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Physiological And Immunohistochemical Investigation Of Silymarin Effect On The Prolactin Gene Expression In Pituitary And Mammary Glands During Lactation Period In Female Rats

Thesis Submitted to the Council of the Faculty of Veterinary Medicine / University of Kerbala in Partial Fulfillment of the Requirement for the Master Degree of Science in Veterinary Medicine / physiology

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Declaration

I hereby declare that this thesis is my origin work except for equations and citations which have been fully acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at University of Kerbala or other institutions.

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/ / 2022

Dedication

To my leader on whom my difficulties based, Imam Hussein (AlayhiAlsalam)

For those who strive to console me and make me happy.... My father

For whom is that paradise under her feet...My mother

To my husband who supported me and encouraged me in every step and My children (Hassan – Mokhalad – Asenat)

To those whom gave me the strength and support… "My sisters and my brothers"

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List of contents

List of tables

List of figures

List of Abbreviations

Abbreviation	Meaning
ACTH	Adrenocorticotropin
ATP	Adenosine Triphosphate
B.W	Body weight
CAT	Catalase activity
cDNA	Complementary Deoxyribonucleic acid
CYP-A	Cyclophilin A
GSH	Glutathione concentration
MDA	Malondialdehyde
mRNA	Messenger Ribonucleic acid
PCR	Polymerase chain reaction
PLs	Placental lactogens
PRL	Prolactin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SLM	Silymarin
SOD	Superoxide dismutase
TIDA	Tuberoinfundibular dopamine

Abstract

The current study was carried out to investigation the effects of Silymarin on prolactin hormone gene expression in female rats during lactation period.

Twelve lactating rats were randomly divided into two groups (six /group), control group normal saline , while the silymarin group was intubated orally with (200 mg/kg/day B .W) of Silymarin for ten days

Fasting blood samples were collected by cardiac punctures technique and tissue from the pituitary gland after ten days of experimental for measuring serum biomarker levels of the Prolactin,Estrogen and Progesterone hormone pituitary tissue biomarker of oxidative stress including Glutathione (GSH) and Malondialdehyde (MDA) concentration.Superoxide Dismutase (SOD) and Catalase (CAT) activities, furthermore a sample of the anterial lobe of Pituitary gland was taken to measure gene expression and also taken mammary gland and anterial lobe of Pituitary gland were assessed for histopathological changes and immunohistochemistry.

The result of our study showed a significant ($P<0.05$) increase of serum prolactin and estrogen hormones, GSH concentration, SOD and CAT activities in the treated group with 200 mg/kg B .W of Silymarin compare to control group , while showed a significant decrease ($P<0.05$) in the serum progesterone hormone and MDA concentration in silymarin compare to control group .

The results of the present study indicated a positive correlation ($P<0.05$) between gene expression and prolactin and estrogen hormone, while showing a negative correlation between gene expression and progesterone hormone.

A positive correlation ($P<0.05$) was found between gene expression and GSH concentration, SOD and CAT activities ,while showing negative correlation between gene expression and MDA concentration in lactating rats

Our study found that when silymarin was given daily for ten days in lactation period, it increased the gene expression of the prolactin gene responsible for the production of the prolactin hormone compared to the control group.

The present study explained there were numerous histological and immunohistochemical changes occurred in pituitary and mammary glands, as well as pituitary and mammary glands immunoreactive of prolactin.One of these changes was observed in the density of lactotroph cells in the pars distalis of the pituitary gland and activity of these cells during followed production of prolactin also.

In control group the lactotrophs in pituitary gland appeared less in activity and paucity in reaction stain , while, in treated group Immunocytochemistry performed with the Prolactin showed a positive reaction throughout the anterior part of pars distalis, the lactotrophs cells appeared oval in shaped and closed to a sinusoid with density in secretory granules seemed in cytoplasm and interstitial spaces .

In control animals, the mammary glands demonstrated lobules distributed among amount of adipose tissue. The mammary alveoli seemed empty from milk and the alveolar cells weakly reaction by antibody of prolactin stain. In addition, the interlobular ducts it narrow and few in number,

The silymarin-treated group displayed enlarged lobules alveoli, with a commensurate decrease in adipose tissue. A thin band of fibrous connective tissue divides the mammary lobes. In comparison to the control group, the treated animals' intra lobular ducts had such a large lumen and were bordered by plain cuboidal cells. A flawless single layer spindle was produced in its place by (myoepithelial) cells when the basement membrane was reattached to the alveoli and alveolar duct.

In condition, the findings of the present investigation support the significance of Silymarin in raising prolactin gene expression and in decreasing oxidation status with raised lactotroph activity.

Chapter One: Introduction

1. Introduction

Prolactin (PRL), is a major polypeptide lactogenic hormone, that is synthesized and secreted by lactotroph cells of the anterior pituitary gland in response to various stimuli. The varied effects of prolactin include the growth and development of mammary alveolar cells (mammogenesis), stimulating them to synthesize milk components (lactogenesis), maintaining milk secretion (Akers, 2017). Prolactin contributes to hundreds of physiologic functions, but the two primary responsibilities are milk production and the development of mammary glands within breast tissues. PRL promotes the growth of mammary alveoli, which are the components of the mammary gland, where the actual production of milk occurs. Prolactin stimulates the breast alveolar epithelial cells to synthesize milk components. PRL plays a role in the regulation of the immune system, osmotic balance, and angiogenesis (Dong *et al*., 2013).

It is common knowledge that the anterior pituitary synthesizes and secretes prolactin and dopamine-mediated hypothalamic regulation; however, the central nervous system, the immune system, the uterus, and the mammary glands all are capable of producing prolactin, nipple stimulation, light, olfaction, and stress can all contribute (Akers, 2017).

Lactotropic cells are rounded cells with an oval cellular nucleus; mainly during pregnancy and lactation there are many large secretory granules. These granules are acidophilic, the lactotrope produces the PRL, which is a 199-amino acid single-chain protein, the lactotrope differs from the other endocrine cell types of the adenohypophysis in two major ways. The lactotrope is not part of an endocrine axis. This means that PRL acts directly on nonendocrine cells to induce physiologic changes (Martín-Pérez *et al.,* 2015) .

The concentration of circulating prolactin increases during pregnancy so that by the end of gestation, levels are 10 to 20 times over normal amounts, Prolactin plays a role in the establishment of mother/child relations (Skorupski and Kmieć, 2012).

Galactagogues may be synthetic, plant-derived (because these are easily available, cheap and with a hope that they may not leave any toxic residues in milk)

The use of plant-derived products to improve performance and health of animals and humans has gained popularity in recent years,or endogenous products(Gabay, 2002). They act through exerting an influence on adreno-hypothalamo-hypophyseal-gonadal axis by blocking hypothalamic dopaminergic receptors or by inhibiting dopamine producing neurons. These medications increase prolactin secretion by antagonizing dopamine receptors (Mohanty *et al* ., 2014).

For nearly 2,000 years, milk thistle (Silybum marianum L. Gaertn, Asteraceae) has been utilized in medicine. It's been widely utilized to treat a variety of liver problems,anti-inflammatory and antioxidant properties (Wu *et al*., 2011). Silymarin is made up of 4 flavonolignans in a complex mixture (isosilybin- silybin- silychristin and silydianin). The most important and active component of silymarin is silybin, which is essential for the pharmacological properties of silymarin (Farmer *et al* ., 2014). Silymarin has a variety of benefits for treating liver disorders, including the protect the liver from injury and ability to inhibit hepatotoxin binding to receptor sites, the ability to decrease glutathione oxidation and increase its own level in the liver, increase hepatocyte protein synthesis. additionally and the ability to stabilize the liver cell membrane , by lowering blood lipids and decreasing platelet aggregation, silymarin can help to avoid diabetes and atheroma (Farmer *et al* .,2017). Its content varies significantly amongst silymarin extracts, potentially altering its biological function. However, silymarin extract has been shown to boost milk yield in nursing mothers, as well as to improve bovine , β-casein gene expression and murine mammary cell proliferation (Ghosh *et al*., 2010). Because silymarin dramatically elevated prolactin in female rats, this effect is done in part through dopamine receptors (Wu *et al*., 2011). The observed favorable effect of silymarin on breastfeeding performance could be attributed to higher prolactin concentration (Feng *et al*., 2016). There are numerous mechanisms by which silymarin can oxidative state and increase antioxidant defense mechanisms, and its protective impact against systemic oxidative stress has recently been established in late-pregnancy animals (Farmer *et al* ., 2017).

Therefore the experiment was designed to investigated the effects of Silymarin on expression of prolactin gene and effect on oxidation ,antioxidation and female reproductive hormones during lactation period by measuring the following :-

1- serum levels of prolactin , estrogen and progesterone hormone .

2- A-Glutathione (GSH) concentration in the pituitary tissue .

B- Malondialdehyde (MDA) concentration in the pituitary tissue.

C-Superoxide dismutase (SOD) activity in the pituitary tissue.

D-Catalase (CAT)activity in the pituitary tissue.

3- Genetic study to determine the changes in the gene expression level of the gene Prolactin, which is responsible for the production of the hormone prolactin, through the use of technology RT- PCR.

4- Histological change to know the changes taking place in the mammary gland and Pituitary gland tissue of experimental animals during treatment.

5- Immunohistochemistry study of the pituitary gland and mammary glands.

Chapter Two: Review of the Related Literature

2. Review of the Related Literature

2.1. Pituitary gland

The pituitary is an organ of dual origin. The anterior lobe (adenohypophysis) is derived from oral ectoderm and is epithelial in origin, whereas the posterior lobe (neurohypophysis) derives from the neural ectoderm. The composite nature of the pituitary requires that the neural and oral ectoderm interact physically and developmentally. Precise spatial and temporal co-ordination and regulation of signals from both structures is critical for pituitary formation and the differentiation of the various hormone-producing cell types in the anterior lobe. The expression of transcription factors that control cell lineage commitment in the developing anterior lobe must be precisely regulated to ensure correct differentiation of hormone-producing cell types; the iterative and cumulative nature of this regulation renders it extremely sensitive to perturbation. Disruption of this process, for instance by mutation, can lead to numerous developmental disorders from congenital forms of hypopituitarism to pituitary tumors (Larkin and Ansorge, 2017) .

The pituitary gland, or hypophysis cerebri, is an oval body approximately 12mm in transverse and 8mm in anterior-posterior diameter weighing approximately 500mg. The anterior lobe of the pituitary is generally smaller in men than women, and nullipara than multipara; during pregnancy the gland may increase by approximately 30% due to lactotroph hyperplasia. The hypophysis is connected to the brain via the infundibulum, a tubular structure arising from the tuber cinereum and median eminence of the hypothalamus. The gland rests in the sella turcica (pituitary fossa) of the sphenoid bone and is covered superiorly by the diaphragma sellae (dura), laterally by the wall of the cavernous sinus, and antero-inferiorly by the posterior wall of the sphenoid sinus, which is used as the standard route for pituitary surgery transsphenoidal adenectomy. Antero-superior the pituitary lies in close proximity to the optic chiasm; this explains why space-occupying lesions of the pituitary commonly present with bitemporal hemianopia (Larkin and Ansorge, 2017).

The anterior pituitary gland enlarges by an average of 36% during pregnancy, primarily because of a 10-fold increase in lactotroph size and number.This enlargement results in an increase in height and convexity of the pituitary on magnetic resonance imaging (Elster *et al*., 1991). There are reduced numbers of somatotrophs

and gonadotrophs and no changes in corticotrophs or thyrotrophs (Scheithauer *et al* ., 1990). The posterior pituitary gland diminishes in size during pregnancy (Larsen, 2003).

The anterior lobe of the pituitary gland composed from special characterized cells called somatotrope, lactotrope , thyrotrope , corticotropes and gonadotropes .These cells are responsible for secretion of the following growth hormone, PRL and thyroid stimulating hormone, adrenocorticotropin, gonadotropic hormones, which include both luteinizing hormone and follicle stimulating hormone respectively (AL-Selawy, 2019).

Lactotrophs, the PRL -secreting cell is a second but distinct acidophil- staining cell randomly distributed in the anterior pituitary. These cells account for 10–25% of anterior pituitary cells. Granule size averages approximately 550 nm on electron microscopy. There are two types of lactotrophs: sparsely granulated and densely granulated. These cells proliferate during pregnancy as a result of elevated estrogen levels and account for the two fold increase in gland size (Martín-Pérez *et al.*, 2015) .

The anterior lobe of the pituitary gland is the principal source of circulating PRL in rodents and the hormone is secreted from a specialized group of cells, lactotrophs. The gland develops from oral ectoderm with proliferation and commitment to the hormone-secreting cell types of the anterior pituitary controlled by a cascade of transcription factors (Kelberman *et al* ., 2009) after exit from the cell cycle (Davis *et al* .,2011) . At birth only a few cells expressing PRL can be detected but there is a rapid post-natal expansion (Islam *et al* ., 2010). The post-natal expansion of lactotrophs is regulated by a number of peripheral, hypothalamic and intrapituitary factors, including insulin-like growth factor 1, epidermal growth factor and estradiol (Hikake *et al* ., 2009). In the adult, it is clear that dopamine , acting through the dopamine 2 receptor is a major suppressor of lactotroph proliferation (Numan and Woodside , 2010).

2.2. hypothalamus-pituitary axis:

The endocrine system composed of many peripheral and central endocrine glands. Peripheral endocrine glands work under control of central endocrine glands, which is also known hypothalamus-pituitary axis. The hypothalamus is the major controlling center that translates brain signals into secreted chemical substances called

releasing hormones that act on special secretory group of cells in the anterior lobe of the pituitary gland (Amar and Weiss , 2003).

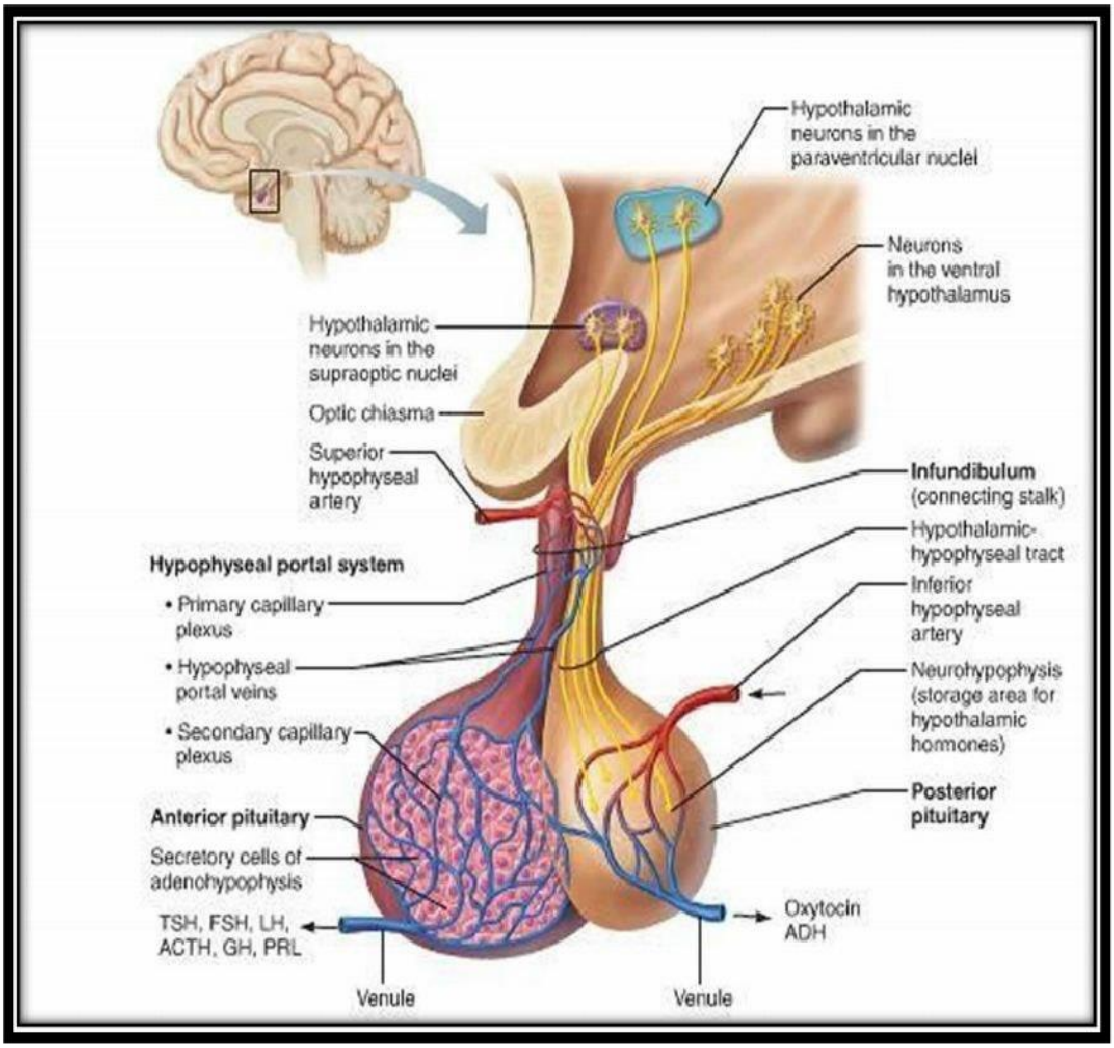
The releasing hormones transmitted from the hypothalamus to the pituitary gland to stimulate releasing another type of chemical substances called stimulating hormone that controls peripheral endocrine glands .In addition to the releasing hormones, hypothalamus releases vasopressin and oxytocin hormones via neurons to store in the posterior lobe of the pituitary gland (Hameed *et al* ., 2016). Furthermore, the anterior lobe of the pituitary gland composed from special characterized cells called somatotrope, lactotrope , thyrotrope , corticotropes and gonadotropes, these cells are responsible for secretion of the following growth hormone, prolactin, thyroid stimulating hormone, adrenocorticotropin (ACTH) and gonadotropichormones, which include both luteinizing hormone and folliclestimulating hormone respectively (Genuth ,1998).

About 30 to 40 % of the anterior pituitary cells are somatotropes that secrete growth hormone, and about 20 % are corticotropes that secrete ACTH. Each of the other cell types accounts for only 3 to 5 % of the total; nevertheless, they secrete powerful hormones for controlling thyroid function, sexual functions ,and milk secretion by the mammary gland (Guyton and Hall , 2016).

Interestingly the physiological functions of endocrine glands represent like hierarchal arrangements, which allow small chemical substances releasing from hypothalamus to stimulate a large group of cells in the pituitary gland to release stimulating substances or hormonal substances. The hormonal substances then travel through the blood stream to stimulate hormone-producing cells in the peripheral endocrine glands that release hormones to the target tissues, which translate into different biological effects figure (2-1).

The hormones release by peripheral glands such as adrenal glands, ovaries and thyroid gland target different types of tissue and talk to the hypothalamus and pituitary gland in the negative or positive feedback loops to insure that hormones level stay in balance in the blood stream. The negative and positive feedback loops can be affected by different factors like hormones peaking change during the daytime and night cycle, and hormones level change according to the different physiological stages

and to the different developmental phases (Kleine and Rossmanith, 2016 ; AL-Selawy, 2019).



Figure(2-1) Hypothalamic pituitary gland axis (Kleine and Rossmanith, 2016)

2.3.The mammary glands

The mammary glands are one of few organs in the body which are able to undergo repeated phases of growth, differentiation and regression. At the onset of puberty in the female, the increase in ovarian steroids induces elongation and side branching of the rudimentary mammary gland ductal system. Some differentiation of the ductal system occurs at this stage, resulting in a compact glandular structure. The gland, then remains relatively inactive until pregnancy (Mohammad *et al* ., 2019)

The mammary gland forms as an appendage of the skin and has its evolutionary origin in skin glands. The number and location of glands vary among different classes of mammals. In rats, five pairs of glands develop along a line that runs slightly ventral to the limb buds, whereas only one pair develops in the thoracic region in humans, Development of the mammary gland commences in the foetus. The initial cues that induce the formation of small buds on the ventral surface of and surrounding mesenchyme (the embryonic stroma) direct the outgrowth of a small duct into deeper layers of the dermal mesenchyme and formation of the nipple, the opening for milk removal. Through further elongation and bifurcation a small ductal system forms that associates with the subdermal fat pad (Sternlicht, 2005).

The alveolar epithelial cells are the functional unit of the mammary gland that synthesize and secrete milk (Anand *et al*., 2012). Functional differentiation of the mammary gland occurs with apparent morphological changes of the epithelial cells and allows milk production and secretion (Richert *et al*. 2000). After parturition with few days, growth and proliferation of the mammary gland cells start to be increased. While during the lactation period, there is a little growth, and a slow proliferation rate of the mammary gland cells (AL-Selawy, 2019).

Epithelial mesenchyme transition plays an important role in the initiation of mammary gland development during embryonic development. Interestingly the mammary glands grew asymmetrically with the other body organs until the puberty time. At the puberty time, the mammary development mostly depends on the hormonal effect. For this reason, this stage of mammary development called hormone dependent actions drive mammary glands development (Brisken and O'Malley, 2010) .

During puberty the cyclical production of ovarian estrogen and progesterone accelerates ductal outgrowth and branching. In the mature animal, the entire fat pad is filled with a regularly spaced system of primary and secondary ducts that are decorated with side branches, which form and disappear during each estrous cycle. Proliferation and maturation of the alveolar compartment occurs during pregnancy and is controlled mainly by prolactin and placental lactogens. At term, the mammary gland reaches maturity and produces and secretes milk to support the young. In mice, mammary tissue produces milk equivalent to 20% of the body weight of the

dam. At the end of lactation, the loss of suckling stimuli and the pressure build-up on cessation of milk removal initiates a remodelling programme called involution. This causes massive cell death, the collapse of the alveoli and the remodelling of the epithelial compartment to restore a simple ductal structure again. A new round of alveolar expansion, maturation and lactation is initiated with the next pregnancy (Hennighausen and Robinson, 2005) .

Two tissue compartments constitute the mammary gland: the epithelium, which consists of ducts and milk-producing alveolar cells; and the stroma, or connective tissue, which is also called the mammary fat pad , in general, the epithelial cells form ducts and alveoli with a central lumen that opens to the body surface through the nipple. By contrast, monotremes have mammary glands without a nipple or central lumen and the ducts open directly to a confined area known as the milk patch, Most epithelial cells are luminal, secretory cells, which undergo functional differentiation in pregnancy to produce milk. They are encased by a mesh-like system of basal, myoepithelial cells, which are contractile and partici pate in the delivery of milk (Sternlicht , 2005) . The extensive system of ducts and alveoli is embedded in the stroma, the main components of which are adipocytes, but fibroblasts, cells of the haematopoietic system, blood vessels and neurons are also present (Hennighausen and Robinson, 2001; Hennighausen and Robinson, 2005) .

2.4. Milk production

Milk serves as an important economic resource. It is vital for animal growth and wellbeing as it is essential product during neonatal life and provides the essential immunoglobulins during early life period as well as later in life (Mohammad *et al* ., 2019) .

Breastfeeding is widely acknowledged to have important health benefits for infants and mothers, a variety of herbal and phytopharmaceutical products have been recommended as galactogogues, substances that promote lactation (Zapantis *et al* .,2012; Capasso, 2014).

The production of breast milk is controlled by an interplay of various hormones, with prolactin being the predominant hormone involved. The maturation of breast tissue, resulting in milk production, is controlled by many other factors besides prolactin, including estrogen, progesterone, insulin, growth hormone, cortisol,

thyroxine, and human placental lactogen (Gabay, 2002). During pregnancy, high levels of estrogen and progesterone inhibit the effects of prolactin on breast milk production (Forinash *et al* ., 2012) .

A dramatic reduction in progesterone following delivery triggers lactation. Nipple stimulation, via suckling or manual stimulation, initiates release of prolactin from the anterior pituitary and oxytocin from the posterior pituitary gland (Gabay, 2002). Other sensory pathways may affect the release of oxytocin beyond nipple stimulation. The release of prolactin from the anterior pituitary stimulates the production and secretion of breast milk, while the release of oxytocin from the posterior pituitary aids in the contraction of myoepithelial cells within the breast, resulting in milk letdown (Goff and Griffiths, 2006) The release and production of prolactin is dependent on the inhibition of a factor known as prolactin inhibitor factor, which is produced by the hypothalamus, and dopamine-releasing neurons. The activation of dopamine receptors on prolactin-secreting cells inhibits the release of prolactin. Prolactin does regulate the volume of milk produced; however, once lactation is established, infant demand drives the process. In the absence of suckling, lactation ceases in 2 to 3 weeks (Gabay, 2002).

Maternal milk is currently considered the optimal feeding for all babies. Breast-feeding is associated with better nutritional and non-nutritional outcomes compared with formula feeding even in case of preterm birth (Chatterton Jr *et al*., 2000). Poor breast milk production is the most frequent cause of breastfeeding failure (Eidelman *et al* .,2012). A reduced breast milk production can occur in many circumstances, such as preterm birth, illness of the mother or the child, mother-baby separation, re-lactation after a prolonged suspension and indirect lactation (breast pump or manual milk expression). Anxiety, fatigue, and emotional stress are also powerful inhibitors of lactation. Educational programs should be offered to all breastfeed mothers to address problems such as inadequate frequency and duration of breastfeeding, incorrect breast attachment, inadequate breast emptying during manual or mechanical milk expression. Milk production can be increased in several other ways, such as psychological support and relaxation techniques (for example using books or audio/video supports). Nevertheless, many mothers ask quite often their physician for medications or other products to increase their breast milk production (Zuppa *et al* ., 2010)

Milk production (lactogenesis), which is a consequence of neuro-endocrine event, is a complex neurophysiological process that involves interaction of a number of physical and emotional factors along with action of multiple hormones, mainly prolactin. During parturition and expulsion of the placenta, progesterone concentration reduced resulting in initiation of full milk supply (Neville *et al* .,2004). Dopamine agonists and antagonists regulate prolactin synthesis and secretion through interaction with the hypothalamus and anterior pituitary and thereby control milk production. Thereafter, prolactin levels gradually decrease but milk supply is maintained or increased by local feedback mechanisms . Therefore, an increase in prolactin levels is mandatory to increase milk production but not to maintain its supply (Behera *et al* ., 2013) . The anterior pituitary plays a significant role for the development of the mammary gland, initiation of lactogenesis and lactation that has been described following the administration of pituitary extracts. So the term 'prolactin'has been described for pituitary substance. It is assumed that, pituitary extract is rich in growth hormone responsible for milk production in lactating cattle. Lactogenesis and ejection both are stress for lactating cows and in turn are affected by stress of any other reason. Because hormone action is completely dependent on emotion and stress, so control of stress is the primary factor on the way of lactogenesis (Mohanty *et al* ., 2014).

Breast feeding process ready flow by letdown reflex. The signs of letdown reflex are ticklish or tingling feelings in the breasts, pressure on the breasts, breasts feel full, the mother feels thirsty and even milk drips into the breast that the baby does not suck. Letdown reflex can occur when the mother hears, sees or even thinks of her baby. and triggered hormone by touching the breast in the nipple area (Putri and Utami, 2021).

2.5. Lactation cycle;

Lactation cycle begins with growth of the breast (mammogenesis), initiation of milk synthesis and secretion (lactogenesis 1 and lactogenesis 2), established lactation (galactopoiesis), regression of the breast during and after weaning (involution) (Hartmann *et al*., 1996). Figure (2-2) presents different stages in the lactation cycle starting from mammogenesis (getting ready for lactation) to lactogenesis (initiation

and establishment of lactation) to galactopoiesis (maintenance of lactation) (Truchet and Honvo-Houéto, 2017).

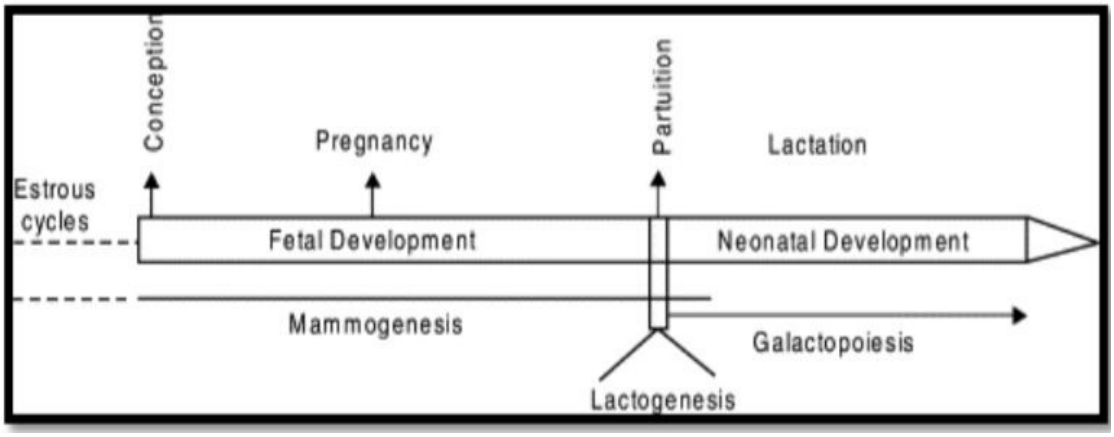


Figure (2-2) Presents different stages in the lactation cycle (Truchet and Honvo-Houéto, 2017)

Mammogenesis is a stage when the mammary gland develops its histological and bio-chemical capacity to synthesize milk. This stage is characterized by increase in number and size of the alveoli, where milk is secreted and stored (Neville, 2006). Lactogenesis is a stage where the body secretes and produces milk for the fetal (Paintal, 2011). Lactogenesis has 2 stages: the first stage, lactogenesis 1 occurs by mid pregnancy, when the mammary gland becomes competent to secrete milk. The secretion of milk, however, is held in check by high circulating levels of progesterone and estrogen (Neville, 2006). The second stage, lactogenesis 2 occurs around the time of delivery and is defined as the onset of copious milk secretion (Robinson, 2009). The hormonal changes during parturition and the subsequent removal of the placenta act as a lactogenic trigger which is necessary for initiation of milk secretion (Neville and Morton, 2001). For the ongoing synthesis and secretion of animal milk, continuous hormonal signals received by the mammary gland (due to stimulation of nipple), relayed to the central nervous system are essential to induce milk secretion (Pillay and Davis, 2020). Milk is secreted more or less continuously into alveolar lumens and stored until the let-down reflex induces milk ejection where it exits through ducts into small sinuses near the areola and then opens directly on the nipple (Madill, 2010). When the baby starts suckling at the breast, it causes the stimulation of touch receptors that are densely located around the nipple and areola (Leung and Sauve, 2005). Tactile sensations create impulses that ascend the spinal cord, creating a

neuronal pathway between the hypothalamus and pituitary gland which release oxytocin resulting in the expulsion of stored milk from alveoli into the sinuses through the nipple pore (Huggins, 2017). Once milk secretion has started, suckling by the baby influences subsequent functioning of the mammary that is jointly controlled by the nervous/endocrine systems through the release of appropriate hormones as well as transfer of nerve impulses (Cahill and Wagner, 2002).

2.6. Prolactin hormone

Prolactin (PRL) is a peptide hormone primarily synthesized and secreted by adenohypophysis. PRL is secreted into blood stream in response to the suckling stimulus on the maternal nipple (Zuppa *et al* ., 2010) .

The output of PRL is highly dynamic with dramatic changes in its secretion from the anterior pituitary gland depending on prevailing physiological status. In adult female, there are three distinct phases of output and each of these is related to the functions of PRL at specific stages of reproduction. During lactation, coordinated activity increases due to the changes in structural connectivity, and this drives large elevations in PRL secretion. Surprisingly, these changes in connectivity are maintained after weaning, despite reversion of PRL output to that of virgin animals, and result in an augmented output of hormone during a second lactation. At the level of the hypothalamus, tuberoinfundibular dopamine (TIDA) neurons, the major inhibitors of PRL secretion, have unexpectedly been shown to remain responsive to PRL during lactation. However, there is an uncoupling between TIDA neuron firing dopamine secretion, with a potential switch to enkephalin release. Such a process may reinforce hormone secretion through dual disinhibition and stimulation of PRL cell activity. Thus, integration of signalling along the hypothalamo-pituitary axis is responsible for increased secretory output of PRL cells during lactation, as well as allowing the system to anticipate future demands (Le Tissier *et al* ., 2015).

PRL hormone affects metabolic homeostasis by regulating key enzymes and transporters that are associated with glucose and lipid metabolism in several target organs, in the lactating mammary gland, PRL increases the production of milk proteins, lactose and lipids. In adipose tissue, PRL generally suppresses lipid storage and adipokine release (Ben-Jonathan *et al* ., 2006). PRL supports the growth

of pancreatic islets, stimulates insulin secretion and increases citrate production in the prostate. A specific case is made for PRL in the human breast and adipose tissue, where it acts as a circulating hormone and an autocrine or paracrine factor. Aside from its actions on reproductive processes, prolactin plays a role in maintaining the constancy of the internal environment by regulation of the immune system, osmotic balance, and angiogenesis, Actions of prolactin on luteal function depend on species and the stage of the estrous cycle. In rodents, prolactin can either be luteotrophic after mating or luteo- lytic in the absence of a mating stimulus (Akers, 2017).

PRL is a hormone/cytokine responsible for the coordination of a wide range of biological processes in vertebrates. In the mouse, rat, cow, and likely other mammalian species, there are large families of paralogous genes closely related to PRL (Soares and Linzer , 2001). The proteins encoded by the PRL family genes have been given a variety of names, including placental lactogens (PLs), PRL-like proteins, PRL-related proteins, proliferin, and PLFrelated protein. Unfortunately, in some instances, the literature contains nomenclature that is confusing and/or incorrect. Members of the PRL family are expressed in celland temporal-specific patterns in the uteroplacental compartment and anterior pituitary (Soares and Linzer , 2001). An overriding theme characteristic of the PRL family is its association with pregnancy and regulatory mechanisms controlling viviparity. The initial identification of a substance extracted from the anterior pituitary possessing actions on the mammary gland and the initiation of lactation and its subsequent naming as PRL occurred over 70 yr ago (Toft and Linzer , 2000)The notion that there may be other hormones related to PRL developed from studies with the pregnant mouse and pregnant rat. Removal of the anterior pituitary during the second half of gestation in the mouse or rat was consistent with continued development of the mammary glands and corpus luteum (Colosi *et al* ., 1987). Their relationship with PRL was apparent following the cloning and sequence analyses of PRL cDNAs and the initial PL cDNAs , Existence of an expanded PRL family became evident during a search for mRNAs in fibroblasts whose expression was dependent upon growth factor stimulation and as a byproduct of cloning rat placental lactogen (PL)-II (Wiemers *et al* ., 2003).

2.6.1. Sites of Synthesis and Secretion of prolactin

2.6.1.1. Anterior Pituitary Gland

The cells of the anterior pituitary gland which synthesize and secrete prolactin were initially described by light microscopy using conventional staining techniques (Freeman *etal* ., 2000). These cells, designated lactotrophs or mammotrophs, comprise 20–50% of the cellular population of the anterior pituitary gland depending on the sex and physiological status of the animal. Lactotrophs were subsequently identified unequivocally by immunocytochemistry in the anterior pituitary gland of the mouse , rat and human using speciesspecific prolactin antibodies. Ontogenetically, lactotrophs descend from the pituitary dependent lineage of pituitary cells, together with somatotrophs and thyrotrophs (Gonzalez-Parra *et al* ., 1996). The morphology and distribution of lactotrophs have been best described in the rat, where prolactincontaining cells are sparsely distributed in the lateroventral portion of the anterior lobe and are present as a band adjacent to the intermediate lobe (Freeman *et al*., 2000). Their shapes are heterogeneous, appearing as either polyhedral or angular but at times rounded or oval (De *et al* ., 1997). With the use of either velocity sedimentation at unit gravity or discontinuous percoll gradients to separate cell populations, it has been shown that lactotrophs vary based on their secretory granule size and content as well as on the amount of prolactin and prolactin mRNA present (Freeman *et al.,* 2000).

Functional heterogeneity of lactotrophs aside from morphological heterogeneity, lactotrophs display functional heterogeneity as well. Development of the reverse hemolytic plaque assay led to a more precise description of functional heterogeneity in lactotrophs (Luque *et al* ., 1986). Although prolactin is largely found and secreted from a distinct cell type in the pituitary gland, the lactotroph, both prolactin and growth hormone can also be secreted from the intermediate cell population called mammosomatotrophs (Frawley *et al* ., 1985). These bifunctional cells, which predominate in the pituitary of neonatal rats, differentiate into lactotrophs in the presence of estrogen (Boockfor *et al* ., 1986).

Mammosomatotrophs also differentiate into lactotrophs in pups in the presence of a maternal signal that appears in early lactation and is delivered to the pups through the mother's milk (Freeman *et al*.,2000).

There also appears to be functional heterogeneity among lactotrophs with regard to their regional distribution within the anterior lobe as well as to the nature of their

responsiveness to secretagogues; that is, lactotrophs from the outer zone of the anterior lobe respond greater to thyrotrophin releasing hormone than those of the inner zone, adjacent to the intermediate lobe of the pituitary gland. On the other hand, dopamine-responsive lactotrophs are more abundant in the inner than the outer zone of the anterior pituitary. Surprisingly, functional heterogeneity is also reflected in the discordance between prolactin gene transcription and prolactin release in some lactotroph p lations (Castan *et al* ., 1997). Taken together, it is clear that lactotrophs are not homogeneous in their morphology, hormonal phenotype, distribution, or function (Freeman *et al*., 2000) .

2.6.1.2. Brain

The first observation that prolactin is produced in the brain was by (Fuxe *et al*., 1977) who found prolactin immunoreactivity in hypothalamic axon terminals. Prolactin immunoreactivity was subsequently found in the telencephalon in the cerebral cortex, hippocampus, amygdala, septum , caudate putamen , brain stem, cerebellum , spinal cord, choroid plexi, and the circumventricular organs (Freeman *et al*., 2000).

2.6.1.2. 1. Hypothalamus

Prolactin immunoreactivity is found within numerous hypothalamic areas in a variety of mammals (Griffond *et al* ., 1993). Within the rat hypothalamus, prolactin immunoreactivity is detectable in the dorsomedial, ventromedial (Griffond *et al* ., 1994), supraoptic, and paraventricular nuclei. Several approaches have been taken to prove that prolactin found in the hypothalamus is synthesized locally, independent of prolactin synthesis in the pituitary gland. Indeed, hypophysectomy has no effect on the amount of immunoreactive prolactin in the male hypothalamus and only diminishes but does not abolish the quantity of immunoreactive prolactin in the female rat hypothalamus (Emanuele *et al* ., 1992). With the use of conventional peptide mapping and sequencing of a polymerase chain reaction product of hypothalamic cDNA from intact and hypophysectomized rats (1882), it has been established that the primary structure of prolactin of hypothalamic and pituitary origin is identical. Thus it seems that the prolactin gene expressed in the rat hypothalamus is identical to the prolactin gene of the anterior pituitary (Freeman *et al*., 2000).

2.6.1.2.2. Regulation of hypothalamic prolactin synthesis: Some well-established stimulators of pituitary prolactin secretion also affect hypothalamic prolactin production. For example, ovarian steroids modulate hypothalamic synthesis and release of prolactin (Devito *et al* ., 1992). Suggesting that these neurons have estrogen receptors. Ovariectomy lowers hypothalamic prolactin content, whereas estrogen replacement elevates it (Freeman *et al*., 2000).

2.6.1.3. Placenta, Amnion and Uterus

The placenta, in addition to its bidirectional fetomaternal metabolic transport functions, has a wide array of endocrine functions as well. Among its many secretory products are a family of placental lactogens found in the rat, mouse, hamster, cow, pig, and human (Shida *et al* ., 1992). The rat placenta produces a bewildering array of prolactin-like molecules that bear structural similarity to pituitary prolactin (Linzer and Fisher, 1999). Progesterone has also been identified as a potent stimulator of decidual prolactin production. Finally, the nonpregnant uterus has been shown to be a source of prolactin (Freeman *et al*., 2000).

2.6.1.4. Mammary Gland and Milk

Prolactin can be detected in epithelial cells of the lactating mammary gland as well as in the milk itself (Grosvenor and Keenan , 1992). There is little doubt that a portion of the prolactin found in the milk originates in the pituitary gland and reaches the mammary gland through the circulation. Thus some of the prolactin found in milk is taken up rather than produced by the mammary epithelial cells , apparently, prolactin reaches the milk by first crossing the mammary epithelial cell basement membrane, attaches to a specific prolactin binding protein within the mammary epithelial cell, and is ultimately transported by exocytosis through the apical membrane into the alveolar lumen. In addition to uptake of prolactin from the blood, the mammary epithelial cells of lactating animals are capable of synthesizing prolactin. The presence of prolactin mRNA as well as synthesis of immunoreactive prolactin by mammary epithelial cells of lactating rats (Freeman *et al*.,2000).

2.7.Prolactin gene and structure

Prolactin, also known as lactotropin, is a polypeptide hormone (Skorupski and Kmieć, 2012).

PRL gene is located on chromosome 17 in rats and chromosome 23 and comprises five exons and 4 introns. spanning a 10 kb genomic segment and encodes a 199 amino acid mature protein in cattle (Uddin *et al.,* 2013). Previously several polymorphic sites have been detected within PRL gene and statistically significant associations between PRL variants and milk production traits (Dong *et al*., 2013).

Based on its genetic, structural, binding and functional properties, prolactin belongs to the prolactin/growth hormone/placental lactogen family [group I of the helix bundle protein hormones (WojdakMaksymiec *et al*., 2008). Significant associations between PRL variants and milk production (Weikard *et al*., 2005 ; Akyuz *et al*., 2012). Several genes are involved in milk production. Among them, casein is the main component of the total milk yield. The main milk protein of all mammals made up of the four casein ins (Alipanah *et al.,* 2008).

The rat preprolactin gene contains at least two intervening sequences, one of which is 597 nucleotides in length and whose sequence was determined. The intervening sequences were found to contain DNA sequences repeated elsewhere in the rat genome (Wiemers *et al* ., 2003).

2.7.1.Prolactin gene Structure:

Genes encoding prolactin, growth hormone, and placental lactogen evolved from a common ancestral gene by gene duplication (Forsyth and Wallis, 2002).

The divergence of the prolactin and growth hormone lineages occurred ;400 million years ago (Takahashi *et al*., 2013). Transcription of the prolactin gene is regulated by two independent promoter regions. The proximal 5,000-bp region directs pituitary-specific expression , while a more upstream promoter region is responsible for extrapituitary expression. The human prolactin cDNA is 914 nucleotides long and contains a 681-nucleotide open reading frame encoding the prolactin prohormone of 227 amino acids. The signal peptide contains 28 amino acids; thus the mature human prolactin is composed of 199 amino acids. The prolactin molecule is arranged in a single chain of amino acids with three intramolecular disulfide bonds between six cysteine residues (Cys^{4}-Cys^{11}, Cys^{58}-Cys^{174}, and Cys^{191}-Cys^{199} in humans). The sequence homology can vary from the striking 97% among primates to as low as 56% between primates and rodents. In rats and mice, pituitary prolactin consists of 197

amino acids, whereas in sheep, pigs, cattle, and humans it consists of 199 amino acids with a molecular mass of ~23,000 Da (Marc *et al*., 2000).

Study on the secondary structure of prolactin have shown that 50% of the amino acid chain is arranged in a-helices, while the rest of it forms loops (Baumbach *et al*., 2020).

2.8.Galactagogues

Galactagogues are medications that aid in initiating, maintaining, and augmenting of adequate milk production. The term galactagogue refers to substances that augment established lactation, whereas the term galactopoietic is used independently to describe the hormone preparations which enhance milk production in an animal already in lactation (Gabay, 2002) . Galactagogues may be synthetic, plant-derived(because these are easily available, cheap and with a hope that they may not leave any toxic residues in milk). The use of plant-derived products to improve performance and health of animals and humans has gained popularity in recent years,or endogenous products. They act through exerting an influence on adreno-hypothalamo-hypophyseal-gonadal axis by blocking hypothalamic dopaminergic receptors or by inhibiting dopamine producing neurons. These medications increase prolactin secretion by antagonizing dopamine receptors (Mohanty *et al* ., 2014).

2.9.Silymarin

Silymarin (SLM) is seeds of milk thistle (Silybum marianum L. Gaertn, Asteraceae) have been used in medicine for over 2,000 years. SLM the main active flavonoid extract in the dried fruits of *S. marianum* and has been widely used in the treatment of various liver diseases, anti-inflammatory and antioxidant properties (Gupta *et al*., 2000; Giese, 2001; Wu *et al*., 2011). Silymarin is a complex mixture of the following four flavonolignans: silybin, isosilybin, silydianin, and silychristin. Among these compounds, silybin is the major and most active component of silymarin; it is responsible for silymarin's pharmacological activity (Ghosh *et al*., 2010). Silymarin has many advantages for treating liver disorders, including its ability to inhibit hepatotoxin binding to receptor sites and to protect the liver against injury, its ability to reduce glutathione oxidation to enhance its own levels in the liver, and its

ability to stabilize the cell membrane of the liver and increase hepatocyte protein synthesis. Furthermore, silymarin can be used to prevent diabetes syndrome and atheroma by decreasing blood lipids and inhibiting platelet aggregation (Dixit *et al* ., 2007; Sonali *et al*., 2010; Farmer *et al* ., 2014).

The silymarin is well tolerated and has relatively few adverse effects. However, the effectiveness of silymarin has been discounted due to its poor water solubility and low bioavailability after oral administration (Theodosiou *et al*.,2014). The absorption of silymarin by the gastrointestinal tract is only between 20–50% due to its poor water solubility and partial degradation by gastric fluid, both of which limit its application (Li *et al.,* 2010) . To overcome these problems and improve silymarin's bioavailability, many approaches have been investigated, including incorporating it into solid dispersion systems (Sonali *et al* ., 2010) ; forming polyhydroxyphenyl chromanone salts, soluble derivatives and complexes with phospholipids (Zhang *et al* ., 2010) ; encapsulating it into liposomes (Elmowafy *et al* ., 2013); and solubilising it in self-microemulsifying drug-delivery systems (Wu *et al* ., 2006) and nanoparticles (Wang *et al* ., 2015).

The active component of silymarin is silybin and its concentration can vary greatly between silymarin extracts, thereby potentially affecting its biological effects. Nevertheless, silymarin extracts were reported to increase milk yield in lactating cows (Tedesco *et al*., 2004) and women (Kroll *et al*., 2007) , and to enhance bovine and murine mammary cell proliferation as well as β-casein gene expression (Starvaggi Cucuzza *et al*., 2010). The observed beneficial effects of silymarin on lactation performance could be linked to increased prolactin concentrations because silymarin significantly increased prolactin in female rats, and this effect was achieved in part through dopamine receptors (Capasso *et al*., 2009; Wu *et al*., 2011;Farmer *et al* ., 2017).

There are many possible mechanisms by which silymarin can improve the antioxidant defense mechanisms and oxidative status (Gabrielova *et al.,* 2015; Surai, 2015; Feng *et al.,* 2016), and its beneficial effect in protecting against systemic oxidative stress was recently demonstrated in late-pregnant animal (Farmer *et al*., 2014). It is likely that the dose of silymarin needed to elicit antioxidant effects is lower than that required to stimulate milk yield (Farmer *et al* .,2017) .

Silymarin's hepatoprotective effects are accomplished by several mechanisms which include antioxidant, inhibition of lipid peroxidation, enhanced hepatocyte regeneration, enhanced liver detoxification and protection from glutathione depletion, anti-inflammatory effects including inhibition of leukotriene, prostaglandin synthesis and Kupffer cells, mast cell stabilization. slowing of fibrosis by reducing conversion of hepatic stellate cells (Starvaggi Cucuzza *et al*., 2010) .

2.9.1.structure of silymarin

Various flavonoids are made and stored in milk thistle fruits, the amount of which varies and depends on location, climate, and the type of plant. The main composition of the plant is a mixture of flavonolignans, generally called silymarin, which has very strong antioxidant effects (Attia *et al*., 2019).

The major flavonolignans in silymarin is silybin, which accounts for 50%, followed by sily chrysanthemum at 20%, silydianin at 10%, and isosilibine at 5% (Kordi *et al.,* 2013). Silydianin levels are higher in plant stems and seed compounds (Shaker *et al*., 2010). Not only does it conjugate harmful free radicals, but it also suppresses pre-inflammatory responses resulting from increased levels of transforming growth factor beta-1 and tumour necrosis factor alpha (Pradeep *et al*., 2007).

After oral administration, the seed extract is absorbed at a rate of about 20% – 50%. However, phosphatidylcholine complexes have higher absorption (Schandalik *et al*., 1992). Flavonoids and antioxidants in plants such as milk thistle can play an important role in improving the body’s immune system, as the vitamin content of medicinal plants and the presence of iron are effective in increasing the level of haematopoiesis (Khazaei *et al*., 2021).

This plant has flavonoid and antinutritional compounds of nitrate and phenol such as tannin (Hervas *et al.,* 2003). Tannins can bind to extracellular enzymes. Therefore, substances such as hemicellulose, whose digestion is dependent on extracellular enzymes, are more affected by tannin (Salem *et al*., 2005) The presence of tannins in milk thistle may also affect protein digestion. Tannins and fatty acids in milk thistle can be a limiting factor in feed consumption (Khazaei *et al*., 2022).

The main component of the silymarin is silybinin, which is a mixture of 2 diastereomers, A and B (1:1 proportion) (Fig2-3). Other flavonolignans in silymarin, namely isosilibin, dehydrosilibin, silychristin, silydianin and taxifolin, are all mainly found in the seeds of Silybum marianum (Křen and Walterová, 2005 ; Radko and Cybulski, 2007).

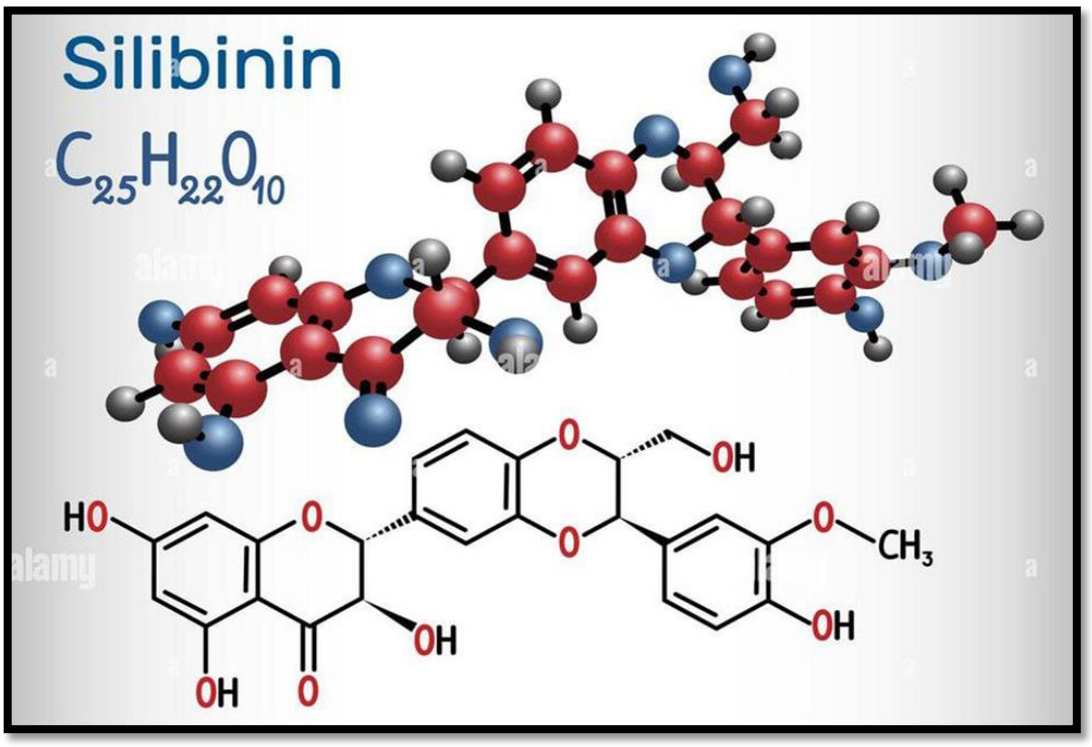


Figure (2-3) Chemical structure of silybinin (Radko and Cybulski,2007)

2.9.2. Pharmacokinetics

Silymarin is insoluble in water and is often administered in a capsulated form. It is absorbed orally, with peak plasma concentration in 6-8 hr. But, the oral absorption of silymarin is only about 23-47% leading to low bioavailability. The poor water solubility and bioavailability led to the development of enhanced formulations like silipide (Siliphos) a complex of silymarin and phosphatidylcholine which is ten times more bioavailable (Khazaei *et al*., 2021). Silymarin is usually administered in capsule form with a standard extract containing 70-80% silymarin. Pharmacokinetic studies have shown that there is low absorption of silybinin after an oral dose. Peak plasma concentrations are reached after 2 h and the elimination t1/2 is 6-8 h. Elimination takes place in the urine (3-8%) after an oral dose, while 20-40% is recovered from the bile as glucuronide and sulfate conjugates. Silybinin reaches peak levels in bile within

2-9 h, and biliary excretion continues for 24 h after a single dose (Fraschini *et al*.,2002; Filburn *et al*.,2007).

Moreover, silymarin is also devoid of embryotoxic potential. efforts have been made to increase its oral bioavailability by combining its active component , silybinin, with phosphatidylcholine, with liposomes containing variables amount of cholesterol and phospholipids, or with lipid microspheres formed by an internal oils core, surfactans (e.g. soybean lecithin) and different co-surfactants (e.g. propylene glycol) (Maheshwari *et al*.,2003 ; Crocenzi and Roma, 2006).

2.9.3. Indication

In the United States, milk thistle is most commonly used to treat viral infections and cirrhosis of the liver. Milk thistle (Silybum marianum) was used in classical Greece to treat liver and gallbladder diseases and to protect the liver against toxins. It recently has been investigated for use as a cytoprotectant, an anticarcinogen, and a supportive treatment for liver damage from Amanita phalloides poisoning. Its active ingredient is silymarin, found primarily in the seeds. Silymarin undergoes enterohepatic recirculation, which results in higher concentrations in liver cells than in serum. It is made up of components called flavonolignans, the most common being silybin (Abrol et al., 2004). Study in laboratory animals have shown the therapeutic effect of milk thistle in diseases caused by high blood lipids (fat), vascular obstruction and atherosclerosis plaque formation ,toxicity and kidney disorders , drug poisoning , liver disorders ,feed poisoning, chemical toxicity, viral diseases, neurological disorders(Khazaei *etal*.,2022).

Chapter Three: Methodology

3. Methodology:

3.1. Chemicals and Kits

Table (3.1) Used chemicals according to the company and origin

No	Materials	Company	Origin
1	Agarose	Promega	USA
2	Aluminum and potassium sulphate	BDH	England
3	CAT ELIZA Kit	monobind	USA
4	Chloroform	BDH	England
5	D.P.X	Thomas Baker	India
6	Dako EnVision detection immunohistochemistry kit	Dako	Denmark
7	Easy-spin™ (DNA free) total RNA extraction Kit	Intron	Korea
8	Eosin Stain	Himedia Lab Put. Ltd	India
9	Estrogen ELIZA Kit	monobind	USA
10	Ethanol	Labort	India
11	Ethidium Bromide	Promega	USA
12	Formalin	BDH	England
13	Glacial Acetic Acid	BDH	England
14	GoTaq® 1-Step RT-qPCR System	Promega	USA
15	GSH ELIZA Kit	monobind	USA
16	Hematoxylin Stain	Himedia Lab Put. Ltd Company	India
17	Isopropanol	Labort	India
18	MDA ELIZA Kit	monobind	USA
19	Normal saline	Labort	India
20	Nuclease free water	Bioneer	Korea
21	Paraffin Wax	Histo- Line Lab,OWax	Italy
22	Progesterone ELISA Kit	monobind	USA
23	Primers	Macrogen	Korea
24	Red Mercuric oxide	BDH	England
25	RNase free water	ABM	Canada
26	Silymarin	Bio-Botanica Inc.	USA
27	SOD ELIZA Kit	monobind	USA
28	Xylene	Scharlau	Spain

3 . 2. Devices and instruments

Table (3.2) the instruments and devices used in present study with manufacture company and Origin

NO.	Devices	Company	Origin
1	Autoclave	TOMY® Vertical Autoclave	Germany
2	Balance	Sartorius	Germany
3	Beaker	HAILAO	Italy
4	Centrifuge	Heraeus Christ	Germany
5	Cold Eppendorf Centrifuge	Hermle	Germany
6	Compound Light microscope with camera	MEIJI	Japan
7	Cylinder	Boeco	Italy
8	digital camer	Mettler	Germany
9	Disposable Syringes	Medical ject	S.A.R.
10	Dissecting tool	S.I.E.	Pakistan
11	Dry microtubes incubator	ae	UK
12	ELISA - Reader and washer	BioTek	USA
13	Eppendorf centrifuge	Fisons	England
14	Eppendorf tube	----------	---------
15	Filter Paper	Turck 0.33 Zelpa	Belgium
16	Hot Plate	Lassco	India
17	Incubator	Memmert	Germany
18	Jell test tube	Gold Star	Jordan
19	Kern PFB balance	Kern & Sohn	Germany
20	Latex gloves	Great glove	Malaysia
21	Liquid nitrogen	Rockefeller	USA
22	Microcentrifuge tubes	Eppendorf	Germany
23	Microfuge IB Centrifuge	Beckman Coulter	Germany
24	Mixer	Exispin	Korea
25	Mx3005P Stratagene Real-Time system	Agilent	USA
26	Oven	Daihan- Lab. Tech	Korea
27	Pipette tips and Pipette filter tips	Axy Gen	USA
28	Pyrex	Volac	England
29	Refrigerator	concord	lebanon
30	Rotary Microtome	Histo-Line Lab. Mod.MRS 3500	Italy
31	Sensitive Balance	Sartorius	Germany
32	Slides and cover slip	China MHECO	China
33	Spectrophotometer	Apple 203	Japan
34	Staining Gar	Harshman	Germany
35	Water Bath	Memmert	Germany

3.3. physiology examination methods

3.3.1. Experimental protocol

Twelve female rats and six male rats were used for insemination only ,weight of rat (190-250 g) and their ages between (8 -10 weeks) and were placed in the animal house of the College of Veterinary Medicine / University of Karbala in special plastic cages and provided the animals with the appropriate conditions In terms of temperature around (25 ±5 C°) and ventilation and The light system was 14/10 hrs light/dark cycle with a relative humidity of 50±5%. They were kept for 2 weeks for adaptation with standard experimental condition.

3.3.1. 1.Experimental Design

Two sexually mature females were placed with one male in each cage throughout the night, and it was confirmed that mating took place the next morning through gross examination and observation of the presence of the vaginal plug (Nau, 1992). The vaginal plug consists of a mixture of secretions of the vesicular glands and glands Coagulatory glands for the male, as this substance appears after 16-24 hours and remains about 48 hours. The percentage of dependence on this method to ensure pregnancy ranges from 80-90%, after which the females who owned the vaginal plug were isolated and the day on which they were observed is considered the zero day of Pregnancy and the next day is the first day of pregnancy (Waterman, 1976), or vaginal swabs were taken to detect the presence of male sperm in the vaginal swab, as its presence in the swab was considered a sign of pregnancy (Eveline *et al*., 2002). And the female that gave a positive result (the presence of male sperm in the vaginal swab) was taught and isolated in a cage alone provided with water and fodder for the purpose of conducting subsequent experiments on it.

Twelve lactating female rats were randomly divided into two groups (6 each group) and treated as follows for ten days.

1. Control Group:- Animals in this group were intubated orally distal water .

2. Silymarin Group:- female rats of this group were intubated orally silymarin (200 mg\kg\day) (1-10day) lactation period (Farmer *et al* ., 2017).

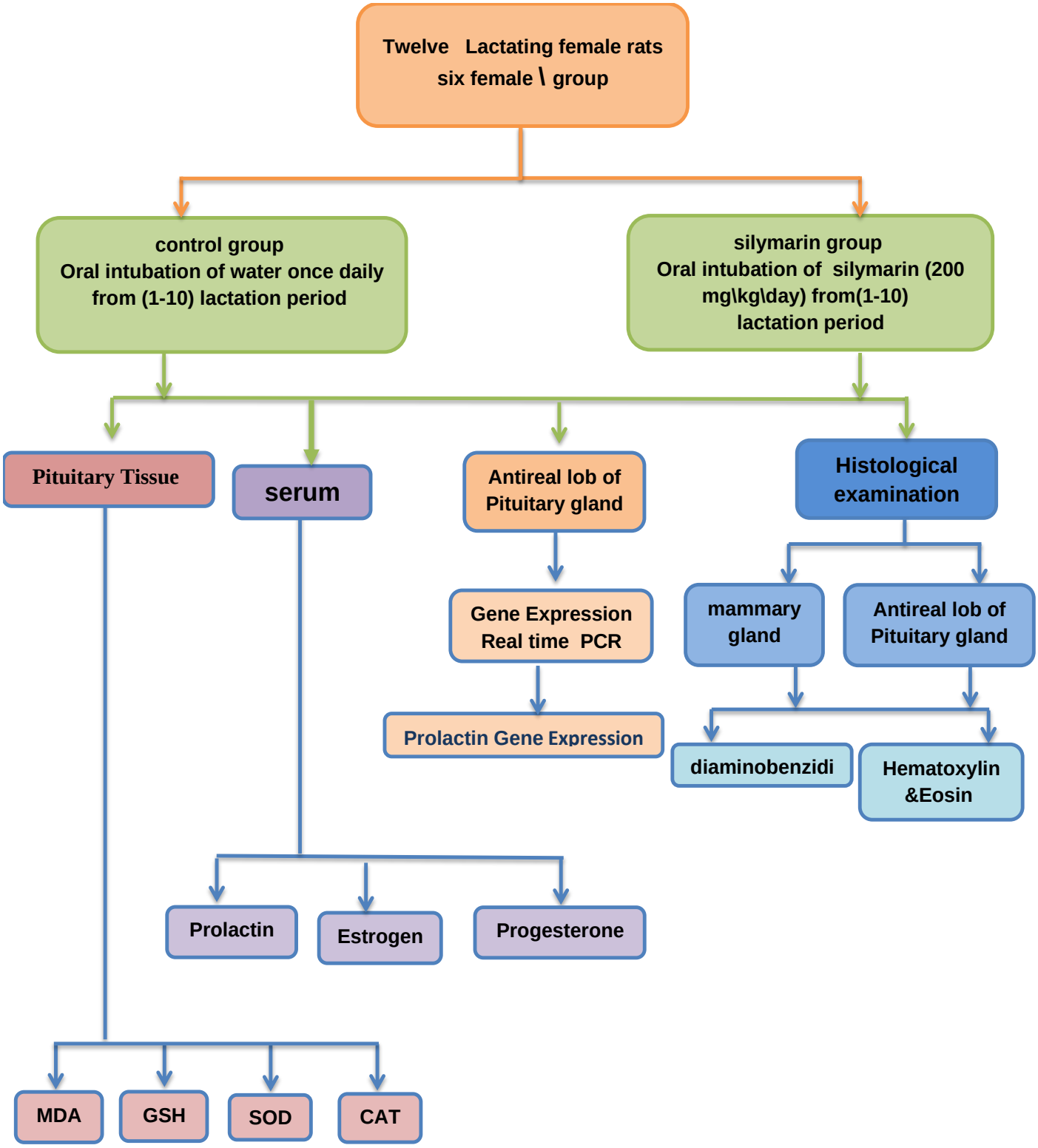


Figure (3-1) Experimental design

3.3.2. Collect of the blood samples

Blood samples were drawn after starving the animals throughout the night after ten days of the experiment taken six sample from each group, the animals anesthetized by chloroform inhalation in order to control and calm the animal before the blood draw. Where 3 ml of blood was drawn from the heart by means of a heart puncture and sterile medical syringes of 3 ml were used, then the blood was placed in gel tubes not containing an anticoagulant, the serum was separated by a centrifuge at a speed of 3000 rpm / min for 15 minutes, and the sera were kept in a freeze at - 20 ° C until complete the measurements.

3.3.3. Collect of the tissue samples

Weighed tissue samples were homogenized in 200 m1 of 0.1 n perchloric acid for 150 second with squishers to ensure that the tissue was evenly distributed the homogenates were then centrifuged for 30 minutes in a cool environment 4◦C and the supernatants were carefully removed the supernatants can either be taken straight for analysis or refrigerated at -80◦ C at this point calculating the glutamate content of pituitary tissue (rat pituitary tissue was evaluated using the ELISA technique) (Kit *et al*., 2011) .

3.3.4. Collection of tissue samples for RNA extraction

Samples were collected after the animals were killed. The pituitary gland was taken placed in liquid nitrogen in order to be preserved and transported to the laboratory for extraction and examination.

3.3.5. Organs collection for histological section

After the end of the experiment, animals (female rats) were sacrificed by anesthesia using chloroform, and the animals were dissected to remove sections (mammary and pituitary gland), and each of them was isolated separately, and the organs were preserved in formalin at a concentration of 10% in clean plastic containers after numbering them for until perform the histological section. The two stain, eosin and hematoxylin, were prepared by following the steps recommended by the researcher and the steps used for staining according to the method (Suvarna *et al*., 2013).

3.4 . Measurement of biochemical parameters

3.4.1. Estimation of prolactin hormone in the serum

The level of the prolactin hormone was measured using a kit specific to measure the hormone level depending on the method of using kit according to the instructions of the producing company , as shown (appendix I) .

3.4.2.Estimation of Estrogen Hormone Concentration in the serum

The level of the estrogen hormone was measured using a Kit Specific to measure the hormone level depending on the method of using Kit According to the instructions of the producing company , as shown (appendix II).

3.4.3. Estimation of Progesterone Hormone in the serum

The level of the progesterone hormone was measured using a kit specific to measure the hormone level depending on the method of using Kit According to the instructions of the producing company , as shown (appendix III).

3.4.4. Determination of tissue MDA concentration

Measure the CAT depending on the method shown in (appendix IV).

3.4.5. Determination of tissue GSH concentration

GSH was measured following the method in (appendix V).

3.4.6. Estimation of tissue superoxide dismutase activity

Measure SOD concentration depending on the method (Younus, 2018) , shown in (appendix VI).

3.4.7. Estimation of tissue Catalase activity

Measure the CAT depending on the method shown in (appendix VII).

3.5.Gene Expiration

3.5.1.RNA extraction

Total RNA extraction using Easy-spin™ (DNA free) total RNA extraction Kit , as shown (appendix VIII) .

3.5.2.Preparation of primers

According to instruction of the primer synthesizer company, the primers (originally lyophilized), were dissolved in the free ddH_2O to obtain a final concentration of 100 pM/µl which served as a stock solution that stored at -20 ^{o}C. A

concentration of 10 pM/µl was prepared from the stock primers to be used as a work primer.

3.5.3.Primers used in this study

Table (3-3) Primers of gene expression experiment

Target gene	Primer name	5'-3'	PCR Product	Reference	Accession number
Prolactin	F	CCTGAAGACAAGGAACAAGCC	344 bp	Iwasaka *et al.*, 2000	XM_03288 5212.1
	R	TGGGAATCCCTGCGCAGGCA			
CYPA	F	TATCTGCACTGCCAAGACTGAGT G	127 bp	Peinnequi n *et al.*, 2004	M19533
	R	CTTCTTGCTGGTCTTGCCATTCC			

3.6. Immunohistochemistry

The stain with immunohistochemistry for detection of apoptosis by use kit, as shown (appendix IX).

3.7. Histological section method

The samples (mammary and pituitary gland) the organs were preserved in formalin at a concentration of 10% in clean plastic containers after numbering them for until perform the histological section.

3.7.1. Dehydration and Clearing

Water was withdrawn from the tissue by passing it through a series of ascending consternation of alcohol (70%, 80%, 90%, 95%, and 100%) for two hours for each concentration. And after that the tissue placed in xylene for 5 min .

3.7.2. Infiltration

After completion of the lamination process, the samples were transferred to glass bottles containing a mixture of Paraffin wax with a melting point of 57-60 °C, molten, filter and xylene at a ratio of 1:1 for half an hour inside an electric oven at a temperature of 60 °C in order to keep the wax melted and to ensure the complete impregnation process For wax models, they were transferred to other bottles containing paraffin wax inside the oven for two hours, then transferred again to other bottles containing paraffin wax for two hours as well.

3.7.3. Embedding

Wax molds were made to contain the samples by pouring the wax into special iron molds in which the models were buried and left at the laboratory temperature to harden, then separated from the mold and preserved until the time of cutting.

3.7.4. Sectioning

A rotary manual microtome was used to cut the models with a thickness of 5 micrometers, then the sections tapes were loaded onto clean glass slides after they were placed in a water bath at a temperature of 45-50 ° C for a period of one minute - two minutes to ensure that the sections were brushed and then left on a hot plate to dry at 37°C .

3.7.5. Staining

The following special dyes were used to color the tissue sections of pituitary gland and mammary gland .

3.7.5.1. Harris' Hematoxylin

The stainer was prepared and based on (Suvarna *et al*., 2013), as illustrated in appendix X.

3.7.5.2. Eosin stain

The stainer was prepared based on (Suvarna *et al*., 2013) as illustrated in appendix XI.

3.8. Statistical analysis

Data are reported as means ± standard error of the mean and data were normally distributed, as tested using the D'Agostino and Pearson normality test. Statistical significance between two conditions (e.g. control vs. treated) was determined using Student's t-test. The data were analyzed using Graph Pad Prism Version 9.0 for Windows and the criterion for statistical significance is ($P < 0.05$) (Ghetti *et al* ., 2009).

Chapter Four: Results and Analysis

4. Results and Analysis

4.1. Effect of Silymarin on some serum hormones concentration during the lactation period on the rats

4.1.1. Effect of Silymarin on serum prolactin hormone concentration during the lactation period on the rats

The result from the figure (4-1) showed oral intubation of (200 mg\kg\day) for ten days of silymarin for lactating rats showed a significant ($P<0.05$) increase in the serum level prolactin hormone (211.16 ±9.00) compare with control lactating rats (31.66 ± 1.20) .

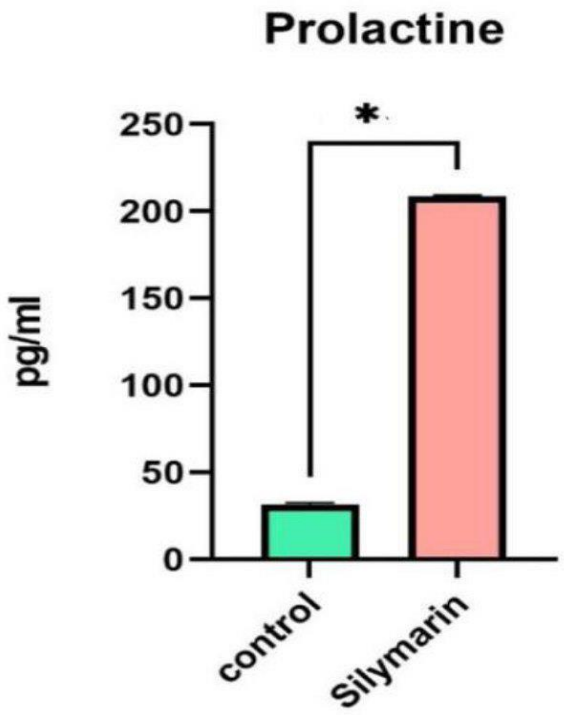


Figure (4-1) Effect of oral intubation of (200 mg\kg\day) Silymarin on serum prolactin hormone (Pg/ml) during the lactating period in the rats

expressed as mean ± SE. n=6/ group control= considered as control group. Silymarin = rats of this group were intubated orally silymarin (200 mg\kg\day) (1-10day) postnatal (* $P \leq 0.05$) refer to values significantly difference

4.1.2. Effect of Silymarin on serum estrogen hormone concentration during the lactation period on the rats

Lactating rats were intubated silymarin for ten days showed a significant ($P<0.05$) increase in the serum estrogen hormone (97.41 ± 16.86) compere with lactating rats of control group (74.41 ± 21.45) . Figure (4-2).

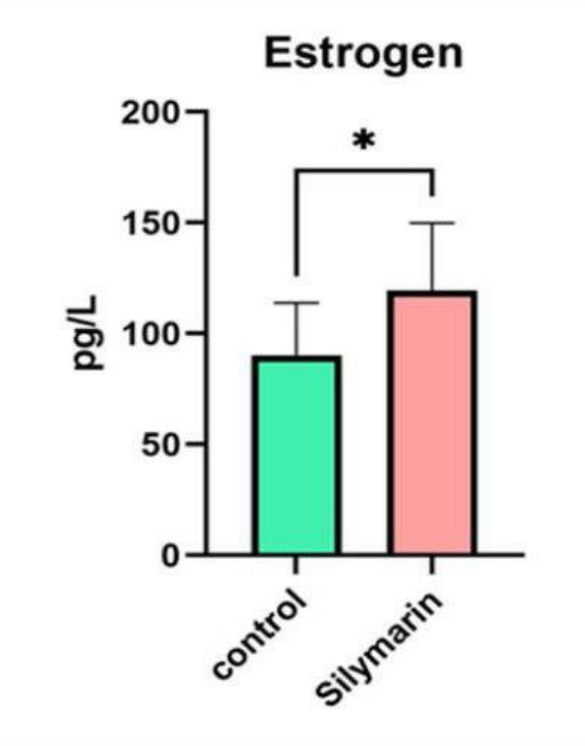


Figure (4-2) Effect of oral intubation of (200 mg\kg\day) Silymarin on serum estrogen hormone (pg/L) during the lactating period in the rats

expressed as mean ± SE. n=6/ group control= considered as control group. silymarin = rats of this group were intubated orally silymarin (200 mg\kg\day) (1-10day) postnatal (* $P \leq 0.05$) refer to values significantly difference

4.1.3. Effect of Silymarin on serum progesterone hormone concentration during the lactation period on the rats:

Figure (4-3) showed a significant decrease ($P \leq 0.05$) in serum progesterone hormone level in the lactating rats received (200 mg\kg\day) for ten days (10.78 ± 2.04) compared with control group (18.13 ± 1.99).

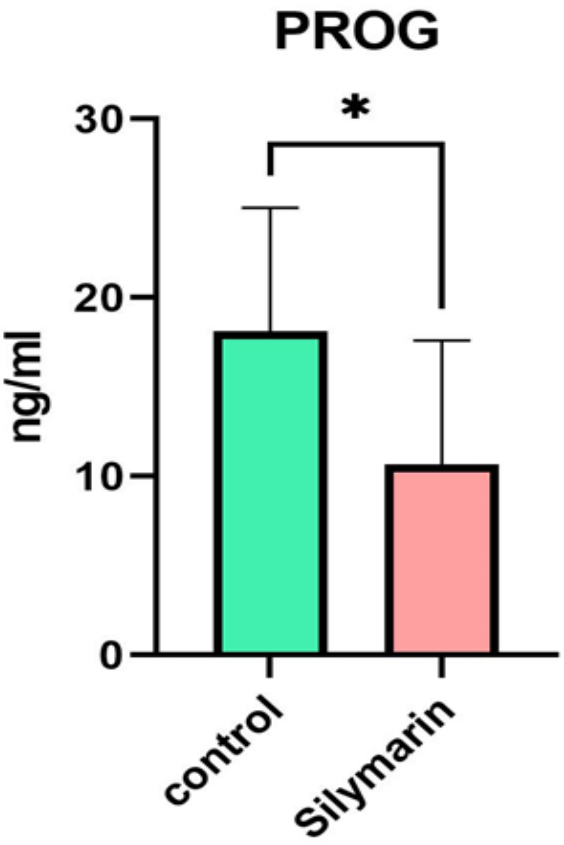


Figure (4-3) Effect of oral intubation of (200 mg\kg\day) Silymarin on serum progesterone hormone (ng/ml) during the lactating period in the rats

expressed as mean ± SE. n=6/ group control= considered as control group. silymarin = rats of this group were intubated orally silymarin (200 mg\kg\day) (1-10day) postnatal (* P ≤ 0.05) refer to values non significantly difference

4.2. Effect of Silymarin on the pituitary tissue oxidant and antioxidant parameter during the lactation period on the rats

4.2.1. Effect of Silymarin on the pituitary tissue of MDA concentration during lactation period on the rats

Figure (4-4) illustrated the significant decrease (P≤0.05) of MDA concentration in the pituitary tissue during the lactation period in the group treated with (200 mg\kg\day) silymarin for ten days (12.30 ± 1.38) compared with group received normal saline only (17.84 ± 1.40) .

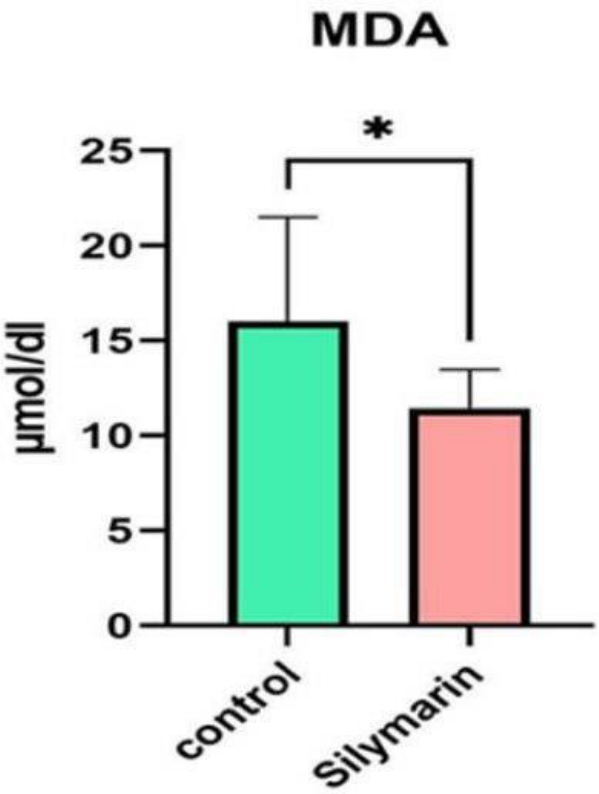


Figure (4-4) Effect of oral intubation of (200 mg\kg\day) Silymarin on MDA (µmol/dl) during the lactating period in the rats

expressed as mean ± SE. n=6/ group control= considered as control group. silymarin = rats of this group were intubated orally silymarin (200 mg\kg\day) (1-10day) postnatal. (* P ≤ 0.05) refer to values non significantly difference

4.2.2. Effect of Silymarin on the pituitary tissue of some antioxidant parameter during lactation period on the rats:

4.2.2.1. Effect of Silymarin on the pituitary tissue of GSH concentration during lactation period on the rats:

Oral intubation of (200 mg\kg\day) silymarin for ten days showed a significant increases (P≤0.05) in the GSH concentration in the pituitary tissue (16.01 ± 1.58) compared with control lactating rats (11.43 ± 0.59) Figure (4-5) .

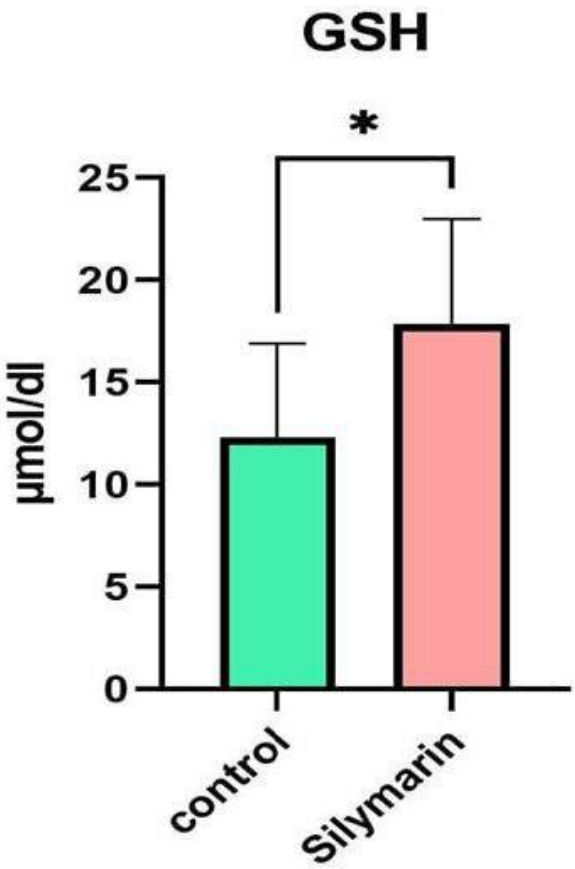


Figure (4-5) Effect of oral intubation of (200 mg\kg\day) Silymarin on GSH(µmol /dl) during the lactating period in the rats

expressed as mean ± SE. n=6/ group control= considered as control group. silymarin = rats of this group were intubated orally silymarin (200 mg\kg\day) (1-10day) postnatal. (* P ≤ 0.05) refer to values significantly difference

4.2.2.2. Effect of Silymarin on the pituitary tissue of SOD activity during the lactation period on the rats

Figure (4- 6) showed a significant increases (P≤0.05) in the SOD activity in the treated group with silymarin (67.85 ± 2.12) compared with lactating rats received normal saline only (42.22 ± 4.65) .

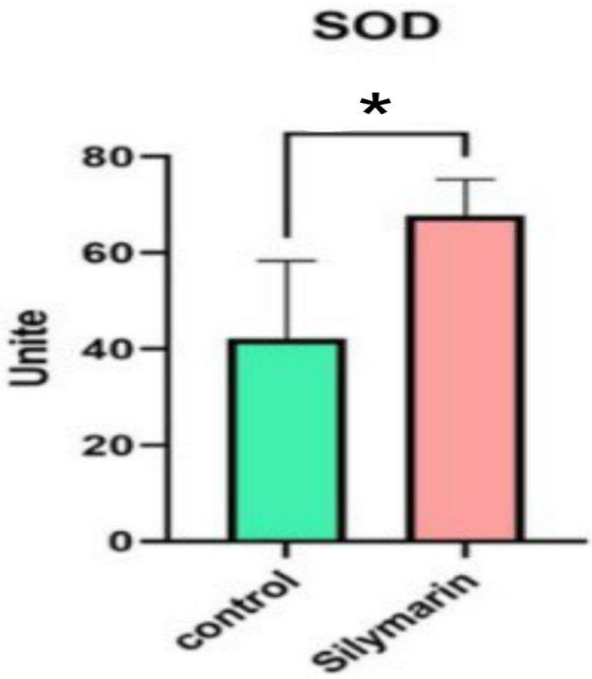


Figure (4- 6) Effect of oral intubation of (200 mg\kg\day) Silymarin on SOD (U\ mg)activity during the lactating period in the rats

expressed as mean ± SE. n=6/ group control= considered as control group. silymarin = rats of this group were intubated orally silymarin (200 mg\kg\day) (1-10day) postnatal
(* P ≤ 0.05) refers to values significantly difference

4.2.2.3. Effect of Silymarin on the pituitary tissue of CAT activity during the lactation period on the rats:

Animal received (200 mg\kg\B.W\ day) silymarin for ten days during the lactation period showed a significant increases ($p \leq 0.05$) in the CAT activity in the pituitary tissue (69.48 ± 5.12) compared with control group (56.03 ± 2.76) Figure (4- 7) .

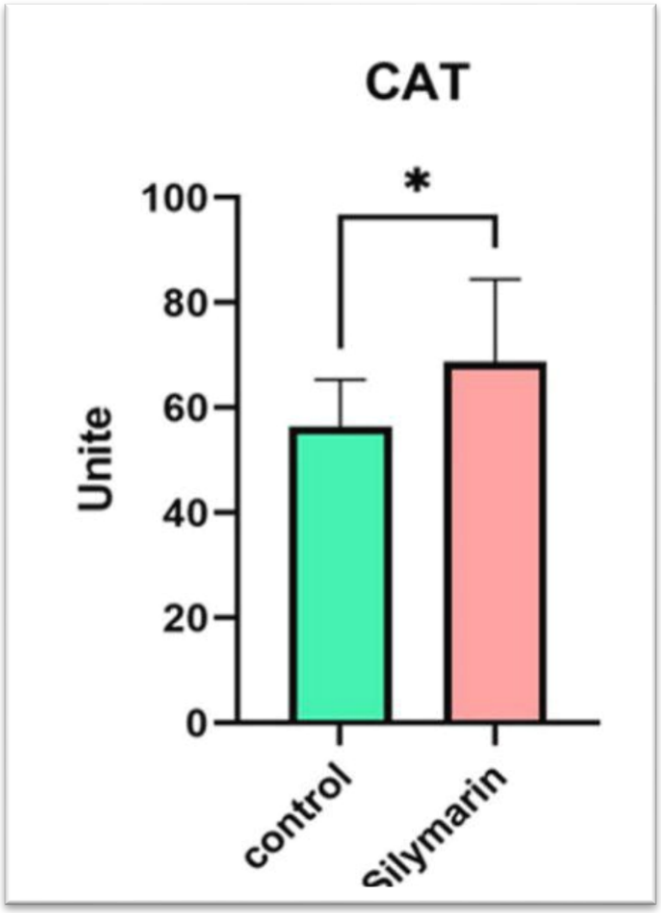


Figure (4-7) Effect of oral intubation of (200 mg\kg\day) Silymarin on CAT activity (U\mg) during the lactating period in the rats

expressed as mean ± SE. n=6/ group control= considered as control group. silymarin = rats of this group were intubated orally silymarin (200 mg\kg\day) (1-10day) postnatal. (* P ≤ 0.05) refer to values significantly difference

4.3. Effect of Silymarin on Gene expression for prolactin gene during lactation period on the rats:

Through our results shown in the figure (4-8) (4- 9), we found that when silymarin was given on a daily basis for ten days in lactation period, it increased the gene expression of the prolactin gene responsible for the production of the prolactin hormone compared to the control group.

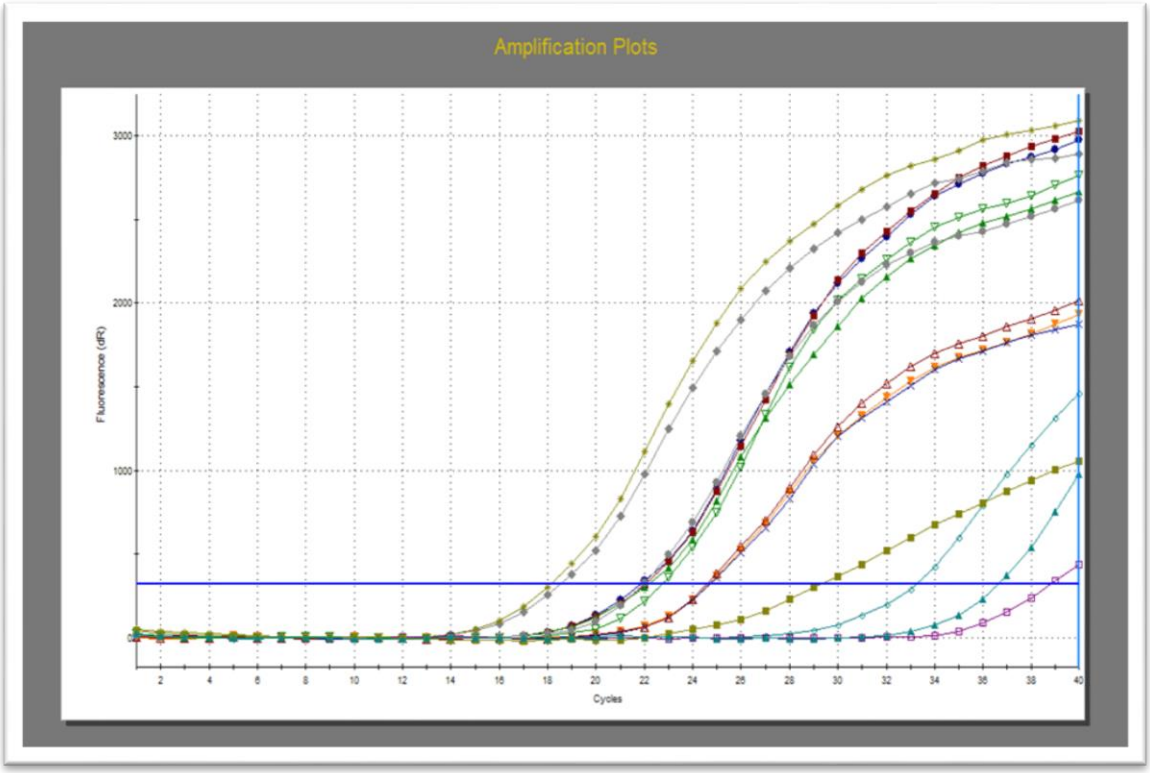


Figure (4-8): Amplification plot of two genes, Prl as a target gene; and CypA as a house-keeping gene by the Mx3005P Stratagene system

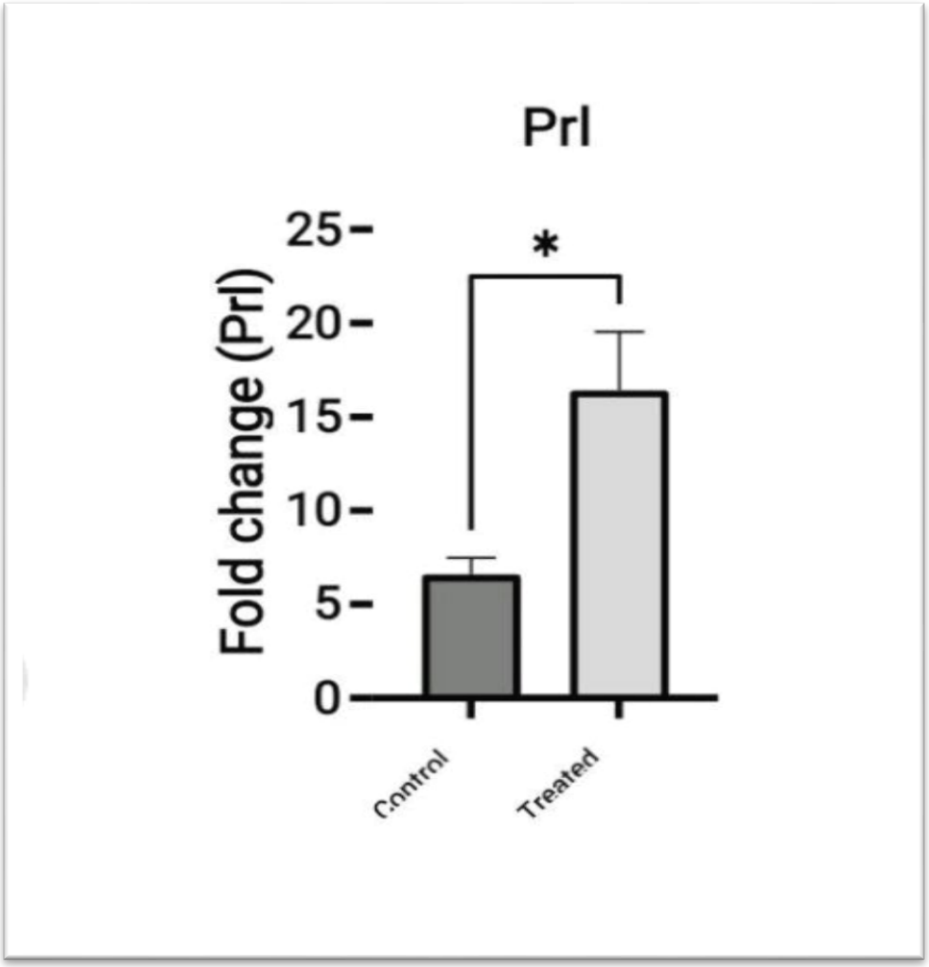


Figure (4-9) Effect of Silymarin on Gene expression for prolactin gene during lactation (* $P \leq 0.05$) refer to values significantly difference

4.4. Correlation between prolactin gene expression and some serum hormones intubated Silymarin during lactation period in the rats:

4.4.1. Correlation between prolactin gene expression and serum prolactin hormone concentration

The result of the present study indicated a positive correlation ($P<0.05$) between prolactine gene expression serum prolactin hormone in treated lactating group ($y = 5.3354x + 86.263$) ($R^2 = 0.8591$) .

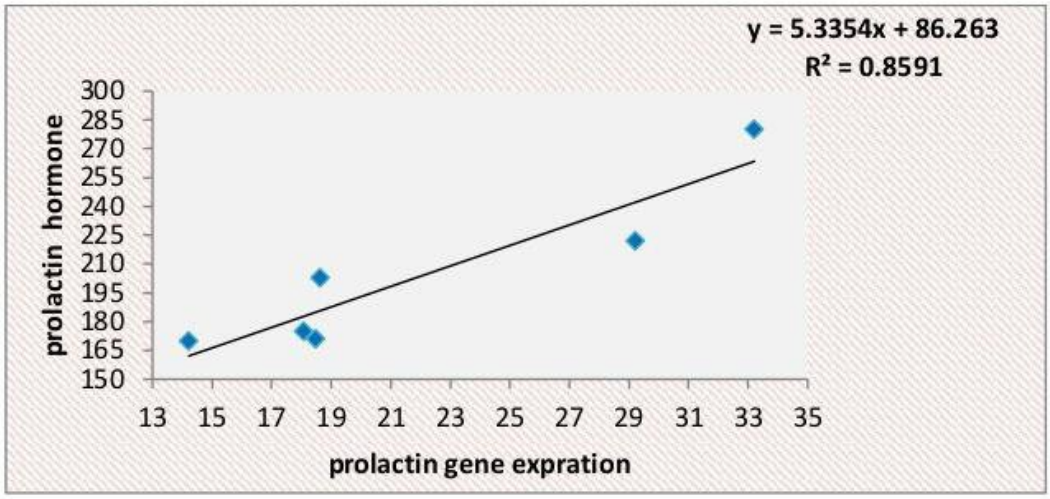


Figure (4-10) Correlation between prolactin gene expression and serum prolactin hormone in the lactating rats during lactation period

4.4.2. Correlation between prolactin gene expression and serum estrogen hormone cocentration

Figure (4-11) showed a significant positive correlation ($P<0.05$) ($y = 4.8621 x$) ($R^2 = 0.7347$) between estrogen hormone concentration and prolactine gene expression in the lactation period in the rats .

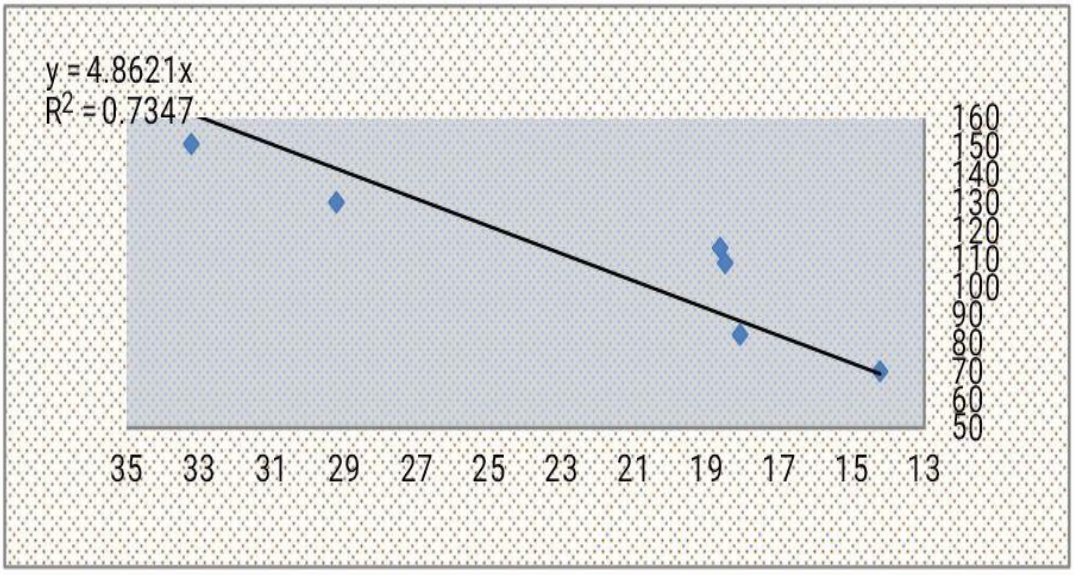


Figure (4-11) Correlation between prolactin gene expression and serum estrogen hormone in rats during lactation period

4.4.3. Correlation between prolactin gene expression and serum progesterone hormone concentration

The result of the Figure (4-12) of the present study indicated a significant negative correlation ($P<0.05$) ($y = - 0.4616 + 17.576$) ($R^2 = 0.6064$) between serum progestron hormone concentration in the animal recived silymarin during lactation period and prolactine gene expression .

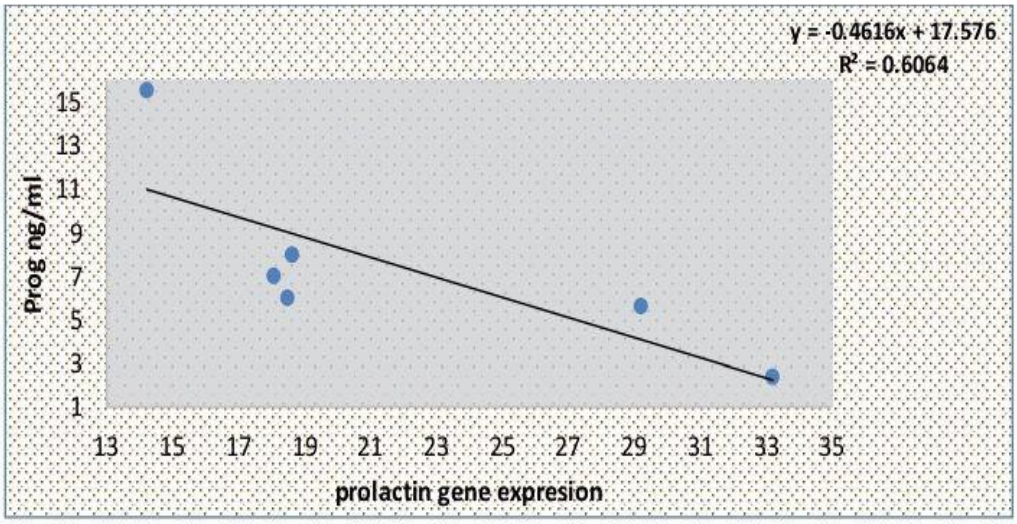


Figure (4-12) Correlation between prolactin gene expression and serum progestron hormone in rats during lactation period

4.4.4. Correlation between prolactin gene expression and GSH concentration in the pituitary tissue of lactation period in the rats

Figure (4 - 13) of The result of the present study indicated a significant positive correlation ($P < 0.05$) ($y = 0.565x + 5.6761$) ($R^2 = 0.7549$) between GSH concentration and prolactine gene expressionin the lactation period in the female rats.

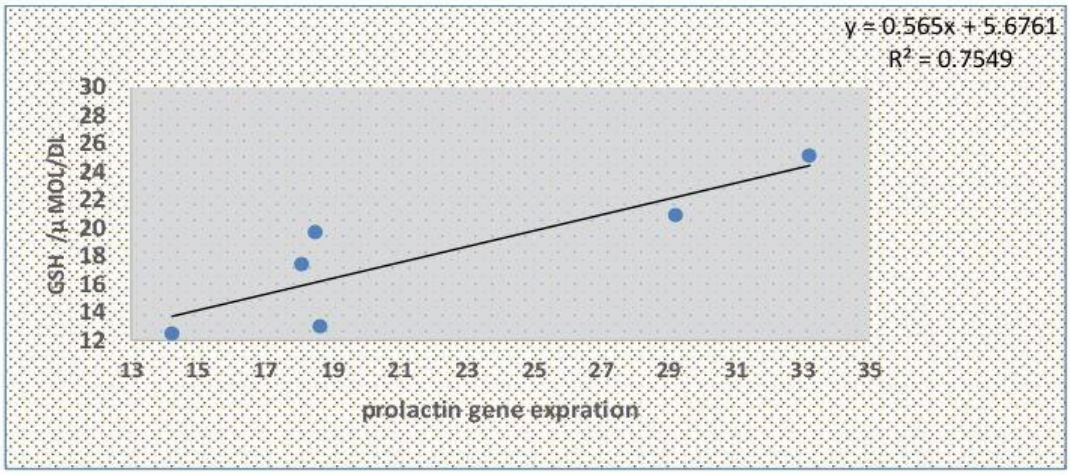


Figure (4-13) Correlation between prolactin gene expression and GSH concentration in rats during lactation period

4.4.5. Correlation between prolactin gene expression and MDA concentration in the pituitary tissue

Our result showed a significant negative correlation ($P<0.05$) ($y = -0.4603x + 21.314$) ($R^2 = 0.7399$) in the MDA concentration in the pituitary gland and prolactine gene expression in the lactating rats recived (200 mg/kg B.W\daily) for ten days of silymarin Figure (4-14).

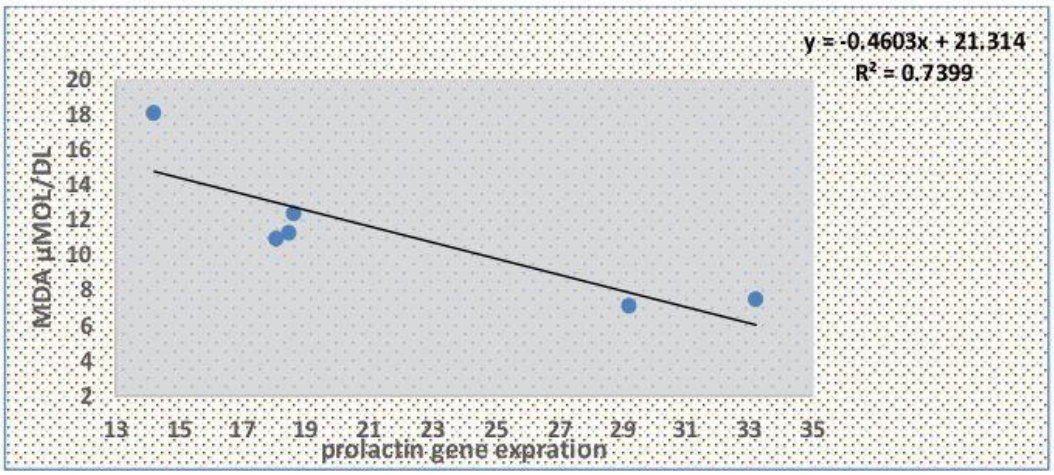


Figure (4-14) Correlation between prolactin gene expression and MDA concentration in rats during lactation period

4.4.6. Correlation between prolactin gene expression and SOD activity in pituitary gland tissue

Figure (4-15) illustented a significant positive correlation ($P<0.05$) ($y= 0.8253x + 42.85$) ($R^2 = 0.6244$) between prolactine gene expression and SOD activity in the pituitary gland tissue in the treated female rats .

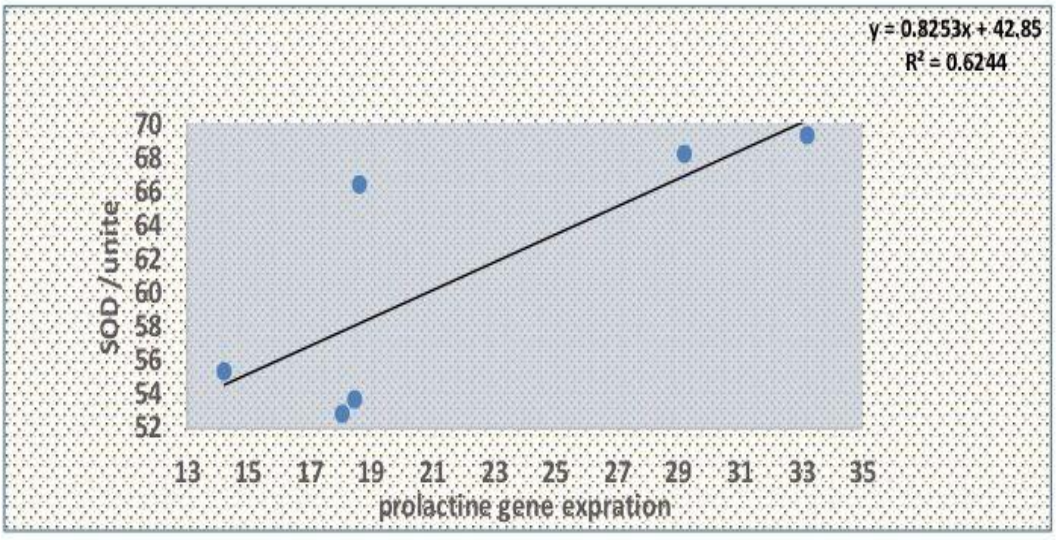


Figure (4-15) Correlation between prolactin gene expression and SOD concentration in rats during lactation period

4.4.7. Correlation between prolactin gene expression and CAT activity in pituitary gland tissue

Figure (4-16) showed a significant positive correlation ($P < 0.05$) ($y = 1.5653 x + 26.712$) ($R^2 = 0.7486$) between prolactine gene expression in the female rats recived (200 mg/kg B.W\ daily) for ten days of silymarin and CAT activity Figure (4-16) .

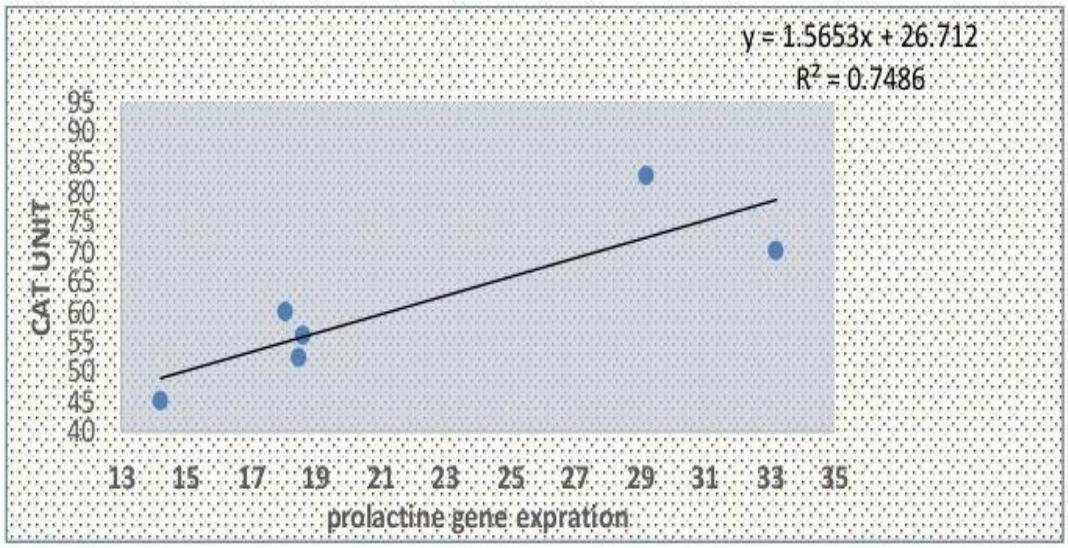


Figure (4-16)Correlation between prolactin gene expression and CAT concentration in rats during lactation period

4.4.8. Diameter of alveolar:

A substantial difference between the control group and the Silymarin groups was found in the analysis of the mean alveolar diameter in treatment groups. In completely treated groups, the mean alveolar diameter was (25 µm), whereas in the control group, it was around (15 µm).

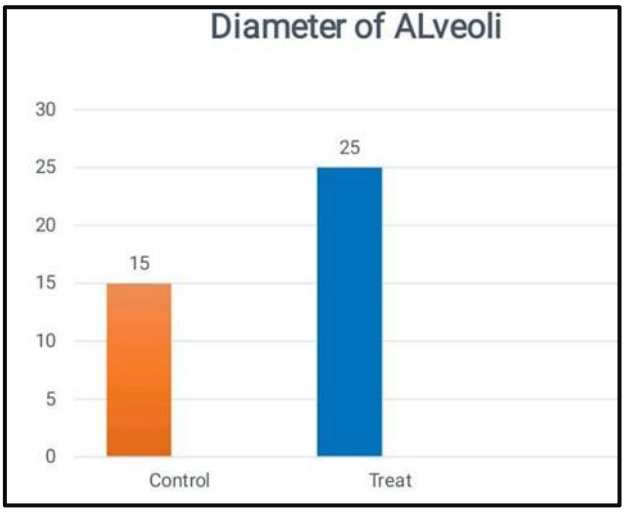


Figure (4-17) A standard chart show different diameter of alveoli between Control and treatment group

expressed as mean ± SE. n=6/ group control= considered as control group. silymarin = rats of this group were intubated orally silymarin (200 mg\kg\day) (1-10day) postnatal

4.5. Effect of Silymarin on Immunohistochemical in the Pituitary gland and mammary gland tissues on lactating rats in during lactation

The present study explain there are numerous of histological and Immunohistochemical changes occurred in pituitary and mammary glands. One of these change observed in density of lactotrophs cells in pituitary gland and activity of these cells during followed production of prolactin also, observation the mammary gland alveoli by effect of Silymarin on the target cells.

4.5.1.Pituitary glands

The pituitary gland in rats were looked as disk in shape, located in oval - conical cavity called sella turcica. The sella turcica appeared as a shallow depression in the base of the brain.

In control group the lactotrophs in pituitary gland appeared less in activity and paucity in reaction stain (Fig 4-18) while, in treated group Immunocytochemistry performed with the Prolactin showed a positive reaction throughout the anterior part of pars distalis, the lactotrophs cells appeared oval in shaped and closed to sinusoid with density in secretory granules seemed in cytoplasm and interstitial spaces (Fig 4-19 , 4-20).

4.5.2.Mammary glands

In control animal mammary glands demonstrated slight lobules distributed among amount of adipose tissue. The mammary alveoli seemed empty from milk and the alveolar cells weakly reaction by antibody of prolactin stain. In addition, the interlobular ducts it narrow and few in number, the diameter of alveoli was approximately (15 µm) (Fig 4-21).

In silymarin group showed increase in size of lobules and crowded through alveoli. Thin fibrous connective tissue that separates the mammary lobes from one another (Fig 4-22). Show overexpression of prolactin hormone in epithelial cells (Fig 4-23). Immune prolactin stain, which is brown in color, found these cells to be extremely alluring, the diameter of alveoli was approximately (25 µm) (Fig 4-22).

In silymarin group, the intra lobular duct in the treated animals had a large lumen and was bordered by simple cuboidal cells (Fig 4-24, 4-25). These alveoli and alveolar duct were reconnected to the basement membrane, and increase number of myoepithelial cells formed a flawless single layer spindle in their place (Fig 4- 26).

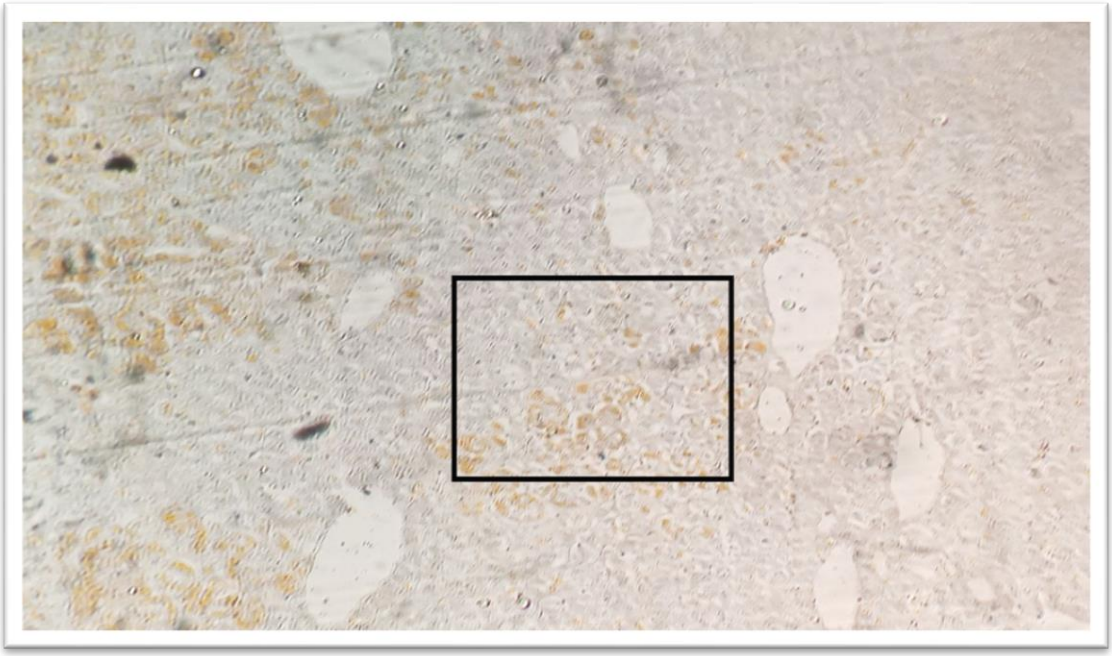


Figure (4-18) Immune histochemical section in the anterior lobe of pituitary gland of control group during lactation period , show normal expression of prolactin observed in of lactotrophs cells (Black Square). Few amount of prolactin filled in sinusoids. DAB and Hematoxylin. 100X.

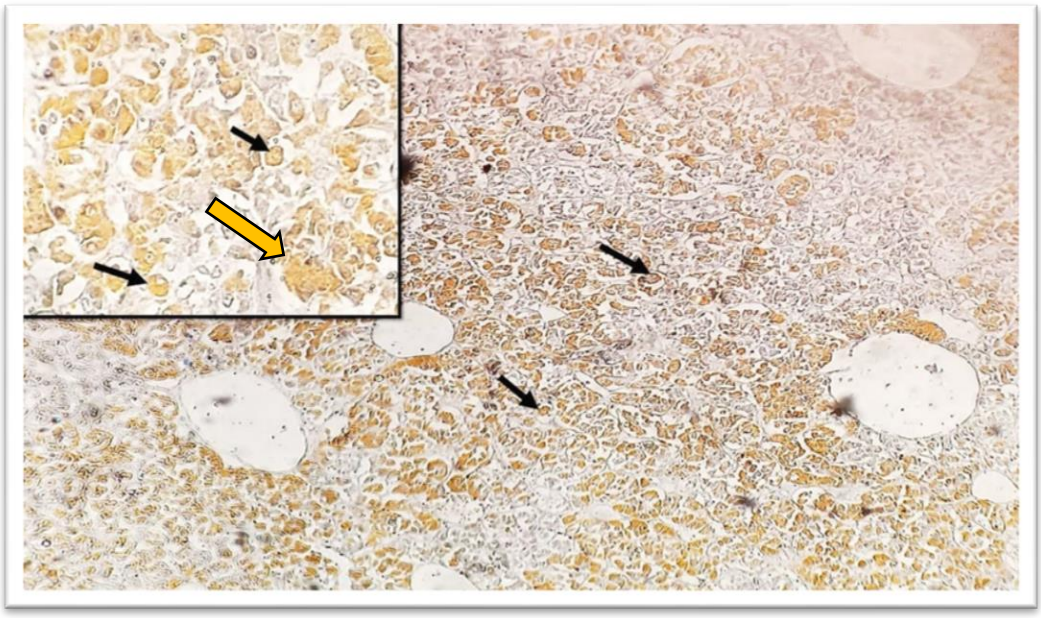


Figure (4-19) Immune histochemical section in the anterior lob of pituitary gland of silymarin group (200 mg\kg\day) during lactation period , show overexpression of prolactin observed in of lactotrophs cells (black arrows). That filled with prolactin in sinusoids (yellow arrows). DAB and Hematoxylin 100X.

Figure (4-20) Immuno histochemical section in the anterior lob of pituitary gland of silymarin group (200 mg\kg\day) during lactation period , show overexpression of prolactin observed in of lactotrophs cells (black arrows). That filled with prolactin in sinusoids (red arrows). DAB and Hematoxylin. 400X.

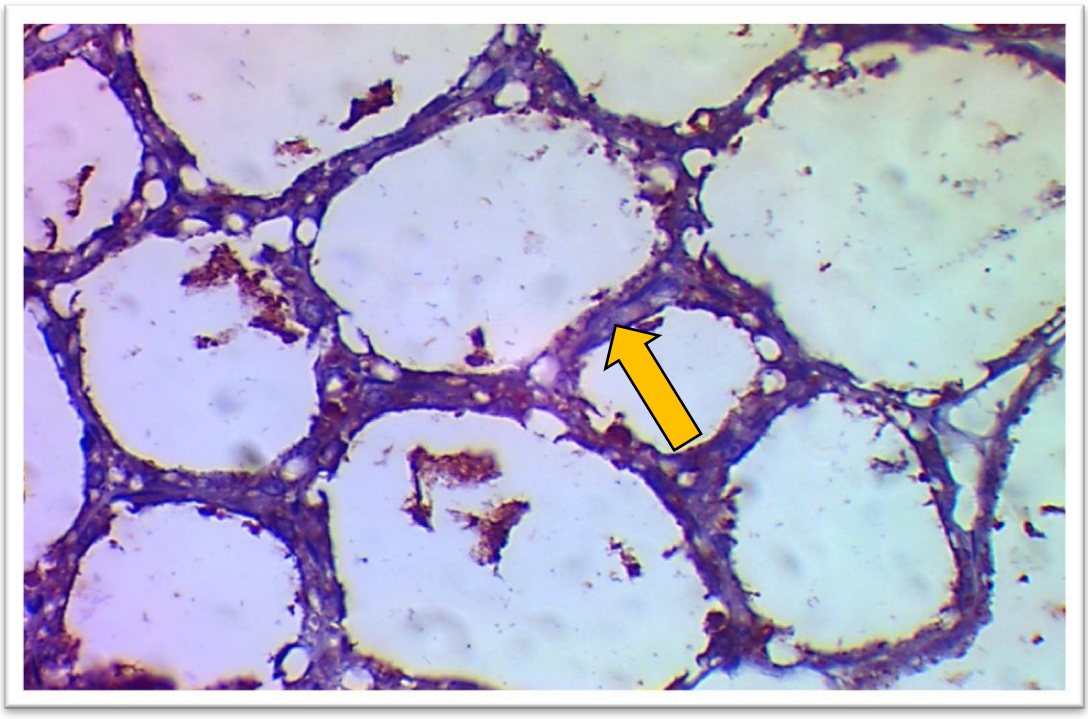


Figure (4-21) Immune histochemical section in mammary gland of control group during lactation period . show normal expression (Yellow arrows) of prolactin hormone in epithelial cells of acinus. DAB and hematoxylin. 100X.

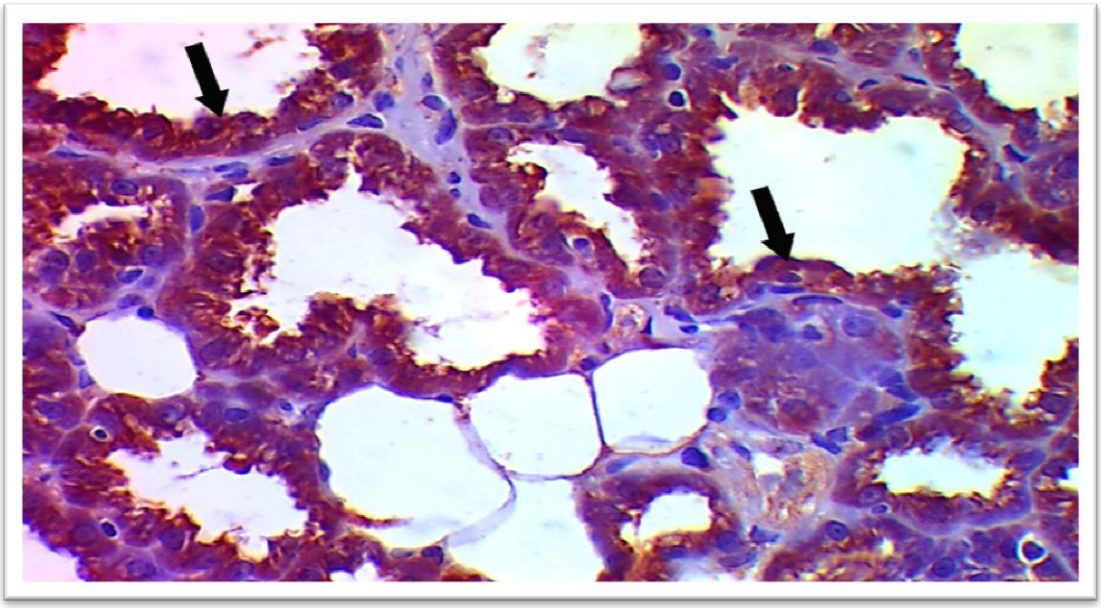


Figure (4-22 Immuno histochemical section in mammary gland of silymarin group (200 mg\kg\day) during lactation period. Show overexpression (arrow) of prolactin hormone in epithelial cells of acinus .DAB and hematoxylin. 400X.

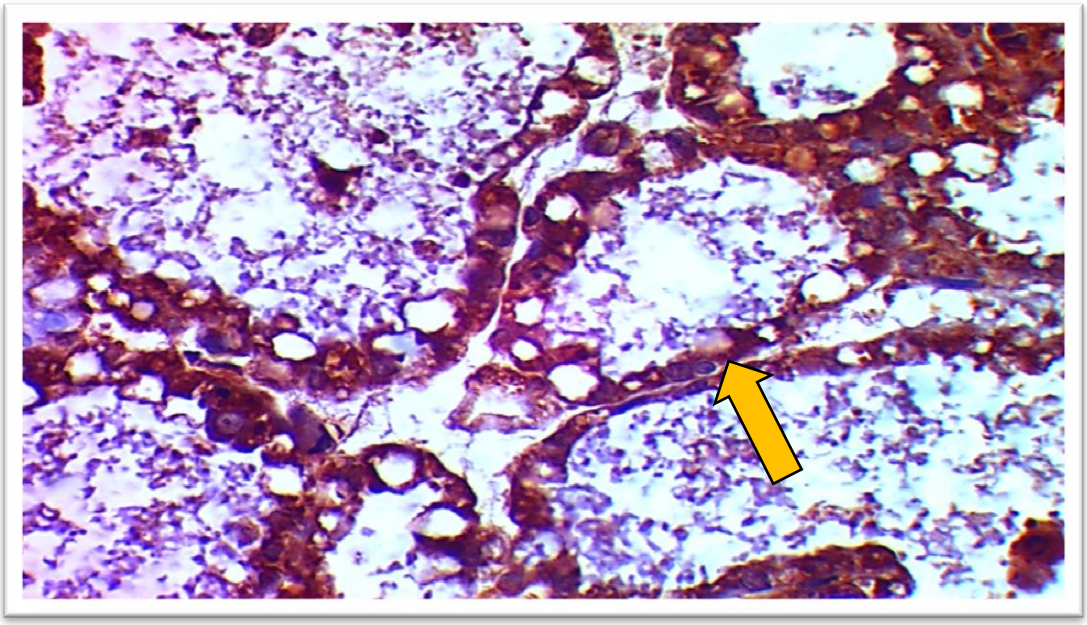


Figure (4-23) Immune histochemical section in mammary gland of silymarin group (200 mg\kg\day) during lactation period. Show overexpression (Yellow arrows) of prolactin hormone in epithelial cells of DAB and Hematoxylin. 100X.

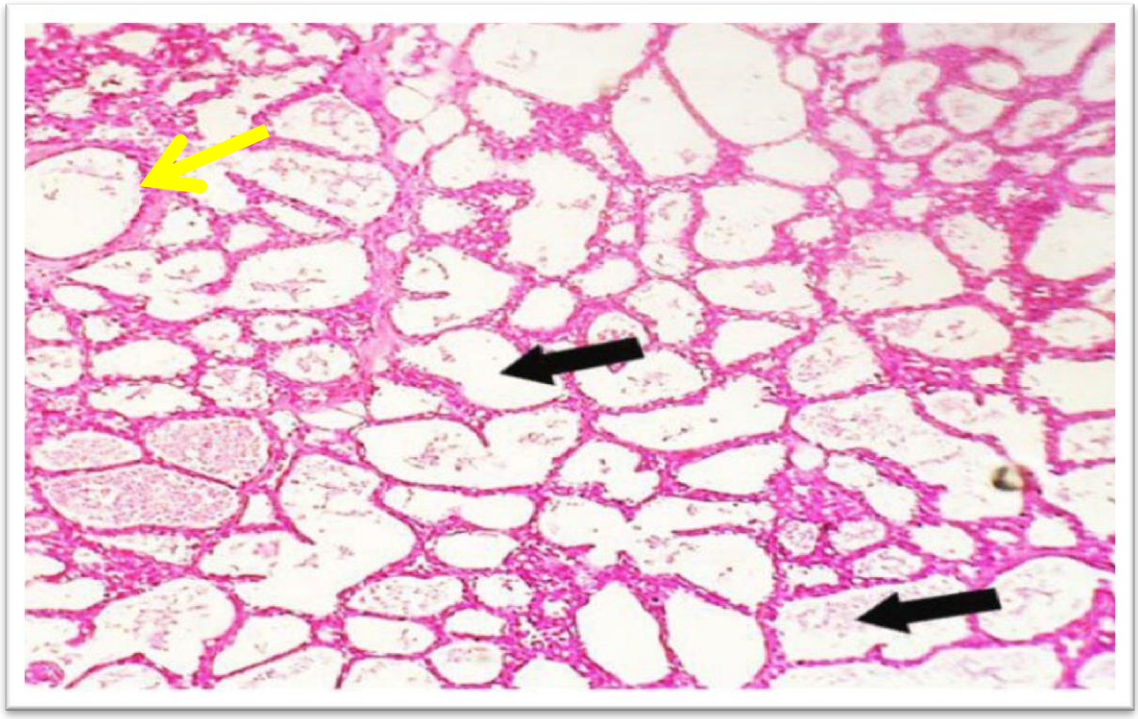


Figure (4-24) Histological section in mammary gland of control group during lactation period . That interlobular duct (Yellow arrows). Show normal quantity of milk in acini (black arrows) H&E. 100X.

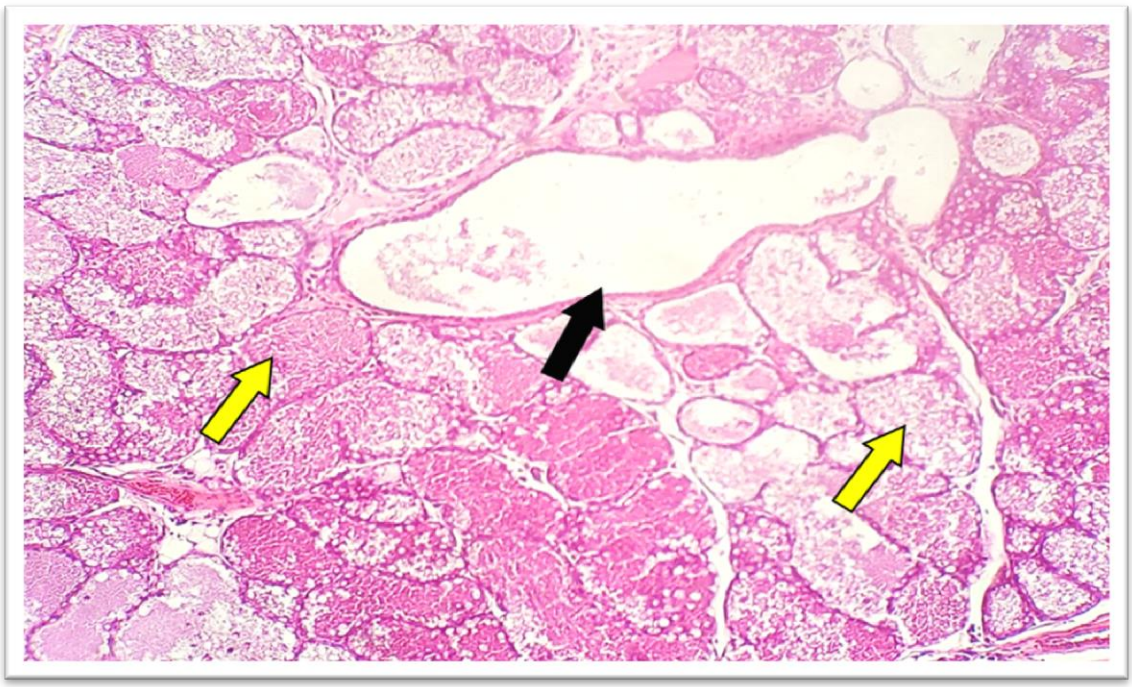


Figure (4-25) Histological section in mammary gland of silymarin group (200 mg\kg\day) during lactation period . show Acini appeared filled by milk (Yellow arrows) that opened in large interlobular duct (black arrow) H&E. 100X.

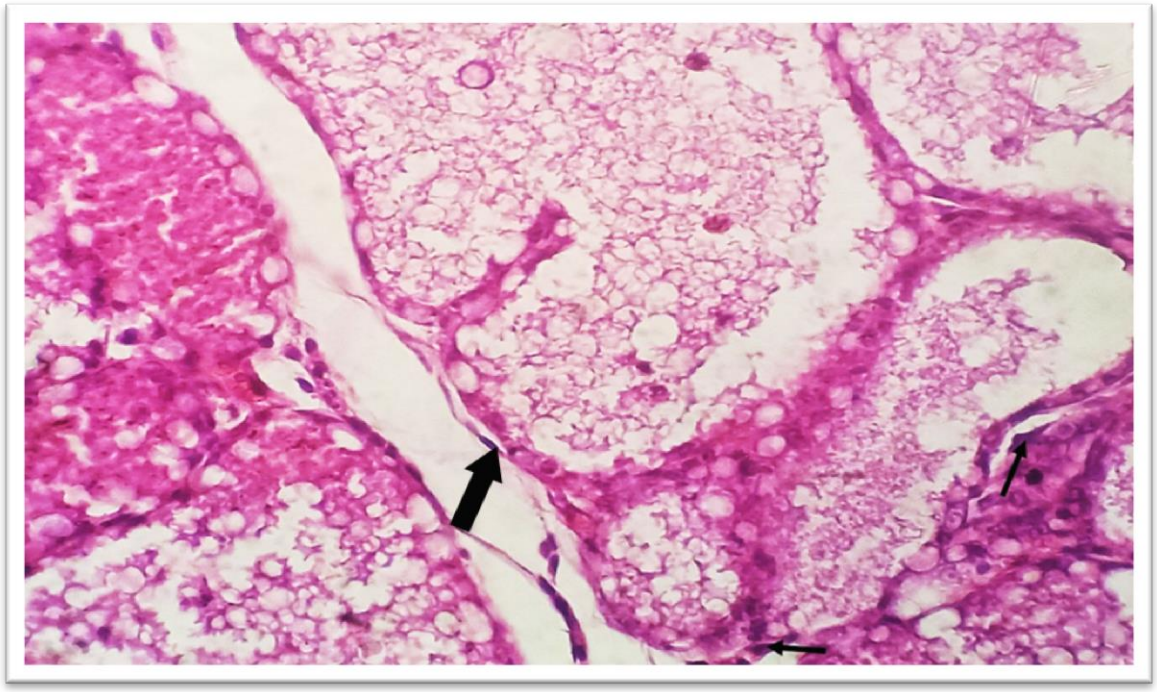


Figure (4-26) Histological section in mammary gland of silymarin group (200 mg\kg\day) during lactation period. Acini surrounded by enumerate of Ductal myoepithelial cells.H&E.400x

Chapter Five: Discussion

5. Discussion:

5.1. Effect of Silymarin on some serum hormones concentration during lactation period in the female rats

5.1.1. Effect of Silymarin on some serum prolactin hormones concentration during lactation period in the female rats

Oure results showed a significant increase in prolactin hormone in serum lactating rats intubated Silymarin (200 mg\ kg B.w\ daily) for ten days comported with control group , this result agreement with (Capasso, 2014 ; Demirci *et al* ., 2014 ; farmer *et al* ., 2014 ; Farmer *et al* ., 2017 ; Mohammad *et al* ., 2019) .

The extract of milk thistle seed increase lactation in cows (Tedesco *et al* ., 2004) and women (Carotenuto and Pierro, 2005). This observation has gained a renewed interest in its galactogenic properties (Capasso, 2014).

This may be attributed to the antidopaminergic activity of silymarin (Capasso, 2014). And the estrogenic effects of silymarin (Demirci *et al* ., 2014) . Moreover, these findings explain the positive effects of silymarin on serum prolactin during the lactation. This fits with the mammogenesis processes (The major portion of mammary growth occurs during pregnancy and is controlled by hormones) (Mohammad *et al* ., 2019).

Therefore, drugs stimulating prolactin production are generally used to enhance milk production (Zuppa *et al* ., 2010). Silymarin increase serum prolactin levels in female rats during lactation. Other studies declared that the estrogen is a positive regulator of prolactin production and release (Le Tissier *et al* ., 2015).

Therefore, Silymarin stimulating prolactin production are generally used to enhance milk production (Zuppa *et al* ., 2010).

Mammogenesis, which takes place in the third trimester of pregnancy, depends on prolactin (Farmer and Palin, 2005), and exogenous prolactin impacts the mRNA expression of both prolactin and its receptor in mammary tissue (Farmer *et al*., 2000; Farmer and Petitclerc, 2003). Recently, it was discovered that hyperprolactinemia in late-pregnant gilts stimulated mammary growth and subsequent milk production (VanKlompenberg *et al*., 2013) .

Recent research on how PRL regulation varies during breastfeeding, when it produces the most, has revealed modifications at the anterior pituitary and brain levels. In virgin animals, the anterior pituitary's PRL-secreting cells are arranged into a homotypic network that facilitates coordinated bursts of activity amongst connected PRL cells (Le Tissier *et al* ., 2015).

Prolactin and placental lactogen, which bind to prolactin receptor, act during three stages: lobulo budding during organogenesis, lobuloalevoler expansion during pregnancy, and lactational differentiation and maintenance milk secretion during lactation (Akers, 2017). Prolactin hormone produced by the pituitary gland enhances two activities in the mammary glands such as stimulation of mammary gland growth during pregnancy and stimulation of milk production while nursing infant (Ben-Jonathan *et al* ., 2006).

5.1.2. Effect of Silymarin on serum estrogen hormone concentration during lactation period in the rats

The current study revealed to intubation of (200 mg\ kg B. W\ daily) Silymarin for ten days in the lactating rats to a significant increases in the serum estrogen concentration comparted with control group , this result agreement with (Capasso, 2014 ; Demirci *et al* ., 2014 ; farmer *et al* ., 2014 ; Farmer *et al* ., 2017 ; Mohammad *et al* ., 2019)

The marked increase in the serum estrogen concentration during pregnancy enhances prolactin synthesis and secretion, and maternal prolactin serum levels increase in parallel with the enlargement of the lactotrophs (Stefaneanu *etal* ., 1992 ; Larsen, 2003)

In addition, estrogen decreases the expression of dopamine (DA-D2) receptors and number of immunoreactive lactotrophs that inhibit prolactin release (Stojilkovic *et al* ., 2017). These results could support the notion that the increment in milk production previously observed in cow and human (Carotenuto and Pierro, 2005). Our data showed that the silymarin increases the prolactin hormone concentration and activity of mammary gland and thus milk production (Capasso *et al* .,2009).

5.1.3. Effect of Silymarin on serum progesterone hormones concentration during lactation period in the rats

The current study showed decrease in serum progesterone levels in lactating female rats compare with control group , this result agreement with (Capasso, 2014 ; Demirci *et al* ., 2014 ; farmer *et al* ., 2014 ; Sun *et al* ., 2015; Farmer *et al* ., 2017 ; Mohammad *et al* ., 2019)

The findings demonstrate that progesterone levels are declining whereas estrogen levels are significantly gained in postnatal with Silymarin group comparison to the control group . This work supports the findings of (Daniel *et al* ., 2013 ; Kinn *et al* ., 2017; Mohammad *et al* ., 2019) showed a significant increase in the serum prolactin concentration and has a variety of functions, including maintaining mammary gland production, acting in tandem with androgens, and affecting androgen metabolic activity. Furthermore, many studies have found that oral administration of aqueous extracts of medicinal herbs causes a considerable drop in progesterone levels, which is consistent with our findings (Peinnequin *et al* ., 2004) .

Since structural differentiation of the mammary gland, is in the direction of hormonal balances in adult mammals, major steps of this differentiation include, sequentially, formation of a lobulo-alveolar structure. Appearance of specific secretory activity, hypertrophy of epithelial cells of mammary gland characterized by an intense synthesis and secretion of milk, is related to such hormones responsible for these changes including estrogen, progesterone, prolactin, placental lactogen as well as other hormones related to the biochemical activity of mammary alveolar secretary cells (Li *et al* ., 2016 ; Mohammad *et al* ., 2019).

5.2. Effect of Silymarin on some oxidant and antioxidant during lactation period in the rats

5.2.1. Effect of Silymarin on GSH in the pituitary gland tissue during lactation period in the rats

The current study showed a significant increases in GSH concentration in the pituitary gland tissue in lactating rats received 200 mg/kg B.W\daily for ten days silymarin group when compared with control group. These results agreement with

(Starvaggi *et al* .,2010; Khastar *et al* .,2011; farmer *et al* .,2014;Wang *et al* .,2015; Kinn *et al* ., 2017; Farmer *etal* ., 2017 ;Akbari *et al* ., 2019)

Silymarin has many advantages for treating liver disorders, including its ability to inhibit hepatotoxin binding to receptor sites and to protect the liver against injury, its ability to reduce glutathione oxidation to enhance its own levels in the liver, and its ability to stabilize the cell membrane of the liver and increase hepatocyte protein synthesis ,in addition to its anti-oxidant effect (Farmer *et al* .,2014).

Silymarin beneficial effect in protecting against systemic oxidative stress was recently demonstrated in late-pregnant animal (Farmer *et al*., 2014). It is likely that the dose of silymarin needed to elicit antioxidant effects is lower than that required to stimulate milk yield (Farmer *et al* ., 2017) .

Accumulated evidence suggests that reactive oxygen species (ROS) can be scavenged through utilizing natural antioxidant compounds present in foods and medicinal plants. In current study, silymarin an antioxidant, present in the milk of thistle Silymarin was found to be successful in upregulating the antioxidant status and lowering the apoptotic responses (Raza *et al* ., 2011).

Silymarin has a regulatory action on cellular and mitochondrial membrane permeability in association with an increase in membrane stability against xenobiotic injury (Gabrielova *et al.,* 2015). It can prevent the absorption of toxins into the hepatocytes by occupying the binding sites as well as inhibiting many transport proteins at the membrane (Radko and Cybulski, 2007). These actions, together with the antiperoxidative property, make silymarin a suitable candidate for the treatment of toxic liver diseases In in vitro studies, silymarin was a potent inhibitor of cyclic AMP (cAMP)-phosphodiesterase (Surai, 2015), Thus, an increase in the hepatic cAMP levels induced by silymarin is likely and may act as a second messenger of some beneficial effects of silymarin (stabilization of cellular membranes). Silymarin exerts strong anticancer activity against human (hepatocellular carcinoma) cells by modulating cell cycle and associated proteins, causing growth inhibition as well as apoptotic death, as it does for many other human cancer cells (prostate, skin, kidney, colon). (Leyon *et al*., 2005; Radko and Cybulski, 2007).

Silymarin was also shown to maintain an redox balance in cells by activating a range of antioxidant enzymes and non-enzymatic antioxidants via the activation of specific transcription factors (Zhao *et al*., 2015). Study showed a beneficial effect of silymarin on oxidative stress conditions. Indeed, silymarin decreased the liver protein carbonyl content, without affecting mitochondrial oxidative status and gene expression of the principal cellular antioxidants, and reduced the accumulation of circulating protein carbonyls. Similar actions of silymarin on protein carbonyls were also observed in rodents (Farmer *et al* ., 2017).

The silymarin decreased the levels of oxidative damage in liver and different tissue but that this effect was not modulated by endogenous antioxidants. Additional work is needed to fully identify the mechanisms underlying the antioxidant properties of silymarin in gestating gilts but it is evident that such effects would be highly beneficial (Feng *et al.,* 2016). Indeed, the increased liver energy metabolism and oxidative stress conditions that normally occur during the periparturient period are known to be associated with various physiological disorders as well as poor litter performance (Farmer *et al* ., 2014) .

5.2.2. Effect of Silymarin on MDA concentration in the pituitary gland tissue during lactation period in the rats

The current study showed a significant decrease in MDA in lactating rats received 200 mg/kg B.W\daily for ten days Silymarin group when compared with Lactating rats served as control group. This results agreement with (Starvaggi *et al* ., 2010; farmer *et al* ., 2014;Wang *et al* ., 2015; Kinn *et al* ., 2017; Farmer *etal* ., 2017 ;Akbari *et al* ., 2019)

Silymarin could decrease the level of MDA, while increasing antioxidant enzyme activities via scavenging free radicals and elevating antioxidant gene expression (Sun *et al* .,2015 ; Akbari *et al* ., 2019).

In the lactotrophs cell , mitochondria are remarkably dynamic organelles that are important for generating energy. Mitochondrial energy metabolism, however, is tightly associated with the production of toxic ROS that are susceptible to induce oxidative stress conditions in periods of high metabolic activity. Furthermore, due to their localization, mitochondrial enzymes are particularly vulnerable to ROS, leading

to mitochondrial dysfunction and increases in oxidative strees during critical reproductive periods in animal (Lapointe, 2014).

Silymarin used as cytoprotectant, an anticarcinogen , antiinflammatoy 50% of flavonolignan in Silymarin is silybin it is conjugate harmful free radical . flavonoid and antioxidant in Silymarin is improving the hoody is immune system (Paradeepetal ., 2007).

Taking into account that increased systemic oxidative stress is observed throughout lactation in sows (Berchieri Ronchi *et al*., 2011), that the lactation process is associated with high energy demands (Hoving *et al*., 2012), and that mitochondrial ATP production is an essential source of energy (Lapointe, 2014), it is imperative to avoid mitochondrial oxidative stress during lactation. There are many possible mechanisms by which silymarin can improve the antioxidant defense mechanisms and oxidative status (Gharagozloo *et al*., 2010). Recent studies revealed that silymarin can scavenge free radicals in the gut, prevent the formation of free radicals by inhibiting specific ROS-producing enzymes and improve the integrity of electron-transport chain of mitochondria in stress conditions (Gabrielova *et al.,* 2015; Surai, 2015; Feng *et al.,* 2016). Silymarin was also shown to maintain an optimal redox balance in cells by activating a range of antioxidant enzymes and non-enzymatic antioxidants via the activation of specific transcription factors (Zhao *et al*., 2015). The study previously carried out with gestating sows showed a beneficial effect of silymarin on oxidative stress conditions. Indeed, silymarin decreased the liver protein carbonyl content, without affecting mitochondrial oxidative status and gene expression of the principal cellular antioxidants, and reduced the accumulation of circulating protein carbonyls. Similar actions of silymarin on protein carbonyls were also observed in rodents(Farmer *et al* ., 2017) .

5.2.3. Effect of Silymarin on CAT activity in the pituitary gland tissue during lactation period in the rats

The current study showed a significant increases in CAT activity in lactating animal received (200 mg\ kg B.W \ daily) Silymarin group when compared with Lactating rats served as control group. This results agreement with (Starvaggi *et al*

.,2010; farmer *et al* .,2014; Sun *et al* .,2015 ;Wang *et al* .,2015; Kinn *et al* .,2017; Farmer *etal* .,2017 ;Akbari *et al* ., 2019)

Silymarin could improving CAT activity ((Ligeret *et al* .,2008 ; Sun *et al* .,2015). Also could increasing antioxidant enzyme activities via scavenging free radicals and elevating antioxidant gene expression (Akbari *et al* .,2019)

The endogeans enzymic such as CAT neutralize the reactive components, because of the large amount of free radicals that exceeded the body's antioxidant capacity (Khastar *et al* .,2011)

5.2.4. Effect of Silymarin on SOD activity in the pituitary gland tissue during lactation period in the rats

The current study showed a significant increases in SOD activity in the pituitary gland tissue in lactating animal received (200 mg\ kg B.w. daily) Silymarin group when compared with control group. This results agreement with (Starvaggi *et al* ., 2010; farmer *et al* .,2014;Wang *et al* .,2015; Kinn *et al* .,2017; Farmer *etal* ., 2017 ;Akbari *et al* ., 2019)

Silymarin can prevent the absorption of toxins into the hepatocytes by occupying the binding sites as well as inhibiting many transport proteins at the membrane (Radko and Cybulski, 2007). These actions, together with the antiperoxidative property, make silymarin a suitable candidate for the treatment of iatrogenic and toxic liver diseases In in vitro studies, silymarin was a potent inhibitor of cyclic AMP (cAMP)-phosphodiesterase (Leyon *et al.*,2005); thus, an increase in the hepatic cAMP levels induced by silymarin is likely and may act as a second messenger of some beneficial effects of silymarin (stabilization of cellular membranes) (Leyon *et al*.,2005; Radko and Cybulski, 2007).

5.3. Effect of Silymarin on gene expression during lactation period in the rats

The influence of Silymarin on the gene transcription of the prolactin gene in the pituitary gland was investigated via real-time PCR in this study. In compared to the control group, Silymarin significantly accelerated Prl gene expression in dosage . The focus of this study was to see how Silymarin extract affected milk supply in

breast tissue. Silymarin raised the level of prolactin in all the other mother rats in this investigation, according to the findings. The amount of prolactin hormone rises throughout pregnancy and after birth, and it has a variety of functions, including secretory activities of the lactotrophic cell in the pituitary glands and stimulation of the pituitary glands . This corresponds to the findings of (Iwasaki and Kelsall, 2000) who found that herbal extracts raised the quantity of prolactin in female mice via acting on endocrinological glands such like different regions of the breast tissues. In addition, this investigation (Dill and Walker , 2017) confirmed that herbal medication had an influence on prolactin concentration, and exogenous prolactin affects mRNA expression for both prolactin and its receptor in mammary tissue (Farmer and Palin, 2005).

5.4. Effect of Silymarin on the histological section of pituitary gland and mammary gland during lactation period in the rats

The present study pointed that intubation of female rats with Silymarin (200 mg/kg B.W/daily) for ten days, to the histological sections change of pituitary and mammary glands, showed overexpression of prolactin observed in of lactotrophs cells, that filled with prolactin in sinusoids, overexpression of prolactin hormone in epithelial cells of acinus that filled with milk . The result of the present study was agreement with (BolzÁn *et al.,* 1997; Oakes *et al* . , 2008 ; Lan *et al* . , 2017) .

The most crucial aspect of a baby's nutrition is provided through nursing; yet, one of the major problems that has to be solved is the lack of nutritious and sufficient nourishment for infants (Tombs *et al.,* 2018). Treatments are therefore required to enhance lactation and nursing. Different bodily organs may respond differently to herbal extracts (Jalili *et al.,* 2015).

Endocrine and exocrine glands, like numerous breast tissues, are one of the target organs for herbal extracts. The current study set out to look into the effects of Silymarin extract on breast tissue milk production characteristics. The current study demonstrated that Silymarin enhanced prolactin levels in the administered group to the study's mother rats. Prolactin levels rise physiologically throughout pregnancy and after birth. It has a variety of functions, including stabilizing the secretory activity of the pituitary mammary glands and milk production. Consequently, higher levels of

prolactin may be linked to increased breast milk production (Dill and Walker, 2017). The high antioxidant properties of Silymarin extract demonstrated in the present study appear to be another factor contributing to the elevated prolactin level in the animals studied, which demonstrated that antioxidants can increase the prolactin blood level (BolzÁn *et al.,* 1997).

The present study explain that the Silymarin increased the number and diameter of the alveoli in the treatment groups. This indicates that Silymarin has benefited the mammary glands. Because prolactin levels in the blood and gene expression during breastfeeding are linked to the formation of mammary lobule alveoli, these result akin with (Oakes *et al*., 2008), it appears that this rise in serum and gene expression of prolactin was accompanied by an increase in the number and width of alveoli. Which really share the same structural characteristics as estrogen; hence, the increased diameter and quantity of lobule alveoli may be linked to estrogen processes, which increase lobule alveoli via boosting prolactin levels(Oakes *et al*., 2008) .

The current study mention in treated group the lactotrophs cells in pituitary gland appeared highly activity in cytoplasm granules and heavily secretion in sinusoids while in contrast in control group appeared weakly, also the increased in number, diameter of alveoli in treatment group. These findings support Jalili *et al* 2015 .'s study, which "showed that the herbs extract for many reasons, such as the growth in diameter and number of alveoli in the breast tissue, which validates the current study's findings." The results of comparing the level of gene expression between axis pituitary- mammary tissue of the control and treatment rats showed that the significantly increased in target tissue of Silymarin treated rats.

The current study demonstrated that the treatment group of lactating rats' treated mammary glands had decreased lipid tissue and increased blood levels of prolactin, which corresponded with (Lan *et al.,* 2017). The importance of Silymarin extract in enhancing milk production characteristics in breast tissue is now further supported by the current study, although identifying the molecular variables and more exact mechanisms at play will necessitate further research in this area.

5.5. Correlation Between prolactin gene expression and serum prolactin, estrogen and progesterone hormone concentration during lactation period in the female rats

The current study found positive correlation ($p<0.05$) between serum prolactin, estrogen hormone and prolactin gene expression This result consist with (Le Tissier *et al* ., 2015; Mohammad *et al* ., 2019). silymarin increase serum prolactin in rat during lactation. This may be attributed to the antidopaminergic activity of silymarin and the estrogenic effects of silymarin (Demirci *et al* .,2014) . Other studies declared that the estrogen is a positive regulator of prolactin production and release (Le Tissier *et al* .,2015). and negative correlation with Progesterone, the results agreement with (Li *et al* ., 2016 ; Mohammad *et al* ., 2019).

5.6. Correlation Between prolactin gene expression and serum oxidant And antioxidant during lactation period in the female rats

Gene expression positive correlation with GSH, SOD and CAT the results agreement with (Farmer *et al* .,2014 : Farmer *et al* ., 2017) and negative correlation with MDA, the results agreement with (Sun *et al* .,2015 ; Akbari *et al* .,2019).

Chapter Six: Conclusions and Recommendations

6.1.Conclusions:

From the results of current study were concluded the oral intubation of (200 mg/kg/dl B.W.) of silymarin for ten days on lactating rats showed:-

1-A significant increase in the serum prolactin and estrogen hormones and a significant decrease progesterone hormone.

2-A significant increase in GSH concentrations , CAT and SOD activities, while a significant depression in MDA concentration in pituitary gland tissue .

3- Over expression on the prolactin gene observed on the lactotrophs cells were showed filled with prolactin hormone production in the anterior lobe of pituitary gland and sinusoids also showed myoepithelial cells and sinusoids in the mammary gland on the female rats treated with silymarin during lactation period .

4- A significant increase in the prolactin hormone gene expression.

6.2.Recommendations

To assure the beneficial effect of silymarin on the body, current further studies are required :-

1. Effect of silymarin on the nervous system (neurohypophysis).
2. Conducting a histopathological study of the heart , blood vessels, reproductive system(ovary , uterus) and nervous system of silymarin in animal models.
3. Addition of silymarin to the diets of poultry and ruminants in order to study its effect.
4- Further studies on out effect of silymarin on the another gene hormone related with milk production.
5- Study effect of the silymarin on the erythropoietin gene expiration.
6- Electron microscope study about effect of silymarin on the lactotrophs cells in the lab animal .

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Appendices

Appendix I

Assessment of Prolactin concentrations (ng/dl) :

1. Standard tube: the original density standard was illustrated as follow table: (1)

Original density standard	Number of Standard Absorption	Concentration
0.125 OD	1 standard	0 ng/dl
0.243 OD	2 standard	50 ng/dl
0.356 OD	3 standard	100 ng/dl
0.594 OD	4 standard	200 ng/dl
1.141 OD	5 standard	400 ng/dl
1.574 OD	6 standard	800 ng/dl

2- Sample adding: the blank wells were set separately (blank comparison wells don't add sample and HRP-Conjugate reagent, other each step operation is same), 40µl of Sample dilution was added to testing sample well, then add testing sample 10µl (sample final dilution is 5-fold), add sample to wells, and Gently mix.

3- Enzyme adding: 100 µl of HRP-conjugate enzyme was added to each well except blank well.

4- Incubate: after closing plate with Closure plate membrane, incubate for 60 min at 37 °C.

5. Configurate liquid: 30-fold wash solution was diluted 20-fold with distilled water and reserve.

6. Washing: uncover Closure plate membrane, discard Liquid, dry by swing, add washing buffer to every well, still for 30s then drain, repeat 5 times, dry by pat.

7. Color: 50ul of Chromogen Solution A was added and Chromogen Solution B to each well, the light preservation was evaded for 15 min at 37 °C.

8. Reaction stopping: 50µl of Stop Solution was added to each well, Stop the reaction (the blue color change to yellow color).

9. Assay: the blank well was taken as zero, absorbance at 450nm was

Read after Adding Stop Solution within 15min.

10. Results Calculation: the standard density was taken as the Vertical, the OD value for the Horizontal, the standard curve on graph paper was drawn , the corresponding density was Find out according to the sample OD value by the Sample curve, multiplied by the dilution multiple, or calculate the straight line regression equation of the standard curve with the standard density and the OD value, with the sample OD value in the equation, the sample density was calculated, multiplied by the dilution factor, the result was the sample actual density.

(Appendix II)

Assessment of Estrogen hormone concentrations (pg/L)

Standard tube: the original density standard was illustrated as follow

Table: (2)

Original density standard	Number of Standard Absorption	Concentration
0.2 OD	1 standard	50 pg/ml
0.5 OD	2 standard	100 pg/ml
1 OD	3 standard	200 pg/ml
1.5 OD	4 standard	400 pg/ml
2.2 OD	5 standard	800 pg/ml

Assay procedure

1.Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.

2.Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.

3.Add 50µl standard to standard well. **Note**: Don't add antibody to standard well because the standard solution contains biotinylated antibody.

4.Add 40µl sample to sample wells and then add 10µl anti-E2 antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a

sealer. Incubate 60 minutes at 37°C.

5.Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.

6.Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.

7.Add 50µl Stop Solution to each well, the blue color will change into yellow immediately.

8.Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minuets after adding the stop solution.

(Appendix III)

Assessment of Progesterone hormone concentrations (ng/ml)

Standard tube: the original density standard was illustrated as follow

Table: (3)

Original density standard	Number of Standard Absorption	Concentration
0.2 OD	1 standard	4 ng/ml
0.5 OD	2 standard	8 ng/ml
1 OD	3 standard	16 ng/ml
1.5 OD	4 standard	32 ng/ml
2.2 OD	5 standard	64 ng/ml

Assay procedure :

1.Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.

2.Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.

3.Add 50µl standard to standard well. **Note**: Don't add antibody to standard well because the standard solution contains biotinylated antibody.

4.Add 40µl sample to sample wells and then add 10µl anti-E2 antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.

5.Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.

6.Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.

7.Add 50µl Stop Solution to each well, the blue color will change into yellow immediately.

8.Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Appendix IV

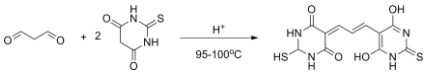
Estimation of tissue Malondialdehyde (MDA):

- **Background**

The body produce oxygen free radicals through the enzyme system and non- enzyme system, which can attack unsaturated fatty acid on biofilm and lead to lipid peroxidation and form lipid peroxide, such as aldehyde group **(MDA),** keto-
, hydroxyl, carbonyl, etc. Oxygen free radicals cause cell damage not only by peroxidation of polyunsaturated fatty acids in biofilm, but also by decomposition products of lipid hydroperoxide. Detection of the MDA content can reflect the level of lipid peroxidation in cells and reflect level of cellular damage indirectly.

- **Detection principle**

MDA in the catabolite of lipid peroxide can react with thiobarbituric acid (TBA) and produce red compound, which has a maximum absorption peak at 532 nm.



- **Kit components & storage**

Item		Component	Specification	Storage
Reagent	1	Clarificant	3 ml x 1 vial	2-8°C , 6 months
Reagent	2	Acid Reagent	1.8 ml x 2 vials	2-*B°C*,6 months
Reagent	3	Chromogenic Agent	Powder x 1 vial	2-8°C , 6 months, shading light
Reagent	4	50 μmol/l Standard	5 ml x 1 vial	2-*B°C*,6 months
		Microplate	96 wells	No requirement
		Plate Sealer	2 pieces	
Note: The reagents must be stored strictly according to the preservation conditions in the above table. The reagents in different kits cannot be mixed with each other.				

- **Materials prepared by users**

Instruments

Microplate reader (530-540 nm), Micropipettor, Vortex mixer, Incubator, Centrifuge

Consumptive material

Tips (10 μl, 200 μl, 1000 μl), EP tubes (1.5 ml, 2 ml)

Reagents

Double distilled water, Normal saline (0.9%NaCl) or PBS (0.01 M, pH 7.4), Acetic acid, Absolute ethanol

- **The key points of the assay**

1. In the incubation of 100°C water bath, the EP tube should not be closed directly. It is recommended to fasten the tube mouth with fresh-keeping film and make a small hole in the film.
2. The temperature of water-bath and the time of incubation should be stabilized (95-100°C, 40 min).

3. The supernatant for colorimetric measurement should not contain sediment, otherwise it will affect the OD values. It is recommended to use a pipette to take the supernatant.

Pre-assay preparation

- **Reagent preparation**

4. Reagent 1 will be frozen when store at 2-8"C for a long time, please warm it in 37°C water-bath until clear.
5. Reagent 2 application solution:

Dilute reagent 2 with double distilled water at a ratio of 1.2: 34 and mix fully.

6. Reagent 3 application solution:

Dissolve the powder with 14 ml of double-distilled water (90-100°C) fully, then add 14 ml of glacial acetic acid, mix fully and cool to room temperature. The prepared solution can be store at 2-8°C with shading light for 1 month. (Glacial acetic acid, analytical reagent, acetic acid concentration 99.5%. This reagent should be self-prepared.)

7. Preparation of 50% acetic acid

Add 8 ml of glacial acetic acid into 8 ml of double distilled water slowlly and mix fully. Stand at room temperature for detection (Note: Glacial acetic acid with high concentrations , please add slowly during the dilution process)

Assay protocol

Ambient temperature	25-30°C
Optimum detection wavelength	532 nm

Instructions for the use of transferpettor:

- Equilibrate the pipette tip in that reagent before pipetting each reagent.
- Don't add the liquid outside the tips into the reaction system when pipetting each reagent.

Operating steps

- **The preparation of standard curve**

Dilute 50 μmol/l Standard with absolute ethyl alcohol to a serial concentration. The recommended dilution gradient is as follows: 0, 5, 10, 15, 20, 25, 30, 40 μmol/l.

- **The measurement of samples**

8. Standard tube: Take 0.02 ml of standard solution with different concentrations into numbered 1.5 ml EP tubes.

Sample tube: Take 0.02 ml of sample into numbered 1.5 ml EP tubes. Control tube: Take 0.02 ml of sample into numbered 1.5 ml EP tubes.

9. Add 0.02 ml of reagent 1 into each tube of Step (1).

- Add 0.6 mL of reagent 2 application solution into each tube of Step (2).
- Add 0.2 mL of reagent 3 application solution into standard tubes and sample tubes, add 0.2 mL of 50% acetic acid to the control tubes.
- Fasten the tube mouth with fresh-keeping film, mix fully, and make a small hole in the film. Then incubate the tubes at 100'C for 40 min.
- Cool the tubes to room temperature with running water, centrifuge the tubes at 9569 g for 10 min.
- Take 0.25 mL the supernatant of each tube to the microplate with a micropipette (the precipitation cannot be added to the microplate).
- Measure the OD value at 532 nm with microplate reader.
- **Calculation**

Plot the standard curve by using OD value of standard and correspondent concentration as y-axis and x-axis respectively. Create the standard curve with graph software (or EXCEL). The concentration of the sample can be calculated according to the formula based on the OD value of sample. If the sample have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. The actual concentration is the calculated concentration multiplied by dilution factor.

The standard curve is: y= ax + b.

1. Serum (plasma) samples:

MDA (μmol/L) = (M-b) +axf

2. Tissue samples:

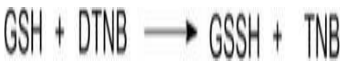
MDA (μmol/gprot) = (M-b) +axf+Cp,

Appendix V

Determination of reduced glutathione (GSH)

Detection principle

Reduced GSH can react with Dinitrobenzoic acid (DNTB) to form a yellow complex which can be detected by colorimetric assay at 405 nm and calculate the reduced GSH content indirectly.



Kit components & storage

Item	Component	Specification	Storage
Reagent 1	Acid Reagent	12 mL × 1 vial	2-8℃ ,6 months, shading light
Reagent 2	Phosphate	12 mL × 1 vial	2-8℃ ,6 months
Reagent 3	DTNB Solution	1.5 mL × 2 vials	2-8℃ ,6 months, shading light
Reagent 4	GSH Standard	3.07 mg × 2 vials	2-8℃ ,6 months
Reagent 5	GSH Standard Stock Diluent	1.5 mL× 2 vials	2-8℃ ,6 months
	Microplate	96 wells	No requirement
	Plate Sealer	2 pieces	
Note: The reagents must be stored strictly according to the preservation conditions in the above table. The reagents in different kits cannot be mixed with each other.			

Materials prepared by users

Instruments

Microplate reader(405-414 nm), Micropipettor, Vortex mixer

Consumptive material

Tips (10 μL, 200 μL, 1000 μL), EP tubes (1.5 mL, 2 mL, 5 mL)

Reagents

Double distilled water, Normal saline (0.9% NaCl) or PBS (0.01 M, pH 7.4)

Pre-assay preparation

Reagent preparation

- Preparation of GSH standard diluent:

Dilute the GSH standard stock diluent with double distilled water at a ratio of 1:9. Prepare the fresh solution before use.

- Preparation of 1 mmol/L GSH standard solution:

Dissolve 3.07 mg of GSH standard with 10 mL of GSH standard diluent and mix fully. Prepare the fresh solution before use.

Dilution of sample

It is recommended to take 2~3 samples with expected large difference to do pre-experiment before formal experiment and dilute the sample according to the result of the pre-experiment and the detection range (2-100 μmol/L).

The recommended dilution factor for different samples is as follows (for reference only)

Sample type	Dilution factor
Human serum	1
Human plasma	1
10% Mouse brain tissue homogenization	1
10% Mouse liver tissue homogenization	1
Hela cell homogenization（0.999 mgprot/mL）	1
Rat serum	1
Rat plasma	1

Mouse serum	1
10% Carrot tissue homogenization	1
293T supernatant	1

Operating steps

- **The preparation of standard curve**

Dilute 1 mmol/L GSH standard solution with GSH standard diluent to a serial concentration. The recommended dilution gradient is as follows: 0, 10, 20, 40, 50, 60, 80, 100 μmol/L.

- **The measurement of samples**

10. Preparation of sample supernatant: take 0.1 mL of sample, add 0.1 mL of reagent 1 and mix fully. Centrifuge at 4500 rpm for 10 min. Take the supernatant for detection.
11. Add 25 μL reagent 3 to each tube.
12. Blank well: Add 100 μL of reagent 1.

Standard well: Add 100 μL of standard solution with different concentration.

Sample well: Add 100 μL of supernatant.

13. Add 100 μL of reagent 2 to each tube.
14. Mix fully for 1 min and stand for 5 min at room temperature. Measure the OD values of each well at 405 nm with microplate reader.

Operation table

	Blank well	Standard well	Sample well
Reagent 3 (μL)	25	25	25
Reagent 1 (μL)	100		
GSH standard solution with different concentration (μL)		100	
Supernatant (μL)			100

Reagent 2 (μL)	100	100	100
Mix fully for 1 min and stand for 5 min at room temperature. Measure the OD values of each well at 405 nm with microplate reader.			

Calculation

Plot the standard curve by using OD value of standard and correspondent concentration as y-axis and x-axis respectively. Create the standard curve with graph software (or EXCEL). The concentration of the sample can be calculated according to the formula based on the OD value of sample. If the sample have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. The actual concentration is the calculated concentration multiplied by dilution factor.

The standard curve is: y= ax + b.

Serum (plasma) and other liquid sample:

GSH content (μmol/L) = $(\Delta A_{405}\text{-}b) \div a \times 2^* \times f$

- Tissue and cells sample:

GSH content (μmol/gprot) = $(\Delta A_{405}\text{-}b) \div a \times 2^* \times f \div C_{pr}$

Appendix VI

Estimation of tissue Superoxide dismutase (SOD) activity

Product Description Superoxide dismutases (SODs) are a family of metal ion-containing enzymes that are found in most organisms, from bacteria to mammals.1- SODs catalyze the dismutation of superoxide (generated by aerobic respiration) to molecular oxygen and peroxide, by the following reaction: 2 O·- + 2 H+ → H2O2 + O2 SODs form the front line of defense against reactive oxygen species (ROS)-mediated injury, 2,3 and harbor anti-inflammatory activities.4- The Superoxide Dismutase (SOD) Activity Assay Kit provides a simple and sensitive procedure for measuring SOD enzymatic activity in various sample types. SOD activity is determined by measuring the decrease in superoxide anions (generated by the enzyme xanthine oxidase [XO]). Since superoxide anions interact with the provided WST dye,

yielding color at 450 nm, the decrease in the color signal is proportional to SOD inhibition activity.

The kit allows the determination of SOD activity in percent inhibition, or in units of activity using the provided SOD Enzyme as a standard. The provided SOD Enzyme can also be used to screen for SOD inhibitors

The kit's lowest limit of detection is 0.3 SOD units/mL. This kit can be used to measure SOD activity in biological samples such as serum, plasma and erythrocytes, tissue homogenates and cell lysates. Components This kit contains sufficient reagents for 500 colorimetric tests. Component Component Number Amount Cap Color/ Container Information Assay Buffer CS0009A 100 mL White cap/bottle Dilution Buffer CS0009B 50 mL White cap/vial SOD Enzyme CS0009C 250 µL Yellow cap/vial WST CS0009D 1 mL Brown vial Xanthine Oxidase CS0009E 100 µL Green cap/vial Components Information

• Assay Buffer (CS0009A): Ready-to-use.

• Dilution Buffer (CS0009B): Ready-to-use.

• SOD Enzyme (CS0009C): 300 units/mL SOD enzyme solution from bovine liver. This component may be used as a positive control or to screen for SOD inhibitors.

It may also be used as a standard to determine SOD activity in samples (see "standard curve preparation" below).

• WST (CS0009D): A 80× solution. Protect from light.

• Xanthine Oxidase [XO] (CS0009E): A 100× solution.

Procedure • All standards should be run in triplicate. Samples can be run either in duplicate or in triplicate.

• The assay is formatted for a 96-well microplate.

• Fresh working solutions should be prepared for every use. Unused working solutions should be discarded.

• Equilibrate all reagents to room temperature before use.

• Briefly centrifuge vials before opening.

• SOD activity can be measured either as percent inhibition activity, which does not require a standard curve, or by a direct comparison to the provided SOD Enzyme, which is used to prepare a standard curve (see "SOD standard curve preparation"). In

case a standard curve is performed, a fresh set of standards should be prepared for every use.

• For convenience, an Excel-based calculation sheet is available on the Product Detail Page. Use this sheet to calculate the amounts of reagents required, as well as to calculate the test results.

Tissue

Tissue should be perfused with PBS to remove any erythrocytes. The tissue should be homogenized (e.g., using a Dounce homogenizer) in ice-cold 0.1 M Trizma®-HCl, pH 7.4, containing 0.5 % Triton™ X-100, 5 mM mercaptoethanol, and protease inhibitors. Centrifuge the homogenate at 4 °C (14,000 × g for 5 minutes) and transfer the SOD-containing supernatant to a new tube.

Calculations:

• An Excel-based calculation sheet is available at the Product Detail Page. Use this sheet to calculate the test results.

• If the Excel-based calculation sheet at the Product Detail Page is not used, calculations should be performed as follows: 1. To calculate the SOD inhibition rate percentage, use the following equation: SOD activity* = (A – B) – (C – D**) x 10 (A – B) * Inhibition rate percentage Where: A: Absorbance value of No SOD control B: Absorbance value of Blank C: Absorbance value of sample D: Absorbance value of No XO. (** Note: If No XO control is not required, use “B” (Blank) instead.) 2. To calculate SOD activity in units/mL, perform the following steps:

• Calculate the average absorbance of each standard, sample and control, and subtract the Blank value from all standards, samples and controls values.

• To obtain the linearized SOD rate, divide No SOD absorbance (maximal absorbance) by itself and by all other blank-subtracted standards and samples absorbance readings. This is the linearized SOD rate.

• Plot the SOD standard concentrations (in units/mL) against the linearized SOD rate of all standards.

• Calculate the activity of the SOD in the sample using the following equation: SOD (units/mL) = (SampleLSR – intercept) x 10 x DF Slope Where: SampleLSR = linearized SOD rate of the sample DF = Sample dilution factor. Note: multiply by 10

to account for the reaction dilution within the well (20 mL sample in a final reaction volume of 200 mL)

Appendix VII

Catalase activity assay

Assay Principle

Catalase enzyme performs a reaction giving rise to a compound that forms a complex with the chromogen. This reaction produces a purple color directly proportional to catalase activity that can be determined by means of a simple and fast spectrophotometrical measure.

Preparation of Reagents Note: All reagents must be brought to room temperature prior to use.

• 1X Assay Diluent: Dilute the 10X Assay Diluent stock to a 1X solution with distilled water. Mix to homogeneity. Store the 1X Assay Diluent at room temperature.

• 1X Sample Buffer: Dilute the 5X Sample Buffer stock to a 1X solution with distilled water. Mix to homogeneity. Use this for all sample and standard dilutions. Store the 1X Sample Buffer at room temperature.

• Hydrogen Peroxide Working Solution: Prepare a 12 mM Hydrogen Peroxide Working Solution by diluting the stock 8.82 M H2O2 solution in Assay Diluent (eg. Add 5 µL of H2O2 stock to 3.67 mL Assay Diluent). Prepare only enough Hydrogen Peroxide Working Solution necessary for immediate applications. This reagent is stable for 2 weeks when stored at 2-8ºC and protected from light.

• Chromogenic Working Solution: Prepare only enough Chromogenic Working Solution necessary for immediate applications. Prepare a Chromogenic Working Solution by diluting Chromogenic Reagent A 1:100 with Chromogenic Reagent B. (Example: Add 0.10 mL of Chromogenic Reagent A to 9.9 mL of Chromogenic Reagent B) Mix thoroughly. Next, add 1 µL of HRP Catalyzer per 1 mL of Chromogenic Working Solution. (Example: Add 10 µL to 10 mL of Chromogenic Working Solution) Mix to homogeneity. This reagent is stable for 2 weeks when stored at 4ºC and protected from light.

Preparation of Samples

Note: Samples should be stored at -70°C prior to performing the assay. Sample should be prepared at the discretion of the user. The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design. Samples should be diluted in Sample Buffer unless noted otherwise. Bovine serum albumin can be added to samples with a protein concentration < 0.050 mg/mL to stabilize the enzyme.

Tissue Homogenate: Prior to dissection, perfuse tissue or rinse with a phosphate buffered saline (PBS) solution. This is to remove any red blood cells and clots. Weigh and homogenize the tissue on ice in 5-10 mL cold PBS with 1mM EDTA per gram of tissue. Centrifuge at 10,000 x g for 15 minutes at 4°C. Remove the supernatant and store on ice. Store any unused supernatant at -70°C for up to one month.

Preparation of Catalase Standard Curve

1. Use only enough Catalase Standard as necessary for immediate applications. Immediately prior to use, vigorously vortex the Catalase Standard stock vial to obtain a homogenous suspension and immediately remove the desired amount. It is recommended to pipette up and down several times prior to removing the suspension. Prepare fresh standards by diluting the 600,000 Units/mL Catalase Standard stock 1:60 in Sample Buffer for a 10,000 Units/mL solution.

(Example: Add 5 µL of Catalase Standard stock tube to 295 µL of Sample Buffer)

Note: One unit of catalase is the amount of enzyme that will decompose 1.0 µmole of H_2O_2 per minute at 25°C.

2. Use the 10,000 Units/mL solution to prepare a series of catalase standards

Assay Protocol

Note: Each catalase standard and samples should be assayed in duplicate or triplicate. A freshly prepared standard curve should be used each time the assay is performed.

1. Add 20 µL of the diluted catalase standards or unknown samples to a 96-well microtiter plate.

2. Add 50 µL of the Hydrogen Peroxide Working Solution (12mM) to each well. Mix thoroughly and incubate at room temperature for exactly 1 minute.

3. Stop the reaction by adding 50 µL of the Catalase Quencher into each well and mix thoroughly.

4. Transfer 5 µL of each reaction well to a fresh well.

5. Add 250 µL of the Chromogenic Working Solution to each well. Incubate the plate at room temperature for 40-60 minutes with vigorous mixing.

6. Read the plate absorbance at 520 nm. Save values for Calculation of Results below.

Appendix VIII

Total RNA extraction using Easy-spin™ (DNA free) total RNA extraction Kit

1. Preparation of 50-100 mg of fresh tissue.

2. Add 1ml of Lysis Buffer (easy-BLUETM reagent) and homogenize tissue sample using a homogenizer or equivalent.

3. Vigorously vortex in room temperature for 10sec.

4. Add 200µl of Chloroform and apply vortex.

5. After centrifuging the solution at 13,000 rpm (4℃) for 10 min, transfer 400µl of the upper fluid to an empty 1.5ml tube.

6. Add 400µl of Binding Buffer and mix it well by pipetting or gently inverting the 2-3 times. Do not centrifuge and leave it for 1min at room temperature.

7. Load the upper solution to the column, but do not load the whole upper solution because the maximum volume of the column reservoirs is 800µl. After loading the optimum of the upper solution to the column, and centrifuge at 13,000rpm for 30sec.

Discard the flow- through after centrifuging and place the spin column back in the same 2ml collection tube. And then repeat this step.

8. Add 700µl of Washing Buffer A to the column. Close the tubes gently, and centrifuge for 30 sec. at 13,000rpm to wash the column. Discard the flowthrough and place the spin column back in the same 2ml collection tube. 9. Wash by adding 700µl of Washing Buffer B to the column and centrifuge for 30 sec. at 13,000rpm. Discard the filtrates and place the spin column back in the same 2ml collection tube.

10. Centrifuge for 1-2 min at 13,000rpm to dry the column membrane

11. Place the column in a clean 1.5ml microcentrifuge tube (not provided), and add 50µl of Elution Buffer directly onto the membrane. Incubate at RT for 1min, and centrifuge for 1min at 13,000rpm to elute

Protocol of GoTaq® 1-Step RT-qPCR System for Real-Time qPCR (Gene expression assay):

1. Program a real-time instrument for standard or fast mode one-step RTqPCR (Table 4).

2. Thaw the components of the GoTaq® 1-Step RT-qPCR System, the RNA templates and the primer pair on ice, at room temperature or at 37°C. Immediately mix each thawed component thoroughly. If using a vortex mixer, mix at low speed to minimize aeration. Keep thawed reagents on ice.

3. Prepare the RNA samples (mRNA [500fg–100ng]) in water or other qPCR compatible diluent

4. Combine reaction components (Table 1) in a non-stick, sterile tube on ice. Mix gently after each addition. Carefully pipet reaction volumes to plate on ice.

5. Transfer plate from ice into the pre-programmed instrument. Start the run immediately.

6. When the run is complete, collect the data and analyse the results.

Table 4: Preparation of Real-Time PCR solutions

Components	**Concentration**	**Volume (20µl)**
GoTaqTM qPCR master mix, 2X	1X	10 µl
Forward primer	10 µM/µl	2µl
Reverse primer	10 µM/µl	2µl
GoScriptTM RT mix for 1-step RT-qPCR	1X	0.4 µl
ddH2O	--	3.6 µl
RNA template	250 ng	2µl

Table 5: Real-Time PCR conditions (According to the instruction of GoTaq® 1-Step RT-qPCR System)

Stage	**Ta(°C)**	**Time**	**Cycles**
Reverse transcription	42	15 min	1
RT inactivation/Hot-start activation	95	10 min	1X
Denaturation	95	10 sec	40X
Annealing/data collection	60	30 sec	
Extension	72	30 sec	
Dissociation	72	2 min	1X

Appendix IX

Immunohistochemical study

The immunohistochemistry was performed using Dako EnVision detection immunohistochemistry kit (Envision FLEX, Dako, K8000, Denmark) and as per manufacturer's instruction. The kit contained:

1. EnVision FLEX Peroxidase-Blocking Reagent (SM801),
2. EnVision FLEX /HRP (SM802) as secondary antibody labeled to horseradish peroxidase,

3. EnVision FLEX DAB+ Chromogen (SM827), EnVision FLEX Substrate Buffer (SM803),
4. Antigen retrieval solution (EnVision™ FLEX Target Retrieval Solution, High pH, DM828),
5. EnVision FLEX Wash Buffer 20X (SM831) as Tris-buffered saline (TBS) bath.

Anti-cytokeratin-8 (CK-8) primary Antibody (monoclonal mouse Anti-CK-8 Antibody: E-AB-40342, Elabscience, China) was used for detection the expression of cytoskeratin-8 (CK-8) levels in current study. Anti-CK-8 Primary antibodies was diluted 100 folds with antibody diluent (EnVision FLEX Antibody Diluent, Dako, K8006, Denmark).

Paraffin embedded mammary gland tissue samples were sectioned at 4 µm thickness and placed carefully in water bath (FALC BI, Italy) and mounted on positive charged glass slides (CrystalCruz® Electro-Statically Charged Micro Slides, sc-363562, SANTA CRUZ BIOTECHNOLOGY, USA) using a hot plate (K&K HYSH11, Korea).

The section pre-heated in oven at 55 ^{o}C, deparaffinized in two changes (10 minutes each) of xylene, and rehydrated in four changes of 100%, 90% and 70% of ethanol alcohol for 2 minutes each, respectively. The tissue sections were rinsed in distilled water and immersed in TBS buffer bath (EnVision FLEX Wash Buffer, SM831) bath for 5 minutes. The excessive buffer on the tissue section was tapped off and wiped gently with a tissue paper around the sections.

The tissue sections were rinsed in distilled water and immersed in Tris-buffered saline (TBS) bath for 5 minutes. The tissue sections were placed in a glass jar filled with antigen retrieval solution (EnVision™ FLEX Target Retrieval Solution, High pH, DM828), pre-heated at 60 ^{o}C and incubated in a water bath at 97 ^{o}C for 25 minutes. The tissue sections were left to cool at room temperature in a glass jar for 20 minutes and rinsed with distilled water and immersed in TBS buffer bath for 5 minutes.

The excessive buffer on the tissue section was tapped off and wiped gently with a tissue paper around the sections. The tissue sections on the glass slides were circled with wax using a special wax pen (Gene Tech Pen, Elabscience, E-BC-R531, China)

to ensure the reagent was confined only on the tissue section of the slide. The tissue sections were rinsed and immersed in two change of TBS buffer bath (EnVision FLEX Wash Buffer, SM831) for 5 minutes each.

The excessive buffer on the tissue section was tapped off and wiped gently with a tissue paper around the sections. The tissue sections were the flooded with 100 µL of peroxidase block solution (EnVision FLEX Peroxidase-Blocking Reagent, SM801) as a blocking reagent and incubated in a humidity chamber for 10 minutes, before the sections were rinsed and immersed in two change of TBS buffer bath (EnVision FLEX Wash Buffer, SM831) for 5 minutes each. The excess buffer on the tissue sections was removed by tapping the slides gently and wiped gently with a tissue paper around the sections.

The sections were applied with 100 µL of diluent anti-CK-8 primary antibodies and incubated in a humidity chamber for one hour at room temperature, then the sections were rinsed and immersed in two change of TBS buffer bath (EnVision FLEX Wash Buffer, SM831) for 5 minutes each. The excessive buffer on the tissue sections was tapped off and wiped gently with a tissue paper around the sections.

The tissue sections were then applied with 100 µL of secondary antibody labeled to horseradish peroxidase (EnVision FLEX /HRP, SM802) and incubated in a humidity chamber at room temperature for 30 minutes, before the sections were rinsed and immersed in two change of TBS buffer bath (EnVision FLEX Wash Buffer, SM831) for 5 minutes each. The excessive buffer on the sections were removed by tapping and wiped gently with a tissue paper around the sections.

The tissue sections were applied with 100 µL of freshly prepared DAB+ substrate-chromogen solution (prepared by apply one drop of EnVision FLEX DAB+ Chromogen (SM827) to 1 ml of EnVision FLEX Substrate Buffer (SM803)), and incubated in a humidity chamber for 10 minutes. Then, the sections were rinsed and immersed in two change of TBS buffer bath (EnVision FLEX Wash Buffer, SM831) for 5 minutes each.

The tissue sections were counter stained with Mayer hematoxylin (Mayer hematoxylin, Bio-Optica, 05-06002/L, Italy) for 3 minutes followed by rinsing in tap water. The tissue sections were dehydrated in in three changes of 70%, 90%, and

100% of ethanol alcohol for 2 minutes each, respectively. The tissue sections were immersed in two changes (10 minutes each) of xylene and mounted with mounting media (DPX) and covered with cover slips. The tissue sections were examined under a light microscope at 100x and 400x magnifications.

Appendix X

Harris' Hematoxylin

General base colorant used to color the nucleus in a dark blue color its components are:

Subject Quantity

1- Hematoxylin powder 2.5 g

2- Absolute ethyl alcohol 25 ml

3- Potassium alum AIK(SO4)2.12H2O or ammonia alum

NH4AI(SO4)2.12H2O50 gm

4- warm distilled water 500 ml

5- Red mercuric oxide 1.25 gm

6- Glacial acetic acid 20 ml

The stainer was prepared according to the following steps and based on (Suvarna et al., 2013):Dissolve hematoxylin with absolute alcohol, then add the dissolved alum to it in warm distilled water, put the mixture on fire until it boils, then add red mercuric oxide to it.

Appendix XI

Eosin stain

The stainer was prepared according to the following steps and based on **(Suvarna *et al*., 2013)**

Subject Quantity

1- Eosin powder 1 gm

2- 70% ethyl alcohol 99 ml

3- Glacial acetic acid 1 ml

Dissolve the eosin well in alcohol, then add glacial acetic acid to it and filter it before use the next day.

Tissue sections were stained using hematoxylin-eosin stained according to **(Suvarna *et al.*, 2013)** as follows:

1- The tissue sections were placed in an oven at 60 °C for five minutes, then wax was removed from the slides using xylene, in two stages, for five minutes for each stage, then passed a descending series of ethyl alcohol starting (100%,90%, 80%, 70 %) for five minutes for each concentration

2- Tissue sections were placed in Harris hematoxylin stain for five minutes.

3- Washed with running water for ten minutes.

4- Paint with eosin for seven minutes.

5- Washed with distilled water for two minutes.

6- Then it was transferred to an ascending series of ethyl alcohol (70%, 80%, 90%, 100%) for two minutes in each concentration except for the last concentration and was placed in it for five minutes and then quenched with xylene in two stages in each stage for three minutes.

الخلاصة

أجريت هذه الدراسة الحالية للتحري عن تأثير السيليمارين على التعبير الجيني لهرمون البرولاكتين خلال فترة الرضاعة لاناث الجرذان .

12 من الجرذان المرضعة قسمت عشوائيا الى مجموعتين (6/ للمجموعة)جرعت المجموعة الأولى بالماء المقطر واعتبرت مجموعة السيطرة بينما جرعت المجموعة الثانية بالسيليمارين (200 ملغم /كغم من وزن الجسم) يوميا لمدة عشر أيام.

جمعت عينات الدم بعد تجويع المرضعات بتقنيه ثقب القلب لقياس مستوى هرمون البرولاكتين ، الايستروجين و البروجيسترون من مصل الدم . و جمعت عينات نسيج الغدة النخامية بعد التضحية بالحيوان و ذلك لقياس مستوى المؤكسدات و مضادات الاكسدة أ – تركيز الكلوتاثيون و المالونديالديهايد ب – فعالية انزيم سوبر أوكسيد ديسميوتاز و انزيم الكاتالاز .كما تم قياس مستوى تعبير جين البرولاكتين في نسيج الغدة النخامية . بالإضافة الى جمع مقاطع من الغدة النخامية و الغدد اللبنية لدراسة التغيرات الهيستوكيميائية .

أظهرت النتائج وجود ارتفاع معنوي ($P\leq0.005$) في مستوى هرمون البرولاكتين و الايستروجين ، الكلوتاثيون ، انزيم سوبر أكسيد ديسميوتاز و انزيم كاتالاز في المجموعة المعالجة بالسيليمارين بالمقارنة مع مجموعة السيطرة و كذلك لوحظ انخفاض معنوي في تركيز المالونديالديهايد وهرمون البروجيسترون في المجموعة المعالجة بالمقارنة مع مجموعة السيطرة .

اشارت النتائج الدراسة الحالية الى وجود علاقة ارتباط موجبة بين التعبير الجيني لهرمون البرولاكتين و هرمون البرولاكتين و الاستروجين، بينما أظهرت ارتباط سلبي بين التعبير الجيني و هرمون البروجسترون .

بينما يوجد ارتباط موجب بين التعبيرالجيني وتركيز الكلوتاثيون و سوبر أكسيد ديسميوتاز و انزيم الكاتالاز ، بينما أظهرت ارتباط سلبي بين التعبير الجيني لهرمون البرولاكتين و المالونديالديهايد.

و وجدت الدراسة الحالية ان إعطاء السيليمارين يوميا لمدة عشرة أيام في فترة الارضاع ارتفاع في مستوى التعبير الجيني لجين البرولاكتين في المجموعة الثانية بالمقارنة مع مجموعة السيطرة .

توضح الدراسة الحالية أن هناك العديد من التغيرات الهيستوكيميائية المناعية التي حدثت في الغدة النخامية والثديية. لوحظ أحد هذه التغييرات في كثافة خلايا اللاكتوتروف في الغدة النخامية ونشاط هذه الخلايا أثناء إنتاج البرولاكتين.

في المجموعة المعالجة بالسيليمارين أظهر زيادة في حجم فصيصات الحويصلات الهوائية ، مما أدى إلى انخفاض مماثل في الأنسجة الدهنية. نسيج ضام ليفي رقيق يفصل فصوص الثدي عن بعضها البعض. تضمنت كل فصيص من الثدييات كمية من الحويصلات الثديية المملوءة بالحليب ، وكل حويصلة حلقية بخلايا مكعبة أساسية. على عكس المجموعة الضابطة ، كان للقناة داخل الفصيص في الحيوانات المعالجة تجويف كبير ويحدها خلايا مكعبة بسيطة. تم إعادة توصيل هذه الحويصلات الهوائية والقناة السنخية بالغشاء القاعدي ، وشكلت الخلايا الظهارية العضلية في مكانها طبقة واحدة خالية من العيوب

نستنتج من نتائج الدراسة الحالية أهمية تأثير السيليمارين على زيادة التعبير الجيني للبرولاكتين و انخفاض الحالة التأكسدية مع زيادة في فعالية الخلية اللاكتوتروف .

جامعة كربلاء

كلية الطب البيطري

فرع الفسلجة و الكيمياء الحياتية والأدوية

التحريض الوظيفي والكيميائي المناعي للسيليمارين على التعبير الجيني للبرولاكتين في الغدة النخامية والغدد الثديية خلال فترة الرضاعة في إناث الجرذان

رسالة مقدمة الى مجلس كلية الطب البيطري / جامعة كربلاء وهي جزء من متطلبات نيل درجة الماجستير في اختصاص علوم الطب البيطري/الفسلجة

كتبت بواسطة

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