg b.w. of MOASE compared to dose 0 mg/kg b.w. of MOASE

5.3.6. Kidney histology

The kidney of animals from control group (**A**) and animals submitted to heat stress while receiving 200 mg/kg b.w. of *Moringa oleifera* aqueous seed extract (**C**) showed histological architecture of a normal kidney with normal renal tubules and glomerulus. However, in animals submitted to heat stress and co-treated with 100 mg/kg b.w. of *Moringa oleifera* aqueous seed extract (**B**) there was fibroblast proliferation in the interstitial tissues and multifocal infiltration with mononuclear cells in cortical renal parenchyma. Animals submitted only to heat stress (**D**) showed focal area of fibroblast proliferation and a tubular epithelial cell swelling in medullary renal parenchyma.
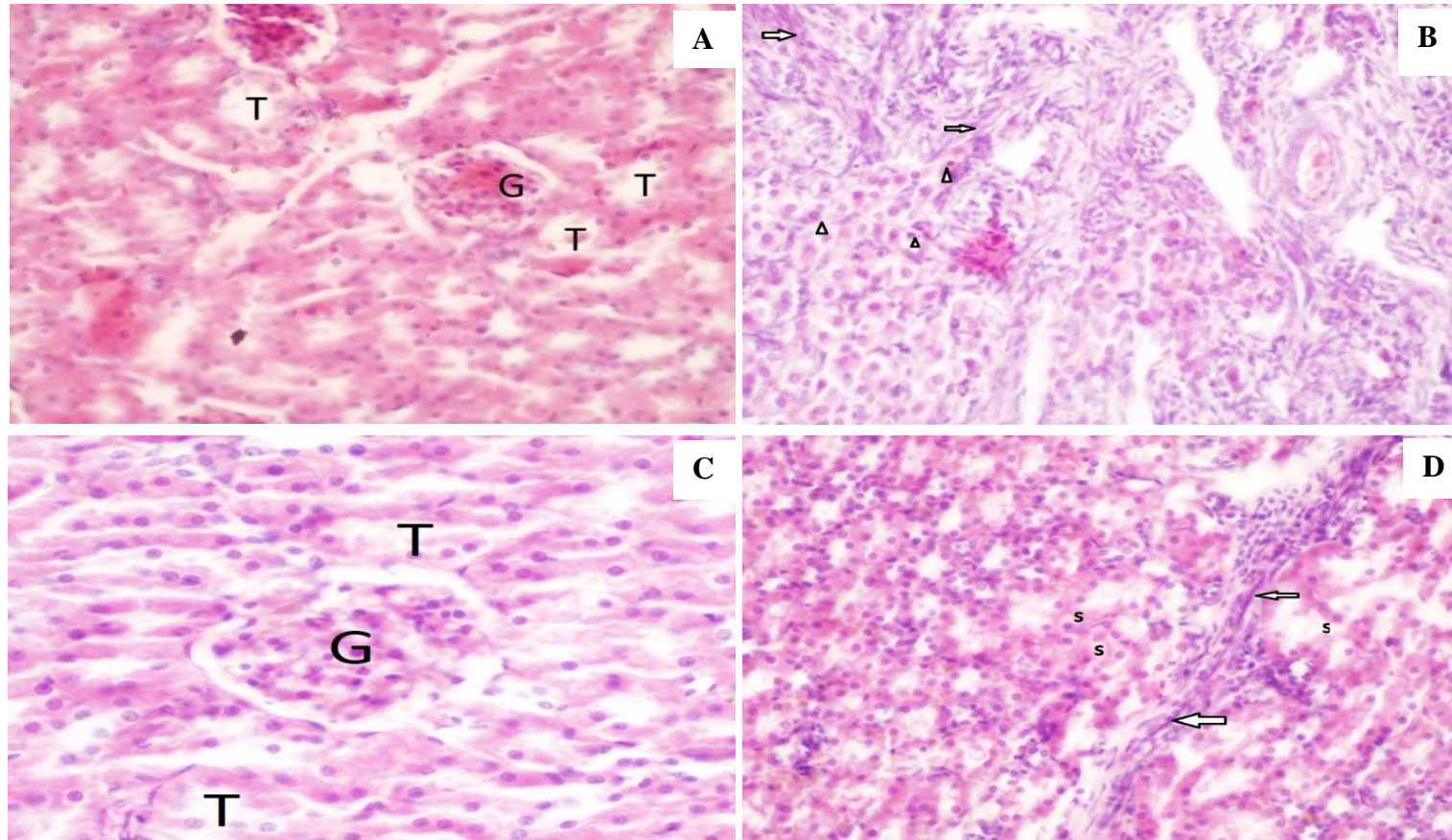
Figure 5.2: Variation in the kidney histological section following different doses of the *Moringa oleifera* aqueous seeds extract in female rabbits exposed to heat stress.

A. Female rabbits' kidney (T0) with normal convoluted renal tubules of kidney nephron (**T**) and glomerulus (**G**). **B.** Kidney (T1) with fibroblast proliferation in the interstitial tissues (**Arrow**) and multifocal infiltration with mononuclear cells (**Arrow head**). **C.** Rabbits' kidney (T2) with normal distal convoluted tubules (**T**) and glomerulus (**G**). **D.** Kidney (T3) with fibroblast proliferation (**Arrow**) and a tubular epithelial cell swelling (**s**).

5.4. DISCUSSION

The investigation on alternative solution to alleviate heat stress impairment in livestock production is one of major challenges in African subtropical regions. Results of the present study indicated an increase in rectal and skin temperature in female rabbits submitted to heat stress compared to the control group. This result agrees with findings of Marai *et al.* (2004) in rabbits affected by heat stress under subtropical conditions of Egypt and in New Zealand White rabbits submitted to 33-35°C for 10 weeks (Balabel, 2004). The increase in rectal temperature of female rabbits submitted to heat stress could be attributed to the failure of the body thermoregulatory mechanisms of the animals to balance the excessive heat produced (Marai *et al.*, 2001). This leads to the increase in the metabolic rate compounded by no-functional sweat glands in rabbits thereby reduced perspiration in areas of the skin covered by fur (Marai *et al.*, 2001; Marai *et al.*, 2004). As skin temperature is directly affected by body temperature, its increase in animals submitted to heat stress may be the result of the insulation effect of the hair coat (Marai *et al.*, 2004). However, administration of *Moringa oleifera* aqueous seeds extract lowered these parameters when compared to animals solely submitted to heat stress. The study observations concur with findings of El-Desoky *et al.* (2021) where 10, 25 and 50 mg/kg body weight of nonencapsulated *Moringa oleifera* leaves ethanolic extract in female rabbits during summer seasons were used. It has been reported the same results in rabbit bucks fed with a basal ration containing 5, 10, 15 and 20% *Cassia tora* leaf meal diets in the Nigeria semi-arid zone (Chintem *et al.*, 2018). The decrease in these parameters could be attributed to several biological activities of phytochemical compounds such as phenols, flavonoids, glycosides contained in *Moringa oleifera*. In fact, these bioactive compounds are able to improve the ability of animals to maintain their body homeostasis including body temperature by provoking endogenous cellular defense mechanisms to cope with oxidative stress under heat stress

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situations (Makkar *et al.*, 2007; Akbarian *et al.*, 2016).

Evaluation of internal organ weights is very important to assess the potential toxicity in animals. In this study, relative live weight of lung, kidney, liver significantly decreased in a dose dependent manner while the weight of ovary increased when aqueous extract of *Moringa oleifera* seeds were administered in animals prior to heat stress compared to heat stress alone treatment group. The increase in the ovary weight is associated with the estrogenic properties of flavonoids, phenols and saponins in the aqueous seed extracts of *Moringa oleifera* (El-Desoky *et al.,* 2021). In fact, mass, size and secretory functions of ovary have been reported to be regulated by estrogens (Gayrad, 2007). Though different experimental set up and different animal species used, the decrease in liver and kidney weight wre also noted in pigs receiving artichoke, celery, beet, onion, garlic, spinach, avocado, oats, and parsley extracts compared to the control during summer conditions of Mexique (Dávila-Ramírez *et al.,* 2020). The decrease in these parameters could be due to the antioxidant activity of flavonoids found in the seeds of *Moringa oleifera* as reportedly flavonoids can interfere with the metabolism of xenobiotics by stimulating detoxification systems (Makkar *et al.*, 2007). The decrease in the weight of these organs were proportionate to a decrease in urea, creatinine, ALAT and ASAT concentration. In fact, urea and creatinine are biochemical markers usually used in the exploration of the renal function while ALAT and ASAT are reported to notify liver diseases such as infectious hepatitis, alcoholic cirrhosis, biliary obstruction, toxic hepatitis and liver cancer (Abdel-Wahab and Aly, 2005). In the present study, an aqueous extract of *Moringa oleifera* seeds decreased, in dose dependent-manner, urea, creatinine, ASAT and ALAT compared to animals submitted only to heat stress. These results corroborate the findings of Thiruchelvi *et al.* (2012) where 200 mg/kg body weight of the aqueous extract of *Terminalia chebula* was administered against Cadmium toxicity in rats for 21 days. As *Moringa oleifera* extract reduce the levels of raised

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serum ALAT, ASAT, urea and creatinine and restored the hepatic and renal functions, it has been indicated that the contents of *Moringa oleifera* not only protected the integrity of kidney but, at the same time, increased its regenerative and reparative capacity (Albrakati, 2017). The decrease in these parameters could also be associated with the decrease in renal tissue injuries reported in animals of control group and those submitted to heat stress and co-treated with 200 mg/kg b.w. of *Moringa oleifera* aqueous seed extract. Indeed, the reduction in the levels of these parameters might be ascribed to the *Moringa oleifera* antioxidant substances present such as flavanone, flavonoids, alkaloids, gallic acid and ellagic acid rich in antioxidant properties (El-Desoky *et al.*, 2017). From the foregoing, *Moringa oleifera* aqueous seed extracts could be used to prevent renal impairment, particularly those induced by oxidative damage.

Additionally, results of the present study showed that glucose and protein levels increased in animals subjected to heat stress and treated with *Moringa oleifera* aqueous seed extract compared to animals subjected to heat stress alone. These results corroborate the findings of Hashem *et al.* (2019) in growing rabbits treated with 250 mg/L of *Moringa oleifera* ethanolic extract in drinking water. The high level of glycerine (11.11%) previously reported in *Moringa oleifera* (El-Desoky *et al.*, 2017) could be the main factor of the increment of glucose content as glycerol is a main component of glycerin that has a high content of energy, and could be absorbed easily and converted to glucose in the liver. This facilitates the production of energy by animals and provides energy for cellular metabolism without additional heat production (Swiatkiewicz and Koreleski, 2009). Moreover, the increase in protein content might be associated with the protein intensive metabolism in rabbits' organs following administration of *Moringa* (Melesse *et al.*, 2013). It can also be associated to a high *Moringa oleifera* antioxidant level which could limit protein catabolism under heat stress condition by decreasing corticosterone secretion and, therefore, increase the total protein concentration (El-Wardany *et*

al., 2012; Asante *et al.*, 2014).

The present study reports a decrease in Hb, RBC and an increase in MCV, WBC and LYM contents in female rabbits exposed to heat stress compared to the control group. Similar observations have been reported in New Zealand White rabbits submitted to 36±3°C (Ondruska *et al.*, 2011). However, Hb and RBC contents increased in animals receiving different doses of *Moringa oleifera* seeds aqueous extract compared to those subjected only to heat stress while the opposite trend was noticed in MCV, WBC and LYM contents. The stability of blood indices such as RBC, Hb in rabbits following administration of *Moringa oleifera* aqueous seed extract reported in this study is an indication that *Moringa oleifera* supports or does not interfere with normal hemopoiesis processes (Oloruntola *et al.*, 2018). In addition, these observed results may be associated with the high content of *Moringa oleifera* from iron (Ogbe and Affiku, 2012) as RBC is in charge of O_2 and CO_2 transportation in the blood as well as manufacture of Hb, indicating therefore a better state of health (Olugbemi *et al.*, 2010). Moreover, the observed increase in WBC in female rabbits following administration of *Moringa oleifera* aqueous seed extract may be the product of immunostimulatory activities of *Moringa oleifera*. In fact, WBC counts possess phagocytic function and act as biomarkers of immune functions (Oloruntola *et al.*, 2018). An immunostimulatory activity in animals is one of the biological activities being associated with phytogenic feed additives (Valenzuela-Grijalva *et al.*, 2017). The level of LYM is an indicator to the body immune responses and the decrease in this parameter is an indicator of animal good health, raising female rabbits' ability to counteract the infection (Ebenebe *et al.*, 2012). The high concentration of Vitamin C in *Moringa oleifera* as earlier reported (Asante *et al.*, 2014) endow this plant with a high antioxidant capacity to alleviate the negative responses to heat stress condition on rabbits to be healthy.

In the present study, the kidney concentration in protein increased while MDA decreased in a

dose-dependent manner following the administration of *Moringa oleifera* aqueous seed extract in heat stressed rabbits. On the other hand, concentration of CAT, SOD and GSH significantly increased in animals of the control group and animals submitted to heat stress and receiving *Moringa oleifera* aqueous seeds extract compared to animal submitted to heat stress only. These results are in agreement with findings of Ngoula *et al.* (2017b) in cavies subjected to 35°C and 45°C and treated with *Psidium guajava* leaves essential oil for 60 consecutive days and Soliman *et al.* (2020) in mice exposed to Methotrexate and later treated with *Moringa oleifera* leaf extract for 12 consecutive days. These antioxidants are used by the biological systems to protect against injuries caused by oxidative damage produced by excessive heat (Li *et al.*, 2018). The decrease in MDA concentration could be associated with the inhibitory effect of *Moringa oleifera* aqueous seeds extract on lipid peroxidation (Soliman *et al.*, 2020). The improvement of these antioxidant enzymes could be attributed to the high concentration of *Moringa oleifera* in phenolic substances such as flavonoids and tannins which are very effective in neutralizing oxygen free radicals (Oloruntola *et al.*, 2018) as well as the ferric reducing antioxidant property and 2,2-diphenyl-1-1-picrylhydrazyl hydrate, as its reducing power (Ngoula *et al.*, 2017a; Oloruntola *et al.*, 2018).

5.5. CONCLUSION

On the basis of the results of the present study, it can be concluded that administration of 200 mg/kg of body weight of *Moringa oleifera* aqueous seed extract in female rabbits exposed to heat stress (35°C-36°C) restored their physiological parameters. This includes the decrease in the skin and rectal temperature, the amelioration of hemato-biochemical parameters, internal organ weights and enzymatic antioxidant biomarkers as well as the regeneration of the kidney tissue structure. The mentioned improvement is due to a wide diversity of bioactive compounds contained in *Moringa oleifera* which interfered with free radicals produced by heat stress. Hence, *Moringa oleifera* seed extract is suggested to be helpful in alleviating heat stress in female rabbits.

CHAPTER SIX: EVALUATION OF THE PHARMACOLOGICAL EFFECTS OF
MORINGA OLEIFERA **AQUEOUS SEED EXTRACTS ON REPRODUCTIVE**
TRAITS OF FEMALE RABBITS EXPOSED TO HEAT STRESS

6.1. INTRODUCTION

Heat stress has been defined as a heat load sustained under the combined effect of environmental factors (air temperature, humidity, air flow and heat radiation) and metabolic heat production (Nardone *et al.*, 2010). It occurs when the environmental temperature exceeds the thermoneutrality zone of the animal (Kumar *et al.*, 2011). Heat stress alter animals' physiology, with the reproduction as the first function to be impaired (Hansen, 2009). In fact, heat stress has been reported to impair embryo development by increasing their mortality in cattle (Hansen, 2007). It is associated with the low fertility rate in dairy cows by poor expression of oestrus due to a reduced estradiol secretion (De Rensis and Scaramuzzi, 2003). Rabbits are reported as one of the animals most affected by thermal stress (Marai *et al.*, 2001) since they do not have enough sweat glands to allow dissipation of excess body heat (Rafel *et al.*, 2012). Rabbit thermoneutrality zone ranges between 16 and 21ºC and above this range, physiological, productive and reproductive performances begin to decrease (Kumar *et al.*, 2011). Marai *et al.* (2001) reported a decrease in rabbit weight, litter size, litter weight at weaning, conception rate and an increase in pre-weaning mortality in adult females exposed to severe heat stress during summer. Decrease in progesterone and estradiol levels have been reported in female rabbits exposed to 41°C (Sabah and Dalal., 2016). Meanwhile rabbits exposed to the temperature between 25 and 36°C compared to those maintained between 14 and 20°C decreased their litter size (9.7 vs 11.4), litter weight (503.0 vs 630.5g) and kit weight at birth (56.6 vs 61.4g) and increased the stillbirth rate (25.4 vs 9.9%) during pregnancy and lactation (Marco-Jiménez *et al*., 2017).

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However, considering the economic benefits and social considerations of the rabbit under the tropics (Schiere and Coustiaensen, 2008; Mutwedu *et al.*, 2015), it is therefore important to look for alternative solutions to minimize the effect of heat stress on its productive and reproductive performances in order to enhance farmers' income.

Phytogenic feed supplements are plant-derived products used in animal feeds to improve the agricultural livestock performance (Svoradova *et al.*, 2021). They are preferred because of their high antioxidant capacity, cheap availability, low toxicity and are ecological friendly for treatment of various ailments (Chumark *et al.*, 2008; Svoradova *et al.*, 2021).

Moringa oleifera, a perennial tree growing up to between 5 and 12 m high and belonging to the *Moringaceae* family, is considered as one of the best antioxidant plants worldwide (Wadhwa, 2013). All parts of this plant including leaves, stembarks, pods and seeds, are reported to be rich in various bioactive compounds such as alkaloids, saponins, phenols, flavonoids, glycosides, terpenoids and tannins (El-Alfy e*t al.*, 2011; Ifesan *et al.*, 2013; Ayirezang *et al.*, 2020). Due to the presence of these compounds, *Moringa oleifera* has been therefore reported to alleviate damages caused by oxidative stress (Sharma *et al.*, 2011; Chisholm, 2015). Odeyinka *et al.* (2008) reported an improvement in litter size, litter weight, gestation length and milk yield following supplementation of leaves of *Moringa oleifera*. In a study that included 10% of *Moringa oleifera* in female rabbits' meal, the litter weight at birth, the litter weight, the weaning weight and the survival rate were improved (Oshibanjo *et al.*, 2018). An improvement in reproductive hormones such as LH, FSH, oestrogen, progesterone and prolactin was reported in female rabbits fed with 5, 10 and 15 g/kg of *Moringa oleifera* leaf powder incorporated into rabbit grower's pellet (Ajuogu *et al.*, 2019). All these findings were performed using *Moringa oleifera* leaves that have been reported to possess better antioxidant activity (Chumark *et al.*, 2008) and therefore overused compared to other parts of

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the plant. It is, therefore, important to explore the effectiveness of other parts of this plant for medicinal value in overcoming health challenges associated with oxidative stress in animals. The pharmacological efficacy of seeds of *Moringa oleifera* on reproductive parameters of female rabbits exposed to heat stress is scant in literature.

It is hypothesized that *Moringa oleifera* aqueous seed extracts have no efficacy on the heat stress damage leading to impaired reproductive performances, altered ovarian histology and reproductive hormonal profile of female rabbits. The present study was designed to evaluate the efficacy of seeds of *Moringa oleifera* aqueous extract on reproductive performances, hormonal profile and ovary histology in the management of heat stress in female rabbits.

6.2. MATERIAL AND METHODS

6.2.1. Experimental location

It is the same as was described previously in section 5.2.1 of the Chapter 5.

6.2.2. Plant material

It is the same as was described previously in section 5.2.2 of the Chapter 5.

6.2.3. Extraction of crude powdered sample

It is the same as was described previously in section 5.2.3 of the Chapter 5.

6.2.4. Phytochemical screening

It is the same as was described previously in section 5.2.4 of the Chapter 5.

6.2.5. Experimental animals and their feeding

It is the same as was described previously in section 5.2.5 of the Chapter 5.

6.2.6. Ethical consideration

It is the same as was described previously in section 4.2.2 of the Chapter 4.

6.2.7. Experimental design

It is the same as was described previously in section 5.2.4 of the Chapter 5 with some additions. Two weeks after subjecting animals to heat stress and gavaging *Moringa oleifera* aqueous seed extract, each female rabbit was transferred to a single proven fertile males' cage for mating. Ten days after mating, pregnancy was evaluated by abdominal palpation by hands feeling around until finding of little lumps that can pass through fingers as gently probe and search (Marai *et al.*, 2004). The females failing to conceive were immediately returned to the same buck for another service.

In pregnant and lactating female rabbits, feed intake (F.I.) and weight gain (W.G.) were measured daily and daily body gain was calculated for each female rabbit according to the method described by Sabah and Dalal (2016).

6.2.8. Reproductive performances

The gestation length was obtained by calculating the duration (in days) between the date of the coupling and the date of farrowing (Musa *et al.*, 2014; Attia *et al.*, 2015). The conception rate was estimated as number of services per conception and type of mating observed was classified as voluntary mating (i.e. mating receptive does) and hand mating (i.e. mating unreceptive does) (Marai *et al.*, 2004). The litter size was obtained by counting the number of young rabbits of each female at farrowing and weekly until weaning (Musa *et al.*, 2014; Attia *et al.*, 2015; Celia *et al.*, 2015). The stillbirth rate was evaluated as the number of stillborn young rabbits on the litter size at birth (Celia *et al.*, 2015) and the survival rate calculated as the litter size at weaning on the litter size at birth (Attia *et al.*, 2015; Celia *et al.*, 2015)**.** The young rabbits weight evolution was obtained by weighing young rabbits at birth and every week until 5 weeks post-partum (weaning) (Musa *et al.*, 2014; Attia *et al.*, 2015; Celia *et al.*, 2015).

For each female, milk yield was evaluated as the difference in weight of the kids before and after suckling. Milk intake per kit was calculated as the milk yield on the number of kits in the litter for each female rabbit and the efficiency of converting milk into body-weight gain calculated as the kit weight gain on the milk intake per kit (Marai *et al.*, 2004).

The litter weight was evaluated by weighing the whole kids of the litter, the kit body weight estimated by weighing separately each kid and the kit body weight gain evaluated as the difference between the actual kit weight and the kit weight of the previous week (Marai *et al.*, 2001). These parameters were estimated at weekly intervals between birth and 35 days of age (weaning).

6.2.9. Sacrifice of rabbits, blood collection and processing

It is the same as was described previously in section 4.2.6 of the Chapter 4.

The obtained blood was then centrifuged, and the resultant serum kept at -20°C for hormones

assessment.

6.2.10. Hormone evaluation

The principal hormones analyzed at the end of the experiment (80 days) were: Estradiol, Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), Progesterone, Prolactin (PL) and Cortisol. Cortisol was estimated by the radioimmunoassay (RIA) technique using coated tubes kits (Diagnostic Systems Laboratories, Inc. Webster, Texas 77598-4217, USA) and the tracer labelled with iodine-125 (I^{125}). Quantitative determination of serum Estradiol, FSH, LH, Progesterone and PL was performed using the solid-phase Enzyme-Linked Immunosorbent Assay (ELISA) as described by the commercial kit Omega Diagnostic (23961 Craftsman Road, Suite D/E/F, Calabasas, CA 91302). All analyses were performed following the manufacturers' recommendations.

6.2.11. Ovary histology

Immediately after sacrifice, the left ovary of each female rabbit was removed and prepared following the procedure previously described in section 5.2.12 of the Chapter 5.

6.2.12. Statistical analysis

Data were compiled into a database using Excel package. For the analysis of the different treatments, effects among experimental groups and control were assessed using one-way ANOVA. The differences in mean values were compared using the Tukey HSD test at $P < 0.05$. Data analysis were performed using XLSTAT for Windows 10 Software. Results are expressed as the means $\pm$ SD, graphs and charts. The Effect Rate (ER) was calculated as the ratio between the value of the control group and the value of the group with the most relevant impact. Histological parameters were observed by comparing the changes in different ovary tissues of each treatment with normal ovary tissues.

6.3. RESULTS

6.3.1. Growth performances in pregnant and lactating female rabbits

Results on food consumption (Figure 6.1) showed decrease of 21% and 34% respectively in pregnant (A) and lactating (B) female rabbits submitted to heat stress compared to those of the control group. However, in *Moringa oleifera* aqueous seed extract-treated animals, this parameter increased in a dose dependent manner. Similar results have been reported in body weight of both pregnant (C) and lactating (D) female rabbits by 8% and 26% respectively, except at the 5th week where body weight of animals submitted to heat stress and treated with 200 mg/kg b.w. of *Moringa oleifera* aqueous seed extract decreased. In these animals, body weight was slightly higher by 4% compared to that of the control group in pregnant females (C) and of animals submitted to heat stress and treated with 100 mg/kg b.w. of *Moringa oleifera* aqueous seeds extract which suddenly decreased by 3% in lactating females (D). There was a dose-dependent effect of *Moringa oleifera* aqueous seeds extract on body weight gain in pregnant females (E) except at the 2nd week where animals of the control group and those submitted to heat stress alone showed a greater body weight gain. However, in lactating females (F), the control group showed the highest body weight gain (8%) compared to those submitted to heat stress, except at the first week of lactation where animals treated with 200 mg/kg b.w. of *Moringa oleifera* aqueous seeds extract had the highest body weight gain. In these animals, body weight gain decreased weekly until weaning while animals solely submitted to heat stress showed loss in body weight from the 2nd week of treatment.
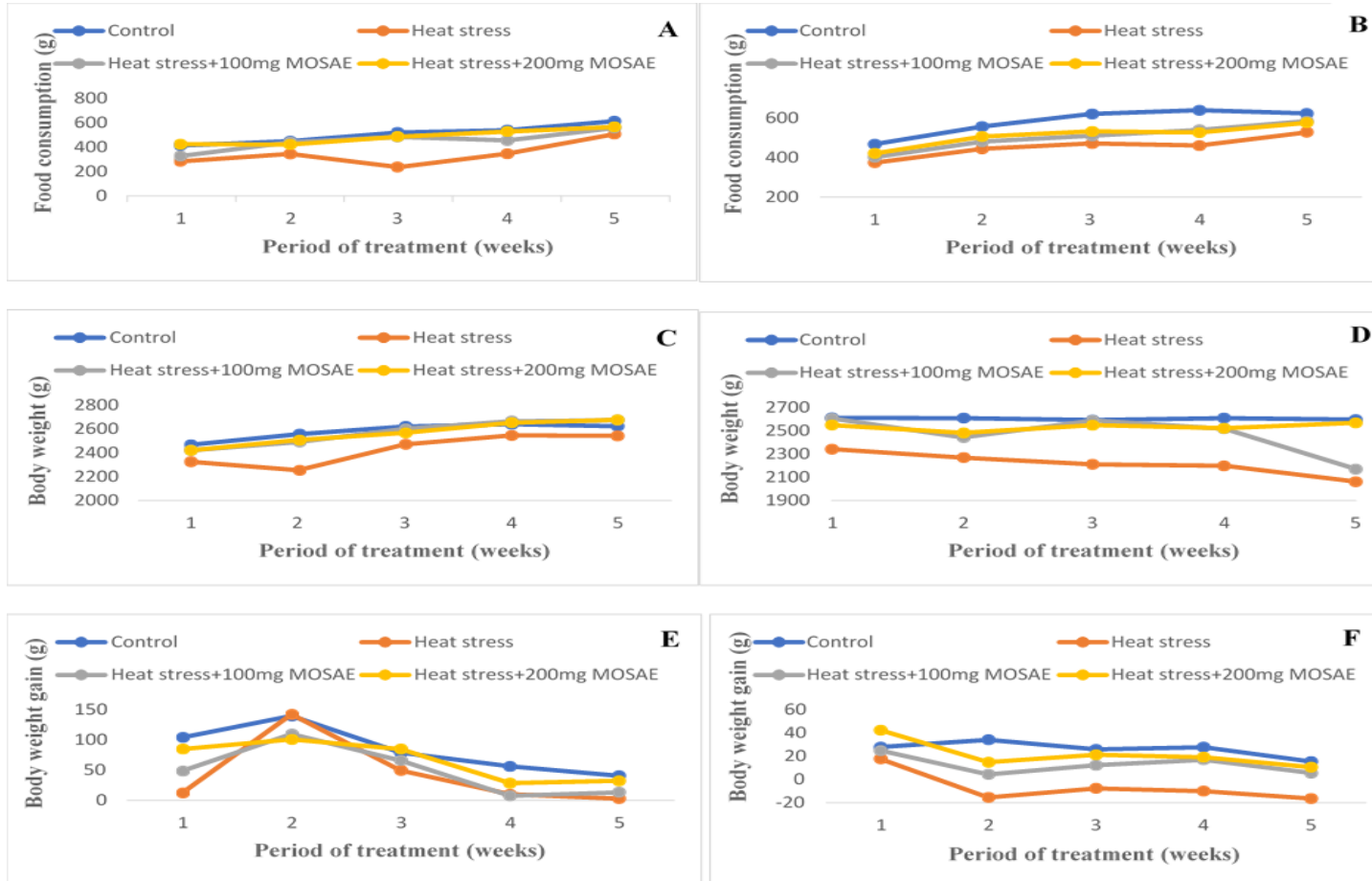
Figure 6.1: Variation of the food consumption, body weight and body weight gain following different doses of the *Moringa oleifera* aqueous seed extract in pregnant and lactating female rabbits exposed to heat stress.

Control: ambient temperature; Heat stress: 35–36°C, MOSAE: *Moringa oleifera* seeds aqueous extract, **A** and **B**: Food consumption in pregnant and lactating female rabbits respectively; **C** and **D**: Body weight in pregnant and lactating female rabbits respectively; **E** and **F**: Body weight gain in pregnant and lactacting female rabbits respectively. Note a significant increase in food consumption, body weight and body weight gain in pregnant and lactacting female rabbits receiving 100 and 200 mg/kg b.w. of MOASE compared to dose 0 mg/kg of b.w. of MOASE

6.3.2. Reproductive performances in female rabbits

Results showed that voluntary mating was the most observed type of mating in female rabbits even though hand mating increased with the heat stress. Generally, most of animals of the control group required one service to achieve pregnancy (71.4%) while 42.9% of animals submitted to heat stress and co-treated with 100 and 200 mg/kg b.w. *Moringa oleifera* aqueous seeds extract respectively required two services to achieve pregnancy and most of animals submitted to heat stress alone required two services to become pregnant (71.4%). The self fur removal was intense in most animals of control group (71.4%), lightly intense in animals co-treated with 100 mg/kg b.w. (57.1%) and 200 mg/kg b.w. *Moringa oleifera* aqueous seeds extract (85.7%) while most of animals of the heat stressed group did not remove their fur (57.1%). A significant decrease in the litter size ($P<0.05$) was observed in animals submitted to heat stress compared to those of control group. However, this parameter increased in a dose dependent manner following administration of *Moringa oleifera* aqueous seeds extract. There was no group significant difference in the gestation length.

Table 6.1: Reproductive performances following administration of different doses of the *Moringa oleifera* aqueous seeds extract in female rabbits exposed to heat stress.

Parameters	T0	T1	T2	T3
Type of mating (%)				
Voluntary mating	85.7	71.4	71.4	71.4
Hand mating	14.3	28.6	28.6	28.6
Number of services per conception (%)				
One	71.4	57.1	57.1	28.6
More	28.6	42.9	42.9	71.4
Self plucked fur (%)				
Dense	71.4	14.3	14.3	0
Light	28.6	57.1	85.7	42.9
Without plucking	0	28.6	0	57.1
Gestation length	30.8±0.3	31.1±0.4	31.1±0.2	31.1±06
Litter size	8.3±1.2^a	6.2±1.1^b	7.6±1.4^{ab}	5.6±1.3^b

The superscripts a, ab, b: means the mean values are significantly different at $P < 0.05$; T0: control group, T1: 35–36°C+100 mg *MO*, T2: 35–36°C+200 mg *MO*, T3: 35–36°C; n=7. Note a decrease in the number of services per conception as well as an increase in fur removal and litter size in rabbits submitted to heat stress and receiving doses at 100 mg and 200mg/kg b.w. of MOASE compared to dose 0 mg/kg of b.w. of MOASE

6.3.3. Litter size at different ages of litter from birth to weaning

The number of kids was significantly higher ($P<0.05$) in female rabbits from the control group from birth to weaning (35 days) compared to those submitted to heat stress. However, in animals submitted to heat stress and co-treated with *Moringa oleifera* aqueous seeds extract, this parameter increased in a dose dependent manner without reaching the values of the control group.

Table 6.2: Litter size at different ages of litter from birth to weaning following administration of different doses of the *Moringa oleifera* aqueous seeds extract in female rabbits exposed to heat stress.

Parameters	T0	T1	T2	T3	p-value
Birth	8.3±1.2[a]	6.2±1.1[b]	7.6±1.4[ab]	5.6±1.3[b]	0.029*
7 days	7.2±0.3[a]	4.4±0.3[b]	6.5±1.6[a]	4.1±0.2[b]	0.044*
14 days	6.9±0.3[a]	4.4±0.3[c]	5.8±0.5[b]	3.5±0.3[d]	<0.001***
21 days	6.9±0.3[a]	3.5±0.3[c]	4.5±0.4[b]	2.7±0.2[d]	<0.001***
28 days	6.9±0.3[a]	2.9±0.2[c]	4.0±0.3[b]	1.6±0.2[d]	<0.001***
35 days	6.9±0.3[a]	2.9±0.2[c]	4.0±0.3[b]	1.6±0.2[d]	<0.001***

The superscripts a, b, c: means the mean values are significantly different at P <0.05; T0: control group, T1: 35–36°C+100 mg *MO*, T2: 35–36°C+200 mg *MO*, T3: 35–36°C. *, ***: significant at P < 0.05 and 0.001, respectively; n=7. Note a significant increase in litter size from birth to weaning in rabbits submitted to heat stress and receiving doses at 100 mg and 200mg/kg b.w. of MOASE compared to dose 0 mg/kg of b.w. of MOASE

6.3.4. Litter weight, kit body weight and kit body weight gain from birth to weaning

Results showed a significant increase ($P<0.05$) in litter weight of the control group from birth to weaning (35 days) compared to groups submitted to heat stress. However, when co-treated with *Moringa oleifera* aqueous seed extracts, this parameter increased in a dose dependent manner but without achieving the values of the control group. The kit body weight at weaning

(35 days) and the daily kit weight gain indicated a significant increase ($P<0.05$) in the control group compared to groups submitted to heat stress. Nevertheless, in heat stressed animals, these parameters were increased in female rabbits co-treated with 100 and 200 mg/kg b.w. *Moringa oleifera* aqueous seeds extract respectively ($P <0.05$) compared to those submitted to heat stress alone.

Table 6.3: Evolution of the litter weight, kit body weight and kit body weight gain from birth to weaning following administration of doses of the *Moringa oleifera* aqueous seed extract in female rabbits exposed to heat stress

Parameters	T0	T1	T2	T3	p-value
Litter weight (kg)					
Birth	332.6±17.3[a]	241.8±18.2[b]	253.5±14.7[b]	218.4±16.3[b]	0.038*
7 days	553.6±26.8[a]	277.2±31.4[c]	449.5±14.7[b]	230.8±12.5[d]	<0.001***
14 days	1092.1±23.1[a]	804.3±14.6[c]	891.7±28.8[b]	351.2±22.3[d]	<0.001***
21 days	1485.7±31.5[a]	616.4±17.7[c]	850.5±23.5[b]	434.7±13.4[d]	<0.001***
28 days	2435.7±31.5[a]	925.1±24.1[c]	1321.9±22.7[b]	476.8±22.7[d]	<0.001***
35 days	3081.8±57.4[a]	1220.9±34.5[c]	1752.1±34.3[b]	592.4±23.7[d]	<0.001***
Kit body weight (g)					
At birth	41.6±1.3	39.6±1.7	39.2±1.1	39.3±1.5	0.066
35 days	448.7±12.5[a]	428.1±10.4[b]	431.1±6.9[b]	373.2±11.4[c]	0.027*
Kit weight gain (g/day)	14.4±0.4[a]	12.8±0.4[b]	13.2±0.7[b]	9.8±0.6[c]	0.034*

The superscripts a, b, c, d: means the mean values are significantly different at $P <0.05$; T0: control group, T1: 35–36°C+100 mg *MO*, T2: 35–36°C+200 mg *MO*, T3: 35–36°C. *, ***: significant at $P < 0.05$ and 0.001, respectively; n=7. Note an increase in litter weight, kit body weight and kit weight gain in rabbits submitted to heat stress and receiving doses at 100 mg and 200 mg/kg b.w. of MOASE compared to dose 0 mg/kg b.w. of MOASE

6.3.5. Milk yield, milk intake and milk efficiency

Results of Table 6.4. indicate a significant increase ($P<0.05$) in milk yield in female of the control group from birth to weaning (35 days) compared to groups submitted to heat stress. However, in animals submitted to heat stress and co-treated with *Moringa oleifera* aqueous seeds extract, this parameter increased in a dose dependent manner without reaching the values of the control group. The daily kit milk intake of the first and second weeks was high ($P <0.05$) in animals of the control group and those receiving 100 and 200 mg/kg b.w. *Moringa oleifera* aqueous seeds extract respectively ($P<0.05$) compared to those only submitted to heat stress; the opposite trend was observed at the fourth and fifth weeks while there was not any significant difference at the third week. Except the third week where there was no significant difference, the milk efficiency significantly increased ($P<0.05$) in females of the control group and those co-treated with 100 and 200 mg/kg b.w. *Moringa oleifera* aqueous seeds extract respectively during the first, second, fourth and fifth weeks compared to the group submitted to the heat stress alone.

Table 6.4: Evolution of the milk yield, milk intake and milk efficiency from birth to weaning following administration of doses of the *Moringa oleifera* aqueous seed extract in female rabbits exposed to heat stress

Parameters	T0	T1	T2	T3	p-value
Milk yield (g per litter per day) per day					
7 days	75.8±2.8[a]	54.6±3.6[c]	68.3±2.5[b]	48.1±3.1[d]	<0.001***
14 days	99.3±8.4[a]	76.4±7.7[bc]	80.6±6.5[b]	65.3±7.1[c]	0.019*
21 days	94.1±9.5[a]	59.8±7.4[c]	71.7±7.9[b]	44.3±6.5[d]	<0.001***
28 days	90.1±7.3[a]	42.6±5.3[c]	53.1±3.8[b]	33.7±4.9[d]	<0.001***
35 days	79.2±5.5[a]	34.7±4.8[c]	46.1±5.9[b]	31.8±3.4[c]	0.033*
Milk intake (g per litter per day) per day					
7 days	10.6±0.8[a]	8.9±0.5[b]	10.4±1.0[a]	8.6±1.1[b]	0.040*
14 days	14.1±1.0[b]	16.4±1.1[a]	13.8±0.7[b]	14.8±0.4[b]	0.021*
21 days	13.4±1.1	14.3±1.8	14.9±1.3	14.1±0.6	0.081
28 days	13.1±1.5[b]	14.1±1.5[b]	13.3±3.8[b]	19.4±0.2[a]	0.033*
35 days	11.6±0.8[b]	10.9±4.8[b]	11.4±0.6[b]	18.7±0.7[a]	0.029*
Milk efficiency per day					
7 days	1.4±0.3[a]	1.4±0.1[a]	1.3±0.1[a]	1.1±0.1[b]	0.040*
14 days	1.0±0.1[a]	0.7±0.1[b]	0.9±0.1[a]	0.6±0.1[b]	0.021*
21 days	1.1±0.2	0.9±0.2	0.8±0.1	0.7±0.2	0.081
28 days	1.1±0.3[a]	0.9±0.2[a]	1.0±0.2[a]	0.5±0.2[b]	0.033*
35 days	1.2±0.2[a]	1.2±0.2[a]	1.2±0.1[a]	0.5±0.2[b]	0.029*

The superscripts a, b, c, d: means the mean values are significantly different at P <0.05; T0: control group, T1: 35–36°C+100 mg *MO*, T2: 35–36°C+200 mg *MO*, T3: 35–36°C. *, ***: significant at P < 0.05 and 0.001, respectively; n=7. Note an increase in weekly milk yield and daily milk efficiency followed by a decrease in milk intake in rabbits submitted to heat stress and receiving doses at 100 mg and 200mg/kg b.w. of MOASE compared to dose 0 mg/kg of b.w. of MOASE

6.3.6. Reproductive hormones profile

Results indicated that serum levels of Estradiol and FSH significantly decreased ($P<0.05$) in the heat stressed animals compared to the control group. These parameters increased in heat stressed animals, co-treated with 100 and 200 mg/kg b.w. *Moringa oleifera* aqueous seeds extract respectively ($P<0.05$) compared to those submitted to heat stress alone. The opposite trend was observed in serum Progesterone and Cortisol levels. On the other hand, LH content was decreased ($P<0.05$) in animals submitted to heat stress alone compared to those of the control group and those receiving 100 and 200 mg/kg b.w. *Moringa oleifera* aqueous seeds extract respectively. There was no significant difference in serum Prolactin content.

Table 6.5: Reproductive hormones profile following administration of different doses of the *Moringa oleifera* aqueous seed extract in female rabbits exposed to heat stress

Parameters	T0	T1	T2	T3	p-value
Estradiol (pg/ml)	68.41±5.74[a]	40.44±5.51[b]	44.92± 9.10[b]	16.22±3.81[c]	0.021*
LH (mIU/mL)	3.40±0.51[a]	2.73±0.31[a]	3.12±0.62 [a]	1.94±0.37 [b]	0.046*
FSH (IU/mL)	2.73±0.21[a]	1.50±0.11[b]	1.71±0.41[b]	0.66±0.22[c]	0.038*
Progesterone (ng/mL)	0.41±0.03[c]	0.56±0.02[b]	0.55±0.09[b]	2.41±0.06[a]	0.032*
Prolactin (ng/ml)	1.53±0.26	1.52±0.31	1.35±0.15	1.14±0.37	0.087
Cortisol (ng/ml)	12.27±0.85[a]	9.7±0.32[b]	9.46±0.47[b]	7.1±0.41[c]	0.019*

The superscripts a, b, c: means the mean values are significantly different at P <0.05; T0: control group, T1: 35–36°C+100 mg *MO*, T2: 35–36°C+200 mg *MO*, T3: 35–36°C. *: significant at P < 0.05; LH: Luteinizing Hormone; FSH: Follicular Stimulating Hormone; n=7. Note an increase in Estradiol, LH, FSH, Cortisol and a decrease in Progesterone serum level in rabbits submitted to heat stress and receiving doses at 100 mg and 200mg/kg b.w. of MOASE compared to dose 0 mg/kg of b.w. of MOASE

6.3.7. Ovarian histology

The ovary of animals from control group (A) was characterized by the presence of a dominant growing follicles with oocyte, forming antral cavity, and surrounded by atretic follicles. However, the ovary of animals submitted to heat stress and co-treated with 200 mg/kg b.w. of *Moringa Oleifera* aqueous extract (C) showed multifocal areas of follicular degeneration characterized with oocyte lysis in graaffian follicle, degeneration of granulosa cells and theca cells and disorganization of corona radiate and cumulus oophorous cells. Animals submitted to heat stress and receiving 100 mg/kg b.w. of *Moringa Oleifera* aqueous extract (B) showed a marked degeneration of graaffian ovarian follicle having eosinophil amorphous material (oocyte lysis), severe degeneration of corona radiata cells, granulosa cells and cumulus oophorous. The tissues from the animals exposed only to heat stress (D) showed focal areas of proliferation of fibroblast in the interstitial tissues, in addition to severe follicular degeneration characterized by oocyte lysis and accompanying enhanced general follicular cell degeration.

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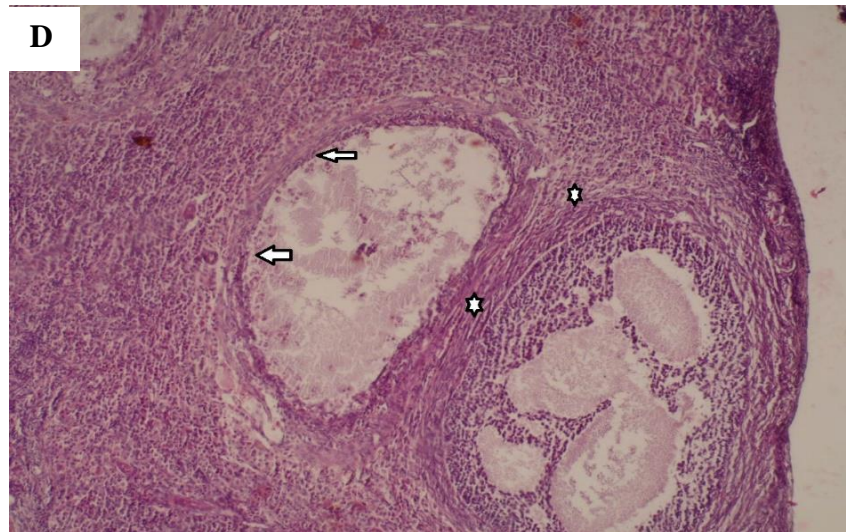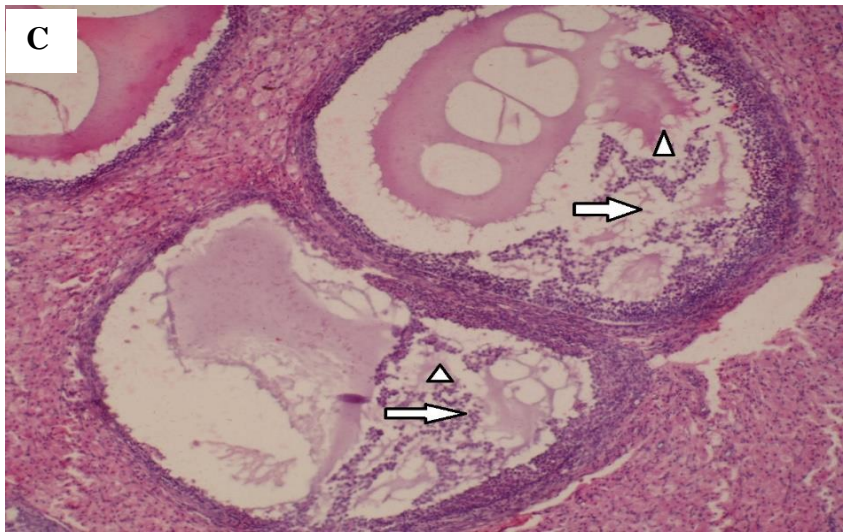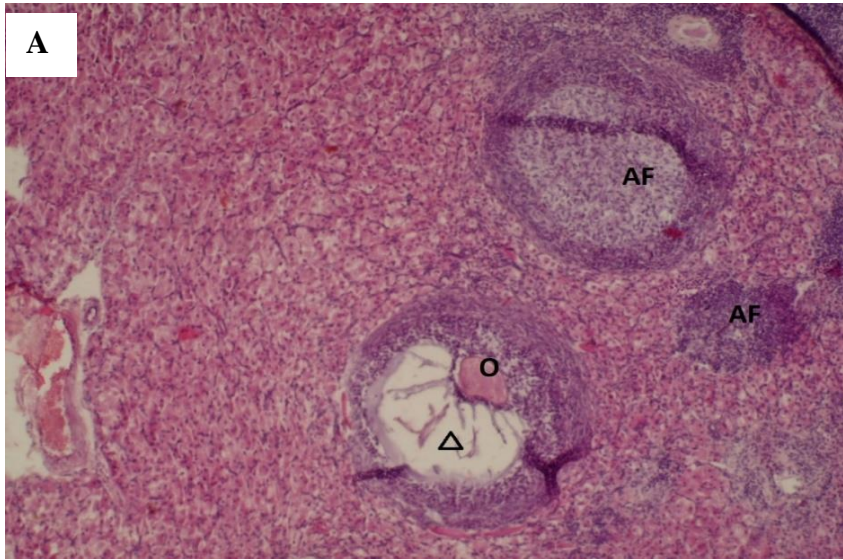
Figure 6.2: Histological changes in animals following administration of different doses of the *Moringa oleifera* seeds aqueous extract in female rabbits after exposure to heat stress.

A. Ovary with a dominant growing follicle with oocyte (**O**), forming antral cavity (**Arrow head**), and surrounded by atretic follicles (**AF**) (H/E X40). **B.** Marked degeneration of Graafian follicle with oocyte lysis (**Arrow head**), severe degeneration of granulosa cells and *cumulus oophorous* (**Arrow**) (H/E X100). **C.** Multifocal areas of follicular degeneration characterized with oocyte lysis (**Arrow head**), Degeneration of granulosa cells and theca cells (**Arrow**) (H/E X100). **D.** Focal areas of proliferation of fibroblast in the interstitial tissues (*****), in addition to severe follicular degeneration characterized by oocyte lysis and degeration of follicular cells (**Arrow**) (H/E X100)

6.4. DISCUSSION

In the present study, feed consumption, body weight and body weight gain in both pregnant and lactating female rabbits have shown to be altered with the exposition to 35-36°C. This observation is in accordance with findings of Marai *et al.* (2004) in female rabbits submitted to heat stress under subtropical conditions of Egypt. It has been observed that in hot environmental temperatures, the appetite center of the hypothalamus is negatively affected and, therefore, decreases feed intake especially in pregnant animals prone to environmental stressors (Das *et al.*, 2016). The reduction in feed consumption in the present study is expected since the homeostatic thermoregulatory mechanism tend to decrease endogenous heat production which might be produced by the body metabolism if feed intake is high (Kadzere *et al.*, 2002) in an already heat stressed animal. Lactating animals solely submitted to heat stress showed negative body weight gain from the 2nd week. Rhoads *et al.* (2013) reported a decrease in feed intake by around 40% in lactating sows exposed to temperatures above 35°C where negative energy balance was observed and consequently body weight substantially decreased (Hooda and Singh, 2010). However, in the present study, administration of *Moringa oleifera* aqueous seed extract increased these parameters. The increase in feed consumption observed in the present study could be explained by the presence of flavonoids found in the aqueous extract of *Moringa oleifera* seeds (Wadhwa, 2013). Flavonoids have been implicated in protection of tissue from the destructive effects of external agents and, therefore, the structures of the central nervous system that regulate appetite (Weston and Mathesius, 2013). Stimulation of this region induces an increase in food intake while bilateral damage induces rather a complete cessation of food intake (Ganong, 2001).

Results of the present study indicate a decrease in voluntary mating in female rabbits, an increase in number of services to achieve pregnancy and the absence of plucking fur in female

rabbits exposed to heat stress. These observations are similar to those of Marai *et al.* (2004) in Egypt that female rabbits decreased receptivity and percentage of voluntary mating frequently in heat stress situations under subtropical conditions. Plucking fur in female rabbits means they are actively searching for materials, including their own fur to build a kid's nest; which if not done kids shall be exposed to several harmful climatic conditions that can culminate into death (Marai *et al.,* 2004; Balabel *et al.*, 2004). In animals, female receptivity is influenced by physiological and environmental factors such as heat stress (Grant *et al.*, 2002). When rejecting male mating advances, female fertilization may be, at least in part, controlled by female acceptance of copulation (Hingle *et al.*, 2001).

The reduction in receptivity, voluntary mating and plucking fur are considered as factors indicating the drop in fertility in female rabbits exposed to hot temperatures (Marai *et al.*, 2007b) and may be the cause of the low litter size reported in females submitted to heat stress in this study. Litter size has been previously linked to decreased animal fertility and conception rate with accompanying significant reduction in total young born and in an increase in percentage of young born dead in animals affected by environmental stress (Balabel *et al.*, 2004). In addition, heat stress decreases length and intensity of estrus while increasing anestrous incidence and silent heat in females (Singh *et al.*, 2013). The improvement on litter size due to administration of *Moringa oleifera* aqueous seeds extract in this study may be attributed to vitamin C and polyphenols found in Moringa (Wadhwa, 2013). In this regard, these compounds have been previously reported to improve oocytes development and fertilization rate (Agarwal *et al.*, 2012) by increasing distribution of compact follicular oocytes in female rabbits (Younan *et al.,* 2015).

There was a significant decrease in litter weight from birth to weaning, individual kid body weight at weaning and daily kid weight gain in animals exposed to heat stress. The decrease in

litter weight may be associated with the reduction in food consumption in lactating female rabbits which impact their milk production and lack or reduced nourishment of the kids. The decrease in the litter weight directly impact other related parameters such kid body weight at weaning and daily kid weight gain. These results are similar to those observed in litter weight at 21 days by Marai *et al.* (2001) and at 35 days by Marai *et al.* (2004). The amelioration of these parameters, following the administration of *Moringa oleifera* aqueous seeds extract in the present study, has also been demonstrated by El-Desoky *et al.* (2021) in female rabbits receiving respectively 10, 25 and 50 mg/kg body weight of nonencapsulated *Moringa oleifera* leaves ethanolic extract during summer seasons. Moreover, various phenol compounds and vitamin C found in *Moringa oleifera* have the ability to prevent the digestive system cell membranes from oxidative stress by scavenging O_2 radicals and, therefore, restoring feed utilization (Abou-Zeid *et al.*, 2000). This action mechanism by these compounds may partly explain the rise in pre-weaning weight gain, as well as the higher weight at weaning and the decrease in pre-weaning deaths (Yassein *et al.*, 2008). Indeed, in the present study, kids' weight is strongly correlated with milk production, milk intake and milk efficiency. The results demonstrate that the low energy intake from food consumption is not enough to cover for both animal body metabolism and daily requirements for milk production (Das *et al.*, 2016). It can be argued that, on the one hand, the low daily milk production reported in the present study is probably due to the decrease in feed consumption as a result of body mechanism to decrease heat production and, on the other hand, heat stress reduced milk production by decreasing mammary cell proliferation (Tao and Dahl, 2013). The drop in litter weight week after week is the result of the observed low milk production as kids become more and more exigent in milk quantity for their metabolic needs, and this situation leads to the daily milk efficiency of converting milk into body-weight gain. The improvement of these parameters could be associated with the antioxidant activity of

bioactive compounds present in *Moringa oleifera* that act against lipid oxidation in the cell membrane (Svoradová *et al.*, 2021). This is also important in pre-weaning kids which have been reported to be highly sensitive to oxidative stress than adults, and for the development of the immune system in young animals (Debier *et al.*, 2005).

The elevated daily milk intake in litter submitted only to heat stress compared to those receiving *Moringa oleifera* and the control group is mainly due to the high kids' mortality rate reported in this group. It is noted that at weaning, there was only 28.6% kids alive in the group submitted to heat stress alone compared to 83.1% of the control group. Therefore, the little quantity of milk produced by these females was enough to meet kids' requirements in contrast to animals of the control group that produced a large quantity of milk but insufficient to meet kids' nutritional needs.

All these reported parameters are under the influence of reproductive hormones as their main function is to regulate reproductive system and therefore ensure the success of such reproductive event (Svoradová *et al.*, 2021). An increase in ambient temperature of more than 2°C is responsible for low or desynchronized endocrine activities in females, mainly the pineal-hypothalamo-hypophyseal-gonadal axis, followed by the impairment of the respective reproductive hormone functions (Upadhyay *et al.*, 2009). A deterioration of reproductive hormones (LH, FSH, Estradiol, Progesterone) as well as stress hormone (Cortisol) following exposition to heat stress was observed in the present study. Heat stress is responsible for the impairment of the follicular estradiol synthesis activity and the reduction of LH activity by decreasing its receptor level (Ozawa *et al.*, 2005). The decrease in the fertilization rate due to the inhibition of estrus signs, gonadotrophin decrease and ovulation disruption has been associated with low estradiol secretion (Wolfenson *et al.*, 2000). Moreover, the decrease in FSH serum content in heat stressed female rabbits is mostly a consequence of decrease in the

inhibition of negative feedback from smaller follicles which finally impair the animal reproductive efficiency (Khodaei-Motlagh *et al.*, 2011). The low progesterone production observed in heat stressed female rabbits impair the embryo development by disrupting endometrial function (Wolfenson *et al.*, 2000; Khodaei-Motlagh *et al.*, 2011) while acyclicity and infertility as well as mammary gland development impairment in pregnant females have been associated with the increased prolactin in heat stressed females (Singh *et al.*, 2013). Cortisol is a stress hormone released by the adrenal gland and is important in enabling the body to deal with stressful situations by enhancing blood sugar production, converting fats, proteins and carbohydrates into usable energy (Marai *et al.*, 2007a). However, an increase in levels of these hormones in the present study has been observed following administration of *Moringa oleifera* aqueous seeds extract. Several compounds found in this plant have been reported to stimulate reproductive hormones production. For instance, phytosterols have a chemical structure similar to that of cholesterol that can be used as precursors of steroid hormones (testosterone, estradiol and progesterone) while isoflavones are one of the flavonoid compounds, also having estrogenic activity and are able to bind with estrogen receptors such as ER-α and ER-β (Setiasih *et al.*, 2021). Phenolic compounds and alkaloids have been reported to protect embryonic tissue against reactive oxidative stress impairment by enhancing the ovarian hormones serum concentration (Grzanna *et al.*, 2005). Phytosterols, polyphenols, saponins and flavonoids present in *Moringa oleifera* seeds have been previously associated with the increase in female reproductive hormones (Estrada *et al.*, 2001; Khan *et al.*, 2005).

Ovary histology is usually used to assess its morphological changes leading to cells and reproductive hormones impairment (Sorelle *et al.*, 2019). Analysis of the micrographs showed damages of ovary of female rabbits solely exposed to heat stress. These histopathologies were characterized by focal areas of proliferation of fibroblast in the interstitial tissues evidenced by

the presence of large opened spaces. However, the administration of *Moringa oleifera* aqueous seeds extract showed minimal alterations with atresic follicles.

6.5. CONCLUSION

The aqueous extract of the seeds of *Moringa oleifera* minimizes the adverse effects of heat stress inducing reproductive impairments in female rabbits mainly due to a large variability of its bioactive compounds thus providing this plant with a high antioxidant activity. From the foregoing, *Moringa oleifera* aqueous seeds extract could be used as a mitigation measure for managing heat stress and associated complications in rabbit farming with sole aim of improving on reproductive performance and production.

CHAPTER SEVEN: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMANDATIONS

7.1. GENERAL DISCUSSION

Moringa oleifera is recognized as an excellent source of essential amino acids, protein, vitamins A, B and C, mineral elements (Fe, P, Cu and Ca), bioactive compounds (phenols, alkaloids, saponins, flavonoids, terpenoids, tannins) located in its different parts and varying from geographical region to another (Hamza, 2010; Wadhwa, 2013; Tlili *et al.*, 2014; Agamou *et al.*, 2015). In the present study, the evaluation of phytochemical profile and antioxidant activities of aqueous extract of *Moringa oleifera* leaves, barks and seeds collected from Bukavu city of DRC and Machakos county of Kenya revealed the presence of many bioactive compounds in leaves compared to seeds and barks from the same countries. Similar results have previously been reported by others (Ifesan *et al.*, 2013; Ayirezang *et al.*, 2020).

The result of the 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity showed that aqueous extract of *Moringa oleifera* leaves from DRC and Kenya have the highest inhibition percentage of free radicals depending on the concentration used. These results are comparable to those of Unuigbe *et al.* (2014) in leaves and seeds of *Moringa oleifera*. At the highest concentration (10 µg/mL), the percentage inhibition was 88.29±1.12, 80.17±3.59 for leaves of Kenya and DRC respectively, 30.08±0.80, 33.21±2.62 for seeds of Kenya and DRC respectively and 37.34±10.26, 41.86±2.01 for barks of Kenya and DRC respectively, whereas the reference standard (ascorbic acid) had a percentage inhibition of 92.63 ± 2.76. This result is in agreement with findings of Unuigbe *et al.* (2014), Sharma *et al.* (2011) and Tlili *et al.* (2014) who reported that, in most cases, sample concentrations do not exceed the ascorbic acid concentration (considered as the standard). The presence and concentration of these bioactive compounds in different parts of this plant endows the plant with a lot of pharmacological proprieties (Tlili *et*

al., 2014; Tsabang *et al.*, 2015; Da Costa *et al.*, 2018) which can act against toxicity induced by heat stress in female rabbits. In fact, this study revealed that, when submitting animals to temperatures of 27–28°C, 31–32°C and 35–36°C for 30 consecutive days, there was a decrease in a dose dependent manner in the final body weight and body weight gain. The opposite trend was observed in feed conversion ratio (FCR) and water intake compared to those from the control. Similar results have been reported in New Zealand White rabbits exposed for 30 consecutive days to hot ambient temperature ranges from 33 to 38°C (Sabah and Dalal, 2016) and 36 ±3°C (Ondruska *et al.*, 2011). A rise in FCR with the increasing temperature indicates poor digestibility of feed and absorption of the resultant nutrients in heat stressed rabbits. Poor digestibility, in this context, has been explained in previous findings where elevated temperature suppressed enzymatic activities at the brush border and pancreatic secretions on duodenum, which led to a weakened digestion and nutrients assimilation (Jinap and Hajeb, 2010). The decrease in final body weight and body weight gain can be ascribed to the observed increase in the FCR in the present study which might have led to less protein biosynthesis and less fat deposition (Marai *et al.*, 2001, 2004; Ogunjimi *et al.*, 2008). Moreover, in the present study there was a significant decreased in serum protein content in animals exposed to 31–32°C and 35–36°C compared to those of the control group. The decrease in total protein content might be associated with the increase in protein utilization and amino acid transamination caused by the dilution of plasma proteins due to the increase in water consumption (Ayyat *et al.*, 2002). The rise in water consumption in heat stressed rabbits observed in the present study may be a result of the normal homeostatic mechanisms governing thermoregulation, as rabbits are almost entirely dependent on pulmonary ventilation for regulation of body temperature in hot conditions and so the increase in water consumption may help them to increase heat loss through pulmonary ventilation mechanisms (Marai *et al.*, 2001; Badr, 2015). On the other hand, results

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of the present study indicated an increase in these parameters when exposed to 35–36°C and administered 100 mg/kg b.w. and 200 mg/kg b.w. of *Moringa oleifera* aqueous seeds extract subsequently. The increase in feed consumption may be a result of the presence of flavonoids in the aqueous extract of *Moringa oleifera* seeds as observed in the present study. These compounds act by protecting the central nervous system that regulate appetite (Weston and Mathesius, 2013) and therefore increase food intake. Impairment of this region induces rather a complete cessation of food intake while its stimulation induces an increase in food intake (Ganong, 2001) and consequently the body weight and body weight gain.

The increase in skin temperature as reported in animals submitted to 27–28°C, 31–32°C and 35–36°C might be attributed to the insulating effect of the hair coat (Marai *et al.*, 2008). In addition, the increase in rectal temperature could be the result of increasing metabolic rate and ultimately hyperthermia as a result of impaired thermoregulation (Shafie *et al.*, 1982). It was reported that at temperatures above 30°C, rabbits stretch out in an attempt to lose as much heat as possible by radiation and convection with significant rises in rectal temperature (Marai and Rashwan, 2004). When administered 100 mg/kg b.w. and 200 mg/kg b.w of *Moringa oleifera* aqueous seeds extract, these parameters tended to be reduced. These findings are similar to those observed by El-Desoky *et al.* (2021) when 10, 25 and 50 mg/kg body weight of nonencapsulated *Moringa oleifera* leaves ethanolic extract were administered in female rabbits during summer seasons. The improvement in these parameters is associated with a large biological activity of phytochemical compounds such as phenols, flavonoids, glycosides contained in *Moringa oleifera* seeds reported in this study. Indeed, these bioactive compounds are able to improve the ability of animals to maintain their body homeostasis including body temperature by provoking endogenous cellular defense mechanisms to cope with oxidative stress under heat stress situations (Makkar *et al.*, 2007; Akbarian *et al.*, 2016).

The increase in relative weight of kidney and liver reported in animals submitted to 35-36°C in the present study has previously been reported on studies in New-Zealand White and Baladi Black rabbits exposed to Egyptian hot conditions (Badawi and El-Aasar, 2018). However, the diminution of the weight of these organs in animals co-treated with *Moringa oleifera* in the present study could be associated with the antioxidant activity of flavonoids found in the seeds. Flavonoids are well known to protect enzyme systems and oxidative damages (Mehta and Agarwal., 2008) and can interfere with the metabolism of xenobiotics by stimulating detoxification systems (Makkar *et al.*, 2007). The decrease in the weight of these organs following treatment with *Moringa oleifera* aqueous seeds extract directly decreased urea, creatinine, ALAT and ASAT concentration in a dose dependent manner. Urea and creatinine are biochemical markers usually used in the exploration of the renal function while ALAT and ASAT have been reported to notify liver diseases such as infectious hepatitis, alcoholic cirrhosis, biliary obstruction, toxic hepatitis and liver cancer (Abdel-Wahab and Aly, 2005). In the present study, *Moringa oleifera* aqueous seed extract restored the hepatic and renal functions by reducing the levels of raised serum ALAT, ASAT, urea and creatinine and accompanying renal tissues injuries. In a related study, it was indicated that the contents of *Moringa oleifera* not only protected the integrity of liver and kidney but, at the same time, increased its regenerative and reparative capacity (Albrakati, 2017). The reduction in the levels of these parameters might be related to the presence of antioxidant substances in *Moringa oleifera* such as flavanone, flavonoids, alkaloids, gallic acid and ellagic acid which are rich in antioxidant properties (El-Desoky *et al.*, 2017). From the findings, *Moringa oleifera* aqueous seeds extract could be used to prevent renal impairment, particularly those induced by oxidative damage.

The decrease in all the above mentioned parameters might be due to the reduction in oxidative stress biomarkers following the administration of *Moringa oleifera* aqueous seeds extract in

female rabbits. Oxidative stress is a consequence of the increase in Reactive Oxygen Species (ROS) in body cells. This has been reported to impair both productive and reproductive functions in animals (Celi, 2011; Toboła-Wróbel *et al.*, 2020; Mutwedu *et al.*, 2021). The decrease in kidney MDA followed by the increase in protein, CAT, SOD and GSH concentrations as the result of *Moringa oleifera* aqueous seeds extract administration in heat stressed female rabbits might be linked to effect of several bioactive compounds found in *Moringa oleifera* seeds. In fact, when screening the phytochemical characteristics *Moringa oleifera* aqueous seeds extract in the present study, various bioactive compounds were detected, among them phenols, flavonoids and tannins. These compounds are known to be effective in neutralizing oxygen free radicals (Oloruntola *et al.*, 2018). In addition, the high antioxidant activity found in *Moringa oleifera* seed extracts resulting in its high DPPH activity and a higher concentration that will scavenge 50% of the initial DPPH radicals (IC_{50}), plays an important role in improving these parameters in heat stressed female rabbits. It has been reported that the ferric reducing antioxidant property and 2,2-diphenyl-1-1-picrylhydrazyl hydrate improve oxidative stress biomarker levels in stressful situations (Ngoula *et al.*, 2017b; Oloruntola *et al.*, 2018). The improvement of these oxidative stress biomarkers has previously been reported in other plants having similar bioactive compounds than *Moringa oleifera* such as *Psidium guajava* leaves in cavies exposed to 35°C and 45°C and treated with essential oil for 60 consecutive days (Ngoula *et al.*, 2017b).

The amelioration of the oxidative stress biomarkers following the administration of *Moringa oleifera* aqueous seeds extract has promoted the improvement of reproductive performances in female rabbits exposed to heat stress. Therefore, feed consumption, body weight and body weight gain in both pregnant and lactating female rabbits have shown to be improved following administration of *Moringa oleifera* aqueous seeds extract in animals submitted to heat stress.

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The amelioration of receptivity, voluntary mating and plucking fur are considered as indicators of improved fertility in female rabbits (Marai *et al.*, 2007b). In the present study, the increment in fertility associated to the rise in food consumption, body weight and body weight gain following administration of *Moringa oleifera* aqueous seeds extract could be the reasons of the elevated litter size reported in these female rabbits. The improvement of litter size due to administration of *Moringa oleifera* aqueous seeds extract in this study may be attributed to the large quantity of polyphenols and vitamin C found in Moringa (Wadhwa, 2013). In a related earlier study, Agarwal *et al.* (2012) observed an improvement of the oocytes development and the fertilization rate while Younan *et al.* (2015) observed an increase in the distribution of compact follicular oocytes in female rabbits following the action of these compounds.

In female rabbits exposed to heat stress (35-36°C), a decrease in litter weight, kit body weight at weaning and daily kit weight gain was observed. The decrease in litter weight may be attributed to the reported reduction in food consumption in lactating female rabbits, which impacts their milk production and consequently decrease food intake of the kits. In fact, the low daily milk production reported in the present study is due either to the decrease in feed consumption as a result of body's mechanism to decrease heat production or due to reduced milk production by decreased mammary cell proliferation (Tao and Dahl, 2013). The administration of *Moringa oleifera* aqueous seeds extract to female rabbits exposed to heat stress improved these parameters. These results are in agreement with findings of El-Desoky *et al.* (2021) in female rabbits receiving respectively 10, 25 and 50 mg/kg body weight of nonencapsulated *Moringa oleifera* leaves ethanolic extract during summer seasons. The causes of this improvement are numerous. First, the presence of phenol compounds and vitamin C found in *Moringa oleifera* seeds in the present study have the ability to prevent the digestive system cell membranes from oxidative stress by scavenging on O_2 radicals and therefore

restoring feed utilization (Abou-Zeid *et al.*, 2000). Second, the improvement of these parameters could be associated with the antioxidant activity of bioactive compounds encountered in *Moringa oleifera* that protect against lipid peroxidation in the cell membrane (Svoradová *et al.*, 2021).

Reproductive hormones are known to regulate reproductive system and thereby ensure the success of each reproductive event (Svoradová *et al.*, 2021), thus their important role in the regulation of the reproductive parameters. In fact, LH acts in stimulating the ovaries to produce Estradiol, influences the ovaries to release follicles during ovulation and stimulates the corpus luteum, which produces progesterone to sustain the pregnancy (Khodaei-Motlagh *et al.*, 2011). The decrease in the fertilization rate due to estrus signs suppression, gonadotrophin decrease, ovulation disruption has been associated with low estradiol secretion (Wolfenson *et al.*, 2000). Low production of progesterone led to impairment of the embryo development by disrupting endometrial function (Wolfenson *et al.*, 2000; Khodaei-Motlagh *et al.*, 2011). Moreover, the decrease in FSH serum content is mostly a consequence of decrease in the inhibition of negative feedback from smaller follicles which finally impair the animal reproductive efficiency (Khodaei-Motlagh *et al.*, 2011). Acyclicity and infertility as well as mammary gland development impairment in pregnant females have been associated with the increased prolactin level (Singh *et al.*, 2013). Cortisol, a stress hormone, plays an important role in helping body to deal with stressful situations by enhancing blood sugar production in liver, converting energy stored in form of fats, proteins and carbohydrates into usable energy (Marai *et al.*, 2007a).

An increase of more than 2°C in the environment is responsible for low or desynchronized endocrine activities in females, mainly the pineal-hypothalamo-hypophyseal-gonadal axis, followed by impairment of the respective reproductive hormones (Upadhyay *et al.*, 2009). In

the present study, these hormones were impaired following the exposition of female rabbits to heat stress but the pattern reversed following administration of *Moringa oleifera* aqueous seeds extract. This enhancement is mainly attributed to numerous compounds found in *Moringa oleifera* seeds. It is reported that phytosterols can act as precursors of steroid hormones (testosterone, estradiol and progesterone) while isoflavones are one of the flavonoid compounds, with estrogenic activity and are known to bind with estrogen receptors such as ER-α and ER-β (Setiasih *et al.*, 2021). Phenolic and alkaloid compounds have been reported to protect embryonic tissue against reactive oxidative stress impairment by enhancing the ovarian hormone serum concentration (Grzanna *et al.*, 2005). On the other hand, phytosterols, polyphenols, saponins and flavonoids present in *Moringa oleifera* seeds have been previously reported to cause increase in female reproductive hormones (Estrada *et al.*, 2001; Khan *et al.*, 2005).

7.2. GENERAL CONCLUSIONS

The following conclusions can be made based on the obtained results:

1. The composition and concentration of bioactive compounds as well as antioxidant capacity of *Moringa oleifera* vary significantly across geographical regions and based on different plant parts of the same plant used. Leaf extract from DRC and Kenya showed better performances compared to seeds and barks and are recommended as a satisfactory antioxidant. However, despite their low composition in bioactive compounds and antioxidant capacity compared to leaves, seeds and barks also have pharmacological proprieties and their use can offer reprieve on the over use of leaves hence conservation of ecosystem.

2. Exposition of female rabbits to 31–32 ◦C and 35–36 ◦C for 30 consecutive days impaired their growth performances, relative organ weights and caused some behavioral abnormalities while it enhanced rectal and skin temperature, impaired hemato-biochemical parameters and the levels of enzymatic antioxidant biomarkers. However, the administration of 200 mg/kg of body weight of *Moringa oleifera* aqueous seed extract in female rabbits exposed to heat stress (35°C-36°C) restored their physiological parameters. These include the decrease in the skin and rectal temperature, the amelioration of hemato-biochemical parameters, internal organ weights and enzymatic antioxidant biomarkers as well as the restoration of the kidney tissues architecture.

3. The aqueous extract of the seeds of *Moringa oleifera* administered at 200 mg/kg of body weight minimizes the adverse effects of heat stress associated with reproductive impairments in female rabbits. This dose of *Moringa oleifera* aqueous seed extract improved feed intake and body weight in both pregnant and lactating female rabbits, litter size, litter weight, kit body

weight and kit body weight gain from birth to weaning, milk yield and milk efficiency, reproductive hormones as well as ovary tissues structure.

7.3. RECOMMENDATIONS

For future studies, it is recommended that:

- Pharmacological effects of *Moringa oleifera* aqueous extract on rabbit buck performances be evaluated

- Other parts of *Moringa oleifera* (pods, stembark) on heat stress disturbances in rabbits be studied

- Pharmacological effects of *Moringa oleifera* aqueous extract on reproductive performances induced by heat stress be evaluated at molecular level

- Bioactive compounds and antioxidant activity of seeds of *Moringa oleifera* using organic solvents be assessed.

- Potential toxicity of *Moringa oleifera* aqueous seed extract be checked through in vitro studies

- Active compounds of the *Moringa oleifera* aqueous seed extract be isolated and identified

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Appendix 1: Dosage of Estradiol

Plasma estradiol levels was determined in plasma using the Enzyme-Linked Immunosorbent Assay (ELISA) which is designed for the measurement of Estradiol in serum and urine and 2–8°C as storage temperature of the kit.

Materials

1. Estradiol ELISA Kit (Calbiotech, ES180S-100). This kit includes the following components:

- ELISA Plate Pre-Coated with Anti-Estradiol Capture Antibodies

- Estradiol Standards (ready to use)

- Assay Diluent (ready to use)

- TMB Substrate Reagent (ready to use)

- Stop Solution (ready to use)

- 20X Estradiol Enzyme Conjugate Concentrate

- 20X Wash Buffer

2. Distilled or deionized water

3. Micropipettes

4. Pipette tips

5. Paper towels

6. Plate reader capable of reading absorbance at 450 nm

Procedure

1. Bring reagents to room temperature (18-26 °C) before use.

2. Prepare working solutions:

3. Pipette 25 µL of standards, controls and specimens into selected wells in duplicate.

4. Add 100 μL of 1X Enzyme Conjugate to each well. Shake the plate 10-30 seconds to ensure proper mixing.

5. Incubate for 2 hours at room temperature, preferably in the dark (cover with aluminum foil, for example).

6. Wash the wells 3 times with 300 μL of 1X wash buffer using either a suitable plate washer or wash bottle taking care not to cross contaminate wells.

7. Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate, does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.

8. Add 100 μL of TMB Substrate Reagent to each well. Gently shake the plate for 10 seconds to mix.

9. Incubate for 30 minutes at room temperature, preferably in the dark.

10. Add 50 μL of Stop Solution to each well. Shake the plate gently to mix the solution for 30 seconds to ensure that the blue color completely changes to yellow.

11. Read absorbance on a plate reader set to 450 nm within 15 minutes.

Calculation of results

The mean absorbance value (A450) for each reference group of standards, controls and samples was calculated. Then a standard curve was drawn by plotting the mean absorbance values obtained for each standard and its concentrations in pg/ml on a graph paper, with the absorbance values on the vertical (Y) and the concentrations on the horizontal (X) axis. Finally, the absorbance value was used for each sample to determine the corresponding concentration of estradiol in pg/ml on the standard curve

Appendix 2: Dosage of Progesterone

Plasma progesterone levels was determined in plasma using the Enzyme-Linked Immunosorbent Assay (ELISA) which is designed for the measurement of Estradiol in serum and urine and 2– 8°C as storage temperature of the kit.

Principle of the test

Progesterone ELISA (Enzyme-linked Immunosorbent Assay) assays are based on the principle of competition for binding sites between the hormone (progesterone) in the test sample and a conjugate of the hormone HRP (Horseradish Peroxidase) for a constant amount of rabbit anti-hormone. In the incubator, goat and rabbit anti IgG coated in wells or tubes are incubated with 10 µl of hormone from standards, controls, samples, 100 µl of hormone-HRP conjugate reagent and 50 µl of rabbit anti-hormone at 37°C for 90 minutes. During incubation, a fixed amount of HRP-labeled hormone competes with the endogenous hormone in the standard, sample, or control sample for a fixed amount of hormone-specific antibody. Thus, the amount of hormone peroxidase conjugate immunologically bound to a well gradually decreases as the concentration of the hormone in the sample increases. The unbound hormone peroxidase conjugate is then removed and the wells washed. Next, a TMB (Tetramethylbenzidine) reaction solution is added and incubated at room temperature for 20 minutes resulting in the development of a blue color. Color development is stopped with the addition of 1N HCl (chloridric acid), and absorbance is measured with a spectrophotometer set at 450 nm. The intensity of the color formed is proportional to the amount of enzyme and is inversely proportional to the amount of the hormone (estradiol or progesterone) without label in the sample. The calibration curve is obtained by tracing the concentration of the standard against the absorbance.

Reagents

- Goat and rabbit anti-IgG coated in wells, 96 wells.

- Reference progesterone standards: 0, 0.5, 3.0, 10, 25, and 50 ng/ml. Liquids, 0.5 ml each, ready to use.

- Rabbit anti-progesterone reagent for progesterone (pink color), 7 ml.

- Progesterone-HRP conjugate reagent for progesterone (blue color), 12 ml.

- Progesterone control 1 liquid (0.5 ml) ready to use.

- Progesterone Control 2 liquid (0.5 ml) ready to use.

- TMB reagent (first step) 11 ml.

- Stop solution (1 N HCl) 11 ml.

Procedure

The desired number of coated wells in the holder was secured, then 10 µl of standards, samples and controls were dispersed into the appropriate wells. Then 100 µl of progesterone-HRP (Horseradish Peroxidase) for progesterone was introduced into each well. Fifty (50 µl) of rabbit anti-progesterone reagent for progesterone respectively was also introduced into each well. The mixture was mixed for 30 seconds and incubated at 37°C for 90 minutes. The wells were rinsed 5 times with distilled water and 100 µl of TMB (Tetramethylbenzidine) reagent was dispersed in each well and mixed for 5 seconds. Once again, the wells were incubated at room temperature (18-25°C) for 20 minutes. The reaction was stopped by adding 100 µl of the stop solution to each well. The whole mixture was mixed for 30 seconds (it was important to make sure that all the blue color changed to yellow) and finally the absorbance was read at 450 nm (Labsystem Multiskan RC) for a maximum of 15 minutes.

Calculation of results

The mean absorbance value (A450) for each reference group of standards, controls and samples was calculated. Then a standard curve was drawn by plotting the mean absorbance values obtained for each standard and its concentrations in ng/ml on a graph paper, with the absorbance

values on the vertical (Y) and the concentrations on the horizontal (X) axis. Finally, the absorbance value was used for each sample to determine the corresponding concentration of progesterone on the standard curve

Appendix 3: Dosage of Luteinizing Hormone (LH)

Luteinizing hormone (LH) is produced in both males and females from the anterior pituitary gland in response to luteinizing hormone-releasing hormone (LH-RH or Gn-RH), that is released by the hypothalamus. LH is glycoprotein with a molecular weight of approximately 30,000 Dalton. It is composed of two noncovalently associated dissimilar amino acid chains, alpha and beta. The alpha chain is similar to that found in human thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), and human chorionic gonadotropin (hCG). LH stimulates ovulation and ovarian steroid production in the female. In the male, LH controls Leydig cell secretion of testosterone. LH is elevated in Luteal phase of menstrual cycle, primary hypogonadism, Gonadotropin-secreting pituitary tumors and menopause. LH is deceased in hypothalamic Gn-RH deficiency, pituitary LH deficiency and ectopic steroid production.

Principle of the test

The LH ELISA kit is an adapted solid phase direct sandwich ELISA. The samples, biotin labeled anti-LH and anti-LH-HRP conjugates are added to the wells coated with Streptavidin. The anti-LH Antibodies form a sandwich around LH in the animal serum. Simultaneously, the Biotinylated Anti-LH antibody binds to the Streptavidin coated well. Unbound protein and excess antibody are washed off during a wash step. Upon the addition of the substrate, the intensity of color is proportional to the concentration of LH in the samples. A standard curve is prepared relating color intensity to the concentration of the LH.

Material

- Microwells coated with Streptavidin: 12x8x1

- LH Standard: 6 vials (ready to use): 0.5ml

- LH Conjugate Reagent: 1 bottle (ready to use): 12 ml

- TMB Substrate: 1 bottle (ready to use): 12ml

- Stop Solution: 1 bottle (ready to use): 12ml

- Wash concentrate 20X: 1 bottle: 25m

- Distilled or deionized water

- Precision pipettes

- Disposable pipette tips

- ELISA reader capable of reading absorbance at 450nm

- Absorbance paper or paper towel

- Graph paper

Procedure

- Prepare 1X Wash buffer by adding the contents of the bottle (25 ml, 20X) to 475 ml of distilled
  or deionized water. Store at room temperature (18-26° C).

- Prior to assay, bring all reagents to room temperature. Gently mix all reagents before use.

- Place the desired number of coated strips into the holder

- Pipette 25 µl of LH standards, control and patient's sera.

- Add 100 µl of Conjugate Reagent to all wells. Mix plate by placing on a plate shaker at
  600rpm for 30 seconds.

- Incubate for 60 minutes at room temperature (18-26° C).

- Remove liquid from all wells. Wash wells three times with 300 µl of 1X wash buffer. Blot on
  absorbent paper towels.

- Add 100 µl of TMB substrate to all wells.

- Incubate for 15 minutes at room temperature.

- Add 50 µl of stop solution to all wells. Shake the plate gently to mix the solution.

- Read absorbance on ELISA Reader at 450 nm within 15 minutes after adding the stopping solution.

Calculation of results

The standard curve is constructed as follows:

1. Check LH standard value on each standard vial. This value might vary from lot to lot. Make sure you check the value on every kit. See example of the standard attached.

2. To construct the standard curve, plot the absorbance for the LH standards (vertical axis) versus the LH standard concentrations (horizontal axis) on a linear graph paper. Draw the best curve through the points.

3. Read the absorbance for controls and each unknown sample from the curve. Record the value for each control or unknown sample

Appendix 4: Dosage of Follicular Stimulating Hormone (FSH)

Follicle-Stimulating Hormone (FSH) is a glygoprotein produced by the anterior pituitary gland. Like other glycoproteins, such as LH, TSH, and HCG, FSH consists of subunits designated as alpha and beta. Hormones of this type have alpha subunits that are very similar structurally; therefore the biological and immunological properties of each are dependent on the unique beta subunit. In the female, FSH stimulates follicular growth, prepares ovarian follicles for action by LH and enhances the LH induced release of estrogen. FSH levels are elevated after menopause, castration and in premature ovarian failure. Although there are significant exceptions ovarian failure is indicated when random FSH concentrations exceed 40 mIU/ml. In

the male, FSH stimulates seminiferous tubule and testicular growth and is involved in the early stages of spermatogenesis. Oligospermic males usually have elevated FSH levels. Tumors of the testes generally depress serum FSH concentrations, but levels of LH are elevated. High levels of FSH in men may be found in primary testicular failure and Klinefelter syndrome. Elevated concentrations are also present in cases of starvation, renal failure, hyperthyroidism, and cirrhosis.

Principle of the test

The FSH ELISA kit is a solid phase assay using streptavidin/biotin method. The samples and Anti-FSH/Anti-Biotin conjugate are added to the wells coated with Streptavidin. FSH in the serum forms a sandwich between specific antibodies labeled with biotin and HRP. Unbound protein and HRP conjugate are washed off by wash buffer. Upon the addition of the substrate, the intensity of color is proportional to the concentration of FSH in the samples. A standard curve is prepared relating color intensity to the concentration of the FSH.

Material

- Microwells coated with Streptavidin: 12x8x1

- FSH Standard: 6 vials (ready to use): 0.5ml

- FSH Enzyme Conjugate: 1 bottle (ready to use): 12 ml

- TMB Substrate: 1 bottle (ready to use): 12ml

- Stop Solution: 1 bottle (ready to use): 12ml

- 20X Wash concentrate: 1 bottle: 25ml

- Distilled or deionized water

- Precision pipettes

- Disposable pipette tips

- ELISA reader capable of reading absorbance at 450nm

- Absorbance paper or paper towel

- Graph paper

Procedure

- Prepare 1X Wash buffer by adding the contents of the bottle (25 ml, 20X) to 475 ml of distilled or deionized water. Store at room temperature (18-26° C).

- Prior to assay, allow reagents to stand at room temperature. Gently mix all reagents before use.

- Place the desired number of coated strips into the holder.

- Pipette 50 µl of FSH standards, control and patient's sera in to selected wells.

- Add 100 µl of enzyme conjugate to all wells.

- Cover the plate and incubate for 60 minutes at room temperature (18-26° C).

- Remove liquid from all wells. Wash wells three times with 300 µl of 1X wash buffer. Blot on absorbent paper towels.

- Add 100 µl of TMB substrate to all wells.

- Incubate for 15 minutes at room temperature.

- Add 50 µl of stop solution to all wells. Shake the plate gently to mix the solution.

- Read absorbance on ELISA Reader at 450 nm within 15 minutes after adding the stopping solution.

Calculation of results

The standard curve is constructed as follows:

1. Check FSH standard value on each standard vial. This value might vary from lot to lot. Make sure you check the value on every kit. See example of the standard attached.

2. To construct the standard curve, plot the absorbance for the FSH standards (vertical axis) versus the FSH standard concentrations (horizontal axis) on a linear graph paper. Draw the best

curve through the points.

3. Read the absorbance for controls and each unknown sample from the curve. Record the value for each control or unknown sample.

Appendix 5: Dosage of Prolactin

Prolactin (lactogenic hormone) is a single chain polypeptide hormone with a molecular weight of approximately 23,000 daltons. Prolactin is secreted from the anterior pituitary gland in both males and females. Female normally have slightly higher basal prolactin levels than males. During and following pregnancy, prolactin, in association with other hormones, stimulates breast development and milk production. Hypersecretion of prolactin can be caused by pituitary tumors, hypothalamic diseases, hypothyroid, renal failure, acute exercise and several medications. Hyperprolactinemia inhibits hypogonadism in males and females with accompanying low FSH and LH levels.

Principle of the test

The Prolactin ELISA kit is a solid phase sandwich ELISA assay method, based on a streptavidin-biotin principle. The standards, samples and a reagent mixture of Anti-Prolactin Enzyme and Biotin conjugates are added into the wells, coated with Streptavidin. Prolactin in the serum forms a sandwich between two highly specific Prolactin antibodies, labeled with Biotin and HRP. Simultaneously, the biotinylated antibody is immobilized onto the well through a high affinity Streptavidin-Biotin interaction. Unbound protein and excess biotin/enzyme conjugated reagent are washed off by wash buffer. Upon the addition of the substrate, the intensity of color developed is directly proportional to the concentration of Prolactin in the samples. A standard curve is prepared relating color intensity to the concentration of the Prolactin.

Material

- Microwells coated with Streptavidin: 12x8x1

- Prolactin Standards: 6 vials (ready to use): 0.5 ml

- Enzyme Conjugate: 1 bottle (ready to use): 12 ml

- TMB Substrate: 1 bottle (ready to use): 12 ml

- Stop Solution: 1 bottle (ready to use): 12 ml

- 20X Wash concentrate: 1 bottle 25 m

- Distilled or deionized water

- Precision pipettes

- Disposable pipette tips

- ELISA reader capable of reading absorbance at 450nm

- Absorbance paper or paper towel

- Graph paper

Procedure

- Prepare 1X Wash buffer by adding the contents of the bottle (25 ml, 20X) to 475 ml of distilled

  or deionized water. Store at room temperature (18-26° C).

- Prior to assay, allow reagents to stand at room temperature. Gently mix all reagents before

  use.

- Place the desired number of coated strips into the holder.

- Pipette 25 µl of Prolactin standards, control and patient's sera.

- Add 100 µl of enzyme conjugate to all wells.

- Cover the plate and incubate for 60 minutes at room temperature (18-26° C).

- Remove liquid from all wells. Remove liquid from all wells. Wash wells three times with 300

µl of 1X wash buffer. Blot on absorbance paper or paper towel.

- Add 100 µl of TMB substrate to all wells.

- Incubate for 15 minutes at room temperature.

- Add 50 µl of stop solution to all wells. Shake the plate gently to mix the solution.

- Read absorbance on ELISA Reader at 450 nm within 15 minutes after adding the stopping solution.

Calculation of results

The standard curve is constructed as follows:

1. Check Prolactin standard values on each standard vial. This value might vary from lot to lot. Make sure you check the value on every kit. See example of the standard attached.

2. To construct the standard curve, plot the absorbance for the standards (vertical axis) versus the standard concentrations (horizontal axis) on a linear graph paper. Draw the best curve through the points.

3. Read the absorbance for controls and each unknown sample from the curve. Record the value for each control or unknown sample.

Appendix 6: Dosage of Cortisol

Cortisol is the most potent glucocorticoid synthesized from cholesterol. Cortisol is found in the blood either as free Cortisol, or bound to corticosteroid-binding globulin (CBG). Cortisol production has an ACTH-dependent circadian rhythm with peak levels in the early morning and a nadir at night. The factors controlling this circadian rhythm are not completely defined. Serum levels are highest in the early morning and decrease throughout the day. In the metabolic aspect, Cortisol promotes gluconeogenesis, liver glycogen deposition, and the reduction of glucose utilization. Immunologically, Cortisol functions as an important anti-

inflammatory, and plays a role in hypersensitivity, immunosuppression, and disease resistance. It has also been shown that plasma Cortisol levels elevate in response to stress. Abnormal Cortisol levels are seen with a variety of different conditions: with adrenal tumors, prostate cancer, depression, and schizophrenia. Elevated Cortisol levels and lack of diurnal variation have been identified in patients with Cushing's disease

Principle of the test

The CBI Cortisol is a solid phase competitive ELISA. The samples and Cortisol enzyme conjugate are added to the wells coated with anti-Cortisol monoclonal antibody. Cortisol in the patient's sample competes with a Cortisol enzyme conjugate for binding sites. Unbound Cortisol and Cortisol enzyme conjugate is washed off by washing buffer. Upon the addition of the substrate, the intensity of color is inversely proportional to the concentration of Cortisol in the samples. A standard curve is prepared relating color intensity to the concentration of the Cortisol.

Material

- Microwells coated with Cortisol Mab: 12x8x1

- Cortisol Standard: 7 vials (ready to use): 0.5 ml

- Enzyme Conjugate (20X): 1.2 ml

- TMB Substrate: 1 bottle (ready to use): 12 ml

- Stop Solution: 1 bottle (ready to use): 12 ml

- 20X Wash concentrate: 1 bottle 25 ml 7. Assay Diluent: 24 m

- Distilled or deionized water

- Precision pipettes

- Disposable pipette tips

- ELISA reader capable of reading absorbance at 450nm

- Absorbance paper or paper towel

- Graph paper

Reagents preparation

- Cortisol-enzyme Conjugate Solution: Dilute the Cortisol enzyme conjugate 1:21 with assay diluent in a suitable container. For example, dilute 100µl of conjugate with 2ml of assay diluent buffer for 10 wells (A slight excess of solution is made).

- Wash Buffer: Prepare 1X Wash Buffer by adding the contents of the bottle (25ml, 20X) to 475 ml of distilled or deionized water. Store at room temperature (18-26°C).

Procedure

- Prior to assay, allow reagents to stand at room temperature. Gently mix all reagents before use.

- Place the desired number of coated strips into the holder

- Pipette 25 µl of Cortisol standards, control and patient's sera.

- Add 200µl of Cortisol Enzyme Conjugate to all wells.

- Thoroughly mix for 10 seconds.

- Incubate for 60 minutes at room temperature (18-26° C).

- Remove liquid from all wells. Wash wells three times with 300 µl of 1X wash buffer. Blot on absorbent paper towels.

- Add 100 µl of TMB substrate to all wells.

- Incubate for 15 minutes at room temperature (18-26° C).

- Add 50 µl of stop solution to all wells. Shake the plate gently to mix the solution.

- Read absorbance on ELISA Reader at 450 nm within 20 minutes after adding the stop solution.

Calculation of results

The standard curve is constructed as follows:

1. Check Cortisol standard value on each standard vial. This value might vary from lot to lot. Make sure you check the value on every kit. See example of the standard attached.

2. To construct the standard curve, plot the absorbance for Cortisol standards (vertical axis) versus Cortisol standard concentrations (horizontal axis) on a linear graph paper. Draw the best curve through the points.

3. Read the absorbance for controls and each unknown sample from the curve. Record the value for each control or unknown sample.

Appendix 7: Organ histology

Fixation

After harvesting, the organ is pre-fixed in 10% formalin, in a volumetric ratio (organ/formalin) of 1/3. Twenty-four (24) hours before dehydration, the pre-fixed organ is soaked in Bouin's solution.

Dehydration

After its stay in Bouin solution, the biopsies of the organ that had been flushed in distilled water for 15 min is dehydrated in ethyl alcohol containers of increasing temperature (70°, 95°, 95°, 100°, 100°, 100°) for 45 min, 45 min, 60 min, 45 min, 60 min and 90 min respectively. Subsequently, these biopsies were introduced into xylene for 2 hours for brightening.

Inclusion and coating

Immediately after being dehydrated, the organ biopsies were embedded in three successive steps in molten paraffin (approximately 60°C) for 60 min, 90 min, and 120 min respectively for soaking. Subsequently, these biopsies placed in molds are embedded in paraffin and then left

on a cooling surface for curing.

Realization of sections

The blocks ara placed on the microtome and cut to a thickness of 5 µm. The sections is then spread on the surface of a gelatin water bath (40°C) for defriping, then placed on slides before being placed in the oven (45°C) to dry for 24 hours.

Dewaxing

After removal from the oven, the slides are dewaxed in three successive xylene containers for 10 min respectively, then rehydrated in decreasing degrees of ethyl alcohol (three times in 100° alcohol, once in 95° alcohol and once in 70° alcohol) (10 min per container), before being flushed with distilled water for another 10 min.

Hematoxylin-Eosin staining

After rehydration, the slides are stained in Mayer's hematoxylin (nuclear stain) for 10 min and flushed with tap water for 10 min for dark blue staining of the cell nucleus. These slides are then successively soaked for 10 min in two containers of ethyl alcohol (70 and 80%) before being stained for 10 min in a 0.5% alcoholic eosin solution, to which a few drops of acetic acid were added at a rate of 40 µl per 100 ml of solution (for pink staining of the cell cytoplasm and orange-red staining of the red blood cells). After quickly flushing the slides with water to remove excess stain, they are dehydrated by successive passages of 10 min each, in two containers of absolute ethyl alcohol and finally, in xylene to make the parts uniformly translucent before being placed on Eukitt.

Observation and photography

After drying the slides at room temperature for 24 h, they are observed using a light microscope (Leica DM 750, X10 and X40) equipped with a DCM35 digital camera (350 Kpixels, USB 2.0), which was connected to the central processing unit of a computer. The images are directly

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observed on the computer equipped with an image capture program. The captured images are magnified 100 times (100X) for histology and cellular integrity.

Appendix 8: Determination of lipid peroxidation

Lipid peroxidation in the organ will be estimated colorimetrically by thiobarbituric acid reactive substances (TBARS) using the modification method of Niehius and Samuelsson (1968). In brief, 0.1 ml of the organ homogenate (10% w/v) will be treated with 2 ml of (1:1:1 ratio) TBATCA- HCl reagent (thiobarbituric acid 0.37%, 15% trichloroacetic acid and 0.25 N HCl). All the tubes will be placed in a boiling water bath for 30 min and cooled. The amount of malondialdehyde formed in each of the samples will be assessed by measuring the absorbance of clear supernatant at 535 nm against reference blank. Percentage inhibition will be calculated using the equation:

% lipids Inhibition = $\{A_o- A_1\}/A_o \times 100$

Where; A_o is the absorbance of the control and A_1 is the absorbance of the sample extract.

Appendix 9: Evaluation of reduced glutathione

Reduced glutathione (GSH) in the organ will be assayed by the method previously described by Ellman (1959). Briefly, 0.02 mL of the homogenate supernatant will be added to 3 mL of Ellman reagent (19.8 mg of 5,5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1 % sodium nitrate). The samples will be mixed and kept at room temperature for at least 1 hour. Changes in absorbance will be read at 412 nm on a spectrophotometer.

Appendix 10: Determination of superoxide dismutase

The ability of superoxide dismutase (SOD) to inhibit the autoxidation of adrenaline at pH 10.2 makes this reaction a basis for a simple assay for dismutase. Superoxide anion (0_{2-}) generated

by the xanthine oxidase reaction causes the oxidation of adrenaline to adrenochrome and the yield of adrenochrome produced by 0_2 increases with pH and concentration of adrenaline. The superoxide dismutase activity in tissues will be determined by the method of Misra and Fridovich (1972). Briefly, the assay mixture consisted of 134 µL tissue homogenate supernatant and 1666 µL carbonate buffer (50 mM, pH 10.2). In the cuvette, 0.2 mL of a freshly prepared adrenaline solution (0.3 mM) will be added to the above mixture. The auto-oxidation of adrenaline will be observed by measuring after one minute intervals, the absorbance at 480 nm. SOD will be quantified as follows:

- % inhibition = $100 - [(\Delta DO_{sample})/\Delta DO_{blanck}] \times 100$

- 50 % inhibition correspond to one unit

- **X%** inhibition to N units

Specific SOD activity or SOD units per mg protein = [(SOD units /mL)/mg protein] X dilution factor.

Appendix 11: Determination of catalase

Catalase (CAT) activity will be determined in tissues according to the method of Sinha (1972). In this method dichromate in acetic acid is reduced to chromic acetate when heated in the presence of hydrogen peroxide (H_2O_2), perchromic acid is formed which is an H_2O_2 unstable intermediate. The chromic acid produced is measured calorimetrically at 570 nm. Briefly, 50 µL of homogenate supernatant will be added to 750 µL of phosphate buffer (0.1 M, pH 7.5). Then, 200 µL of hydrogen peroxide substrate (50 mM) will be added. Exactly 1 min later, the reaction will be stopped by adding 2 mL of dichromate (5%) in acetic acid (300 mL). After that, the tubes will be kept at 100°C for 10 min. After cooling with tap water, changes in absorbance

will be recorded at 570 nm. Hydrogen peroxide will be quantified using a calibration curve and the CAT activity will be expressed as μmol of H_2O_2 per minute per mg of protein.

Tissue protein will be evaluated using the Biuret method of protein assay.

Appendix 12: Evaluation of Glutathione peroxidase

Glutathione peroxidase (GPx) will be measured by the method described by Rotruck et al. (1973). Briefly, the reaction mixture contained 0.2 ml 0.4 M phosphate buffer (PH7.0), 0.1ml 10 mM sodium azide, 0.2 ml tissue homogenized in 0.4M phosphate buffer pH7.0. 0.2 ml tissue homogenized in 0.4 M, phosphate buffer, pH 7.0, 0.2 ml reduced glutathione, 0.1 ml 0.2 mM hydrogen peroxide. The contents will be incubated for 10 min at 37°C, 0.4 ml 10% TCA will be added to stop the reaction and centrifuged at $3200 \times g$ for 20 min. The supernatant will be assayed for glutathione content using Ellman's reagent (19.8 mg 5,5'-dithiobisnitrobenzoic acid [DTNB] in 100 ml 0.1% sodium nitrate). The activities will be expressed as μg of GSH consumed/ min/mg protein.