Comparative Biochemical Effects of Bovine and Camel Whey Proteins against Aflatoxicosis in animals.

A Thesis

Submitted for Partial Fulfillment of the Ph.D. Degree in Biochemistry

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Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Item
α-LA	Alpha-lactalbumin
Acr	Acrolein
Ag R	Antigen R
AFs	Aflatoxins
AFB1	Aflatoxin B1
AFBO	AFB1-8,9-epoxide
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFM1	Aflatoxin M1
AFM2	Aflatoxin M2
AFP	Alpha fetoprotein
AKT	Serine/threonine-protein kinases1
ALEs	Advanced lipo-oxidation end products
AP-1	Activator protein-1
APCs	Antigen presenting cells
β-LG	Beta-lactoglobulin
Bad	Pro apoptotic member of the bcl-2 gene family
Bax	B-cell lymphoma-2 associated x protein
BCL-2	B-cell lymphoma 2
BCR	B cell receptor
BEA	Beauvericin
BFU-E	Burst-forming unit-erythroid

BLF	Bovine lactoferrin
BSA	Bovine serum albumin
BSF-2	B-cell stimulatory factor-2
BWP	Bovine whey protein
CAT	Catalase
Caspase-3	Cysteine-aspartic acid protease-3
cAMP	Cyclic adenosine monophosphate
cGMP	3,5-cyclic guanosine monophosphate
CCR2	C-C Motif chemokine receptor 2
CCR5	C-C Motif chemokine receptor 5
CD4	Cluster of differentiation 4
CEA	Carcino embryonic antigen
CLF	Camel lactoferrin
CLP	Common lymphoid progenitor
CRP	C-reactive protein
Cro	Crotonaldehyde
CFU-GM	Colony-forming unit-granulocyte and macrophage
COX-2	Cyclooxygenase-2
CWP	Camel whey protein
CXCL11/I-TAC	T-cell alpha chemoattractant
CXCR1	C-X-C motif chemokine receptor type 1
CXCR2	C-X-C motif chemokine receptor type 2
CXCR7	C-X-C motif chemokine receptor type 7
CXCR4	C-X-C motif chemokine receptor type 4
CXCL12	C-X-C motif chemokine ligand 12
Cytc	Cytochrome complex
DCs	Dendritic cells
DN-thymocyte	Double negative thymocyte
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
DP-thymocytes	Double positive thymocyte
ENNs	Enniatins
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ETP	Early thymic progenitor
FAS	Cell surface death receptor
FAK	Focal adhesion kinase
FOXO	Forkhead family transcription factors
FUM	Fumonisins
G-CSF	Granulocyte colony-stimulating factor
GDP	Guanine nucleotide diphosphate

GTP	Guanine nucleotide triphosphate
GEF	Guanine nucleotide exchange factor
GPCR	G-protein coupled receptors
GRKs	G-protein coupled receptor kinases
GST	Glutathione-S-transferase
GSH	Glutathione
GSH-Px	Glutathione peroxidase
GSK3	Glycogen synthase kinase 3
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HDL	High density lipoprotein
HepG2	Hepatocellular Carcinoma G2
HGF	Hybridoma growth factor
HIV	Human immunodeficiency virus
HIF-1	Hypoxia-inducible factor-1
HSCs	Hematopoietic stem cells
HPCs	Hematopoietic progenitor cells
HS	Heat stress
HSF	Hepatocyte-stimulating factor
HT-2	Type of trichothecenes toxins
IAPs	Inhibitors of apoptosis
icIL-1Ra1	Intracellular IL-1 Receptor Antagonist
Igs	Immunoglobulins
IgA	Immunoglobulin A
IL-1β	Interleukin-1β
IL-1Ra	Interleukin-1 receptor antagonist
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-21	Interleukin-21
iNOS	Inducible nitric oxide synthase
INF-α	Interferon-alfa
IFN-β2	Interferon-beta 2
IP3	Inositol-1,4,5-trisphosphate
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha
JNK	C-Jun N-terminal kinase
LF	Lactoferrin
LITAF	Lipopolysaccharide-induced TNF factor
LOX-2	Lipoxygenase-2
LDL	Low density lipoprotein
MAPK	Mitogen-activated protein kinase

MDA	Malondialdehyde
MHC	Major histocompatibility complex
Mon	Moniliformin
mTOR	Mammalian target of rapamycin
NO	Nitric oxide
NEMO	Nuclear factor-kappa B essential modulator
NOS	Nitric oxide synthase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NF-κB/Rel	Rel-homology domain of NF-κB
NK cells	Natural killer cells
OTA	Ochratoxin A
PDK1	Pyruvate Dehydrogenase Kinase 1
PPARα	Peroxisome proliferator-activated receptor-α
PBSF	Pre-B-cell growth-stimulating factor
PGRP	Peptidoglycan recognition protein
PI3K	Phosphoinositide 3-kinase
PLC	Phospholipase C
PKC	Protein kinase C
PLCβ	Phospholipase C β subunit
p70S6K	P70 ribosomal protein S6 kinase
pre-TCR	Pre-T-cell receptor
pre-BCR	pre-B-cell receptor
RANKL	Receptor activator of nuclear factor kappa-B ligand
ROS	Reactive oxygen species
SDF-1	Stromal cell-derived factor-1
SFKs	Src family of tyrosine kinases
SIL-1Ra	Secretory IL-1 receptor antagonist
sGC	Soluble guanylate cyclase
SOD	Superoxide dismutase
SP T-cells	Single positive T-cells
TACE	TNF-alpha-activating converting enzyme
TCTs	Trichothecenes
TCR-α	T-cell receptor-alpha
Th1	T- helper cell type-1
Th2	T-helper cell type-2
TNF-α	Tumor Necrosis Factor-α
TNF-β	Tumor Necrosis Factor-β
TNF-R_1	Tumor necrosis factor receptor 1

TRADD	Tumor necrosis factor receptor type 1-associated death domain protein
VEGF	Vascular endothelial growth factor
WPE	Whey Protein Extract
WPs	Whey proteins
ZEA	Zearalenone
ZIP	Zinc importer

Introduction

Natural toxins probably pose a greater threat to human and animal health than synthetic toxins, approximately 4.5 billion people worldwide are exposed to aflatoxin-contaminated food, particularly in low-income countries four decades before (**Bankole & Adebanjo, 2003**). Aflatoxins (AF) are low molecular weight compounds, produced by fungi such as *Aspergillus flavus* and *Aspergillus parasiticus* **(Dai *et al.*, 2017).** The primary disease associated with aflatoxin intake is hepatocellular carcinoma (HCC, or liver cancer), which results from genetic variation in metabolic enzymes, and DNA repair (**Liu *et al.*, 2012**).

The cells of the immune system are continually proliferating, differentiating, and vulnerable to the immunomodulatory, primarily immunosuppressive effects of mycotoxins. Previous studies on the effect of aflatoxin on the immune system in animals and cell cultures examined cell-mediated and antibody responses, natural killer cell activity, macrophage phagocytic function, infectivity and host- resistance challenges. The previous studies found that aflatoxin may have an immunomodulatory rather than simply immunosuppressive role as aflatoxicosis decreases T or B lymphocyte activity, impairs macrophage/neutrophil effector functions, modifies thesynthesis of inflammatory cytokine suppressed natural killer cell-mediated cytolysis, decreases immunity to vaccination, and impaired immune function in developing animals (**Jolly *et al.*, 2008**).

Whey proteins (WPs), while having no direct effects on primary functions of neutrophils like chemotaxis, phagocytosis, oxidative burst, and degranulation, WPs could modulate immune defenses by stimulating the production of certain cytokines by neutrophils (**Rusu *et al.*, 2009**).

Whey proteins activates neutrophils to release interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) which have been shown to prime these cells and mediate an increased innate immunity because these cytokines stimulate immunity. Whey proteins can stimulate other immune cells like monocytes, and lymphocytes that will be involved in immune responses **(Kishimoto, 2006)**.

Whey proteins showed positive effects on different human pathologies (**Krissansen, 2007**). They contain bioactive substances such as alpha-lactalbumin, major whey protein found in milk with potential antiproliferative effects **(Gupta & Prakash, 2017).** The effect of whey proteins on cytokines could be related to neutrophil viability that was dose-dependently increased by whey proteins, they can also stimulate human leukocytes in vitro and has been shown to slightly reduce inflammation in humans (**Poulin *et al*., 2006**)**.**

Camel milk differs from bovine milk in both the composition and structure of its protein components, which influences their functional and biological properties **(El-Hatmi *et al*., 2007**)**.** Camel lactoferrin (CLF) not only inhibits HCV (genotype 4a) entry into HepG2 cells and peripheral blood mononuclear cells (PBMCs) but also suppresses viral replication in infected cells **(El-Fakharany *et al*., 2008).**

Most importantly, CLF has been postulated to have hepatoprotective activity because can improve the levels of Th1/Th2 cytokines (**Saltanat *et al*., 2009**). Lactoferrin can stimulate immune responses involving natural killer cells, neutrophils, and macrophage cytotoxicity **(Keri Marshall, 2004).**

Bovine proteins can have functional effects on human cells. Although most of the proteins are degraded during digestion, certain whey proteins, such as β-lactoglobulin and α-lactoalbumin are resistant to

gastric digestion and remain intact after absorption in vivo **(Yamaguchi & Uchida, 2007).**

It has also been shown that a high-protein dietary supplement (containing 41% whey proteins) had similar immunomodulatory, anti-inflammatory, and antioxidative functions in its undigested and digested form **(Kanwar & Kanwar 2009).**

There is evidence that the hepatic overexpression of iNOS plays an important role in liver damage in various liver injury models (**Sass *et al*., 2001**). In addition, mycotoxins other than AFB are described to modulate in expressions such as the trichothecene, deoxynivalenol that affect iNOS expression by the gut and the brain (**Graziani *et al*., 2015**).

Targeting chemokines and their receptors may serve as a promising strategy in immunotherapy, especially combined with other strategies such as chemotherapy, cyclin-dependent kinase, or immune checkpoints inhibitors. CXCL12/CXCR4 axis plays a crucial role in the homing of stem and progenitor cells in the bone marrow and controls their mobilization into peripheral blood and tissues in homeostatic conditions as well as after tissue injury or stress. The chemokine receptor CXCR4 and its ligand CXCL12 also known as stromal cell-derived factor-1 (SDF-1), which are attractive therapeutic targets in the treatment of cancer as they support migration, proliferation and survival of cancer cells **(Domanska *et al*., 2013).**

NF-κB is an important transcription factor for immune regulation, inflammatory response, apoptosis, and is also involved in gene regulation of various factors. NF-κB affects the expression of cytokine genes and thus mediates inflammatory responses. TNF-α positively regulates NF-κB activation, amplifies the immune response **(Giridharan & Srinivasan, 2018)**. It is of interest to mention that NF-κB is activated by oxidative stress and its activation can be modulated by some antioxidants, possibly

through the involvement of the cysteine moiety in p65 of NF-κB (**Zhang *et al*., 2007**).

The inflammation-associated cytokines include IL-6 and TNF-α, as they are the chief stimulator of the production of most proteins in acute phase inflammation **(Gabay, 2006)**. TNF-α is produced by macrophages and it plays an important role in tumor progression **(Abdel-Wahhab & Aly, 2003).**

Caspase-3 (cysteinyl aspartate proteinase) is one of the cysteine proteases that play a major role in the execution of apoptosis **(Nicholson, 1999**). Several genetic and biochemical studies suggest that caspase activation is essential for the occurrence of the apoptotic phenotype of cell death. A variety of caspase substrates are involved in the regulation of DNA structure, repair, and replication **(McCarthy *et al*., 1998).**

Aflatoxin B could induce apoptosis of hepatocytes, bone marrow cells, lung cells (**Meki *et al,* 2001**), thymocytes, and bursal cell (**Chen *et al*., 2014**). The process of apoptosis was mediated by proteins of the Bcl-2 family and performed in Fas path or caspase-dependent apoptotic pathway which relied on the mitochondrial active control **(Martinou & Youle, 2011)**. Aflatoxin B could be up-regulate cellular apoptosis via the process of a mitochondrial signaling pathway in human hepatocytes and lung cells **(Van Vleet *et al*., 2006).**

Aflatoxin B causes excessive apoptosis through the increase of reactive oxygen species (ROS) which in turn increases caspase-3 and Bax, but decrease Bcl-2 proteins which layered on the surface of mitochondria and can prevent cytochrome c from releasing into plasma, whereas Bax can cause leaking out of cytochrome c by punching holes in the mitochondrial membrane. Once the balance between Bcl-2 and Bax

was broken, caspase-dependent apoptotic pathway could be activated (**Kroemer *et al*., 2007**).

Aim of the work

The present study aimed to investigate the immune-enhancing effects of CWP and BWP supplementation against aflatoxicosis in the exposed intact animals by investigating their effects on the following parameters:

1. Expressions of CXCL12 and NF-κB in the spleen and thymus.
2. Inflammation biomarkers: pro-inflammatory cytokines levels IL-6 and TNF-α (tumor necrosis factor-alpha) in the spleen and thymus.
3. Measuring apoptotic markers: Expression of cleaved caspase-3 in spleen and thymus.
4. NO, MDA levels, and antioxidants levels (GSH, GSH-Px, GST) in liver tissues homogenates that are responsible for AFB metabolism.
5. Histopathological examination of ultrastructure of spleen and thymus to detect changes caused by aflatoxin.

Aflatoxin

1. Mycotoxins

The presence of mold in the environment is a source of contamination for foodstuff and hence mycotoxins are natural contaminants found in human food and animal feed **(Gauthier *et al.*, 2020).** They contaminate many foodstuffs, particularly groundnuts, corn, rice, sorghum, milk, and food oils **(Strosnider *et al.*, 2006).**

The term mycotoxin was coined in 1962 in the aftermath of an unusual veterinary crisis near London, England, during which approximately 100,000 turkeys died. When this mysterious turkey X disease was linked to a peanut (groundnut) meal contaminated with secondary metabolites from *Aspergillus Flavus* (aflatoxins), it sensitized scientists to the likelihood that other occult mold metabolites could be deadly. Global warming climate change scenarios for European regions tend to favor the production of aflatoxins in corn crops **(Bailly *et al.*, 2018)**.

Toxic compounds produced from fungi are not all mycotoxins otherwise, these compounds are of fungal origin. The target and the concentration of the metabolite are both important, antibiotics such as penicillin which cause toxicity to bacteria are also produced by fungi (**Wood *et al.*, 1972**). Plant pathologists define fungal products that are toxic to plants by phytotoxins. Mycotoxins are made by fungi and are toxic to vertebrates and other animal groups in low concentrations, but mycotoxins do not include low molecular weight fungal metabolites like ethanol which is only toxic in high concentrations **(Bennett, 1987).**

Mycotoxins are produced mainly by the secondary metabolism of some filamentous fungi, or molds under suitable temperature and humidity conditions, and should develop on various foods and feeds causing serious risks to human and animal health **(Taniwaki *et al.*, 2019)**. Mycotoxins are varied from simple C4 compounds e.g., moniliformin, to complex substances like phomopsins. Currently, quite 300 mycotoxins are known, scientific attention is concentrated mainly on species who have proven to be carcinogenic and/or toxic **(Smith *et al.*, 2016)**. There are combinations of mycotoxins that frequently occur as established in analytical–chemical monitoring programs. These combinations are summarized in **Table (1).**

Table 1: Frequently occurring combinations of mycotoxins in different plant products **(Smith *et al.*, 2016).**

Mycotoxins
Aflatoxin B1, fumosin B1, zearalenone, deoxynivalenol, nivalenol
Ochratoxin and aflatoxin B1
Ochratoxin and penicillic acid
Ochratoxin and citrinin
Ochratoxin and zearalenone
Patulin and citrinin
Fumonisin B1 and moniliformin
Deoxynivalenol, nivalenol, diacetoxyscirpenol, T-2, HT-2, and other trichothecenes

Aspergillus penicillium and *Fusarium* genera are the molds that produce mycotoxins contaminating foods and having toxic effects on the health of humans and animals (**Joshi *et al.*, 2021**), these molds account

for many dollars annually in losses worldwide in human health, animal health, and condemned agricultural products (**Hussein & Brasel, 2001**). Among the thousands of fungal secondary metabolites currently known, only a few groups of mycotoxins are important from the safety and economic points of view; namely aflatoxins (AFs), mainly produced by *Aspergillus* species; ochratoxin A (OTA), produced by *Aspergillus* and *Penicillium* species, and zearalenone (ZEA), Fumonisins (Fum) and trichothecenes (TCTs) {especially Deoxynivalenol (DON)}, primarily produced by many *Fusarium* species **(Streit *et al.*, 2013)**.

Mycotoxins of greatest public health and agro-economic significance include aflatoxins (AFs), trichothecenes, ochratoxins A (OTA), Zearalenone (ZEA), Tremorgenic toxins, Fumonisins (Fum). Moreover, several species from the *Fusarium* genus can produce other mycotoxins with toxicological properties such as beauvericin (BEA), enniatins (ENNs), and moniliformin (MON), a group of lesser-studied toxins called emerging mycotoxins a non-exhaustive list of mycotoxins producing *Aspergillus*, *Penicillium* and *Fusarium* species, split into eight groups **(Jestoi, 2008)**, as provided in **Table (2).**

Aflatoxin B1 (AFB1), the foremost potent hepatocarcinogenic substance known, which has been recently proven to even be genotoxic. In dairy cows, another problem arises from the transformation of AFB1 and AFB2 into hydroxylated metabolites, aflatoxin M1 and M2 which are found in milk and milk products obtained from livestock that have ingested contaminated feed (**Boudra *et al.*, 2007**).

Table 2: some mycotoxins of interest and their fungal source, with primary food and feed hosts and endemic regions **(Bryden, 2012)**.

mycotoxins	Fungi Source	Product of Primary Concern	Geographical Occurrence
AFs * (B1, B2, G1, G2)	**Aspergillus (bombycis, flavus, nomius, ochraceoroseus, parasiticus, parviclavatigenus**	**Cereals and cereal-based products (mainly corn), nuts, nut products, and**	**Temperate, tropical, and subtropical regions (Southern Asia and**
OTA *	**Aspergillus (alliaceus, auricomus, carbonarius, cretensis**	**Cereals and cereal-based products (mainly rice and wheat), coffee and cocoa**	**From cool-temperate to tropical regions (Northern and Southern**
TCTs * (DON, NIV, T-2, HT-2, DAS)	**Fusarium (acuminatum, armeniacum, culmorum, crookwellense, equiseti**	**All cereals and cereal-based products**	**Northern temperate regions (Europe, America, and Asia**
ZEA *	**Fusarium (crookwellense, culmorum, equiseti, graminearum, incarnatum**	**All cereals and cereal-based products, and banana**	**Northern temperate regions (Europe, America, and Asia)**
FUM * (B1, B2, B3)	**Fusarium (anthophilum, dlamini, fujikuroi, globosum, napiforme, nygamai**	**Corn, millet, sorghum, rice and their derivatives**	**Hot-temperate regions (Europe, Africa)**
BEA *	**Fusarium (acuminatum, armeniacum, anthophilum, avenaceum, beomiforme**	**All cereals and cereal-based products**	**Temperate regions (Europe)**
ENs * (A, A1, B, B1)	**Fusarium.(acuminatum, avenaceum, langsethiae, lateritium, poae, proliferatum**	**All cereals and cereal-based products**	**Temperate regions (Europe)**
MON *	**Fusarium (acuminatum, avenaceum, culmorum, equiseti, fujikuroi, napiforme, nygamai**	**All cereals and cereal-based products**	**Temperate regions (Europe)**

1.1. Aflatoxins

Aflatoxins are toxic metabolites produced via a polyketide pathway by various species and by unnamed strains of *Aspergillus* section Flavi, which include. *Flavus*, *A. Parasiticus*, *A. Parvisclerotegenus*, *A. Minisclerotigenes* **(Cotty & Mellon, 2006)** and less commonly *A. Nomius* **(Kurtzman *et al.*, 1987)**. The exposure to these carcinogenic aflatoxins can be through the ingestion of the contaminated crops that end up in the stomach where it gets absorbed or through the inhalation of dust particles contaminated with AFB_1. Aflatoxins B1 has been documented in several kinds of cells including hepatocytes, intestinal cells, spleen cells, cardiomyocytes, and macrophages **(Liao *et al.*, 2014).**

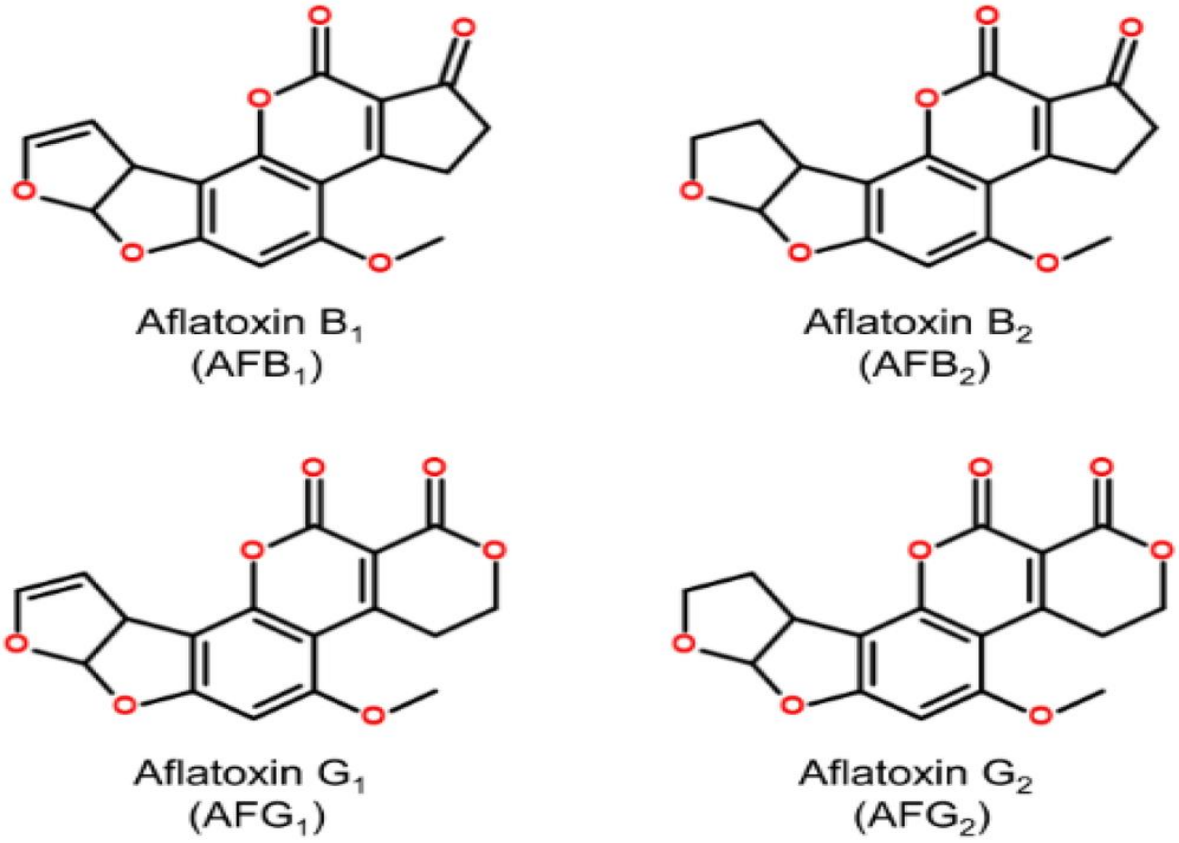

Fig (1): Molecular structures of the four primary aflatoxins **(Monson *et al.*, 2015).**

1.2. Toxic effects of aflatoxins

Aflatoxin B1-contaminated food and feed commodities ingestion cause hepatic toxicity. It has been reported that mycotoxins, AFB1 in particular, exert stressful impacts on the hepatic tissues **(Abdel-Wahhab *et al.*, 2010)**. The target organ of (AFB) poison is the liver, where the poison massively affects the liver and causes a defect in fat and protein metabolites and deposits fat in the liver, which leads to lubrication and then damages liver cells and eventually fibrosis and cancer **(Taheur *et al.*, 2019).**

Aflatoxicosis as a result of consuming AFs-intoxicated foods has two main classes: acute aflatoxicosis and chronic aflatoxicosis **(Williams *et al.*, 2004)**. Acute aflatoxicosis, characterized by hemorrhage, acute liver damage, edema, elevation in transaminases, and death. These effects result from extremely high doses of aflatoxin in the diet **(Abdel-Wahhab *et al.*, 2002)**.

Hepatic carcinogenic effects of AFB are mainly due to the induction of oxidative stress **(Souza *et al.*, 1999)**, Agents that stimulate cellular oxidative metabolism are also apoptosis inducers (**Chandra *et al.*, 2000**). The method of AFB-initiated hepatocyte death was prevalently apoptosis but not necrosis (even though necrosis additionally happened after treatment with AFB), likely because of the generation of massive cellular ROS which was a consequence of uncoupling to help prompt apoptosis **(Malhi *et al.*, 2006)**.

Apoptosis is a gene-directed program implicated in tissue homeostasis, but any mutation in the apoptosis pathway can lead to cancer. Apoptosis was described by its morphological characteristics, including, nuclear fragmentation, membrane blebbing, chromatin

condensation, and cell shrinkage **(Thompson, 1995)**.

P53, the first tumor suppressor gene is linked to apoptosis. Any mutations in P53 lead to human tumors and are also associated with advanced tumor stage and poor patient prognosis **(Wallace-Brodeur & Lowe, 1999)** as illustrated in **figure (2)**.

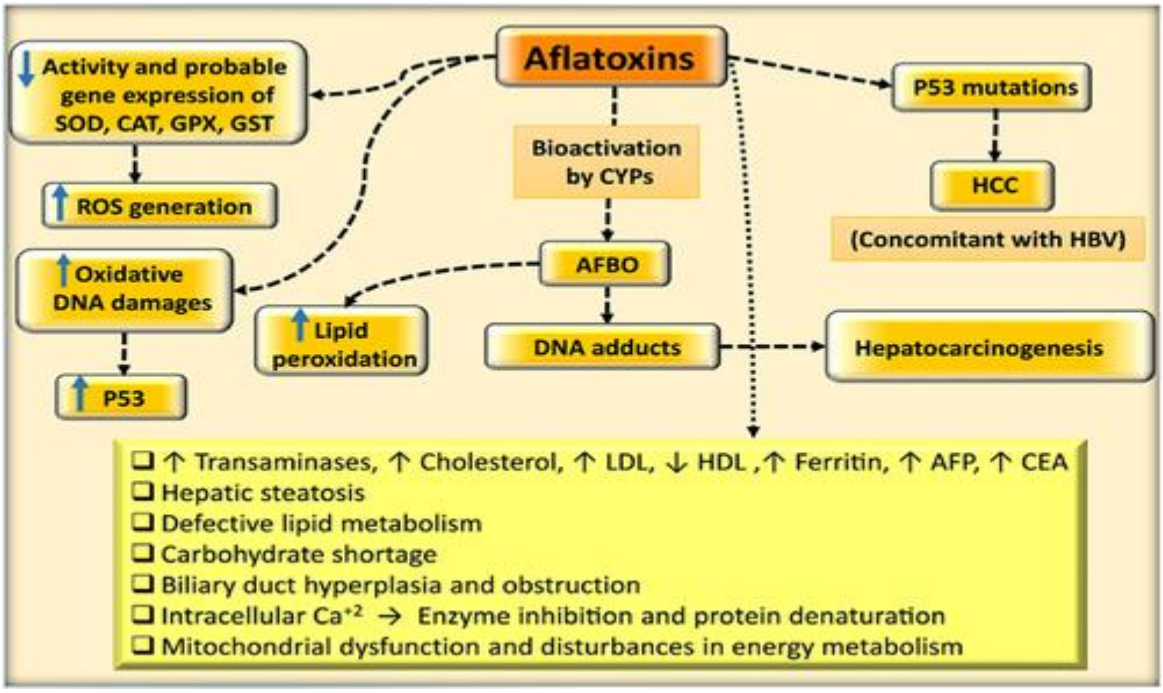

Fig (2): Major hepatotoxic and adverse effects of aflatoxins in the liver. SOD: superoxide dismutase; CAT: catalase; GPH-PX: glutathione peroxidase; GST: glutathione-S-transferase; ROS: reactive oxygen species; CYP: cytochrome P450; AFBO: AFB1-8,9-epoxide; HCC: hepatocellular carcinoma; HBV: hepatitis B virus; LDL: low-density lipoprotein; HDL: high-density lipoprotein; AFP: alpha-fetoprotein; CEA: carcinoembryonic antigen (**Mohajeri *et al.*, 2018**).

1.3. Metabolism of aflatoxins

Once the aflatoxins get into the cells, they are metabolized by the action of cytochrome P-450 enzyme systems into AFB1 8,9-Exo-epoxide which is highly reactive and unstable. AFB1 8,9-Exo-epoxide requires binding to a DNA or the protein molecule to become more stable **(Rawal *et al.*, 2010)**.

Unstable aflatoxin-8, 9-epoxide binds to the DNA molecule with high affinity forming aflatoxin-N7-guanine that cause a transversion mutation of a guanine (G) to a thymine (T) in the P53 gene which carries the codes for tumor suppressor proteins, this will, in turn, affect the cell cycle and promote the development of tumors and cancers (**Riley *et al.*, 2001**).

The mitochondrial respiratory chain is a major source of ROS which can induce cell apoptosis. Different components have been proposed as usable in AFB hepatotoxicity. Among these, mitochondrial impairment mitochondrion, has pulled in much intrigue due to its association with ROS production and oxygen utilization in cells **(Xu *et al.*, 2021)**.

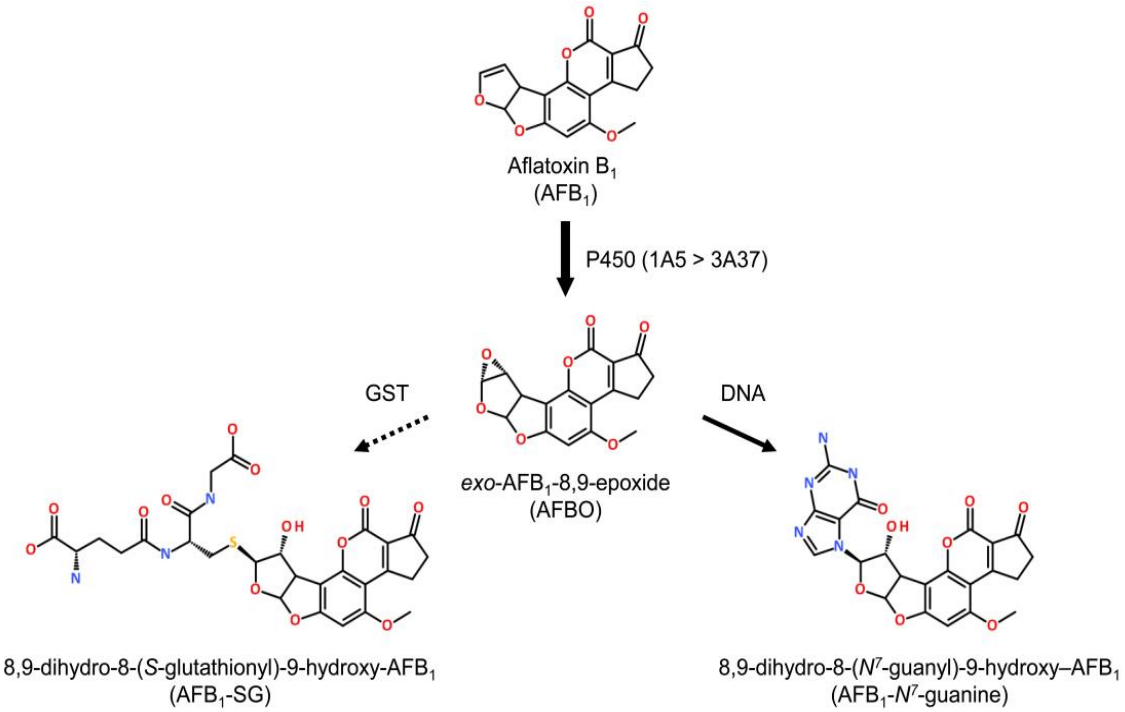

Fig (3): Metabolites and enzymes involved in aflatoxin B1 (AFB1) metabolism in the turkey liver. Arrow width shows reaction efficiency (wide > narrow), while a dashed line indicates that a reaction does not occur in all birds. Cytochrome P450 (*CYP*) enzymes effectively interact with AFB1. Domestic turkey glutathione *S*-transferase (GST) enzymes cannot conjugate *Exo*-AFB1-8,9-epoxide (AFBO), although wild and heritage turkey GST enzymes can have activity **(Monson *et al.*, 2015)**.

1.4. Aflatoxin B toxicity on the immune system

Aflatoxin can damage immune tissues and suppress innate and adaptive immune responses, also AFB consumption during growth can lead to immune tissue atrophy, and reducing relative weights of the bursa, spleen, and thymus **(Hoerr, 2010).**

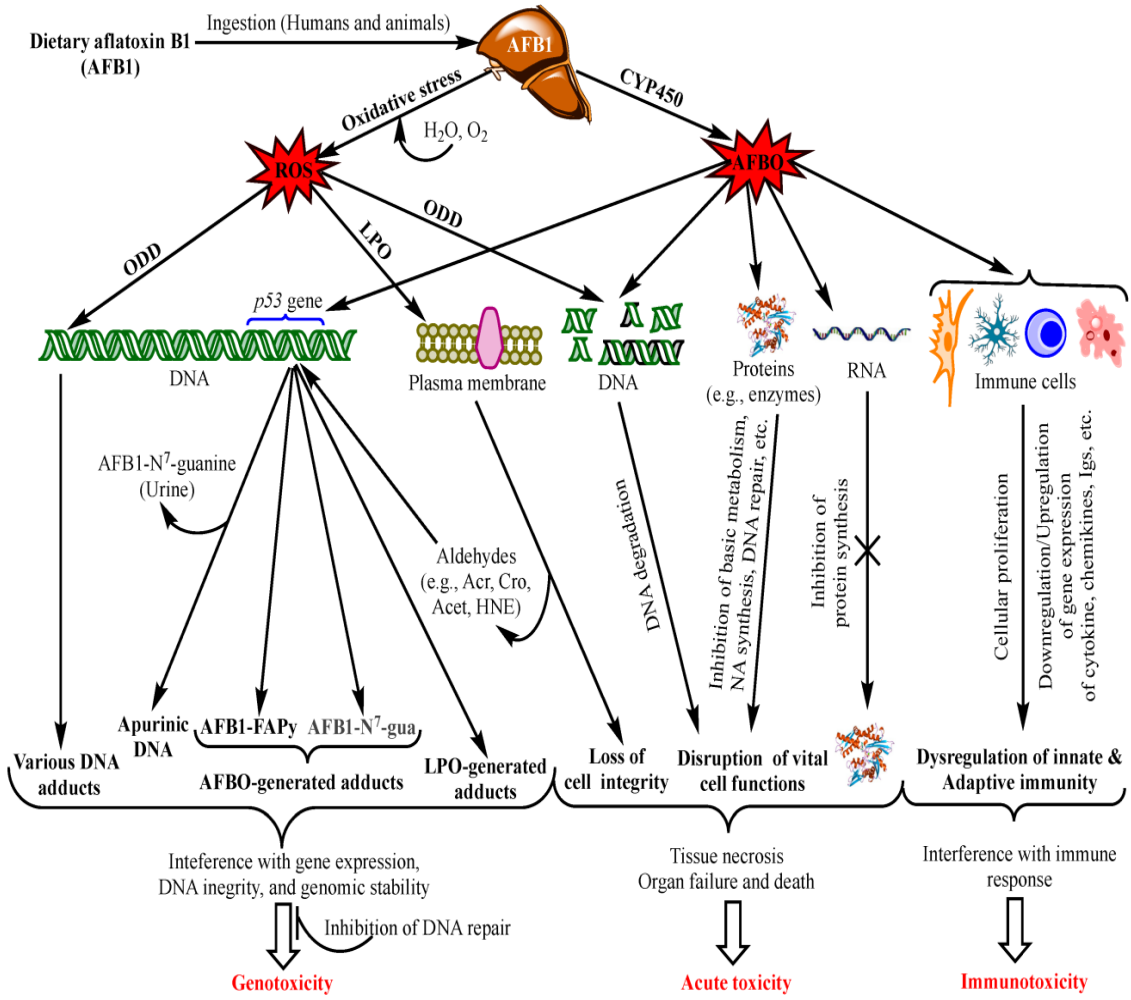

Fig (4): Main aflatoxin B1 toxicity mechanisms mediated by the oxidative stress and AFB1-Exo-8,9 epoxide. NB: ROS also affect proteins, RNA molecules, and immunity as does AFBO **(Benkerroum, 2020)**.

Aflatoxin B restrained the advancement of the thymus, decreased the Ig-containing cell number and splenic plasma cells, discouraged the mitosis of B-cells, immunoglobulin (Ig) as well as antibody production. Aflatoxins have been shown to decrease phagocytic activity in chicken leukocytes, including neutrophils **(Ibrahim *et al.*, 2000)**, macrophages, and monocytes **(Qureshi *et al.*, 1998)**. Following AFB intake, the first

organ exposed to this toxin is the intestine accommodating various T-lymphocyte subpopulations whose functions are mediated by cytokines. A reduced percentage of T-cell subsets is indicative of the impact of AFB on the cellular immunity of broilers' intestines **(Wittig & Zeitz, 2003).**

1.5. Aflatoxins modulate the immune response of the spleen

Other effects of immunity disturbance during aflatoxicosis are suppression of cell-mediated immune response and lymph-blast genesis, damage to delayed cutaneous hypersensitivity and graft-versus-host reaction, a decline in numbers of splenic CD4 (helper T) cell and IL-2 production, and levels of the heat-stable serum factors involved in phagocytosis **(Coulombe, 1993)**. other findings suggestive of AFB interference with immunity: inhibited myelopoiesis in the bone marrow, increased apoptotic thymocytes, and reduced lymphocytes in the peripheral blood (**Cukrova *et al.*, 1991**).

Aflatoxin B_1 has immune-toxic effects as illustrated in **figure (5)**, as exposure to AFB reduced ileac expression of IL-2, IL-4, IL6, IL-10, IL-17, and IFN-γ in chickens. Lipopolysaccharide-induced TNF factor (LITAF) expression also decreased within the intestine because it is employed as a marker for TNF activity since TNF-α has not been identified in birds **(Jiang *et al.*, 2015)**

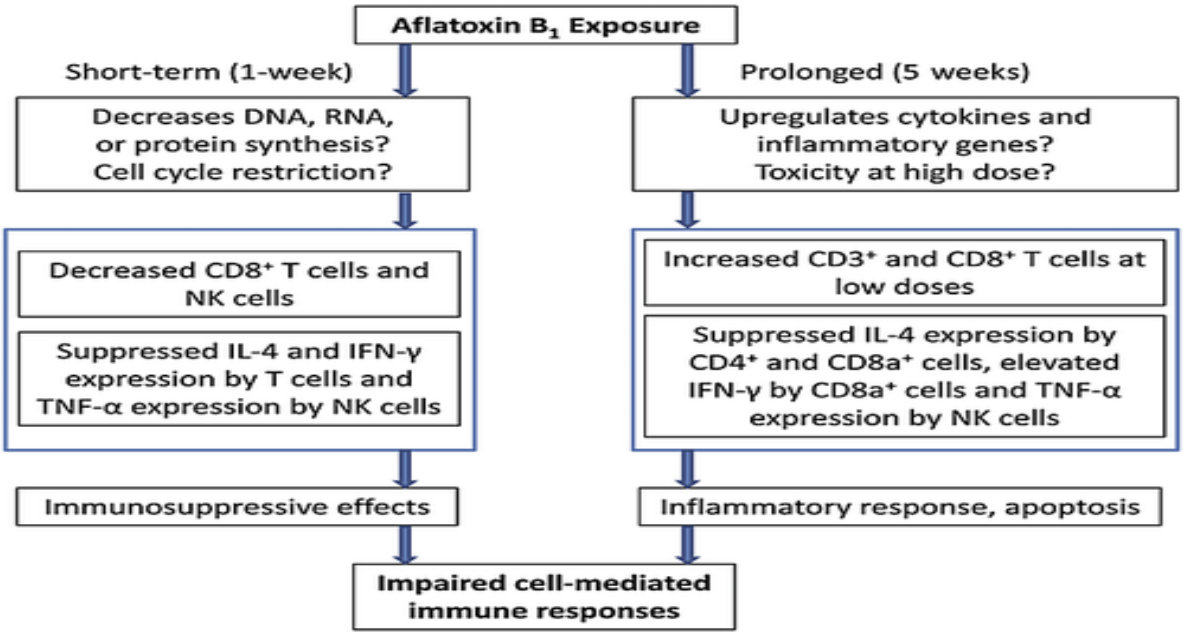

Fig (5): Possible mechanisms of the aflatoxin B_1 (AFB_1) immunotoxic effects in F344 rats **(Qian *et al.*, 2014)**.

1.6. Thymus state in intoxicated rats

The thymus is the primary lymphoid organ, where the T-cells develop in animals. The percentage of thymic T-cell subsets is an important parameter that represents the composition of mature T cells in the body. The number of mature T cells decides the biological function of mature T cells and finally relates to the cellular immune function of the body. Dietary aflatoxin causes a decrease in thymus weight and changes its color to pale **(Chen *et al.*, 2014)**, as AFB could repress the development of the thymus and inhibit the activity of immune cells, such as T lymphocytes and natural killer cells **(Reddy & Sharma, 1989)**.

2. Methods to resist aflatoxicosis

Naturally occurring chemicals with antioxidant properties prevent the oxidative effects of mycotoxins. Much less information is available from studies carried out using dietary antioxidants and mycotoxins. The protective properties of antioxidants probably result from their ability to

act as superoxide anion scavengers, thereby protecting cell membranes from mycotoxin-induced damage **(Gautier *et al*., 2001)**.

Chemoprevention is defined as disease prevention or reversal using nutrients or pharmacologic agents **(Sporn & Liby, 2005)**. Over the last years, milk attracted scientific interest as the primary source of nutrients for young mammalians, because it is nutritionally balanced. The most components that got attention in milk are milk proteins and their properties including mineral binding, antimicrobial, anti-lipidemic and anticancer properties ***(Kanwar et al*., 2015).**

2.1. whey proteins

The concepts in nutrition have changed considerably during the past decade. The growing interest in functional foods, which apart from nutritional values might be health-promoting and reduce the risk of several diseases **(Meisel, 2005)**. Whey Proteins (WPs) is a highly functional food showing high protein quality scores and containing relatively high proportions of essential amino acids. Consequently, increasing attention has been focused on the production of bioactive peptides derived from WPs **(Korhonen & Pihlanto, 2006).**

Whey Proteins represents a heterogeneous group of proteins (β-lactoglobulin, α-lactalbumin, serum albumin, and immunoglobulins). Indeed, recently published data have suggested that WPs has antioxidant activity, probably owing to the abundance of cysteine in WPs or the presence of glutamyl cysteine groups, which are also found in other food proteins. Therefore, WPs may be a therapeutic tool for oxidative-stress-associated diseases **(Balbis *et al*., 2009)**. It has been reported that WPs has immunomodulatory properties and the potential to increase host defense **(Rusu et al., 2010)**. And anticancer effects **(Castro *et al*., 2009)**.

Whey Proteins has utility in many different areas, including effects on bone, muscle blood, brain, pancreas, immune cells, cancer, infection, metabolism, wound healing, learning, and aging **(Krissansen, 2007)**. Moreover, milk WPs decreases free radicals' production in a murine model of chronic iron-overload cardiomyopathy **(Bartfay *et al.*, 2003).**

2.2. Camel milk benefits health

The milk composition of dairy animals has been widely studied throughout the world and thousands of references are available, especially about milk consumed by humans. The literature data mainly concerns cow milk, which represents 85% of the milk consumed in the world and, to a lesser extent, goat and sheep milk. Studies on other dairy animals (buffalo, yak, mare, and camel) are rather scarce, despite their nutritional interest. In this context, camel milk needs to be further investigated. There are only a few references on camel milk, whether they concern production or composition aspects **(Farah, 1993)**.

Camel milk is an important source of proteins for the people living in the arid lands of the world. Also, camel milk is known for its medicinal properties, which are widely exploited for human health, as in several countries from the ex-Soviet Union and developing countries **(Kenzhebulat *et al.*, 2000).** Camel milk is considered to have anti-cancer **(Magjeed, 2005)**, hypo-allergic **(Shabo *et al.*, 2005)**, and anti-diabetic properties **(Agrawal, 2003)**. High content in unsaturated fatty acids contributes to its overall dietary quality **(Karray *et al.*, 2005)**. The low quantity of β-casein and the lack of β-lactoglobulin are linked to the hypo-allergic effect of camel. Other components such as lactoferrin, immunoglobulins, lysozyme, or vitamin C were reported to play a central role in the determination of these properties **(Konuspayeva *et al.*, 2007).**

2.3. Bovine milk benefits to health

Bovine milk comprises a complex mixture of simple, globular, and conjugated proteins. Most of these are susceptible to the degradative effects of gastric processing, and extensive hydrolysis takes place upon exposure to enzymes in the gut **(Meisel, 1997).**

These enzymes may be of endogenous origin (i.e secreted by the digestive system) or exogenous origin (e.g derived from actively metabolizing gut microflora). In either case, many of the peptides released by enzymatic hydrolysis in the gut have specific biological functions through their ability to bind to (and affect) cellular function **(Meisel, 1997).**

Several of the biologically active peptides released by enzymatic hydrolysis of milk proteins are known to affect cells of the immune system, and (as a consequence) to affect downstream immunological responses and cellular functions (**Kayser & Meisel, 1996**).

Bovine milk contains some immunologically active peptides in the preformed or near-formed state i.e those which occur naturally in milk and require no or minimal gastric modification to become biologically active (**Guimont *et al.*, 1997**).

2.4. Comparison between camel milk components and bovine milk components

Table 3: Amino acid composition of camel and cows' milk **(Farah, 1993).**

Amino acid components	Camel	Cow
Alanine	2.7	3.5
Arginine	3.8	3.7
Aspartic acid	6.4	7.9
Cystine	0.6	0.7
Glutamic acid	19.5	21.8
Glycine	1.3	21
Histidine	2.7	2.8
Isoleucine	5	6.4
Leucine	9.5	10.4
Lysine	7.1	8.3
Methionine	3.6	2.7
Phenylalanine	5.6	5.2
Proline	11.1	10.0
Serine	4.2	5.6
Threonine	4.3	5.1
Tryptophan	—	1.4
Tyrosine	4. 0	5.3
Valine	6.9	6.8

Table 4: Vitamin content of camel and cows' milk (**Yadav et al., 2015**)

vitamin	camel milk	cow milk
Vitamin A (µg%)	20.10 ±10.00	99.60 ±62.00
Vitamin B (mg%)	19.60 ±6.40 mg%	34.70 ±8.10 mg%
Vitamin E (µg %)	32.70 ±12.80	171.00 ±114.40
Niacin (mg/ml)	4.60 mg/ml	0.60 mg/ml
B Carotene (µg%)	Absent	99.60± 62.00
Vitamin C (mg/ml)	35.00	10.00

2.5. Protective effects of camel whey proteins

Oxidative stress seems to play a fundamental role in lots of diseases. From here the necessity of natural antioxidants come as they play essential roles in improving immune system function via oxidative stress-dependent mechanisms. The addition of antioxidants to food takes a special concern, particularly the milk and milk-derived peptides, which are consumed every day by humans of all ages, for their overall health and immune system functions **(Kurutas, 2015).**

Camel milk proteins include lactoferrin A, α-lactoserum albumin, lactoperoxidase, and immunoglobulins**.** Three other fractions peptidoglycan recognition protein (PGRP), whey acidic protein (WAP), blood serum albumin **(El-Hatmi *et al.*, 2006).**

2.6. Antiviral effects of camel milk proteins

Camel's milk had antiviral effects and was found to improve biochemical and physical parameters in chronic active liver disease patients. Lactoferrin extracted from camel's milk also had anti-hepatitis C effects, which were found to prevent it from entering the cells **(Konuspayeva *et al.*, 2007)**.

It has also been reported that camel's milk enhances the immune response of human which inhibit chronic hepatitis **(Saltanat *et al.*, 2009)**. Camel's milk has positive effects on chronic hepatitis and lactoferrin in camel's milk is more anti-viral than bovine lactoferrin and lactoferrin extracted from breast milk (**Gader & Alhaider, 2016**).

2.7. Antioxidant effects of camel whey proteins

Camel whey proteins has antioxidant activity due to the existence of cysteine or glutamyl cysteine **(Balbis *et al.*, 2009)**. Camel whey proteins can modulate immune functions through the activation and proliferation of lymphocytes, cytokine secretion, production of antibodies, phagocytic activity, and granulocyte and natural killer (NK) cell activity (**Gauthier *et al.*, 2006)**. It also stimulates IL-1β, IL-6, and TNF-α (**Rusu *et al.*, 2010**). Moreover, WPs stimulates lymphocytes and increases phagocytosis and secretion of immunoglobulin A (IgA) from Peyer's patches. Whey proteins improved immunity during early life and protects against immune disorders during diabetes (**Beaulieu *et al.*, 2006**).

2.8. Anticancer activity of camel whey proteins

Camel whey protein hydrolysates showed potential anticancer activity against human liver cancer HepG2 cells, and the anticancer activity was dose-dependent **(Kamal *et al.*, 2018)**. The most important protein in camel milk is lactoferrin (Lf) which is a glycosylated globular protein with molecular weight of 78 kDa that nearly consists of 690 amino acid residues **(Baker & Baker, 2005)**. It was first known as the "red protein" of milk, which was subsequently defined as an iron-binding protein due to its sequestration of Fe2+ and Fe3+ free ions and is therefore categorized as a metalloprotein **(González-Chávez *et al.*, 2009).**

Cell proliferation is physiological machinery that occurs in almost all tissues under different circumstances. However, free cell division can induce tissue propagation and even metastasis. Therefore, the inhibition of cell proliferation is thought to be a functional method for neoplasm therapy. Anticancer effects of food protein-generated peptides have been extensively explored and there are several approved peptide-based anticancer drugs **(Actor *et al*., 2009)**.

Milk-derived peptides have shown interesting chemoprotective properties. In particular, lactoferrin has been reported to inhibit the growth of a breast cancer cell line (MDA-MB-231) and nasopharyngeal carcinoma cells by arresting the cell cycle at the G1–S transition and suppressing Akt signaling, respectively (**Azhar *et al*., 2021**).

Lactoferrin is also known for its anti-oxidant, anti-bacterial, antiviral, antifungal, antimicrobial, anti-inflammatory, anti-parasitic, anti-allergic, and most importantly anti-cancerous properties. The cellular level lactoferrin significantly affects the differentiation, maturation, activation, migration, proliferation, and functions of immune cells by using nuclear factor-kappa B (NF-κB) and MAP kinase signaling pathway (**Parhi *et al*., 2012**).

2.9. Effect of lactoferrin on immunity

Inflammation is a key risk factor for the development of various chronic illnesses such as obesity, diabetes, and tumor progression. Generally, cells exposed to inflammation release pro-inflammatory cytokines that have damaged effects on DNA, leading to cancer initiation or promotion. Recent reports hypothesized the potential of milk-derived proteins and their hydrolysates against chronic inflammation prompted us to explore the anti-inflammatory potential of camel milk whey proteins and their hydrolysates (**Kamal *et al*., 2018**).

Lactoferrin at the molecular level influences the maturation of lymphocytes and the release of cytokines in the bone marrow microenvironment. The anti-inflammatory action of LF alleviates stress by preventing the excess inflammatory response, it is hypothesized that iron confinement by LF from the microenvironment limits the oxidative damage to bio-membranes by restraining lipid peroxidation, LF minimizes the damage to the surrounding tissues by controlling the systemic inflammatory response (**Actor *et al*., 2009**).

It was revealed that LF Increases NK cells activity, and activates macrophages by increasing the production of cytokines and nitric oxide (NO) which in turn, reduces the proliferation of intracellular pathogens, and potentiates the phagocytic activity of neutrophils, LF is involved in regulating the production of antigen-presenting cells (APCs) like dendritic cells macrophages, and B cells which present the processed antigen to CD4+ T cells via major histocompatibility complex II (MHC II). LF also interacts with specific cell receptors of immune and epithelial cells to modulate immunity **(Damiens *et al*., 1998)**.

2.10. The protective effect of bovine whey proteins

The two major important families in milk proteins are whey proteins (soluble) and caseins (insoluble). Milk globular molecules with a substantial content of a-helix motifs which are called whey proteins have acidic/basic and hydrophobic/hydrophilic amino acids distributed in a fairly balanced way along their polypeptide chains. whey proteins by addition of, or in situ production of acid, or rennet-driven coagulation of the whole milk while caseins account for 80% (w/w) of the whole protein inventory, and can easily be recovered from skim milk via isoelectric precipitation **(Madureira *et al*., 2007)**

Whey proteins include immunoglobulins (IG), β-lactoglobulin (β-LG, for short), bovine serum albumin (BSA), α-lactalbumin (α-LA),

lactoperoxidase (LP), bovine lactoferrin (BLF), and, together with other minor components. The whey protein profile including general physiochemical and chemical properties are depicted in **table (6)** and their main biological activities are summarized in **table (7).**

Table 5: Proteins profile of bovine whey proteins and primary structure basic properties (**De Wit, 1998**)**.**

Protein	Concentration (g/l)	Molecular weight (kDa)	Number of amino acids residues
β-Lactoglobulin	1.3	18**,** 277	162
α-Lactalbumin	1.2	14**,** 175	123
Bovine serum Albumin	0.4	66,267	582
Immunoglobulins (A, M and C)	0.7	25,000 (light chain) + 50,000– 70,000 (heavy chain)	–
Lactoferrin	0.1	80,000	700
Lactoperoxidase	0.03	70,000	612
Glycomacropetide	1.2	6700	64

Table 6: Biological functions of bovine whey proteins **(Madureira *et al.*, 2007).**

Protein	Biological function
Whole whey proteins	Prevention of cancerClick or tap here to enter text. • Breast and intestinal cancer Increment of glutathione levels • Increase in tumor cell vulnerability • Treatment of HIV patients Antimicrobial activities Increment of satiety response • Increment in plasma amino acids, cholecystokinin, and glucagon-like peptide
β-Lactoglobulin	Transporter • Retinol • Palmitate • Fatty acids • Vitamin D and cholesterol Enhancement of pragmatic esterase activity Transfer of passive immunity Regulation of mammary gland phosphorus metabolism
α-Lactalbumin	Prevention of cancer Lactose synthesis Treatment of chronic stress-induced disease
Bovine serum albumin	Fatty acid binding Anti-mutagenic function Prevention of cancer Immunomodulation Disease protection through passive immunity
Immunomodulation	Antibacterial activity Antibacterial activity Opioid activity

2.11. Antimicrobial and antiviral properties of Bovine whey proteins

The main biological property of milk whey proteins that have been most thoroughly examined is their antibacterial function. Whey protein concentrate enriched with Helicobacter pylori-specific antibodies produced by lactating cows prevented infections thereby **(Early *et al.*, 2001).**

2.12. Immune system modulation of bovine whey proteins

Bovine whey proteins suppressed in vitro lymphocyte mitogenesis and alloantigen-induced proliferation when included in mature murine lymphocytes solutions. When added to T and B lymphocyte cultures**,** modified WPC can also inhibit the mitogen-stimulated secretion of γ -interferon, as well as the surface expression of the interleukin-2 receptor. Whey proteins increased chemokine-mediated actin polymerization in T-lymphocytes in the spleen. The most abundant protein in bovine whey protein is β-lactoglobulin, which stimulates the proliferation of spleen cells and lamina propria lymphocytes. The second most abundant protein in bovine whey protein, α-lactalbumin which modulates macrophages and B and T cell functions **(Tsai *et al.*, 2000)**.

Dietary whey protein concentrate supplementation increases the B cell population via Th cell-mediated, increases IL-2 and IL-4 levels and increased humoral immunity, particularly plasma IgG production. Moreover, WPs decreased the IgA levels before the antigen challenge. Whey proteins concentrate prevents antigen exposure by improving innate immunity and also activates adaptive immunity when antigen exposure is increased **(Ha *et al.*, 2021).**

2.13. Effect of whey proteins on glutathione

Whey protein's richness in cysteine and glutamate residues suggests that their intake will increase the level of free cysteine, and therefore produce GSH. In the immune-deficient state, WPs is an effective cysteine donor to supplement GSH in immune deficiency cases; as glutathione is important in immune regulation and cancer prevention in animals, immunity, and liver function enhancement, as well as helping HIV positive patients to overcome glutathione deficiency. Cell culture studies and in vivo experiments have shown that whey proteins can enhance non-specific and specific immune responses **(Micke *et al.*, 2002).**

The amino acids cysteine, glutamic acid, and glycine are part of the primary structure of this peptide; the incorporation of cysteine is the step that limits the speed of its synthesis; cysteine and glutamine in macrophages and lymphocytes are important participants in the coordinated T-cell response **(Grey *et al.*, 2003).**

Whey-based product supplementation increase lymphocyte GSH levels in patients suffering from lung inflammation associated with cystic fibrosis. In addition, the immunization supplement (long-term) is a WPs that is effective in improving liver dysfunctions in patients with chronic hepatitis B. In most patients, serum alanine aminotransferase (ALT) activity decreases, and plasma GSH levels increases in most patients suffering from that condition, 12 weeks after provision of the aforementioned supplement started; on the other hand, serum lipid peroxide levels decreased significantly, natural killer (NK) activity increased significantly and interleukin IL-2 levels which is a cytokine that regulates the activities of white blood cells (lymphocytes) also increased, resulting in enhancement of body's natural response to microbial infection **(Tsai *et al.*, 2000).**

2.14. Anticancer Activity of bovine whey protein

Whey proteins in the diet prevent various metastasis for example, breast cancer and bowel cancer in female rats. The entire whey protein system is resistant to chemically-induced colon and breast tumors in the body **(Yoo *et al.*, 1998)**. In vivo experiments demonstrated anticancer activity determined by WPs through the influence of increasing GSH concentration in related tissues.; stimulation of immunity via the GSH pathway produces anti-tumor effects in low-volume tumors **(Keri Marshall, 2004)**.

Whey Proteins can cause tumor cells with a higher concentration of GSH more vulnerable to chemotherapy in vitro experiments, immunoglobulin causes GSH depletion and inhibits the proliferation of human breast cancer cells, and its concentration can induce GSH synthesis in normal human cells. Hydrolyzed Whey protein isolate (WPI) can prevent oxidant-induced cell death in the human prostate epithelial cell line (RWPE-1), which is also due to increased GSH synthesis **(See *et al.*, 2002).**

Whey protein isolate may also protect against cancer by acting as a co-adjuvant of baicalein – an anticancer drug; the cytotoxicity of this molecule is enhanced by inducing more apoptosis in the human hepatoma cell line Hep-G2 which is in turn associated with depletion of GSH **(Tsai *et al.*, 2000)**.

2.15. Effect of Bovine whey proteins on immunity

The major whey protein β-LG can stimulate interferon- γ (IFN-γ) production by T-lymphocytes present in lamina propia and Peyer's patches while it activates IFN-γ and IL-10 production by human cord lymphocytes **(Ebert & Roberts, 2001).**

Bovine macrophages cultured with α-LA have increased production of IL-1b. on the other hand, α-LA reduced IL-6 release in the

blood of rats that underwent a transient intestinal ischemia/reperfusion **(Yamaguchi & Uchida, 2007).** Bovine LF can enhance phagocytosis and IL-8 production of human neutrophils **(Miyauchi *et al.*, 1998)**. Neutrophils are key players of innate immunity and the first cells that infiltrate inflammatory sites, where they deploy a battery of responses against invading microorganisms **(Nauseef, 2007)**. Neutrophils can also modulate host immune responses by producing multiple cytokines **(Cassatella, 1999).**

Neutrophils can counterbalance the proinflammatory effects of TNF-α and IL-1 by producing soluble cytokine receptors like sTNFp55, sTNFRp75, and IL-Ra **(Malyak *et al.*, 1994)**. IL-1 is the term for 2 polypeptides, IL-1a and IL-1b, that stimulate the same receptor on target cells and share common biologic activities **(Taga *et al.*, 1989)**. IL-1 is a crucial mediator of inflammatory and immune responses. Neutrophils can produce IL-1a and IL-1b, but whereas IL-1a is primarily a cell-associated molecule with little or no release, the active form of IL-1b is secreted **(Lord *et al.*, 1991).**

The biological effects of IL-1b are tightly regulated by IL-1Ra, which blocks the binding of IL-1b to type I and II receptors. The efficacy of IL-1Ra to abrogate the effects of IL-1b was demonstrated in a variety of in vitro systems and animal models of inflammatory diseases **(Arend *et al.*, 1990).**

Furthermore, an imbalance between IL-1Ra and IL-1b plays a key role in the pathophysiology of chronic disorders like inflammatory bowel disease, type 1 diabetes, and rheumatoid arthritis **(Netea *et al.*, 1997)**. Some workers demonstrated that a bovine whey protein extract (WPE) could enhance innate immune defenses through priming human blood neutrophils **(Rusu *et al.*, 2010).** These cells are also known to participate in immune defenses by releasing IL-1Ra (21). IL-1Ra exists as 3

isoforms: the 17-kDa secretory IL-1Ra (sIL-1Ra), 18-kDa intracellular IL-1Ra (icIL-1RaI), and 16-kDa intracellular IL-1Ra (icIL-1RaII). The latter is formed by alternative translation initiation of SIL-1Ra mRNA. Neutrophils synthesize sIL-1Ra and icIL-1RaII only **(Malyak *et al.*, 1998)**.

Other cytokines produced by neutrophils, like IL-8, IL-6, and TNF-α, have been shown to prime these cells and to mediate an increased innate immunity **(Mitchell *et al.*, 2003)**. Therefore, it was hypothesized that WPs, while having no direct effects on primary functions of neutrophils like chemotaxis, phagocytosis, oxidative burst, and degranulation, could modulate immune defenses by stimulating the production of certain cytokines by neutrophils **(Rusu *et al.*, 2009).**

3. Chemokine CXCL12 / CXCR4 axis

3.1. Introduction

There are more than 50 chemokines have been discovered, chemokines are a class of small (8–10 kDa) inflammatory or homeostatic cytokines sharing a common biological activity in stimulating the migration of different types of cells including lymphocytes, monocytes, neutrophils, endothelial cells, mesenchymal stem cells, and malignant epithelial cells **(Smith *et al.*, 2012).**

Chemokines are classified into four conserved groups – CXC, CC, C, and CX3C – based on the number and spacing of their N-terminal cysteine residues: CXC chemokines have a single non-conserved amino acid residue (X) between the first N-terminal cysteine residues (C); CC chemokines have these two cysteine residues adjacent; C chemokines have only one N-terminal cysteine; whereas CX3C chemokines contain three non-conserved amino acid residues separating the N-terminal cysteine pair **(Viola & Luster, 2008).**

Over 20 chemokine receptors have been identified **(Pierce *et al.*, 2002)**, chemokine receptors belong to a family of G protein-coupled receptors (GPCRs) containing seven transmembrane-spanning α-helix domains. One of the intracellular loops of the chemokine receptors couples with heterotrimeric G proteins that mediate a cascade of intracellular signaling following ligand binding **(Gilman, 1987)**.

3.2. CXCR4 /CXCL12 signaling pathway

The heterotrimeric G protein is composed of the Gα, Gβ, and Gγ subunits. Both Gα and Gβ subunits have covalently attached lipid tails that anchor G proteins to the plasma membrane in the inactive or basal state, the Gα subunit contains the guanine nucleotide diphosphate (GDP), upon activation, GPCR acts as a guanine nucleotide exchange factor (GEF) and promotes the conformational change of the Gα subunit and replacement of the bound guanine nucleotide diphosphate (GDP) by guanine nucleotide triphosphate (GTP) **(Milligan & Kostenis, 2006)**.

This exchange triggers the further conformation changes within the Gα subunit, which allows the trimeric G protein to be released from the receptor, and to dissociate into the GTP-bound Gα subunit and Gβ/Gγ dimer. Both the activated components interact with various effector proteins and initiate unique intracellular signaling cascades, such as activation of phospholipase C (PLC), regulation of adenylate cyclase, triggering of different kinase cascades including mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), p38, and the phosphoinositide-3-kinase (PI3K) routes (**Figure 6**) **(Pierce *et al.*, 2002).**

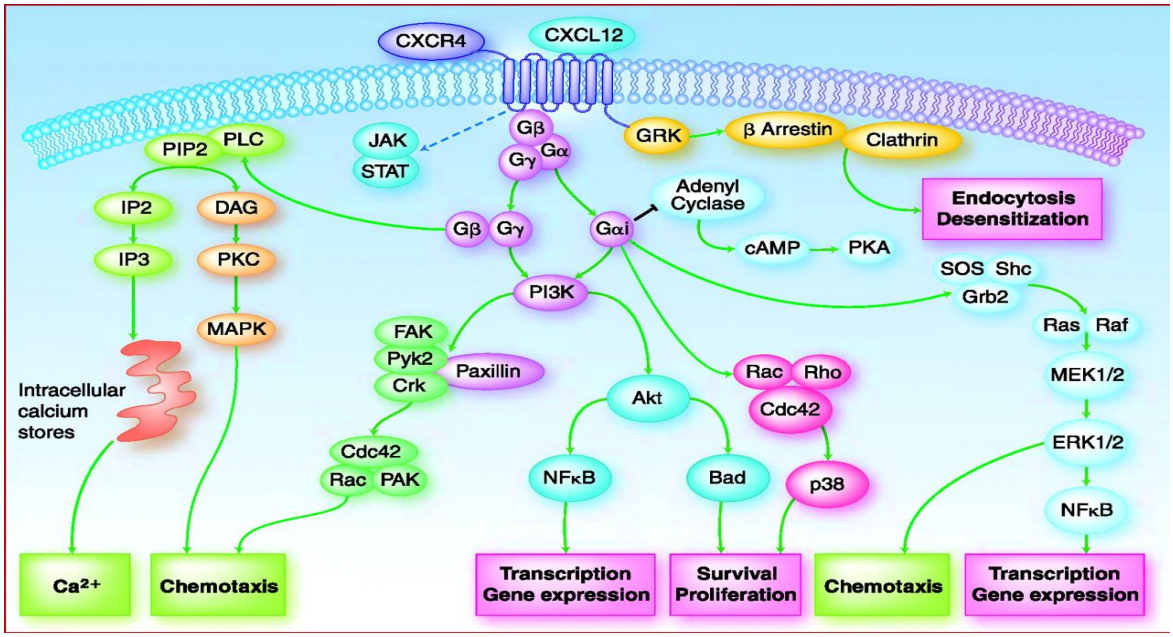

Fig (6): A schematic of the CXCL12/CXCR4 intracellular signal transduction pathways **(Teicher & Fricker, 2010).**

The distinct routes of the GPCRs signaling to depend on the coupled Gα subunits, which are classified into four families; Gαs, Gαi, Gαq, and Gα12. GPCRs coupled to the Gαs stimulate adenylyl cyclase whereas Gαi bound GPCRs to inhibit it, the adenylyl cyclase serves as an effector enzyme that catalyzes 5′adenosine triphosphate into cyclic adenosine monophosphate (cAMP) and thereby activates cAMP-dependent protein kinase, which regulates a host of other downstream effectors including MAPK signaling pathway **(Gerits *et al.*, 2008)**. Activated Gαi is also able to activate the Src family of tyrosine kinases (SFKs), which play an important role in signal integration **(Mohammadi *et al.*, 2014).**

GPCRs coupled to Gαq act through Phospholipase C β subunit (PLCβ), which cleaves phosphatidylinositol 4,5-bisphosphate to form the second messenger molecules called diacylglycerol and inositol-1,4,5-trisphosphate (IP3). Diacylglycerol activates another enzyme called

protein kinase C (PKC), whereas IP3 diffuses to the endoplasmic reticulum where it opens calcium channels and triggers the release of calcium from intracellular stores into the cytoplasm. This intracellular calcium mobilization is frequently used for the analysis of chemokine receptor activity **(Princen *et al.*, 2003)**.

Activation of PI3K by GPCRs is thought to be dependent on the direct binding of Gβγ subunits. PI3K activation triggers a signaling cascade leading to the activation of AKT (also called protein kinase B) and its downstream targets including phosphoinositide-dependent kinase 1 (PDK1), glycogen synthase kinase 3 (GSK3), mammalian target of rapamycin (mTOR), p70 ribosomal protein S6 kinase (p70S6K), forkhead family transcription factors (FOXO), and other signaling proteins. Notably, PI3K activation in response to the GPCR-mediated signaling results in the activation of focal adhesion kinase (FAK), which induces migratory activity in different types of cells, including tumor cells **(Liang & Slingerland, 2003)**.

The duration of the GPCR signaling depends on the Gα subunit lifespan in the GTP-bound state. Hydrolysis of the GTP of Gα-GTP to GDP leads to the inactivation of the Gα subunit and its reassociation with the Gβ/Gγ dimer, which terminates all effector interactions **(Teicher & Fricker, 2010).**

In addition, chemokine receptor signaling is tightly regulated by the process of internalization and lysosomal degradation. Upon GPCR signaling activation, intracellular domains of receptors are phosphorylated by the second messenger kinases such as G protein-coupled receptor kinases (GRKs), followed by the binding of the phosphorylated receptors with regulatory proteins called arrestins. These

arrestins impair communication of GPCRs with the G proteins and target them for lysosomal degradation following protein internalization and trafficking (**Figure 7**) **(Luttrell & Gesty-Palmer, 2010).**

Interestingly, it has been reported that several chemokine receptors including CCR2, CCR5, CXCR1, CXCR2, CXCR4, and CXCR7 can undergo homo- or hetero-dimerization upon ligand binding; a process that was proposed to regulate distinct intracellular signaling pathways **(Décaillot *et al*., 2011)**.

Chemokines and their receptors display a high degree of redundancy in that most chemokines bind to multiple receptors and vice versa. The chemokine stromal cell-derived growth factor-1 (SDF-1), also known as CXCL12, binds primarily to its cognate receptor CXCR4, which is also a coreceptor for the entry of the human immunodeficiency virus (HIV) into the target immune cells (T helper cells) besides the CD4 receptor **(Berger *et al*., 1999).**

The assumption that CXCR4 is the only receptor for CXCL12 was recently challenged since it was demonstrated that this chemokine also binds to the orphan receptor called CXCR7, which is a receptor for the interferon-inducible T-cell chemoattractant CXCL11/I-TAC. Moreover, CXCR7 constitutively forms heterodimers with the CXCR4 receptor. Growing evidence indicates that binding of CXCL12 to CXCR7 does not result in activation of signaling pathways typical of G proteins. It has been proposed that CXCR7 serves as a ligand scavenger or acts as a "decoy" receptor **(Singh *et al*., 2013).**

This receptor has been described as an activator of various signaling pathways in a CXCL12-dependent manner. CXCR7 is broadly expressed in normal tissues including the heart, brain, spleen, kidney, lung, testis, ovary, thyroid, and human placenta **(Sun *et al*., 2010)**.

Germline deletion of CXCR7 resulted in perinatal lethality and expression of CXCR7 was associated with cardiac development. Moreover, CXCR7 is upregulated in many malignant cells including breast, lung, cervical, pancreatic, and prostate cancer cells, and found to be involved in tumor cell growth, survival, and metastasis **(Décaillot *et al.*, 2011)**.

3.3. Physiological function of the CXCL12/CXCR4 signaling

CXCL12 is a small (8 kDa) homeostatic chemokine that was originally described as an efficacious lymphocyte chemoattractant and regulator of hematopoiesis and was soon after also characterized as a modulator of multiple physiological processes **(Kucia *et al.*, 2004)**.

CXCL12 is a pleiotropic chemokine that is widely expressed in different organs including the brain, lung, colon, heart, kidney, and liver where it acts as a chemoattractant for immature and mature hematopoietic cells; it thus plays an important role in inflammation and immune surveillance of tissues. Additionally, CXCL12 serves as an emergent salvage signal for initiating tissue regeneration and repair, various tissues respond to the chemical or physical insults such as toxic agents, hypoxia, and irradiation by increasing the expression and secretion of CXCL12, which is important for the recruitment of CXCR4 positive stem and progenitor cells to a site requiring tissue regeneration **(Kucia *et al.*, 2004)**.

CXCL12 is expressed from a single gene in six splice variant isoforms known as SDF-1α, SDF-1β, SDF-1γ, SDF-1δ, SDF-1ε, and SDF-1ϕ **(Ho *et al.*, 2012)**. These CXCL12 isoforms share the same first three exons but contain different fourth exons. Different splice variants are characterized by distinct properties such as stability and tissue of

origin. SDF-1α is constitutively produced in many organs but tends to undergo rapid degradation in the blood. In contrast, SDF-1β displays high proteolytic stability and is expressed in highly vascularized organs such as the liver, spleen, and kidney, SDF-1γ is present in less vascularized organs such as the heart and brain **(Janowski, 2009).**

The expression of CXCL12 cognate receptor CXCR4 is highest in hematopoietic cells but it is also widely and constitutively expressed by numerous cells types including hematopoietic stem cells, endothelial stem cells, liver oval stem cells, neural stem cells, skeletal muscle satellite cells, primordial germ cells, retina pigment epithelium stem cells, and embryonic stem cells. All of these cells not only express functional CXCR4 on their surface but also follow a CXCL12 gradient **(Tiveron & Cremer, 2008).**

Due to the apparent redundancy within the chemokine system the knockout of one of the chemokines/GPCR axes could be compensated to a large extent by other GPCR routes. However, mice lacking the CXCR4 or CXCL12 genes exhibit a significant defect in the colonization of embryonic bone marrow by hematopoietic stem cells (HSC) and show defects in the development of other organs including the heart, brain, and blood vessels. In the case of CXCL12 and CXCR4, ablation of both gene is lethal and these embryos die in utero, Thus, the CXCL12/CXCR4 axis appears to have a fundamental physiological role in normal tissue development **(Vandercappellen *et al*., 2008)**.

3.4. The role of chemokines in lymphocytes trafficking

Lymphocyte trafficking between blood and secondary lymphoid tissues, for example, is a nonrandom process that is regulated by tissue-specific expression of chemokines **(Campbell *et al*., 1998)**. Circulating

blood lymphocytes interact transiently and reversibly with vascular endothelium through adhesion molecules (selectins, integrins) in a process called rolling. Chemokines on the luminal endothelial surface can activate chemokine receptors on the rolling cells, which triggers integrin activation **(Springer, 1994).**

This results in the arrest, firm adhesion, and trans-endothelial migration into tissues where chemokine gradients direct localization and retention of the cells, these steps collectively referred to as "homing" are essential for the normal development of the organism, organization, and function of the immune system, and tissue replacement **(Campbell *et al*., 1998)**.

Secretion of CXCL12 by marrow stromal cells is a major source for CXCL12 in adults. Stromal cells create cellular niches in which hematopoietic stem cells (HSCs) and progenitors are retained for growth and differentiation **(Fuchs *et al*., 2004)**. Chemotactic responsiveness of hematopoietic stem cells is restricted to CXCL12 **(Wright *et al*., 2002)**. This unique selectivity for CXCL12 may be necessary for the retention of HSCs in the hematopoietic microenvironment and marrow-specific homing of circulating HSCs (**Figure 7**), **(Peled *et al*., 1999).**

Clinical trials have demonstrated that CXCR4 antagonists, alone or in combination with granulocyte colony-stimulating factor (G-CSF), can affect the rapid mobilization of HSCs, supporting the hypothesis that CXCL12 is essential for HSCs retention within the marrow (**Broxmeyer *et al.*, 2005**). Because CXCL12 helps retain B-cell precursors in close contact with protective stromal cells within the hematopoietic microenvironment, its expression is essential for normal B-cell development **(Egawa *et al.*, 2001)**.

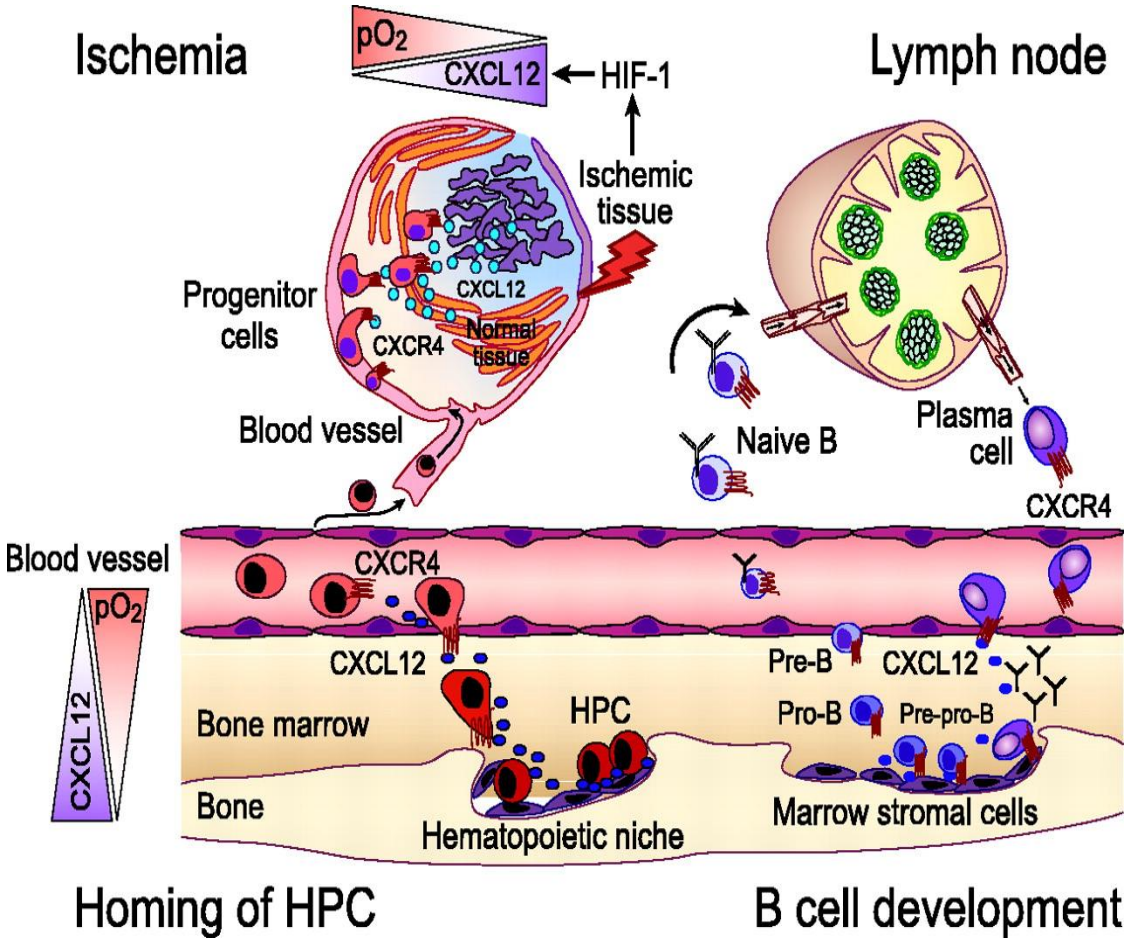

Fig (7):The CXCR4 chemokine receptor in homing of hematopoietic progenitors, B-lymphocyte development, and progenitor recruitment to sites of ischemic tissue damage **(Burger & Kipps, 2006).**

End-stage B cells (plasma cells) also require CXCR4 for homing to CXCL12-rich niches within the marrow (**Figure 7**). Moreover, CXCL12 also may function as a paracrine growth factor for B lymphocytes and other cell types. Initial studies characterized CXCL12 as a pre-B-cell growth-stimulating factor (PBSF) because recombinant CXCL12 supported the proliferation of a stromal cell-dependent B-cell line (**Tokoyoda *et al*., 2004**).

Another highly important function of the CXCR4/CXCL12 axis is related to tissue repair and regeneration. Repair of ischemic injuries involves the selective recruitment of circulating or resident progenitor cells. Hypoxia-inducible factor-1 (HIF-1), a central mediator of tissue hypoxia, induces CXCL12 expression in ischemic areas in direct proportion to reduced oxygen tension in vivo **(Ceradini *et al*., 2004)**.

Hypoxia-inducible factor-1(HIF-1) induced CXCL12 expression on endothelial cells attracts circulating stem and progenitor cells to areas of tissue damage. As such, hypoxia induces a transient, conditional stem cell niche for CXCR4-mediated progenitor cell recruitment for tissue repair (**Figure 7**), **(Li & Rich, 2010)**.

During tissue regeneration, the expression of CXCL12 normalizes after regular oxygen tension has been restored **(Ceradini *et al*., 2004)**. Distinct niches of hypoxia are also present in the normal marrow that display increased levels of CXCL12. Progenitor cells localize with these niches, suggesting that HIF-1-regulated CXCL12 expression plays an important role in generating concentration gradients of CXCL12 within the marrow microenvironment (**Ceradini *et al*., 2004)**.

In addition to recruiting stem cells, CXCL12 also modulates the function of the immune system through lymphocyte migration,

development, and survival. Elevated plasma CXCL12 levels result in up to a ten-fold increase in leukocytes in peripheral circulation **(Hattori *et al.*, 2001)**. CXCL12 also acts as a chemotactic factor for T lymphocytes and monocytes promoting trans-endothelial chemotaxis to local inflammatory responses **(Bleul *et al.*, 1996).**

3.5. Role of the CXCL12/CXCR4 axis in cancer and cancer stem cells

The chemokine CXCL12 and its cognate receptor CXCR4 were first identified as regulators of trafficking and tissue localization of B cells from patients with chronic lymphocytic leukemia **(Möhle *et al.*,1999)**.

C-X-C motif chemokine receptor type 4 (CXCR4) was proposed to regulate the trafficking and invasion of breast cancer cells to sites of metastases. (**Müller *et al.*, 2001**). More recently, it has been established that CXCR4 plays a central role in tumor cell dissemination a fundamental physiological role in normal tissue development **(Ratajczak *et al.*, 2006)** and metastasis development in more than 75% of all cancers including breast, ovarian, lung, colon, prostate, kidney, melanoma, brain, esophageal, pancreatic, and many forms of leukemia **(Darash-Yahana *et al.*, 2004).**

3.6. MDA level affect CXCR4 expression

Malondialdehyde (MDA) is one of the results of lipid peroxidation **(Gönenç et al., 2001)** and, in the last few years, it has come to be considered the main indicator of lipo-peroxidative processes **(Gaweł *et al.*, 2004)**, thus justifying the studies concerning its role as an indicator of oxidative stress **(Del Rio, 2005)** and a precursor of the endothelial disorder **(Polidori *et al.*, 2002).**

The lower total antioxidant capacity and higher malondialdehyde (MDA) levels increase oxidative stress and could be related to breast cancer (**Erten Şener *et al.*, 2007**). Malondialdehyde (MDA), one of the final decomposition products of lipid peroxidation is known to be present in human plasma and to possess biological properties that may be relevant to carcinogenesis. Lipid peroxides and their products can cause damage to membrane-bound enzymes and other macromolecules, including DNA and have been implicated in several disease processes, including cancer. Numerous studies have examined the possibility of a connection between lipid peroxidation and cancer **(Torun *et al.*, 1995)**.

Studies have shown that the regulation of CXCR4 mRNA expression depends on cell activation and oxidative stress, as well as cell type ***(Caruz et al.*, 1998)**. Furthermore, signaling and internalization of the CXCR4 protein can be regulated by receptor phosphorylation-dependent and independent mechanisms **(Haribabu *et al.*, 1997)**.

The reactions of lipid-derived intermediates with proteins lead to the formation of maillard reaction products, which subsequently lead to the formation of advanced lipo-oxidation end products (ALEs) which have pro-inflammatory effects in monocytes. Several key target proinflammatory proteins were significantly induced by MDA **(Shanmugam *et al.*, 2008).**

Nitric oxide and MDA play a significant role in DNA damage, sister-chromatid exchanges, and carcinogenesis. Patients with stage II disease showed the highest levels of both NO and MDA compared with control **(Ray *et al.*, 2001)**. ROS in the extracellular environment of human monocytes can increase the expression of the chemokine receptor CCR5 through redox-sensitive transcription factors, such as NF-Kβ **(Lehoux *et al.*, 2003).**

3.7. Nitric oxide level correlate with cxcr4/cxcl12 axis regulation

Nitric oxide is a short-lived free radical that serves as a signaling molecule in a wide spectrum of pathophysiological and physiological processes including inflammation, apoptosis, regulation of enzyme activity, and gene expression. Nitric oxide promotes T-cell activation at low levels but suppresses T-cell responses at high concentrations (**Thomas *et al.*, 2008**). Within the vasculature, the production of NO from endothelial cells is required for vascular homeostasis **(Moncada & Higgs, 2006)**.

Nitric oxide is produced by NO synthase (NOS) using L-arginine and molecular oxygen as substrates, yielding NO and L-citrulline as product. Three isoforms of NOS are known, two of the isoforms, neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS, NOS3) are constitutively expressed whereas the other isoform is inducible NOS (iNOS or NOS2) is not, but its expression can be induced in tissues undergoing inflammatory responses. The multiple effects of NO are based on its capacity for modifying the function of many proteins, including ion channels, enzymes, and G proteins. Nitric oxide can also regulate gene expression by modulating transcription factor activity, translation, or stability of mRNA **(Bogdan, 2001)**.

These functions are performed through two pathways: NO may interact with soluble guanylate cyclase (sGC) and then activate the guanosine 3, 5-cyclic monophosphate (cGMP) second messenger system, namely the cGMP-dependent protein kinase (PKG) pathway. Also, NO may directly modify target proteins through S-nitrosylation of cysteine residues or nitration of tyrosine residues **(Bogdan, 2001).**

Nitric oxide donors exhibit a variety of effects upon Hematopoietic progenitor stem cells HPCs. They inhibit burst-forming unit-erythroid (BFU-E) formation while displaying inhibitory or stimulatory effects on colony-forming unit-granulocyte and macrophage (CFU-GM) growth ***(Michurina et al.*, 2004).**

It was shown that exposure of mice to NOS inhibitors increases the number of stem cells **(Michurina *et al.*, 2004),** which suggests that NO acts control the proliferation of Hematopoietic stem cells (HSCs). The importance of NO in stem cell biology was also indicated by the fact that mice deficient in eNOS (NOS3) show reduced hematopoietic recovery and impaired mobilization of endothelial progenitors in response to vascular endothelial growth factor (VEGF) suggesting that NO may influence the recruitment of stem and progenitor cells **(Aicher *et al.*, 2004).**

Nitric oxide is recognized as an important regulator of gene expression because a broad number of genes are regulated by NO both in vitro and in vivo, as CXCR4 was included in the NO-regulated genes, as they demonstrate a robust induction of CXCR4 mRNA paralleled by surface protein expression in human CD34 cells after exposure to NO-generating agents **(Shami & Weinberg, 1996).**

It has been shown that cell surface expression of CXCR4 on CD34(+) cells was increased in a dose- and time-dependent manner in response to NO donors, demonstrated that the NO pathway can modulate CXCR4 expression in human CD34(+) cells and suggests that NO may play a critical role in the tracking, regulation of proliferation, and differentiation of hematopoietic progenitors **(Zhang *et al.*, 2004).**

Nitric oxide modulates SDF-1 chemotaxis of human CD34 cells and, thus, further strengthens the link between NO signaling and stem cell

homeostasis. Although increased CXCR4 mRNA expression is a major mechanism by which NO alters CXCR4 membrane expression **(Staller *et al*., 2003).**

Pretreatment of T lymphocytes with NO donors markedly enhances SDF-1-induced chemotaxis **(Cherla & Ganju, 2001)**. Recent studies have reported that hypoxia-inducible factor (HIF-1) proteins induce CXCR4 expression by binding to hypoxia-responsive elements present in the promoter of the CXCR4 gene **(Schioppa *et al*., 2003)**.

Interestingly, NO stabilizes HIF in normal conditions during the last decade, it was also recognized that NO modifies protein activities by binding to sulfhydryl-containing molecules. In many biological systems, nitrosylation reactions transferring NO from an NO donor to a protein, affect protein functions **(Gaston *et al*., 2003).**

The activity of several transcription factors involved in the regulation of CXCR4 (including nuclear factor- κB and activator protein-1) was shown to be modified by S-nitrosylation **(Kelleher *et al*., 2007)**. Whether one of these factors represents the molecular target of NO-mediated effect on CXCR4 remains to be determined **(Marshall *et al*., 2000).**

4- Biological effect of IL-6 on inflammation and immunity

IL-6 is a soluble mediator with a pleiotropic effect on inflammation, immune response, and hematopoiesis. At first, distinct functions of IL-6 were studied and given distinct names based on their biological activity. For example, the name B-cell stimulatory factor 2 (BSF-2) was based on the ability to induce differentiation of activated B cells into the antibody (Ab)-producing cells **(Kishimoto, 1985),** the name hepatocyte-stimulating factor (HSF) on the effect of acute-phase protein synthesis on hepatocytes, the name hybridoma growth factor (HGF) on

the enhancement of growth of fusion cells between plasma cells and myeloma cells, and the name interferon (IFN)-β2 owing to its IFN antiviral activity **(Hirano *et al*., 1986).**

Human IL-6 is made up of 212 amino acids, including a 28-amino acid signal peptide, and its gene has been mapped to chromosome 7p21. Although the core protein is ~20 kDa, glycosylation accounts for the size of 21–26 kDa of natural IL-6. After IL-6 is synthesized in a local lesion in the initial stage of inflammation, it moves to the liver through the bloodstream, followed by the rapid induction of an extensive range of acute-phase proteins such as C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen, haptoglobin, and α1-antichymotrypsin **(figure 8), (Heinrich *et al*., 1990).** On the other hand, IL-6 reduces the production of fibronectin, albumin, and transferrin. These biological effects on hepatocytes were at first studied as belonging to HSF. When high-level concentrations of serum amyloid A (SAA) persist for a long time, it leads to a serious complication of several chronic inflammatory diseases through the generation of amyloid A amyloidosis **(Gillmore *et al*., 2001).**

This results in amyloid fibril deposition, which causes progressive deterioration in various organs. IL-6 is also involved in the regulation of serum iron and zinc levels via the control of their transporters. As for serum iron, IL-6 induces hepcidin production, which blocks the action of iron transporter ferroportin-1 on the gut and, thus, reduces serum iron levels **(Nemeth *et al*., 2004)**.

This means that the IL-6-hepcidin axis is responsible for hypoferremia and anemia associated with chronic inflammation. IL-6 also enhances zinc importer (ZIP) expression on hepatocytes and so induces hypozincemia seen in inflammation. When IL-6 reaches the bone

marrow, it promotes megakaryocyte maturation, thus leading to the release of platelets **(Liuzzi *et al*., 2005)**.

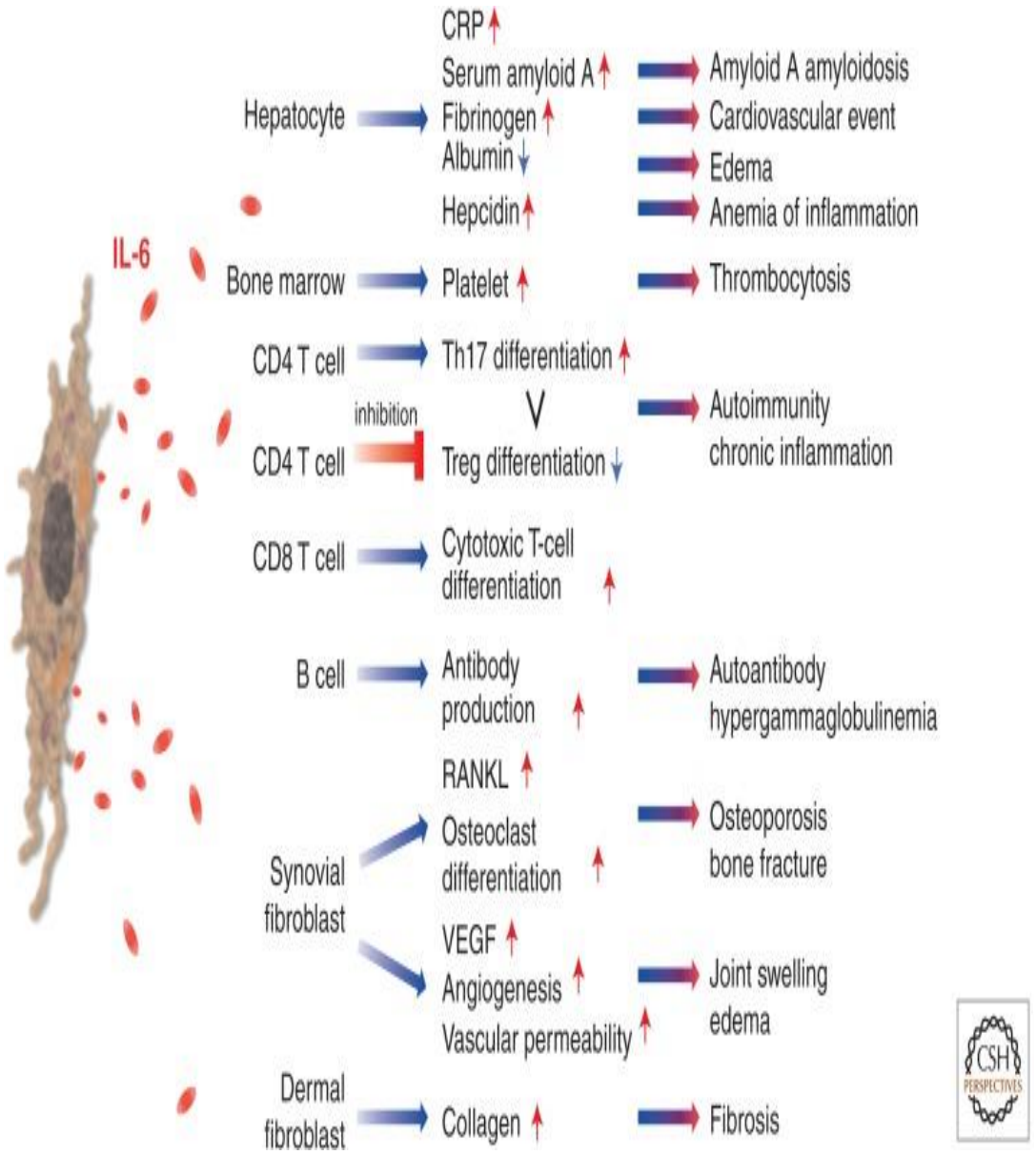

Fig (8): IL-6 in inflammation, immunity, and disease. IL-6 is a cytokine featuring pleiotropic activity; it induces the synthesis of acute-phase proteins such as CRP, serum amyloid A, fibrinogen, and hepcidin in hepatocytes, whereas it inhibits the production of albumin. IL-6 also plays an important role in the acquired immune response by stimulation of antibody production and effector T-cell development. Moreover, IL-6 can promote the differentiation or proliferation of several nonimmune

cells. Because of the pleiotropic activity, dysregulated continual production of IL-6 leads to the onset or development of various diseases. Treg, regulatory T cell; RANKL, receptor activator of nuclear factor κB (NF-κB) ligand; VEGF, vascular endothelial growth factor **(Tanaka *et al.*, 2014)**.

It has been further shown that IL-6 also promotes T-follicular helper-cell differentiation as well as the production of IL-21, which regulates Ig synthesis and IgG4 production in particular. IL-6 also induces the differentiation of $CD8^+$ T cells into cytotoxic T cells, under one of its previous names, BSF-2, IL-6 was found to be able to induce the differentiation of activated B cells into Ab-producing plasma cells, so that continuous over-synthesis of IL-6 results in hypergammaglobulinemia and autoantibody production **(Ma *et al.*,2012)**.

IL-6 exerts various effects other than those on hepatocytes and lymphocytes and these are frequently detected in chronic inflammatory diseases. One of these effects is that, when IL-6 is generated in bone marrow stromal cells, it stimulates the RANKL, which is indispensable for the differentiation and activation of osteoclasts, and this leads to bone resorption and osteoporosis **(Hashizume *et al.*, 2008)**. IL-6 also induces excess production of VEGF, leading to enhanced angiogenesis and increased vascular permeability, which are pathological features of inflammatory lesions **(Hashizume *et al.*, 2009)**.

5-Role of NF-κB in the immune system

Nuclear Factor-κB plays an important role in the development and function of primary (bone marrow, thymus) and secondary (lymph nodes, Peyer's patches, mucosal-associated lymphoid tissue, and the spleen) lymphoid tissues. There is a role for NF-κB in the development and regulation of bone **(Burkly *et al.*, 1995).**

5.1. Role of NF-κB in lymphopoiesis

The development of T and B cells has historically been the subject of much greater scrutiny than the development of cells of the myeloid lineages. NF-κB has been examined in many aspects of lymphopoiesis and found to be vital for the development and function of adaptive immune cells (**figure 9**), **(Siebenlist *et al*., 2005)**. Despite their potential longevity in the periphery, lymphocyte development is characterized by abundant apoptosis. As a consequence, the anti-apoptotic properties of NF-κB play a key role in lymphopoiesis. Indeed, in many instances, the requirement for NF-κB can be overcome by transgenic expression of the anti-apoptotic factor Bcl-2 **(Sentman *et al*., 1991).**

The deficiency in NF-κB function could be circumvented through overexpression of Bcl-2, the necessity of NF-κB for lymphopoiesis is strikingly illustrated in human genetic diseases in which the gene encoding NF-kappa B Essential Modulator (NEMO) is inactivated by mutation. Because the NEMO gene is located on the X chromosome, it is usually subject to random inactivation in individual cells in females. However, in female patients who are heterozygous for a mutant version of NEMO, all peripheral lymphocytes possess an intact NEMO gene, rather than the 50% predicted by random inactivation, suggesting that in the absence of NEMO-dependent NF-κB signaling, B and T cells fail to develop **(Derudder *et al*., 2009)**.

The effects of NEMO inactivation in both mice and humans solidify the role of NF-κB in lymphopoiesis, although the details by which NF-κB functions in this process remain obscure. NF-κB plays diverse roles in lymphocyte development that can be grouped according to timing that is, before, during, or after pre-antigen receptor signaling **(Horwitz *et al*., 1997).**

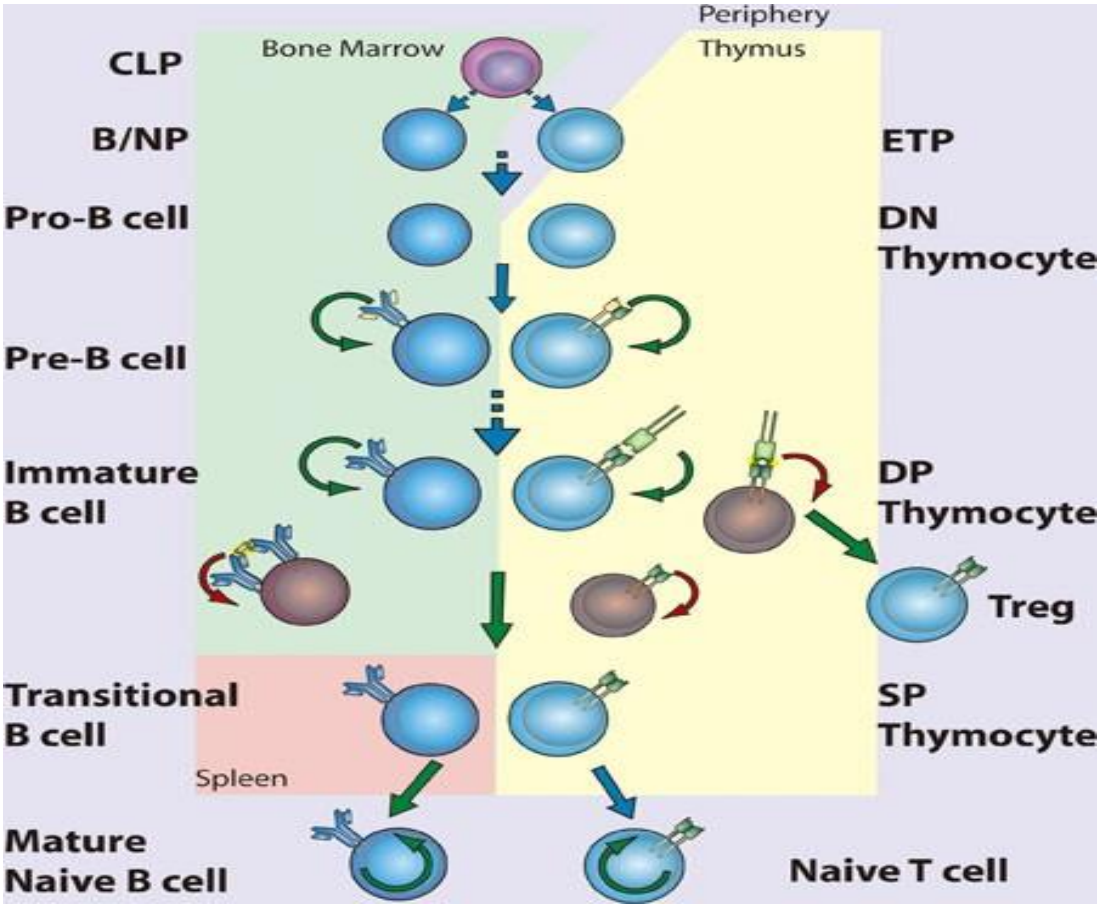

Fig (9): NF-κB in lymphopoiesis. NF-κB plays a pro-survival role in common lymphoid precursor (CLP) cells which give rise to B- and T-cell lineages. B-cell development occurs in the bone marrow, where NF-κB protects pre-B cells from pro-apoptotic stimuli including TNFα. Signaling to NF-κB through the pre-B cell receptor mediates survival of Pre-B cells, which then undergo light chain recombination to produce a functional B cell receptor. NF-κB provides a necessary pro-survival signal during Igλ but not Igκ rearrangement. Expression of BCR leads to NF-κB-dependent differentiation into immature B cells. High levels of BCR signaling, i.e., through recognition of self-antigen, result in negative selection through the loss of NF-κB activity. Transitional B cells exit the bone marrow and migrate to the spleen, where they mature and differentiate, a process that also requires NF-κB. T-cell development occurs following the migration of precursor cells into the thymus. Stimulation of NF-κB through pre-TCRα provides a pro-survival signal allowing recombination of the TCR-α chain and maturation to the double-positive (DP) stage. Optimal signaling through the TCRα/β complex induces NF-κB-dependent survival pathways, while a failure to signal or high-level signaling results in death by neglect or negative selection, respectively. Intermediate high NF-κB activation facilitates intra-thymic regulatory T cell (Treg) development. NF-κB activity is required for the maintenance of long-lived B and T cells. (CLP, common lymphoid progenitor; ETP, early thymic progenitor; DN, double negative – $CD4^{-}CD8^{-}$; DP, double-positive – $CD4^{+}CD8^{+}$; SP, single positive $CD4^{-}CD8^{+}$ **(Hayden & Ghosh, 2011)**.

Although no single NF-κB-subunit knockout mouse has as severe a phenotype as NEMO knockouts about the generation of mature lymphocytes, double knockouts of Rel proteins confirm the essential anti-apoptotic function of NF-κB. For example, loss of both p50 and p65, or both p65 and c-Rel, terminates lymphopoiesis before expression of the pre-antigen receptors, suggesting that NF-κB regulates antiapoptotic factors required for early lymphoid cell survival in response to pro-apoptotic stimuli **(Grossmann *et al.*, 1999)**. Hematopoietic stem cells can activate NF-κB in response to TNF-α, and in these cells, NF-κB acts as a pro-survival factor **(Pyatt *et al.*, 1999)**.

The role of NF-κB in early lymphocyte development seems clear – an expression of either pre-T-cell receptor (pre-TCR) or pre-BCR (pre-B-cell receptor) coincides with increasing NF-κB activity and induction of anti-apoptotic signals through NF-κB **(Kishimoto *et al.*, 1998)**. However, it remains unclear how NF-κB is activated downstream of the pre-AgR as the nature of the signaling pathway at play remains almost entirely uncharacterized. Whether NF-κB contributes to lineage choice made at these early stages or merely promotes survival is also unknown **(Del Rio *et al.*, 2005)**.

The extent of NF-κB activation serves as a rheostat in the selection of DP (double-positive; $CD4^+CD8^+$) thymocytes **(figure 9)**. TCR-mediated NF-κB activation follows binding to peptide: major histocompatibility complex (MHC). A thymocyte that expresses a TCR that cannot bind MHC succumbs to "death by neglect", whereas those that bind peptide: MHC are either positively or negatively selected depending on the strength of signaling. Thymocytes that bind self-peptide: MHC with very high affinity, are likely to be self-reactive, and

hence are deleted through negative selection. Thus, only DP thymocytes that recognize self-peptide: MHC and signal within a defined range are positively selected and become single-positive (SP) T cells. During negative selection, NF-κB facilitates the induction of apoptosis following high-affinity TCR ligation ***(Hettmann et al.*, 1999)**. perhaps by facilitating the expression of pro-apoptotic genes and consequent sensitization to pro-apoptotic signals **(Wu *et al.*, 1996)**.

The role of NF-κB in the positive selection of thymocytes is more in keeping with the better-established role of NF-κB as an inducer of anti-apoptotic genes. Unlike in thymocytes, however, NF-κB functions as a pro-survival factor during the negative selection of B cells. Immature B cells display constitutive NF-κB activity that is downregulated following BCR ligation **(Wu *et al.*, 1996).**

Decreased NF-κB activity might then sensitize these cells to pro-apoptotic signals. Interestingly, some signaling components required for NF-κB activation in mature B and T cells can be genetically disrupted without affecting their development, suggesting that pathways leading to activation of NF-κB in developing B or T cells differ significantly from the pathways engaged following AgR ligation in mature lymphocytes. Following positive and negative selection, DP thymocytes must make a lineage commitment as SP thymocytes ($CD4^+CD8^-$ or $CD4^-CD8^+$) and thereafter emigrate from the thymus. This process requires NF-κB as deletion of NEMO in cd4-Cre mice results in loss of mature peripheral T cells **(Schmidt-Supprian *et al.*, 2004).**

Equivalent deletion of the upstream kinase Transforming growth factor beta-activated kinase 1 (TAK1) has a similar outcome. The exact nature of the NF-κB pathway requirement is somewhat unclear, as Nikki−/− chimeras, or cd4-Cre IKKβ conditional knockouts, are not defective in the production of naive T cells **(Liu *et al*., 2006)**.

Further, the contribution of NF-κB to CD8 and CD4 lineages are not equivalent. CD8 SP cells have significantly higher levels of NF-κB activity than CD4 SP thymocytes, yet the anti-apoptotic factor Bcl-2 is more highly expressed in CD4 than CD8 cells. Therefore, CD8 SP thymocytes are dependent on NF-κB for survival, while CD4 SP thymocytes are not. NF-κB is, however, clearly important in CD4 SP cell development, and forced activation of NF-κB in CD4 SP cells results in negative selection **(Jimi *et al*., 2008).**

NF-κB has a regulatory role in inflammation, innate and adaptive immune response, proliferation and apoptosis, and importantly in cancer. It is a nuclear transcription factor that binds to the promoter region on the immunoglobulin kappa light chain on B lymphocytes cells. Acetylation of NF-κB can be reversed by Histone deacetylase inhibitors (HDACs) and Sirtuin-1 (SIRT1) to suppress the activity of NF-κB. It is evidenced that resveratrol directly or indirectly upregulates the cellular expression of antioxidant defense genes. Antioxidant properties of resveratrol have been reported in many diseases, including cancer and it is proven to be a well-known chemo-preventive and chemotherapeutic molecule **(Truong *et al*., 2018)**.

6-Role of TNF-α in immunity and inflammation induced by aflatoxin:

Tumor necrosis factor-α and TNF-β, produced primarily by monocytes and lymphocytes, respectively, were first isolated in 1984, as cytokines that kill tumor cells in culture and induce tumor regression in vivo **(Aggarwal *et al*., 1984).**

Tumor necrosis factor-α is a transmembrane protein with a molecular mass of 26 kDa that was originally found to be expressed in macrophages and has now been found to be expressed by a wide variety of cells. In response to various stimuli, TNF-α is secreted by the cells as a 17 kDa protein through a highly regulated process that involves an enzyme TNF-α-activating converting enzyme (TACE) **(Aggarwal, 2003).**

The pro-inflammatory effects of TNF- α are primarily due to its ability to activate NF-κB. Almost all cell types, when exposed to TNF-α, activate NF-κB, leading to the expression of inflammatory genes. Over 400 genes have been identified that are regulated by NF-κB activation. These include cyclooxygenase-2 (COX-2), lipoxygenase-2 (LOX-2), cell-adhesion molecules, antiapoptotic proteins, inflammatory cytokines, chemokines, and inducible nitric oxide synthase (iNOS). Tumor necrosis factor-α produced by tumor cells or inflammatory cells in the tumor microenvironment can promote tumor cell survival through the induction of genes encoding NF-κB -dependent anti-apoptotic molecules (**Ahn & Aggarwal, 2005**).

Aflatoxin effect on the secretion and genetic expression of some significant cytokines, such as IL-1a, IL-6, and TNF-α by human

monocytes. A reduction in the release of IL-1, IL-6, and TNF-α was observed after pretreatment of monocytes with AFB1. Treatment with AFB1 affected gene expression of the cytokines. AFB1 entirely blocked the transcription of IL-1a, IL-6, and TNF-α mRNAs **(Rossano *et al.*, 1999).** The effects of AFB1 on the genetic expression of main macrophage-mediated cytokines (IL-1a, IL-6, and TNF-α) showed that it markedly inhibited the production of these cytokines. High concentrations of AFB1 hurt TNF-like substance secretion in peritoneal macrophages **(Cheng *et al.*, 2002).**

Tumor Necrosis Factor-α (TNF-α) activates cell survival signaling pathways, i.e., NF-κB, Akt, and MAPK pathways, as well as apoptotic pathways such as JNK, p38, and AP-1. Hence, inhibitors that target these pathways also have potential against various proinflammatory conditions mediated by TNF-α. For example, TNF-α activates NF-κB, which in turn regulates TNF-α production. Hence various NF-κB blockers (both synthetic and natural) are currently available on the market and are effective against a wide variety of inflammatory conditions ***(Aggarwal et al.*, 2005).**

7-Role of caspase 3 in immunity

In the immune system, apoptosis is involved in the selection of immature T-cells and B-cells during the development of the thymus and bursa of fabricius **(Funk & Palmer, 2003**). Excessive apoptosis of lymphocytes is related to immunosuppression in various circumstances **(Rathmell & Thompson, 2002).**

Aflatoxin-induced apoptosis has been reported in hepatocytes, bone marrow cells, lung cells, or human bronchial epithelial cells **(Yang**

***et al*., 2012)**. Aflatoxin B could induce excess apoptosis of thymocytes and bursal cells in broilers and both early stage and late-stage apoptosis processes were affected **(Peng *et al*., 2014)**.

Caspase-3 (cysteinyl aspartate proteinase) is one of the cysteine proteases that play a major role in the execution of apoptosis **(Nicholson, 1999).** Several genetic and biochemical studies suggest that caspase activation is essential for the occurrence of the apoptotic phenotype of cell death. A variety of caspase substrates are involved in the regulation of DNA structure, repair, and replication. Caspase-3 substrate cleavage has been observed under oxidative stress in different pathological conditions **(Nicholson & Thornberry, 1997).**

Glutathione depletion in aflatoxicosis is sufficient for the onset of apoptosis in cellular systems, especially, in hepatocytes, which has been addressed in several studies (**Sanchez *et al*., 1997**). Intracellular zinc interferes with the apoptosis process, possibly through the regulation of cellular redox potential involving GSH. The caspase-3 activity could be modulated by zinc and by the cell redox state **(Marini *et al*., 2001)**. The changes in the redox state can alter the association of zinc to enzyme antioxidants and consequently impair their activation or may affect zinc binding to intracellular stores, through altering its free to bound ratio and/or its sub-cellular compartmentalization **(Turan *et al*., 1997).**

Nitric oxide activates the transduction pathways leading to apoptosis. NO stimulates the expression of enzymes and transcription factors involved in apoptosis such as the tumor suppressor P53. The latter molecule transactivates the expression of pro-apoptotic genes, such as bax and that of the cyclin-dependent kinase inhibitor P21(p21Cip1),

whereas it down-regulates the expression of the anti-apoptotic protein BCL-2 **(Kolb, 2000).**

Excessive apoptosis in circulating leukocytes can cause sever immunopathologic conditions such as autoimmune diseases and cancer (**Fuchs & Steller, 2011**). Apoptosis is featured with a list of morphological and enzymatic changes including caspase activation, cell contraction, extensive plasma membrane budding, chromatin condensation, fragmented nucleus, formation of macromolecules, enzymatic hydrolysis, and apoptosome **(Torkzadeh-mahani *et al.*, 2012).**

The three highly regulated main pathways of cell death activation are referred to as the mitochondria-associated apoptosome formation (intrinsic), the binding of extracellular death ligand (extrinsic), and cytotoxic lymphocytes-initiated granzyme B with several caspases and adenosine triphosphate (ATP)-related physio-pathological pathways **(Reed, 2002).** Aflatoxin B is an external toxin and has documented pathologic effects on apoptosis in the liver, kidney, thymus, spleen, and gastrointestinal tract immune system **(Lewis *et al.*, 2005)**. Based on the dose and time of effect, one report indicates AFB may reduce the number of B and T lymphocytes and disturb cytolysis activity in neutrophils **(Asadollahi *et al.*, 2015)**. Researcher has been confirmed that AFB can induce apoptosis and potential ATP depletion in various cells, which might occur through caspase-3/7 activation and ATP depletion **(Vahidi-Ferdowsi *et al.*, 2018).**

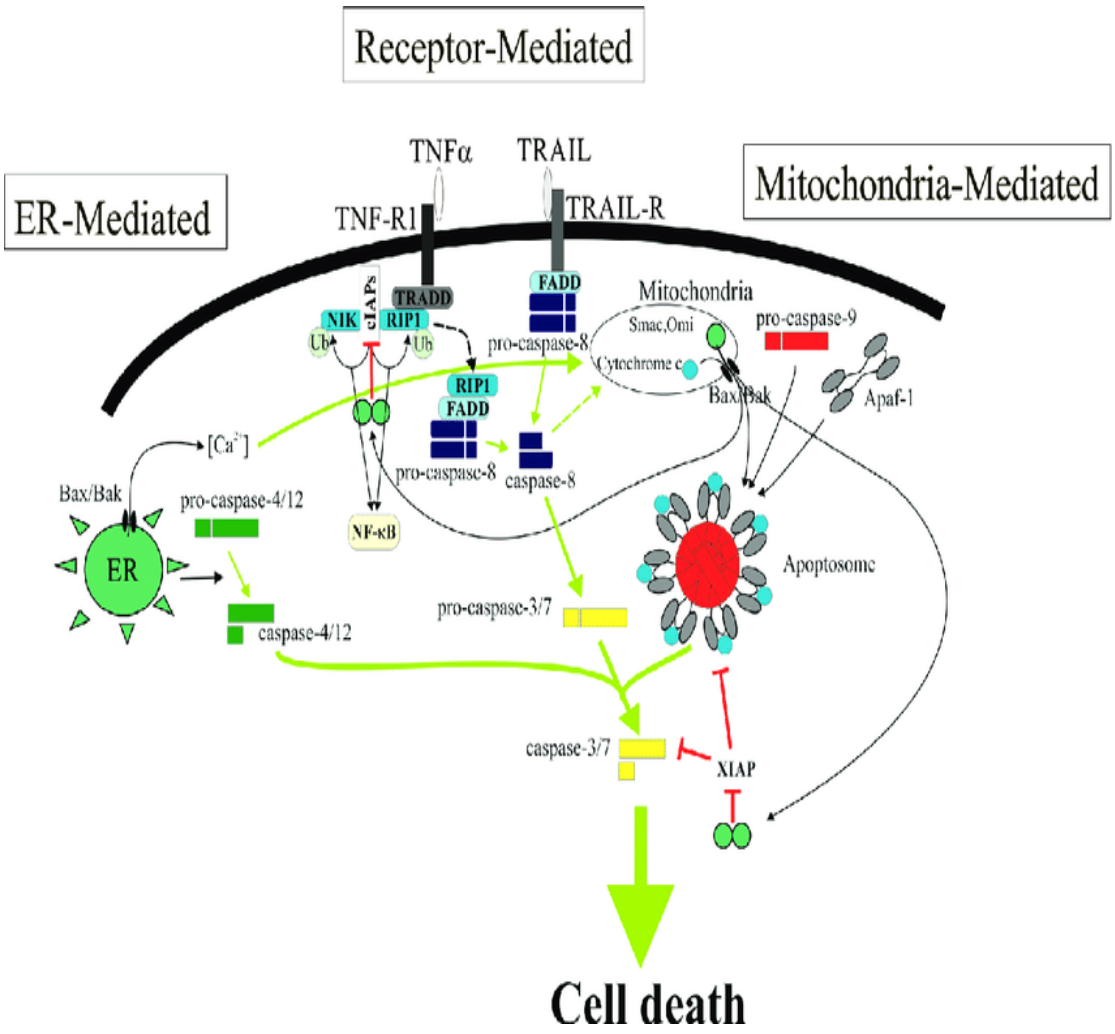

Fig (10): Apoptotic pathways. Apoptotic signals converge on caspases, forming a self-amplifying cascade. External or internal apoptotic triggers evoke distinct cellular responses. Intracellular stress (such as DNA damage) activates the intrinsic (mitochondrial) pathway, which is characterized by the release of cytochrome C followed by apoptosome formation and caspase-9 activation. Similarly, endoplasmic reticulum (ER) stress results in the activation of an intrinsic ER-specific pathway that activates caspase-12 (or a similar caspase in humans) and/or caspase-7 and, subsequently, caspase-3. In addition, the ER-specific pathway triggers the mitochondrial pathway. Extrinsic pathways are activated by extracellular ligand binding to death receptors, which either directly results in the activation of the caspases or, depending on the cell type, requires further amplification through the mitochondrial pathway. Ultimately, all apoptotic signaling pathways converge at the level of effector caspases, such as caspase-3 and caspase-7. Multiple control points exist along these pathways, either by regulating the release of cytochrome C and other apoptogenic factors from the mitochondria (by Bcl-2 family proteins) or by regulating the levels or activity of caspase inhibitors, the inhibitors of apoptosis (IAPs), through their antagonists (such as Smac/DIABLO or HtrA2/Omi) or other regulatory mechanisms, adapted from **(Holcik & Sonenberg, 2005).**

Materials and methods

I-Extraction, purification, and identification of AFB:

I.1.Preparation of AFB for biological use:

Aflatoxin (AFB) laboratory preparations for biological use was done because it was very difficult to get aflatoxin by purchasing from chemical companies.

A1-Fungal cultivation of *Aspergillus flavus* for the biosynthesis of AF:

Aspergillus flavus was obtained from the center of Fungi Research and Botany Department, Faculty of Science, Assiut University, then cultivated by the following steps:

1-The Potato Dextrose Broth media (PDB) preparation: was used for the cultivation of the fungal isolate that was prepared as follows: scrubbed and diced potato (200 gm) was boiled in distilled water (1000 ml) for 1 hour and the mixture passed through a fine sieve then 15 gm dextrose was added, sirred and the medium was supplemented with chloramphenicol (250 mg/ml) as a bacteriostatic agent that autoclaved for 20 min.

2-Cultivation of fungal isolate was performed by transferring the isolate into 50 Erlenmeyer flasks (250 ml), each containing 50 ml of sterilized potato dextrose broth (PDB). The flasks were incubated at 28 ℃ for 10-14 days as a static culture.

3-Extraction of aflatoxins: At the end of the incubation period, 100 ml chloroform was added to each flask and the content of the flask (medium+ mycelium) that was transferred into a metallic blender jar was homogenized for 2 min at low speed and 3 min at high speed (shaken for 2 min then homogenized for 3 min). The extraction procedure was

repeated with the same volume of chloroform. The combined chloroform extracts of each flask were washed with an equal volume of distilled water in a separating funnel where the organic layer was filtered then dried over anhydrous sodium sulfate and evaporated to near dryness by a rotatory evaporator. The total residual remnants were collected, transferred, and rewashed with chloroform that was left for evaporation to near dryness in a funning cupboard **(Booth, 1971)**.

4-Isolation and purification of the crude toxin extract by preparative thin-layer chromatography (TLC):

- Glass plates (20 cm) were cleaned by washing with detergent, distilled water then ethanol, and dried with acetone.
- A thin layer (0.3 mm thickness) of silica gel class plates was prepared; air-dried then activated in an oven at 110 ℃ for 1 hour and left to cool before using.
- The collected aflatoxin samples were applied as 50: l solutions for each in chloroform. The spots were dried during application with a flow of cold air. The plates were developed in developing tanks of 15 cm diameter (Zeiss, Jena, Germany) containing the solvent system of chloroform-methanol (97:3 v/v) for the separation of the different AFs.
- The developed plates were detected by using long-wave UV light (365 nm) where **AFB** was the major component in the mixture and has a fluorescence of bright blue **(Baur & Ensminger, 1977)**. silica gel containing **AFB** was scraped from the chromatoplates and transferred into vials containing 10-15 ml chloroform. This aided in releasing silica bound **AFB** then centrifuged at 3000 r.p.m rounds per min for 20 min, the organic layer was collected and then evaporated to near dryness by the flash evaporator.

- The dried purified sample was applied as 50 solutions in chloroform then give one spot with value = 0.5 in solvent (chloroform: methanol; 97:3) or value=0.77 in solvent (toluene: ethyl acetate: acetic acid; 8:1:1) corresponding to the value as in reference and this sample was ready to use for spectral analysis and intoxication.

B. Extraction, purification, and Identification of Camel whey proteins and Bovine whey proteins

B1-Isolation and purification of Camel whey proteins and Bovine whey proteins from Camel milk and Bovine milk:

The camel whey proteins and the bovine whey proteins were prepared in the same way, fresh milk was transported on ice immediately to the laboratory where fats were removed by centrifugation at 5000 × g for 30 min at 10˚C, the obtained skim milk was acidified to pH 4.3 using 1 N HCl at room temperature and centrifuged at 10,000g for 10 min to precipitate casein. The resultant whey, which contains the WPs, was saturated with ammonium sulfate to a final saturation of 80% to precipitate the WPs. We separated the supernatants (whey proteins) from the precipitate and collected them in sterilized falcon tubes. WPs was freeze-dried and refrigerated until use **(Badr et al., 2012)**.

B2- Identification of camel whey proteins and bovine whey proteins by SDS-PAGE

1- Samples were analyzed by the methode of **Laemmli, (1970**) with few modifications. For preparation 5 ml of stacking gel (4%), the following materials were mixed:

0.5 M Tris-HCL (pH 6.8)	1.25 ml
10% SDS	50 µl
Acrylamide/bis (30%, W/V stock)	0.65 ml
10% APS (fresh)	30 µl
TEMED	6.5 µl
Deionized water	3.05l

- This mixture added directly before use.

2-Separating gel: For preparation 10ml SDS-PAGE separating gel (10%) following materials were mixed:

1.5 M Tris-HCL (pH 8.8)	5 ml
10% SDS	200 µl
Acrylamide/ bis (30% w/v)	6.7 ml
10% APS (fresh)	200 µl
TEMED	14 µl
Deionized water	7.9ml

- added directly before use.

3-Lysing buffer:

Tris-HCL	0.0706
Glycerol	1 ml
SDS	0.2 g

2-mercaptoethanol	0.5 ml
Distilled water	10 ml

4-Sample buffer:

Deionized water	4 ml
0.5 M Tris-HCL	1 ml
10% SDS	1.6 ml
Glycerol	0.8 ml
2-mercaptoethanol	0.4 ml
1%(w/v) BromopHenol blue	5.2ml

5- Gel running protocol:

- An appropriate amount of separating gel was prepared in a small beaker, then add specific volume of ammonium persulphate (APS) and TEMED and gently swirl the beaker to ensure sufficient mixing. The gel solution was pipetted into the gap between the glass plates of gel casting. The rest space was filled with ethanol and allowed for 30 min for complete gelation.
- An appropriate amount of stacking gel was prepared in a beaker and 10% APS and 1% TEMED was added. The ethanol in the first step was to pour out and the separating gel solution was pipetted into the gap and the comb was inserted and allowed 30 min to let it polymerize.
- The sample (10 µl) was mixed with sample buffer (1:1). The mixture was boiled for 5 min at 95 ˚C in a water bath.
- 10 µl sample mixture load in gels hole. The gels were run under constant voltage of 110 V and 120 amber for about 2 h. Electrophoresis was carried out on Biometra co-Mini Electrophoresis System as shown in **figure (11)**.

- The gel was stained using Coomassie Brilliant Blue G-250 solution (0.25 g stain in 8:46:46 acetic acid: methanol: water) for 24 h and destained using 5% acetic acid solution. The protein bands in camel whey were identified by comparison with the result obtained by **(Ebaid et al.,2012),** the protein bands in bovine whey were identified by comparison with the result obtained by **(Bassan et al., 2015).**

Figure (11): Biometra co-Mini electropHoresis system, 110 voltage, 120 amber, time taken: 2 hrs, Commassie blue stain.

C-Experimental animals:

In this study, 40 White Albino male rats were used and obtained from the Animal House of the Faculty of Veterinary medicine, Assiut University, Assiut, Egypt. Rats body weight were 130±10 gm. Age of rats were about a month. Rats were housed in cages, kept at room temperature with a normal 12h light/12h dark cycle, and supplemented with standard commercial pellets for feeding, water, ad libtium. For one week before the start of the study, all of the animal procedures were performed following guidelines for the care and use of experimental animals

established by the committee for control and supervision of experiments on animals and the protocol of the National Institutes of Health (NIH) (**Ragab *et al*.,2015**).

C1- Design of the experiment:

Rats were divided randomly into 4 groups, contained 10 rats each, as follows:

GI (CONT): Reference normal group.

GII (AFB): Intoxicated group with **AFB.**

GIII (CWP+AFB): Intoxicated group with **AFB** and received prophylactic dose of camel whey proteins.

GIV (BWP+AFB): Intoxicated group with **AFB** and received prophylactic dose of bovine whey proteins**.**

C2- Route of administration of intoxicating solutions and the other treating agents:

The animals in the a control group received normal diet, while animals of groups **GII**, **GIII,** and **GIV** were individually intoxicated by oral administration (through esophagus) 3 times per week (500 µg **AFB** suspended in corn oil/ kg b.wt) for successive 4 weeks according to **(Raisuddin et al., 1993)**.

The animals of groups **GIII and GIV** were orally supplemented with CWP and BWP (200 mg/kg body weight dissolved in 250 µl distilled water), respectively. Each rat received 250 µl of distilled water containing 200 mg/kg of CWP and BWP **(Ramadan *et al*., 2018).**

C3- Collection and preparation of the samples for biochemical determinations and histopathological examinations:

Rats of the different groups were killed by cervical dislocation, the spleen and thymus were quickly removed, washed with saline solution, and cut into three equal parts, one part was fixed in formalin for histological examination and the second part was imbedded in liquid nitrogen and kept frozen at -80 ℃ until RNA extraction, the third part was preserved in RIPA lysis buffer for western blots analysis. The liver samples were kept in -20 ℃ until measurement of oxidant/ antioxidant biomarkers.

D- Biochemical determinations:

D1- Homogenization of liver samples methods:

300 mg liver tissue was homogenized in 3 ml phosphate buffer (pH 7.4) then centrifuged at 4000 r.p.m for 10 min. The liver homogenate (supernatant) was used for measuring the following parameters:

D2- Total protein content determination

Total protein in liver homogenate was determined using a Biodiagnostic kit (Spinreact S.A.U / spin react, S, A, Ctra. Santa Coloma,7 E-17176 Sant Esteve de Bas (Girona) ESPANA, (Catalog NO, 265).

Principle:

In the presence of an alkaline cupric sulfate, the protein produces a violet color, the intensity of which is proportional to their concentration according to (**Gornal *et al*., 1949**).

Reagents

1-Standard Albumin 5 g / dL

2- Biuret Reagent:

- Cupric sulfate 6 mmol / dL.
- Sodium potassium tartrate 21 mmol / dL.
- Sodium hydroxide 750 mmol / dL.
- Potassium iodide 6 mmol / dL**.**

Procedure:

	Blank ml	Standard ml	Sample ml
Standard	**-**	**0.025**	**-**
Sample	**-**	**-**	**0.025**
Reagent 2	**1.0**	**1.0**	**1.0**

The tubes were mixed well, incubated for 10 min. at 37°C. The absorbances of the sample (A Sample) and standard (A standard) was read against the reagent blank at 550 nm.

CALCULATION:

Protein concentration g/dl = $5\times \frac{A\ sample}{A\ standard}$

D3- Nitric oxide (NO) Assay in liver homogenate

Principle:

Nitric oxide was determined by **(Ding *et al.*, 1988).** In acid medium and in the presence of nitrite, the nitrous acid diazotize sulphanilimide was formed then the product is coupled with naphthyle ethylene diamine dihydrochloride. The resulting azo-dye has a bright reddish-purple color which can be measured at 540 nm.

Reagents:

-Griess reagent: 1% Sulphanilamide, 0.1% Naphthyl ethylene diamine dihydrochloride and 2.5% phosphoric acid.

- Standard sodium nitrite.

Procedure:

1- 100 µl of griess reagent was added to 100 µl of standards as well as to 100 µl of liver tissue homogenate and incubated for 20 min.

2- The absorbance of samples and standard at 540 nm (Awareness technologist statfax 2100 microplate reader) were recorded.
3- The concentration of nitric oxide was calculated using an external standard curve and expressed as nmol /mg tissue protein was calculated.

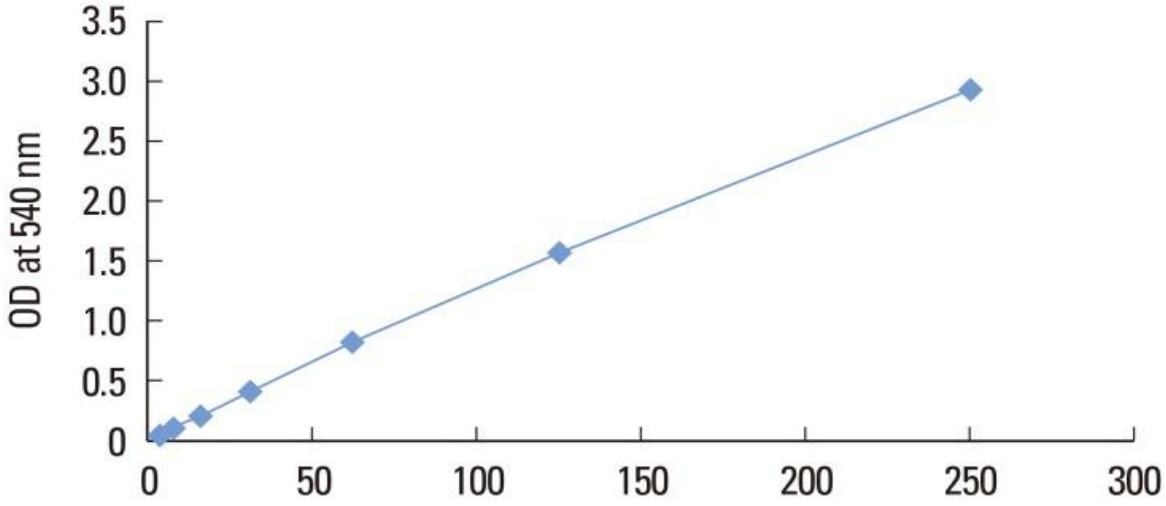

Figure (12): Standard calibration curve for nitric oxide (NO) expressed as µmol/L.

D4- Assay of malondialdehyde (MDA):

Principle:

Thiobarbituric acid (TBA) reacts with MDA in an acidic medium at a temperature of 95°C for 30 min to form TBA reactive product then the absorbance of the result pink product was measured at 532 nm, **(Wills, 1969)**.

Reagent:

Reagent A: Thiobarbituric acid (TBA) (0.375%), Trichloroacetic acid (TCA) reagent 15%) (0.375 and 15%, respectively).

Procedure:

1- 200 µL supernatant of tissue homogenate was mixed with 2ml of Reagent A.

2- The volume was made up to 3ml with distilled water and boiled on a water bath (CW-20G Korea) at 95 °C for 20 min.

3- The solution was cooled under tap water.

4- The reaction product (TBA–MDA complex) was extracted by adding 3 ml of n-butanol to the above solution and vortexed well.

5- The absorbance of the pink-colored extract in n-butanol was measured at 532 nm using a spectropHotometer (Awareness technologist statfax 2100 microplate reader).

6- The amount of MDA was calculated using a molar extinction coefficient of $1.56\times105M^{-1}cm^{-1}$ and expressed as nmoles of MDA formed/ mg tissue protein.

E5- Glutathione Standard (GSH) content assay:

Principle:

The tissue glutathione (GSH) content was determined using Ellman's reagent (5, 5- dithiobis-nitrobenzoic acid solution (DTNB)) that should be freshly prepared as 10 mmol solutions in 1% sodium citrate (**Beutler, 1963)**. Ellman's reagent reacts with GSH and the developed color was measured at 412 nm.

Reagents:

1- The precipitating solution was freshly prepared as follows:

(1.67 gm) glacial metaphosphoric acid (a mixture of HPO3, NaPO3), (0.2 gm) disodium ethylenediaminetetraacetic acid (EDTA.2Na), and (30 gm) sodium chloride per 100 ml of distilled water.

2- Phosphate solution (0.3 M Na_2HPO4) was prepared by dissolving (4.26 gm) of disodium monohydrogen phosphate (Na2HPO4) in 100 ml distilled water then stored at 4ºC until working time, the pH of the solution was adjusted to be 7-8 by 0.1 N HCl.

3- DTNB (5,5- dithiobis-nitrobenzoic acid) reagent:

DTNB reagent was prepared by dissolving (40 mg) of DTNB in 100 ml of 1% sodium citrate.

Procedure:

1- One-tenth milliliters of the tissue sample was added to 0.9 ml of distilled water and then added 1.5 ml of the previously prepared precipitating reagent.

2- The mixture was allowed to stand for 5 min and then centrifuged at 3500 g for 10 min.

3- One milliliter of supernatant was added to 4 ml of the phosphate solution and then (0.5 ml) of the DTNB solution was added to deduce yellow color.

4- The absorbance was measured at 412 nm by using the Awareness technologist statfax 2100 microplate reader.

5- The concentration of glutathione content (GSH) expressed as nmol/ mg tissue protein for each sample was calculated from a standard curve.

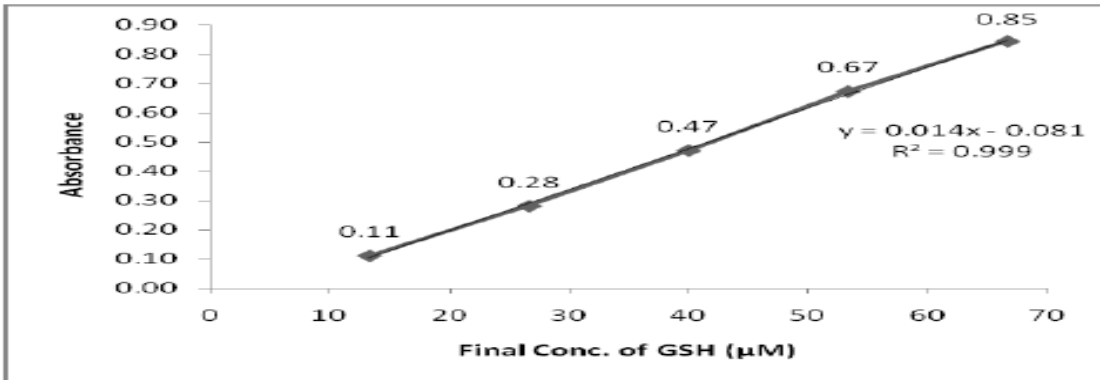

Fig (13): Standard curve for

E6- Glutathione peroxidase (GSH-Px) activity enzyme assay:

Principle:

Glutathione peroxidase (GSH-Px) catalyzes the following reaction:

2 GSH + ROOH → ROH + GSSG + H_2O

The GSH-Px activity was determined by subtracting the excess of GSH after enzymatic reaction from the total GSH in absence or presence of the enzyme. The excess GSH reacts with dithiobisnitrobenzoic acid (DTNB) to form yellow color **(Habig, 1974)**.

Reagents:

1- Tris-HCl buffer (50 mM, pH 7.6 was prepared by dissolving (3.15 gm) of Tris-HCl in (400 ml) H_2O.

2- Tris-HCl buffer, 0.4 M, pH 8.9 was prepared by dissolving (28.06 gm) Tris-HCl in (445 ml) H_2O.

3- Cummen, pH 7.6 was prepared by mixing of 50 µl of cummin with 10 ml Tris-HCl buffer, 50 mM, pH 7.6.

4- GSH, pH 7.6 was prepared by dissolving (5 mg) of GSH in (10 ml) Tris-HCL buffer, 50 mM, pH 7.6.

5- DTNB solution was prepared by dissolving (0.0198 gm) of DTNB in (5 ml) methanol; Absolute methanol.

6- Trichloroacetic acid (TCA), 15 % w/v.

Procedure:

1- The supernatant of tissue homogenate was diluted 10 times with distilled water before the enzymatic assay.

2- The substrate reacting solution was prepared by mixing (100 µL) GSH, (100 µL) cummin, (750 µl) Tris-HCl, pH 7.6 then, incubated at 37 °C for 5 min.

3- 50 µl of the diluted tissue homogenate was added to the incubated substrate solution for another 10 min. at 37.

4- 1 ml of TCA was added after this 10 min. to stop the enzymatic reaction.

5- For control, (50 µl) diluted supernatant and (100 µl) GSH was added to (750 µl) Tris-HCL, pH 7.6 then incubated at 37ºC for 10 min., (100 µl) cummin was added and then added one milliliter TCA.

6- Both tubes containing (sample and control) was centrifuged at 3000 r.p.m for 20 min and then the supernatants were separated off.

7- One ml of each supernatant was mixed with (2 ml) Tris-HCl, pH 8.9 and (100 µL) DTNB then incubated for 5 min at 37 ºC.

8- The absorbance of the tested sample supernatants (A sample) and control (A control) were read at 412 nm by using Awareness technologist statfax 2100 microplate reader against distilled water as a blank.

9- The activity of GSH-Px was calculated by the following equation that was derived from the corresponding calibration curve:

GSH-Px activity in tissue samples, nmol/ min/ gm tissue

= ((E × 6.2 × 10 × 10)/ (0.05 × 10 × gm tissue protein))

where, E = A sample - A control, 6.2 is extinction coefficient and other factors are the dilution factors.

E7- Assay of Glutathione-S-transferase (GST) activity:

Principle:

Conjugated Glutathione as detoxification agent against toxic compounds and metabolites by the action of Glutathione-S-transferase (GST) determined according to **(Habig, 1974)** method. GST activity was assayed spectrophotometrically by monitoring the conjugation of 1-chloro-2,4dinitro benzene (CDNB) with GSH at λmax=340 nm at 37 ºC.

CDNB + GSH → CDNB – S – GSH

Reagents:

- Potassium phosphate buffer (0.1 M, pH= 6.5)
- 100 mM GSH
- 80 mM CDNB
- 10% supernatant

Procedure:

1- 1.85 ml of phosphate buffer was added to 0.1 ml of GSH and 0.025 ml of CDNB (non-enzymatic reaction/ blank).

2- non-enzymatic reaction was recorded change in absorbance at 345 nm using Awareness technologist statfax 2100 microplate reader.

3- 25 µl of supernatant was added to the above reagent then recorded change at 345 nm (enzymatic reaction/ sample) using Awareness technologist statfax 2100 microplate reader.

4- The activity of GST was calculated as the following:

Δ Abs/ min (enzym) = Δ Abs (sample) – Δ Abs (blank)

Increase in conc of CDNB- GSH/ min= Δ Abs/min (enzyme)÷ DCNB

DCNB= 8.5 mM-1.cm-1 then to obtain the specific GST activity, the obtained result was divided by mg protein.

F-Western blotting analysis of cleaved caspase-3 in spleen and thymus:

Reagents:

Rabbit polyclonal anti-cleaved caspase-3 IgG and rabbit anti-β actin IgG antibodies were from ABCAM. Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG was obtained from Santa Cruz Biotechnology. Enhanced chemiluminescent (ECL) substrate was obtained from (Thermo Scientific, Rockford, USA). SDS-PAGE chemicals were from Sigma Aldrich.

Method:

Tissues from spleen and thymus (3 mm^3) were lysed with ice-cold RIPA buffer (50 mM Tris-Cl [pH 7.6], 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, and 0.5% Triton-X-100) containing 1 µg/ml leupeptin and aprotinin, and 0.5 mM PMSF. Lysates were centrifuged at 2,500 rpm for 10 min at 4 ºC. Protein concentrations were measured by Bradford assay. 40 µg protein aliquots were separated by SDS–PAGE using 10% gels then transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk and probed with primary antibodies (anti-caspase 3 and β-actin 1:1000) overnight at 4 ºC. Membranes were then incubated with HRP-conjugated secondary antibody (1:10,000) for 1 h at room temperature. Detection was performed using the ECL substrate. Band densities were measured by ImagJ software and normalized to the control then equalized to the corresponding actin band.

G-Reverse transcription-quantitative polymerase chain reaction (RT-qPCR):

G1- RNA extraction

RNA was extracted from spleen and thymus tissues using GENEzol reagent (Geneaid, Taiwan, Catalog No. GZR100) as the following method:

Reagents: GENEzol Reagent®, chloroform, RNase-free water, ethyl alcohol 70%, isopropanol.

1. Sample Homogenization

Procedure:

I. Sample Homogenization

1. 1 ml of GENEzol™ Reagent was added to each 100 mg of thymus and spleen samples then mixed well by a vortex.
2. The homogenized sample was incubated for 5 min at room temperature.

II. Phase Separation

1. 200 µl of chloroform was added to the sample per 1 ml of GENEzol™ Reagent used in sample homogenization then shacked vigorously for 10 seconds and was incubated at room temperature for 2 min then the sample was centrifuged at 12000 rpm for 15 min at 4 ºC to separate the phases.
2. The upper aqueous phase was transferred to a new 1.5 ml microcentrifuge tube (RNase-free).

III- RNA Precipitation

1. 500 µl of isopropanol was added to the aqueous phase then mixed by inverting the tube several times.
2. The sample mixture was incubated for 10 min at room temperature and was centrifuged at 16000 r.p.m for 10 min at 4ºC to form a tight RNA pellet, then isopropanol was removed.

IV- RNA Wash

1. The RNA pellet was washed with 1 ml of 70% ethanol then vortex briefly and centrifuged the sample at 12000 r.p.m for 5 min at 4ºC.
2. The supernatant was removed with a pipette carefully not to contact the RNA pellet.
3. The RNA pellet let for Air-dry for 5 min at room temperature.

V-RNA Resuspension

1. We added 50 µl of RNase-free water to re-suspend the RNA pellet then the samples were incubated at 60 ºC for 10 min to dissolve the RNA pellet.
2. After RNA extraction, the concentration was determined using (Spectro star nano BMG lab tech).
3. RNA samples were stored at – 80 ºC until cDNA synthesis.

G2- Reverse transcription (cDNA synthesis):

The Topscript ™ cDNA synthesis kit (Enzynomics), Catalog No. (EZ005M) was used to obtain the cDNA samples needed for qRT-PCR (according to the manufacturer's instructions) by using BIO-RAD T100 thermal cycler.

Standard reaction conditions:

Mixture reaction (20 µl) consist of:

a- Ready to use mixture (10 µl) consist of:

1-10× TOPscript ™ RT buffer.

2-TOPscript™ Reverse Transcriptase (200 units/µl).

3-dNTP Mixture (2Mm each).

4-Random hexamer.

5-RNase inhibitor (40 units/µl).

b- Add RNA to ready to use mixture in a volume (1 µg).

c- Sterile water (RNase free) up to (20 µl).

The reactions were loaded into the thermal cycler at 50 ℃ for 60 min, then incubate at 95 ℃ for 5 min to inactivate the reaction. The reaction product was stored at -20°C until the RT-PCR step.

• **Primer design:**

The primers were obtained from Thermo Fisher Scientific (Thermo, US). Primers were designed using the Primer Quest tool from Integrated DNA Technologies® (Illinois, USA). About 1 µl of the primer was taken (from each forward and reverse) in a reaction volume of 20 µl.

Table (7): Primer sequences of related genes for RT-qPCR

Primers	**Primer sequences 5′-3′**
CXCL12 (SDF1)	Forward: TGAGGCCAGGGAAGAGTGAG Reverse: GACACATGGCGATGAATGGA
IL-6	Forward: TCTCTCCGGAAGAGACTTCCA Reverse: ATACTGGTCTGTTGTGGGTGG
NF-κβ	Forward: CATGAAGAGAAGACACTGACCATGGAA Reverse: TGGATAGAGGCTAAGTGTAGACACG
TNF-α	Forward: TCCACGCTCCTTCTGTCACTG Reverse: CTTGGTGGTTTGCTACGAC
GAPDH	Forward: GGTGGACCTCATGGCCTACAT Reverse: GCCTCTCTCTTGCTCTCAGTATCCT

G3- Real-time PCR (qPCR) reaction:

1- All solutions were vortexed briefly after thawing.

2- The mixture is prepared by adding the following components (except template DNA) for each 20 µl reaction to a tube at room temperature:

Table (8): RT-PCR reaction mixture.

SYBR Green	10 µl
Forward Primer	1 µl
Reverse Primer	1 µl
Water nuclease-free	3 µl

3- The mixture was thoroughly mixed and appropriate volumes were dispensed into PCR tubes.

4- Template DNA was added (5 µl/reaction) to the individual PCR tubes containing the PCR mixture.

5- The reactions were gently mixed without creating bubbles, which would interfere with fluorescence detection.

6- The thermal cycler (Applied Biosystems 7500 Fast Real-Time PCR System, USA, Catalog No. 4351104) was programmed to 95 for 10 min, 40 cycles of 95 ℃ for 30 sec, 60 ℃ for 60 sec, and final cycle of 72 for 45 sec. GAPDH is an internal reference primer.

G4- Calculation:

To calculate the mRNA expression of the target genes on relative transcription. The formulas used to be as follows:

ΔCt = Ct of target gene − Ct of internal reference gene. $\Delta\Delta Ct = \Delta$ Ct of treated group - ΔCt of control group. mRNA expression of target gene on the relative transcription = $2^{-\Delta\Delta Ct}$.

H-Histopathological Examinations

Thymus, and spleen tissues were collected for histological examination. Tissues were fixed in 10 % neutral buffered formalin solution, embedded in paraffin wax, cut into 5 μm-thick sections, and stained with hematoxylin and eosin (H&E) for examination by light microscopy according to the method described by **Drury & Wallington, (1980).**

I- Statistical analysis

Statistical differences of parameters are presented as **Mean ± SE**, statistical significance was determined using the one-way ANOVA test followed by Tukey's test for comparison between groups were performed with Graph Pad Prism 8. Values of $P < 0.05$ are considered statistically significant where ^{a} significant of treated group AFB, CWP+AFB, BWP+AFB with respect to reference normal CONT, ^{b} is a significant of treated groups CWP+AFB and BWP+AFB with respect to aflatoxicosed group, and ^{c} is a significant between treated groups CWP+AFB and BWP+AFB.

RESULTS

1- Laboratory biosynthesized and collected a pure sample of AFB:

1.1- Figure (14) shows the TLC separation of aflatoxin that was used in the present study. The biologically synthesized aflatoxins (B_1 and B_2) were characterized and identified then the AFB fraction was separated by TLC.

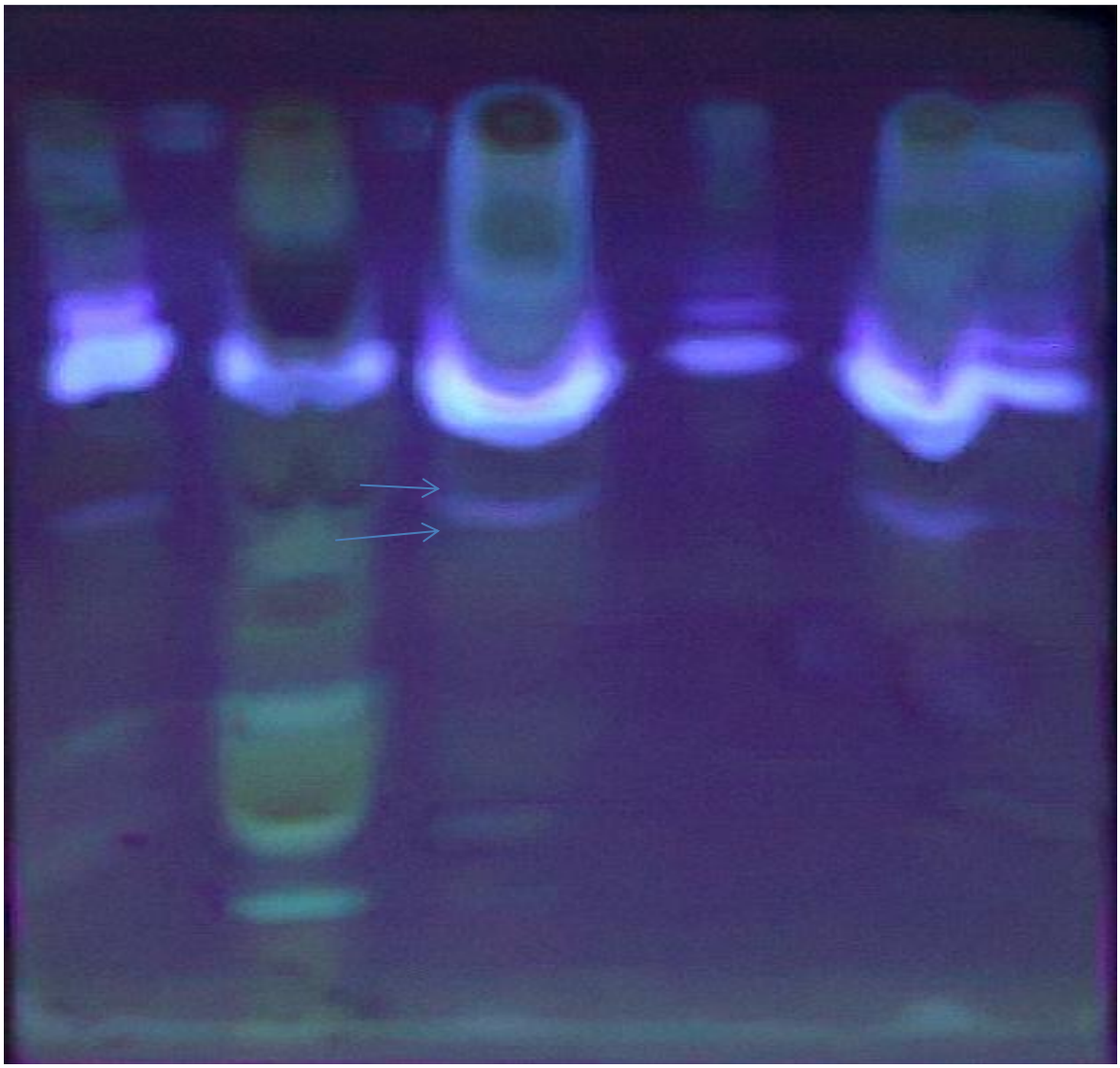

Fig (14): Separation and identification of AFB1 by TLC (arrow) by methanol-chloroform (9:1) from mixture of biologically synthesized aflatoxins illuminated by UV.

2. Camel whey proteins and Bovine whey proteins identification of by SDS-PAGE

2.1. Camel whey proteins identification by SDS-PAGE

The whey protein fractions of camel milk were analyzed by SDS-PAGE and identified based on their apparent molecular mass in comparison with the marker protein ladder (**Figure 15**). The camel whey proteins showed electrophoretic bands and their molecular weights as the following: lactoferrin (71.6-72 kDa) and α-lactalbumin (14.0 KDa).

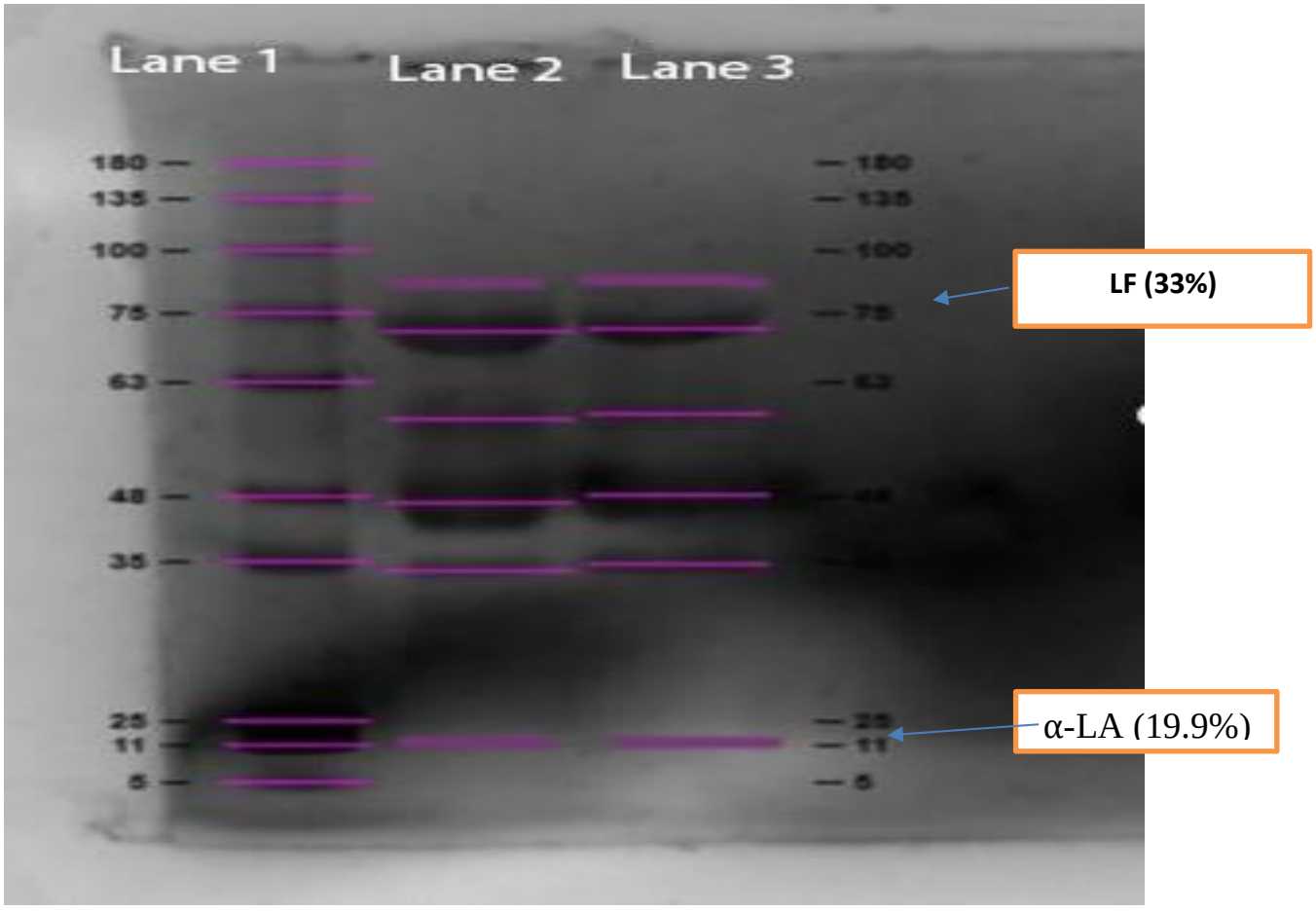

Fig (15): The SDS-PAGE pattern of whey proteins in camel milk, Lane 1: molecular weight marker, lane 2 and 3: camel whey samples; LF: lactoferrin; α-LA: α-lactalbumin.

2.2. Bovine whey proteins identification by SDS-PAGE

The whey protein fractions of bovine milk were analyzed by SDS-PAGE and identified based on their apparent molecular mass in comparison with the marker protein ladder (**Figure 16**). The bovine whey protein showed electrophoretic bands and their molecular weights were identified as the following: lactoferrin (67-71 kDa) and α-lactalbumin (14.0 KDa).

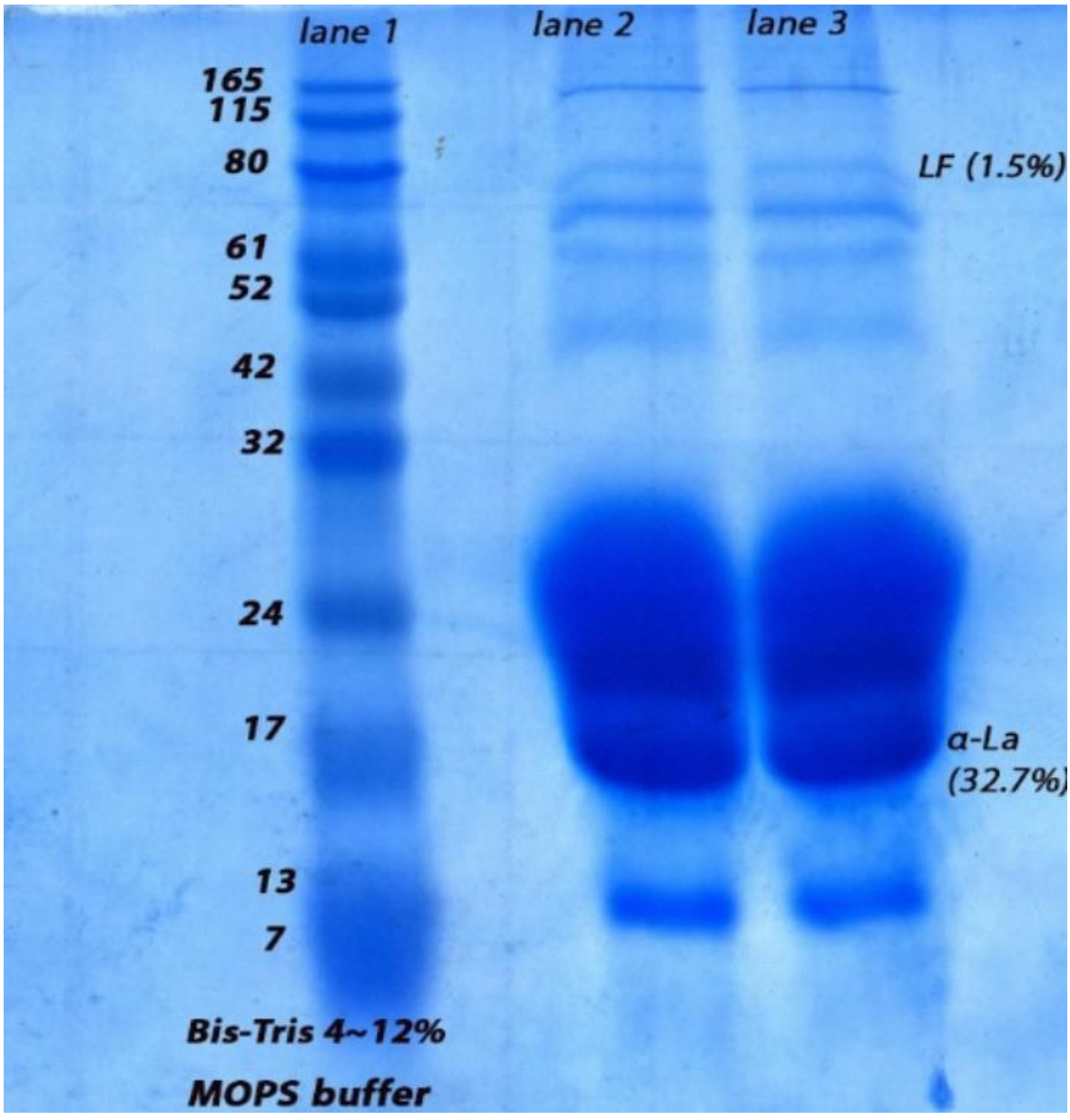

Fig (16): The SDS-PAGE pattern of whey proteins in bovine milk, Lane 1: molecular weight marker, lane 2 and 3: bovine whey samples; LF: lactoferrin; α-La: α-lactalbumin.

3- Biochemical indices

3.1. Nitric oxide

Nitric Oxide (NO) levels (nmol/ mg of tissue protein) in the liver homogenate of the experimental rats were significantly increased in the **AFB** group, **CWP+AFB** group, and **BWP+AFB** group in comparison to the reference normal (**Control) (p <0.001, p<0.05, p<0.001 respectively)**. The prophylactic doses of **CWP** and **BWP** decreased the level of NO significantly in comparison to the **AFB** group (**p <0.001 for each). CWP+AFB** group decreased NO levels significantly in comparison to BWP+AFB group (**p<0.01**) as presented in **Table (9) and Figure (17).**

Table (9): Level of NO (nmol/ mg tissue protein) in the liver of the experimental groups.

Group	Cont	AFB	CWP+AFB	BWP+AFB	P-value
Liver NO (nmol/ mg of tissue protein)	81.9 ±3	956.4± 26.9 a***	158.4±14.5 a*, b***	259.0±11.5 a***, b***, c**	<.001

Data are presented as means ± SE, [a,b,c] significance difference from control (Cont), Aflatoxin B (AFB), between camel whey proteins treated group (CWP+AFB) and bovine whey protein treated group (BWP+AFB). **p (<0.05). **p (<0.01), ***p (<0.001).*

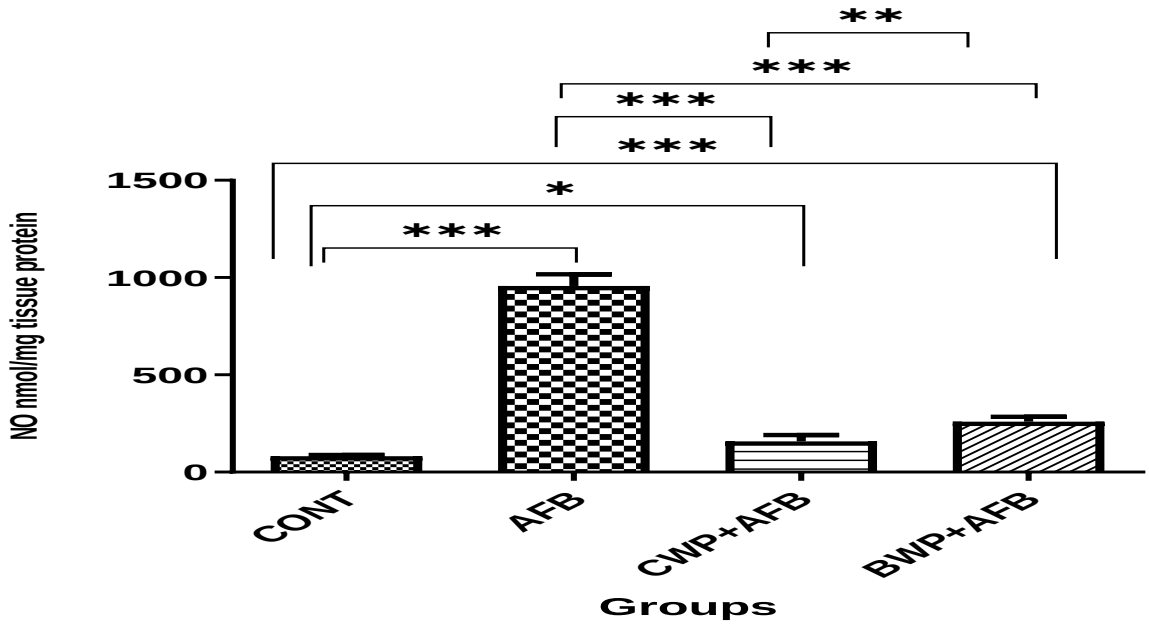

Fig (17): Bar graphs representing mean ± SE of NO level (nmol/ mg of tissue protein) in the liver tissue homogenate of the experimental groups**.** **p (<0.05), **p (<0.01), ***p (<0.001).*

3.2- Lipid peroxide as MDA:

The level of MDA (nmol/ mg of tissue protein) in the liver of experimental rats increased significantly in the **AFB** group and **BWP+AFB** group when compared to the control group (**p <0.001, for each**), while **CWP+AFB** showed no significant difference in comparison to the control group. **CWP+AFB** group **BWP+AFB** showed a significant decrease in MDA in comparison to **AFB (p< 0.001, for each).** CWP+AFB group showed a significant decrease in MDA level compared with BWP+AFB **(p< 0.001)** as presented in **Table (10)** and **Figure (18).**

Table (10): MDA level (nmol/ mg protein) in liver of the experimental groups

Groups	Cont	AFB	CWP+AFB	BWP+AFB	P-value
Liver MDA (nmol/ mg of tissue protein)	14.6 ±0.2	92.3±0.1 a^{***}	14.9±0.3 b^{***}	74.3±3.7 $a^{***}, b^{***}, c^{***}$	<0.0001

Data are presented as means ± SE, ^{a,b,c} significance difference from control (Cont), Aflatoxin B (AFB), between camel whey proteins treated group (CWP+AFB) and bovine whey protein treated group (BWP+AFB). **p (<0.05), **p (<0.01), ***p (<0.001).*

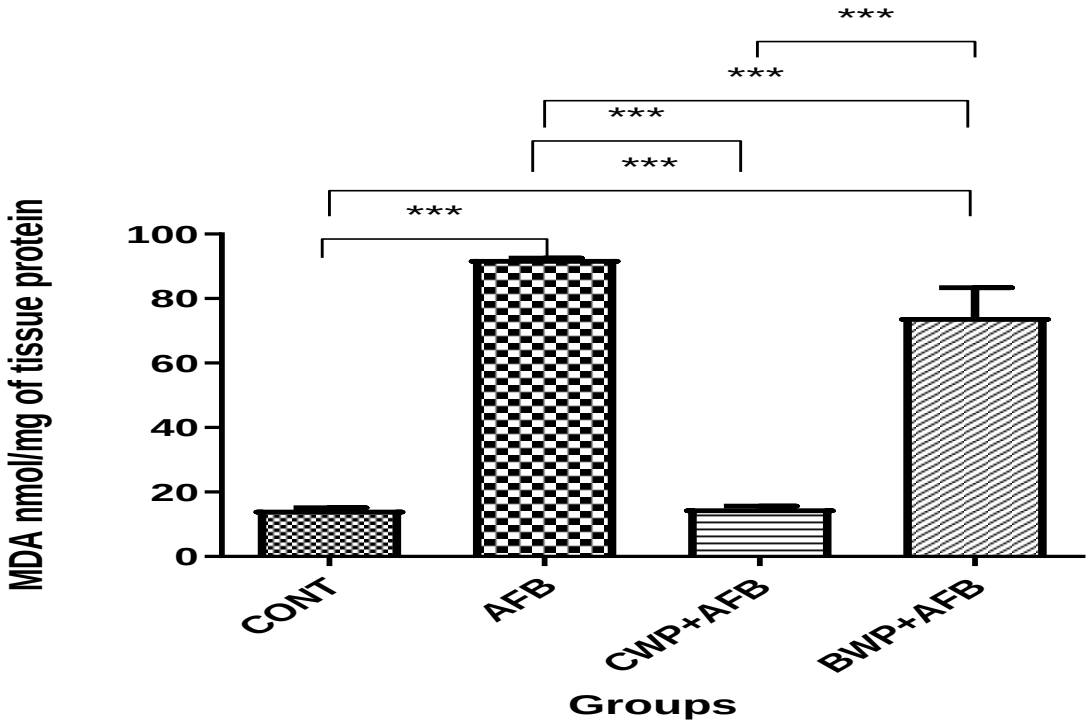

Fig (18): Bar graphs representing mean ± SE of MDA level (nmol/ mg of tissue protein) in the liver of the experimental groups. **p (<0.05), **p (<0.01), ***p (<0.001).*

3.3- Glutathione (GSH) Content

The **GSH** levels (nmol/mg of tissue protein) in the liver of experimental rats were significantly decreased in the AFB group in comparison to the control group (**p <0.001**) while the treatment with **CWP+AFB** significantly increased in comparison to the control group **(p<0.05**). **CWP_AFB and BWP+AFB** treated groups were significantly higher than the AFB group **(P<0.001, for each).** No significant difference between **CWP+AFB** and **BWP+AFB** as presented in **table (11) and Figure (19)**.

Table (11): GSH (nmol/mg of tissue protein) level in the liver of the experimental groups.

Groups	Cont	AFB	CWP+AFB	BWP+AFB	P-value
Liver GSH (nmol/mg of tissue protein)	7.2±0.02	2.8±0.1 a^{***}	8.4±0.3 a^{*}, b^{***}	7.3±0.5 b^{***}	<0.0001

Data are presented as means ± SE, ^{a,b,c} significance difference from Control (Cont), Aflatoxin B (AFB), between camel whey proteins treated group (CWP+AFB) and bovine whey protein treated group (BWP+AFB). **p (<0.05), **p (<0.01), ***p (<0.001)*.

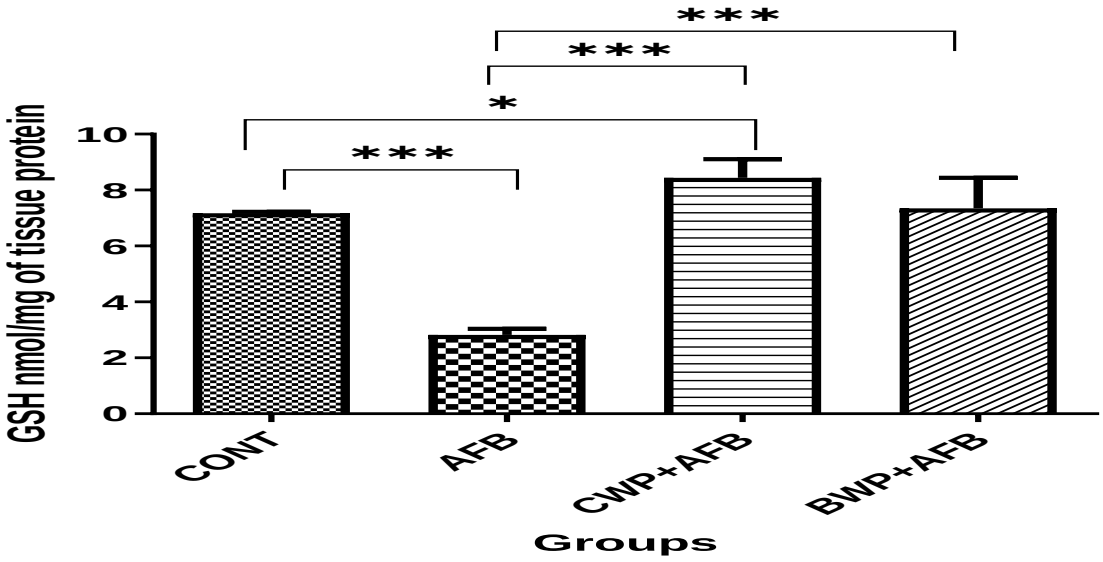

Fig (19): Bar graphs representing mean ± SE of GSH content in the liver tissues of experimental groups. **p (<0.05), **p (<0.01), ***p (<0.001).*

3.4- Glutathione peroxidase (GSH-Px) activity:

The activity of **GSH-Px** (nmol/ min/mg of tissue protein) in the liver of experimental rats was significantly decreased in the AFB group and BWP+AFB when compared with the Control group **(P<0.001, P<0.01, repectively)**. The CWP+AFB and BWP+AFB groups showed a significant increase in comparison to the AFB group (**p<0.001, for each)**. CWP+AFB group showed increased GSH-Px activity significantly in comparison to BWP+AFB group (p<0.01) as presented in **Table (12) and Figure. (20**).

Table (12): GSH-Px activity (nmol/ min/ mg protein) in the liver of the experimental groups.

Groups	Cont	AFB	CWP+AFB	BWP+AFB	P-value
Liver GSH-Px (nmol/ min/mg of tissue protein)	3654 ±96.03	153.9±15.13 a^{***}	3537±466.4 b^{***}	2137±73.4 a^{**}, b^{***}, c^{**}	<.001

Data are presented as means ± SE, ^{a,b,c} significance difference from Control (Cont), Aflatoxin B1 (AFB), between camel whey proteins treated group (CWP+AFB) and bovine whey proteins treated group (BWP+AFB). *p (<0.05), **p (<0.01), ***p (<0.001).

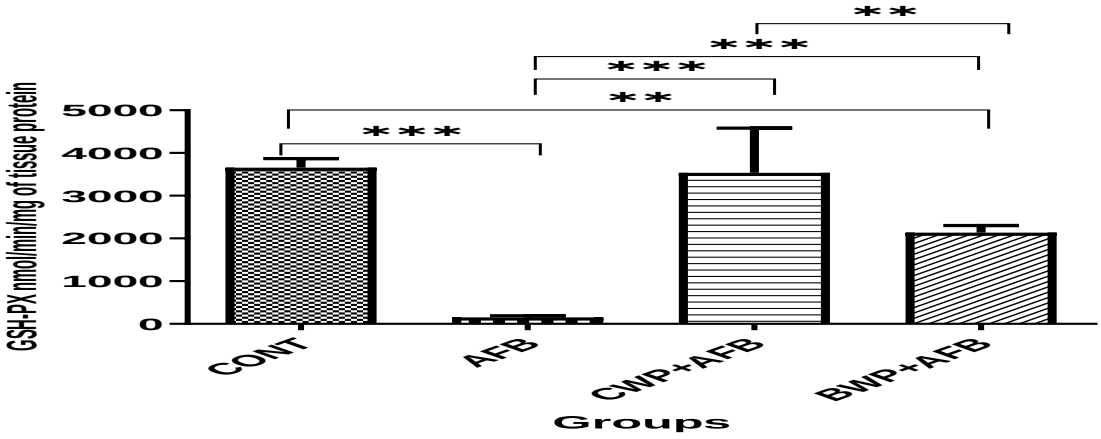

Fig (20): Bar graphs representing mean ± SE of GSH-Px (nmol/ min/mg of tissue protein) activity in liver of the experimentalgroups. **p (<0.05), **p (<0.01), ***p (<0.001).*

3.5- Glutathione S- transferase (GST) activity:

The activity of GST (nmol/ min/ mg of tissue protein) in the liver of experimental rats was significantly decreased in the intoxicated **AFB** group and **BWP+AFB** in comparison to the control group (**p <0.001, for each**), while **CWP+AFB** treatment increased the GST activity to reach the level of the control as they showed no significant difference in comparison to control group**. CWP+AFB** and **BWP+AFB** show a significant increase in comparison to AFB (**p <0.001, P<0.01, respectively**). **CWP+AFB** showed a significantly increase in comparison to the **BWP+AFB** (**P<0.001**) group as presented in **Table (13)** and **Figure (21)**.

Table (13): GST activity (nmol/ min/ mg of tissue protein) in the liver of the experimental groups

Groups	Cont	AFB	CWP+AFB	BWP+AFB	P-value
Liver GST (nmol/ min/ mg of tissue protein)	1.7±0.17	0.1 ±0.04 a^{***}	1.7±0.09 b^{***}	0.7±0.07 a^{***}, b^{**}, c***	< 0.001

Data are presented as means ± SE, ^{a,b,c} significance difference from Control (Cont), Aflatoxin B (AFB), between camel whey proteins (CWP) and bovine whey protein (BWP). **p (<0.05), **p (<0.01), ***p (<0.001).*

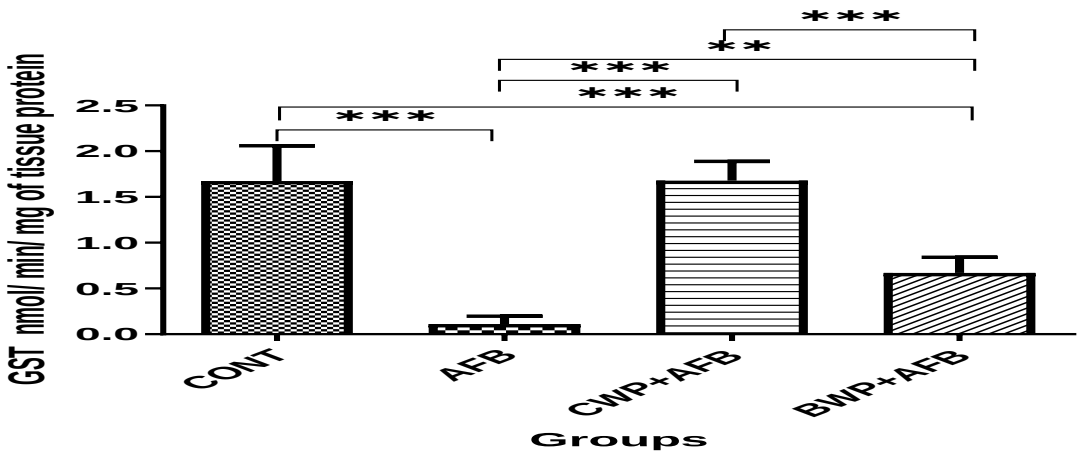

Fig (21): Bar graphs representing mean ± SE of GST (nmol/ min/ mg of tissue protein) activity in liver tissues of the experimental animals. **p (<0.05), **p (<0.01), ***p (<0.001).*

4. The mRNA relative expression of CXCL12 in thymus and spleen

4.1. The mRNA expression of CXCL12 in the thymus

The expression levels of CXCL12 in the thymus showed a significant decrease in the **AFB** group in comparison to the control group (**p <0.01**), while **CWP+AFB** group showed a significant increase in comparison to the control group (**p <0.01**), and **BWP+AFB** showed non significantly increase in comparison to control group. The groups treated with CWP and BWP showed a significant increase in comparison to the **AFB** group (**p <0.001, p <0.05, respectively**). Moreover, the CWP+AFB group had a significantly higher CXCL12 level than the BWP group **(p <0.05)** as presented in **table (14)** and **Figure (22)**.

Table (14): mRNA expression of CXCL12 in the thymus of the experimental groups

Groups	Cont	AFB	CWP+AFB	BWP+AFB	P-value
Relative CXCL12 fold gene expression in the thymus	0.9±0.14	0.07±0.01 a^{**}	1.9±0.3 a^{**}, b^{**}	0.9±0.1 b^{*}, c^{*}	< 0.001

Data are presented as means ± SE, ^{a,b,c} significance difference from Control (CONT), Aflatoxin B (AFB), between camel whey proteins treated group (CWP+AFB) and bovine whey proteins treated group (BWP+AFB). **p (<0.05), **p (<0.01), ***p (<0.001).*

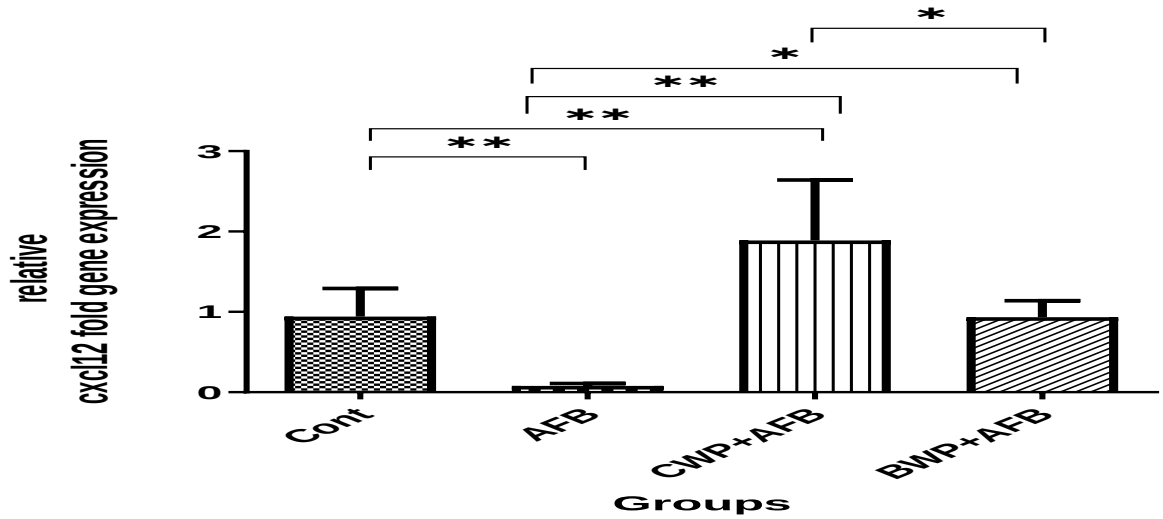

Fig (22): Bar graphs representing mean ± SE of CXCL12 mRNA expression in the thymus of the experimental animals. **p (<0.05), **p (<0.01), ***p (<0.001).*

4.2. The mRNA expression of CXCL12 in the spleen

The CXCL12 expression levels in the spleen of the **AFB** group and BWP+AFB were significantly decreased in comparison to the reference control group (**p <0.05, P<0.01**, respectively), CWP+AFB group showed a non-significant increase in comparison to the reference control group. CWP+AFB group showed a significant increase (p <0.01) in comparison to the AFB group. BWP+AFB treated group showed a significant decrease **(p <0.01)** in comparison to CWP+AFB as presented in **table (15)** and **figure (23)**.

Table (15): mRNA expression of CXCL12 in the spleen of the experimental groups

Groups	Cont	AFB	CWP+AFB	BWP+AFB	P-value
Relative CXCL12 fold gene expression In the spleen	0.26±0.07	0.07±0.002 a^{*}	0.31±0.063 b^{**}	0.004±0.0009 a^{**}, c^{**}	<.001

Data presented as means ± SE, ^{a,b,c} significance difference from Control (C), Aflatoxin B1 (AFB), between camel whey proteins (CWP+AFB) and bovine whey protein (BWP+AFB). **p (<0.05), **p (<0.01), ***p (<0.001)*.

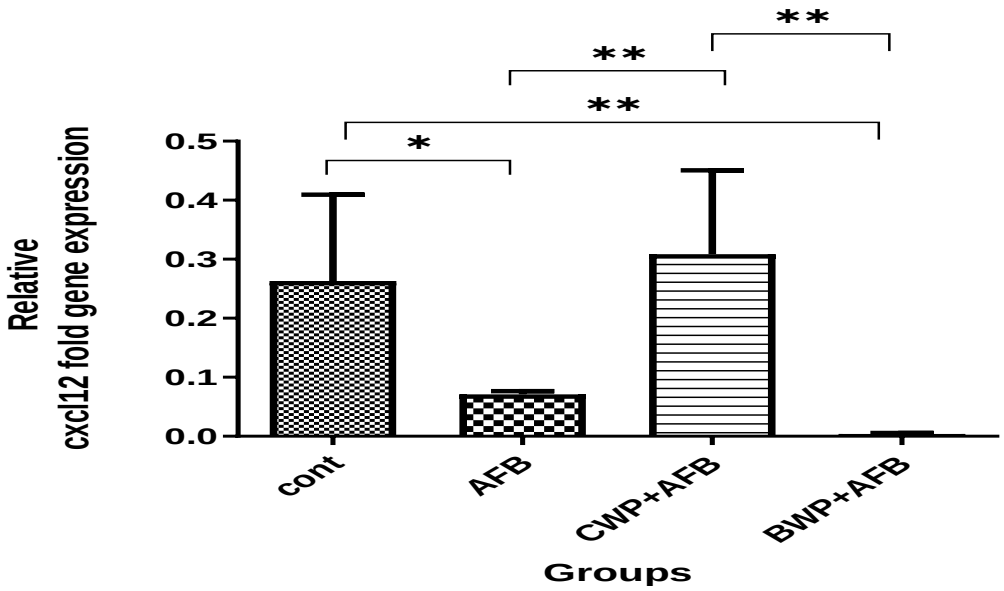

Fig (23): Bar graphs representing mean ± SE of CXCL12 mRNA expression in the spleen of the experimental animals, **p (<0.05), **p (<0.01), ***p (<0.001).*

5. The mRNA expression of NF-κB *in* the thymus and Spleen

5.1. The mRNA expression of NF-κB in the thymus

The expression of *NF-κB* in the thymus showed a significant increase in the **AFB** treated group in comparison to the reference normal group (**p <0.001**). **CWP+AFB** and **BWP+AFB** treated groups had a lower significant **NF-κB** expression level than the aflatoxicosis group (**p <0.001, for each**) as presented in **Table** (16) and **Figure** (24).

Table (16): mRNA expression of NF-κB in the thymus of the experimental group

Groups	CONT	AFB	CWP+AFB	BWP+AFB	P-value
Relative NF-κB fold gene expression in the thymus	0.49±0.16	6.8±0.67 a^{***}	0.049±0.01 b^{***}	0.37±0.015 b^{***}	<.001

Data are presented as means ± SE, ^{a,b,c} significance difference from Control (Cont), Aflatoxin B (AFB), between camel whey proteins treated group (CWP+AFB) and bovine whey protein treated group (BWP+AFB). **p (<0.05), **p (<0.01), ***p (<0.001).* The NF-κB expression was relative to GAPDH as a control gene.

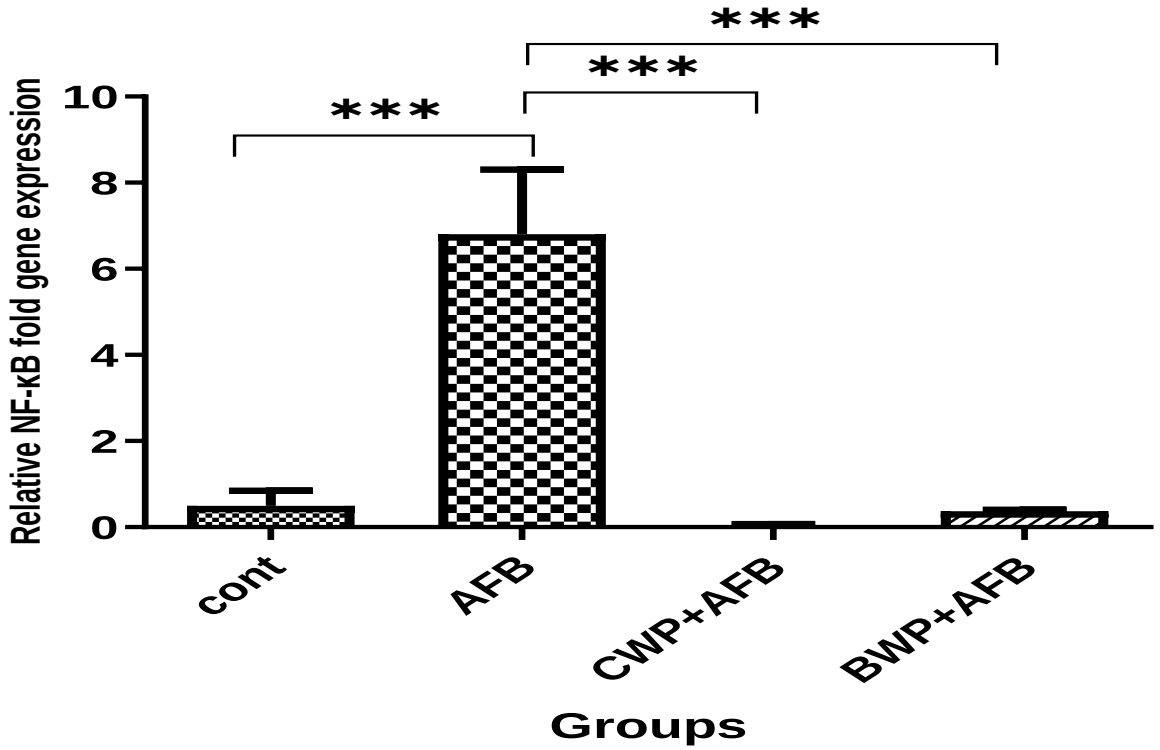

Fig (24): Bar graphs representing mean ± SE of NF-κB expression level of in the thymus of the experimental animals, **p (<0.05), **p (<0.01), ***p (<0.001).*

5.2. The mRNA expression of NF-κB in the spleen

The expression level of NF-κB *in* the spleen was significantly increased in AFB and BWP groups in comparison to the reference control group (**p <0.01, for each**). The groups treated with CWP and BWP showed a significant decrease in comparison to the AFB group (**p <0.001, p<0.01, respectively**). The group treated with CWP decreased significantly NF-κB levels in comparison to BWP **(p <0.05)** as presented in **Table** (17) and **Figure** (25).

Table (17): mRNA expression of NF-κB in the spleen of the experimental animals

Groups	Cont	AFB	CWP+AFB	BWP+AFB	P-value
Relative NF-κB fold gene expression in the spleen	0.95±0.06	6.41±0.6 a***	2.4±0.14 b***	4.19±0.57 a***, b**, c*	<.001

Data are presented as means ± SE, ^{a,b,c} significance difference from control (C), Aflatoxin B (AFB), between camel whey proteins treated group (CWP+AFB) and bovine whey protein treated group (BWP+AFB). **p (<0.05), **p (<0.01), ***p (<0.001)*.

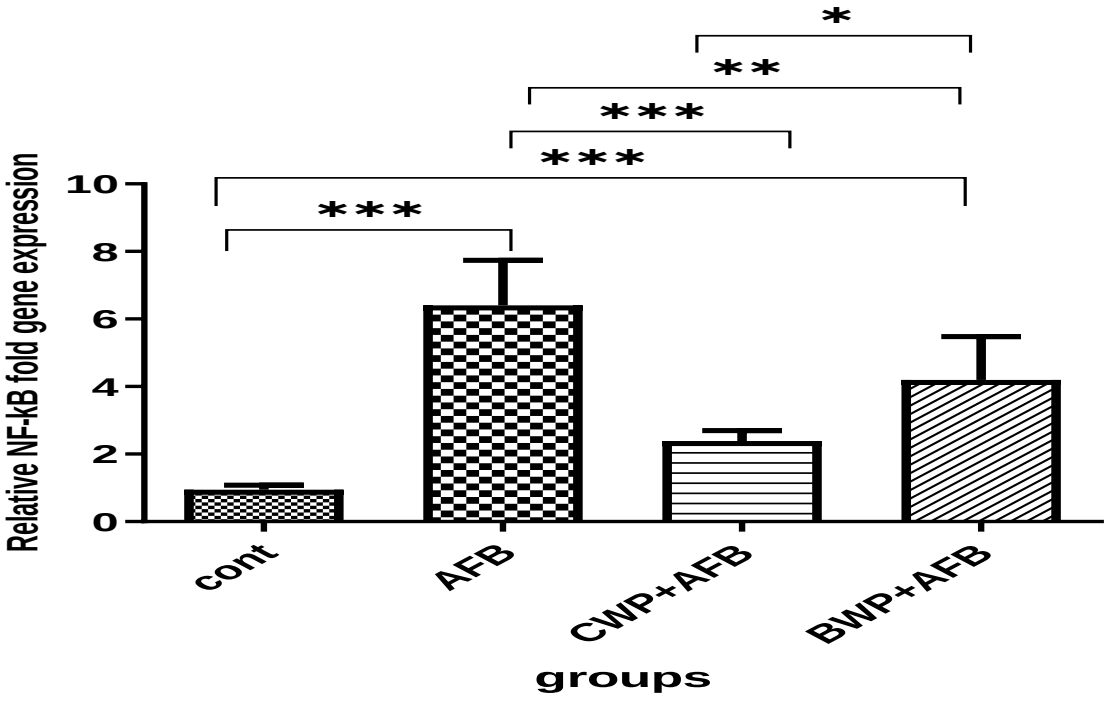

Fig (25): Bar graphs representing mean ± SE of NF-κB expression level of in the spleen of the experimental animals, **p (<0.05), **p (<0.01), ***p (<0.001)*.

6. The mRNA expression of IL-6 in the thymus and spleen

6.1. The mRNA expression of IL-6 in the thymus

The level of IL-6 mRNA in thymus was significantly increased in the groups treated with **AFB**, CWP+AFB, and **BWP+AFB** in comparison to the reference control group (**p <0.001, for each**). IL-6 levels in CWP and BWP group showed a significant decrease in comparison to the AFB group (**P <0.001, for each**). The IL-6 level was significantly higher in the BWP+AFB group than that in CWP+AFB (**p <0.001)** as presented in **table (18)** and **Figure. (25)**.

Table (18): expression of IL-6 in the thymus of the experimental animals

Groups	Cont	AFB	CWP+AFB	BWP+AFB	P-value
Relative IL-6 fold gene expression in the thymus	0.28±0.05	2.78±0.07 a***	0.81±0.03 a***, b***	1.68±0.08 a***, b***, c***	<0.001

Data are presented as means ± SE, [a,b,c] significance difference from Control (C), Aflatoxin B1 (AFB), between camel whey proteins treated group (CWP+AFB) and bovine whey protein treated group (BWP+AFB). **p (<0.05), **p (<0.01), ***p (<0.001).*

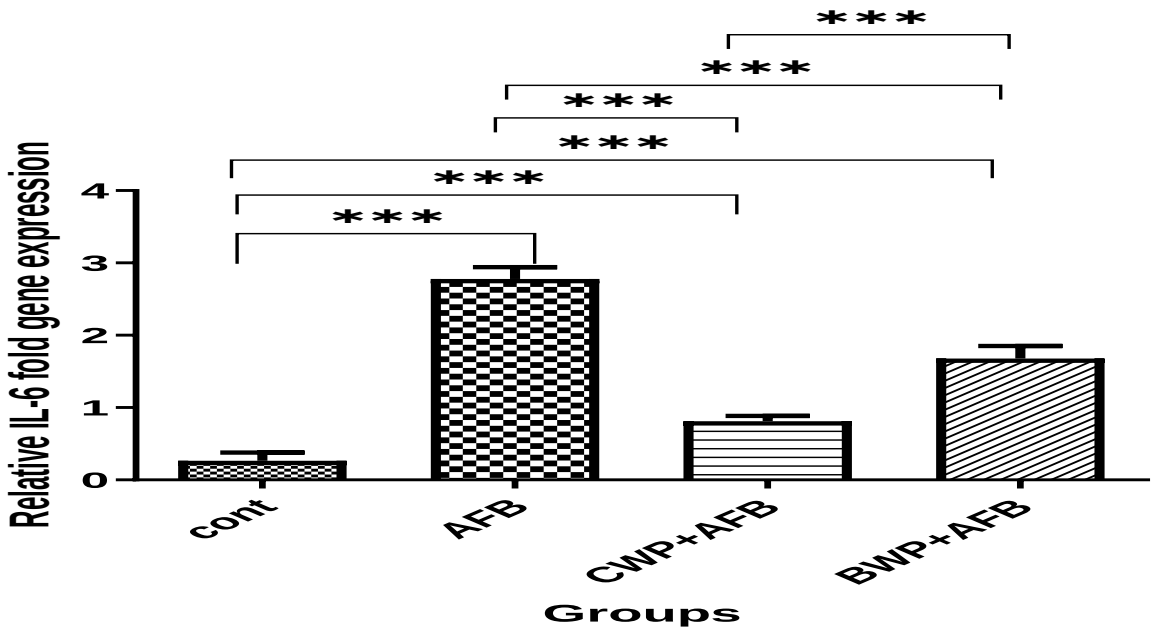

Fig (26): Bar graphs representing mean ± SE of IL-6 expression of in the thymus of the experimental animals**,** p (<0.05), **p (<0.01), ***p (<0.001).

6.2. The mRNA expression of IL-6 in the spleen

The level of IL-6 in the spleen was significantly decreased in group treated with AFB and BWP+AFB in comparison to the reference control group (**p <0.05 and p <0.01**, **respectively**), while the group treated with **CWP+AFB** showed no significant difference in comparison to the reference control group. Group treated with CWP showed a significant increase in comparison to the AFB group (**p<0.01**). CWP+AFB showed a significant increase in IL-6 expression than BWP+AFB **(p <0.001)** as presented in **Table (19)** and **Figure (27)**.

Table (19): mRNA expression of IL-6 in the spleen of the experimental animals

Groups	Cont	AFB	CWP+AFB	BWP+AFB	P-value
Relative IL-6fold gene expression in the spleen	2.09±0.24	0.54±0.08 a*	2.9±0.7 b**	0.14±0.02 a**, c***	<.001

Data are presented as means ± SE, ^{a,b,c} significance difference from Control (C), Aflatoxin B1 (AFB), between camel whey proteins (CWP) and bovine whey protein (BWP). *p (<0.05), **p (<0.01), ***p (<0.001).

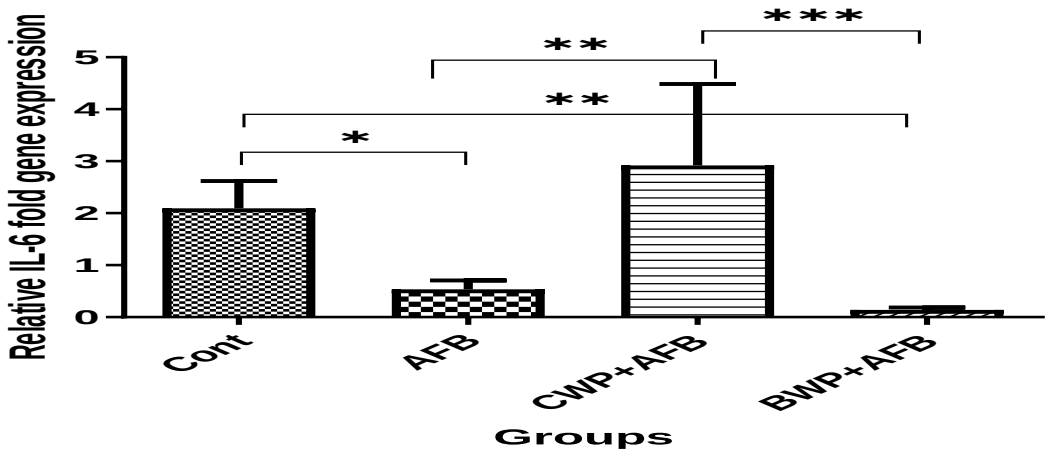

Fig (27): Bar graphs representing mean ± SE of IL-6 expression of in the spleen of the experimental animals. p (<0.05), **p (<0.01), ***p (<0.001).

7. The mRNA expression of TNF-α in thymus and Spleen

7.1. The mRNA expression of TNF-α in the thymus

The expression of **TNF-α** in the thymus tissue of the group treated with **AFB** was significantly increased in comparison to the control group (**p <0.001**), while the groups treated with CWP and BWP showed no significant difference in comparison to the control group. Groups treated with CWP and BWP showed a significant decrease in comparison to the AFB group (**P <0.001**, **for each**). There was no difference in TNF-α levels between **CWP+AFB** and **BWP+AFB** groups as presented in **table (20)** and **Figure (27)**.

Table (20): gene expression of TNF-α in the thymus of the experimental animals

Groups	CONT	AFB	CWP+AFB	BWP+AFB	P-value
Relative TNF-α fold gene expression in the thymus	1± 0.05	2.5±0.11 a***	0.63±0.25 b***	0.92±0.22 b***	<.001

Data are presented as means ± SE, ^{a,b,c} significance difference from Control (Cont), Aflatoxin B1 (AFB), between camel whey proteins (CWP) and bovine whey protein (BWP). **p (<0.05), **p (<0.01), ***p (<0.001).*

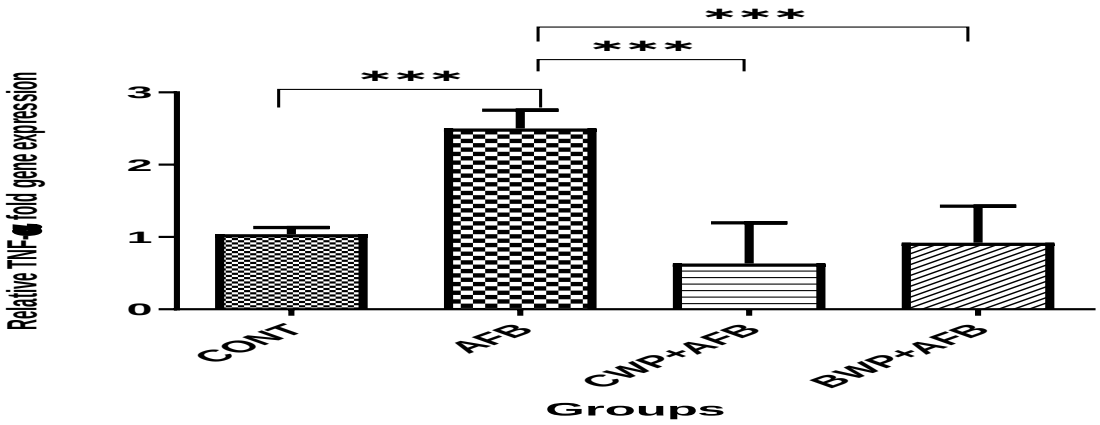

Fig (28): Bar graphs representing mean ± SE of the TNF-α expression level in the thymus of the experimental animals**.** **p (<0.05), **p (<0.01), ***p (<0.001).*

7.2. The mRNA expression of TNF-α in the spleen

The expression levels of TNF-α in the spleen in the groups treated with AFB, CWP, and BWP were significantly increased in comparison to the reference control group (**p. <0.01, p <0.05, p <0.05**, respectively). Groups treated with CWP and BWP showed a significant decrease in comparison to the AFB group **(p <0.01 and p < 0.05,** respectively**)**. No significant difference between group treated with CWP and BWP as presented in **table** (21) and **Figure** (28).

Table (21): Expression of TNF-α in the spleen of the experimental animals

Groups	CONT	AFB	CWP+AFB	BWP+AFB	P-value
Relative TNF-α fold gene expression in the spleen	0.28±0.08	1.62±0.035 a***	0.71±0.07 a***, b***	0.86±0.47 a***, b***	<0.001

Data are presented as means ± SE, [a,b,c] significance difference from Control (CONT), Aflatoxin B1 (AFB), between camel whey proteins treated group (CWP+AFB) and bovine whey protein treated group (BWP+AFB). **p (<0.05), **p (<0.01), ***p (<0.001).*

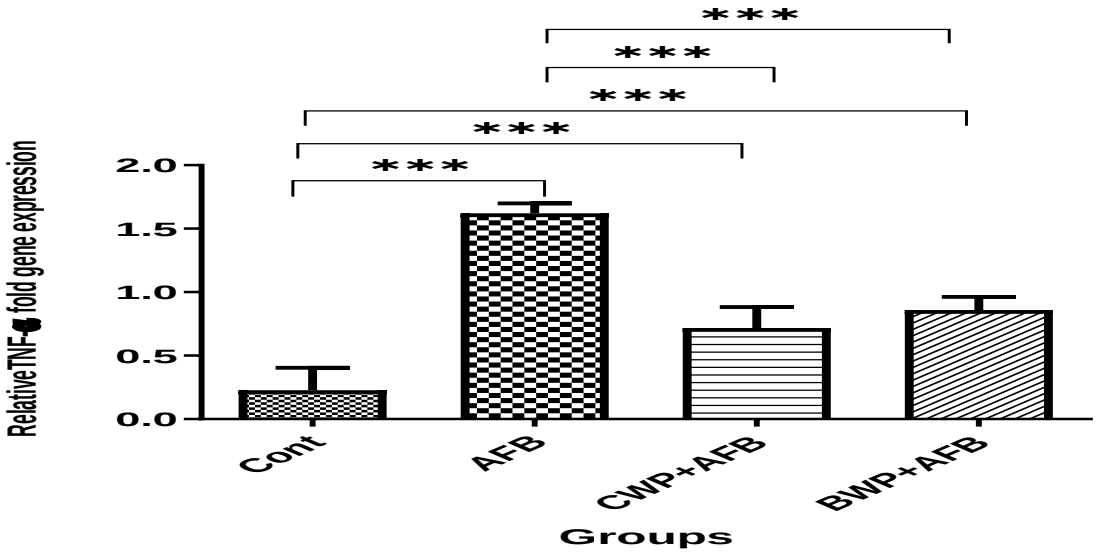

Fig (29): Bar graphs representing mean ± SE of TNF-α expression mRNA in the spleen of the experimental animals. **p (<0.05), **p (<0.01), ***p (<0.001).*

8-Immunodetection of cleaved caspase-3 by western blot in the thymus and spleen

8.1. Immunodetection of cleaved caspase-3 by western blot in the thymus of the experimental animals

The levels of **cleaved caspase-3** in the thymus of the experimental rats were significantly increased in **AFB and BWP+AFB** groups in comparison to the reference normal Control group (**P<0.001, P<0.01,** respectively), while the treatment with **CWP+AFB** showed no significant difference in comparison to control group**. CWP+AFB** decreased the levels of **cleaved caspase-3** when compared to the **AFB** group (**P<0.01)**, while **BWP+AFB** showed no significant difference in comparison to AFB as presented in **Table** (25)**, Figure** (30**) and Figure** (31).

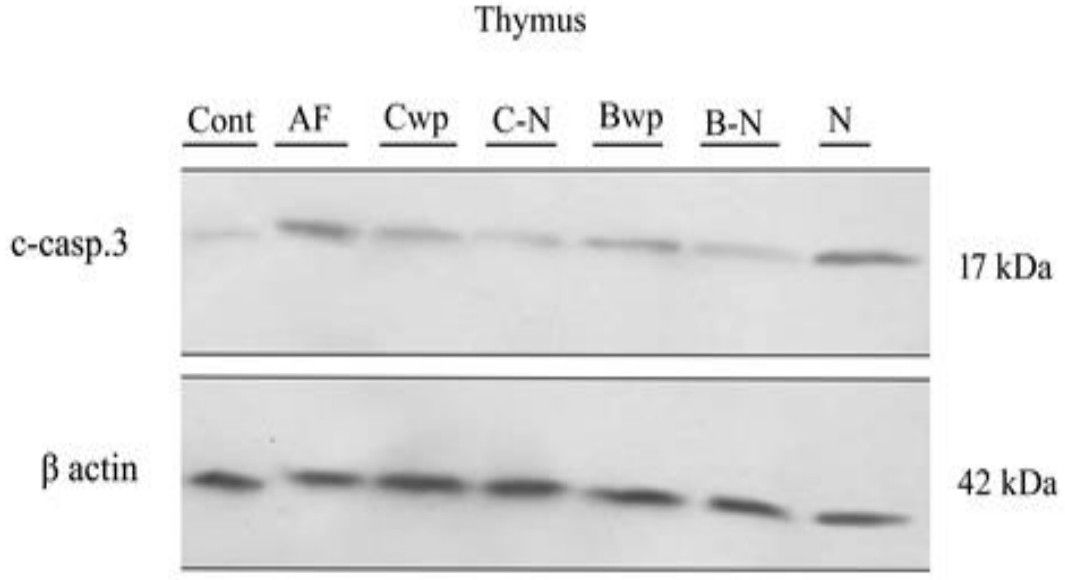

Fig. (30): Cleaved caspase-3 expression in the thymus of control, AFB, CWP, C-N, BWP, B-N, and N groups. The leaved caspase-3 antibody is the large subunit of active/cleaved caspase-3 (~14 to 21 kDa). β-Actin was used as a loading control for normalization purposes, as C-N refers to camel microparticles, B-N refers to Bovine microparticles, N refers to nanoparticles

Table (22): Cleaved caspase-3 in the thymus tissue of the experimental animals

Groups	Cont	AFB	CWP+AFB	BWP+AFB	P-value
Cleaved caspase-3	1	4.4±0.7 a**	2.1±0.2 b*	3.2±0.3 a*	< 0.001

Data are presented as means ± S.E, [a,b,c] significance difference from Control (CONT), aflatoxin B1 (AFB), between camel whey proteins treated group (CWP+AFB) and bovine whey protein treated group (BWP+AFB). **p (<0.05), **p (<0.01), ***p (<0.001).*

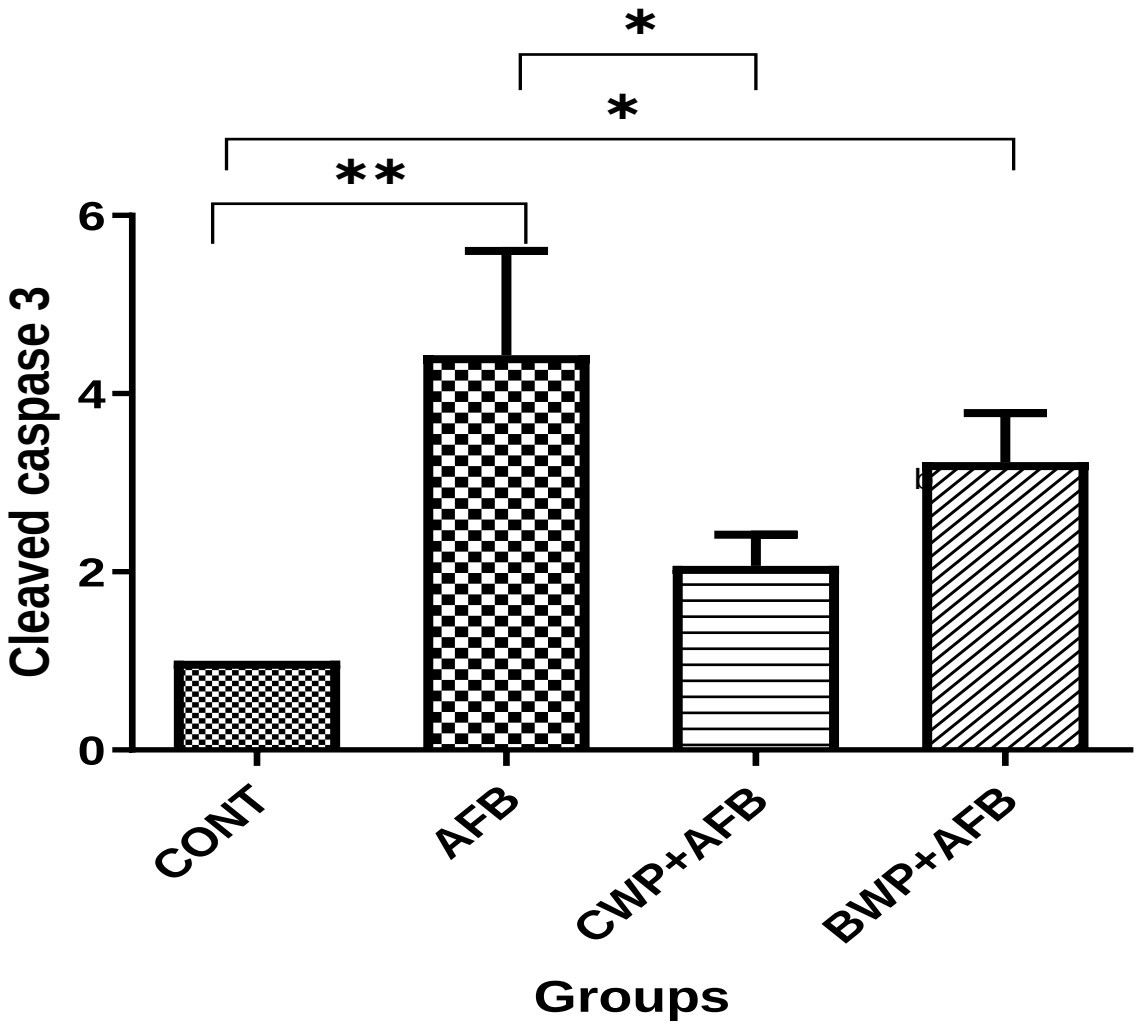

Fig (31): Bar graphs representing mean and SE of Cleaved Caspase- 3 protein expression in the thymus of the experimental groups, **p (<0.05), **p (<0.01), ***p (<0.001).*

8.2. Immunodetection of cleaved caspase-3 by Western blot in the spleen of the experimental groups

The levels of **cleaved caspase-3** in the spleen of the experimental rats were significantly increased in the intoxicated groups **AFB** in comparison to the control group **(p <0.001),** while **CWP+AFB** and **BWP+AFB** group showed no significant difference in comparison to control. The treatment with **CWP** and **BWP decreased the** levels of **cleaved caspase-3** compared to the **AFB** group **(p <0.01, p <0.05**, respectively**)** as presented in **Table (26) and Figure (32).**

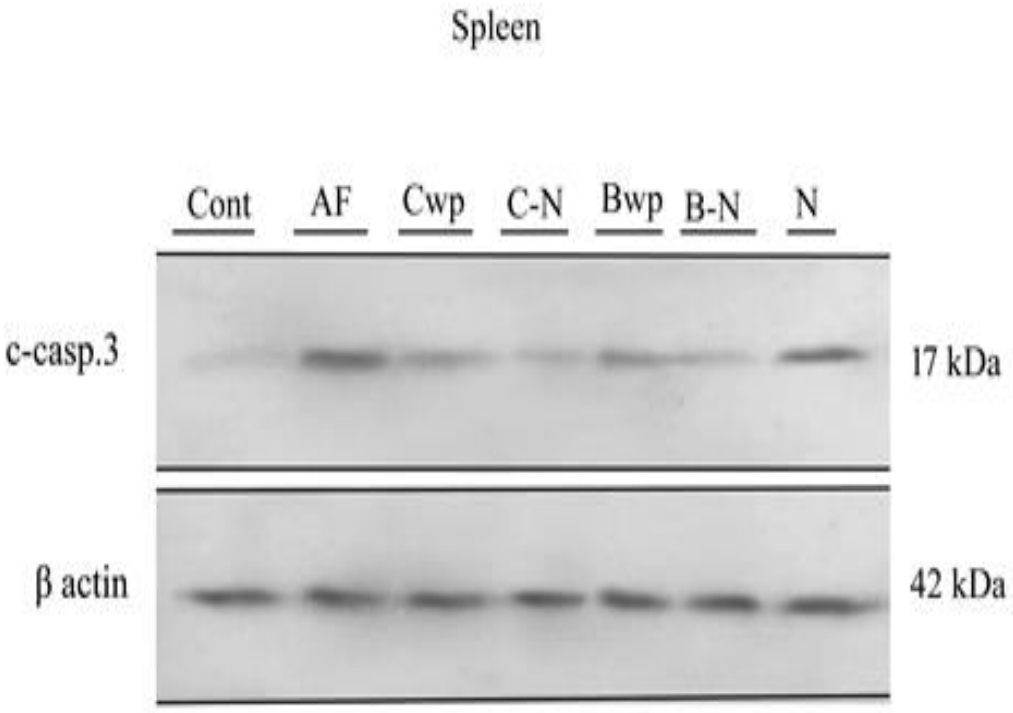

Fig. (32): Cleaved caspase-3 protein expression level in the spleen of the CONT, AFB, CWP, CMP, BWP, BMP, and NPs groups. The cleaved caspase-3 antibody is the large subunit of active/cleaved caspase-3 (~14 to 21 kDa). β-Actin was used as a loading control for normalization purposes. as C-N refers to camel microparticles, B-N refers to Bovine microparticles, N refers to nanoparticles

Table (23): Cleaved caspase-3 expression in the spleen of the experimental groups

Groups	Cont	AFB	CWP+AFB	BWP+AFB	P-value
spleen	1	5.97±0.9 a***	2.2±0.3 b***	3.17±0.3 a*, b*	< 0.001

Data are presented as means ± S.E. [a,b,c] significance difference from Control (CONT), Aflatoxin B1 (AFB), between camel whey proteins (CWP) and bovine whey protein (BWP). *p (<0.05), **p (<0.01), ***p (<0.001).

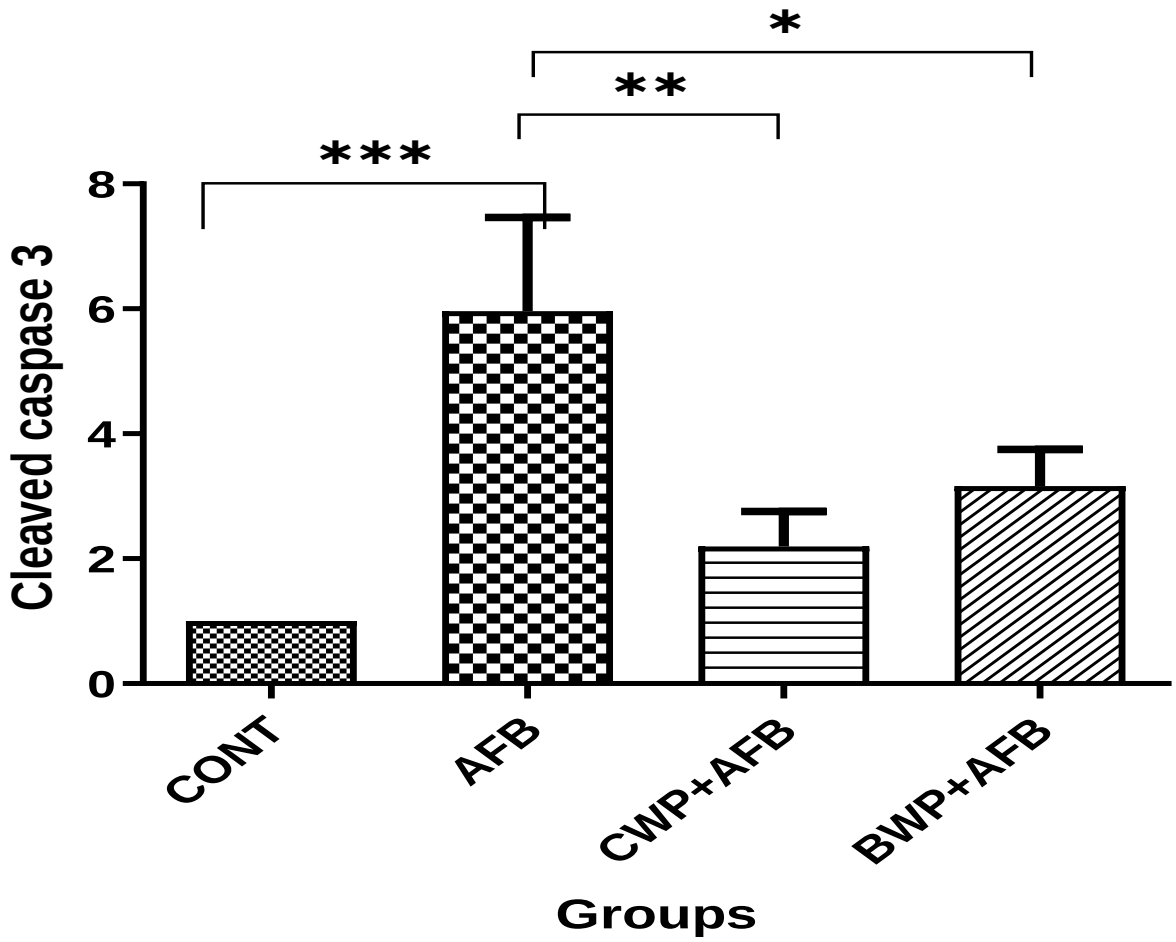

Fig (33): Bar graphs representing mean ± SE of Cleaved Caspase-3 protein expression levels in the spleen of the experimental groups**.** *p (<0.05), **p (<0.01), ***p (<0.001).

Histopathological finding

A-Spleen tissue

Examination of spleen sections from **control group** showing that the spleen is formed of splenic pulps has two components white pulp and red pulp The white pulp consists of the central artery surrounded by the periarterial lymphoid sheath with a normal distribution of lymphocytes in the lymphatic nodule. In between the White pulp is the Red Pulp which is composed of splenic cords (of Billroth) and sinusoids. The red pulp (RP) which is composed of splenic cords and sinusoids, both contain blood cells of all types. Splenic cords that are highly cellular contain plasma cells, white blood cells, and lymphocytes. Splenic sinusoids contain blood cells of all types. **(figure 33 & 34**). In **aflatoxin B group**, loss of architecture with shrinkage of the lymphatic nodules of white pulp and decreased cellularity in the follicle and marginal zone could be detected and wide empty spaces among the cells in both white pulp and red pulp. Most of cells showed degenerative changes, and many cells had pale vacuolated cytoplasm **(figure 35 &36).** In camel whey protein treated group showed significant improvement in splenic tissues in form of normal organization of the structure of white pulp and red pulp nearly close to control group (**figure 37)**. Also, white pulp and red pulp showed normal appearance of the cells **(figure 38)**. In bovine whey protein treated group there is slight improvement but still there is vacuolation of splenic cells, degeneration of lymphocytes in the white pulp and red pulp is observed (**figure 39 & 40**).

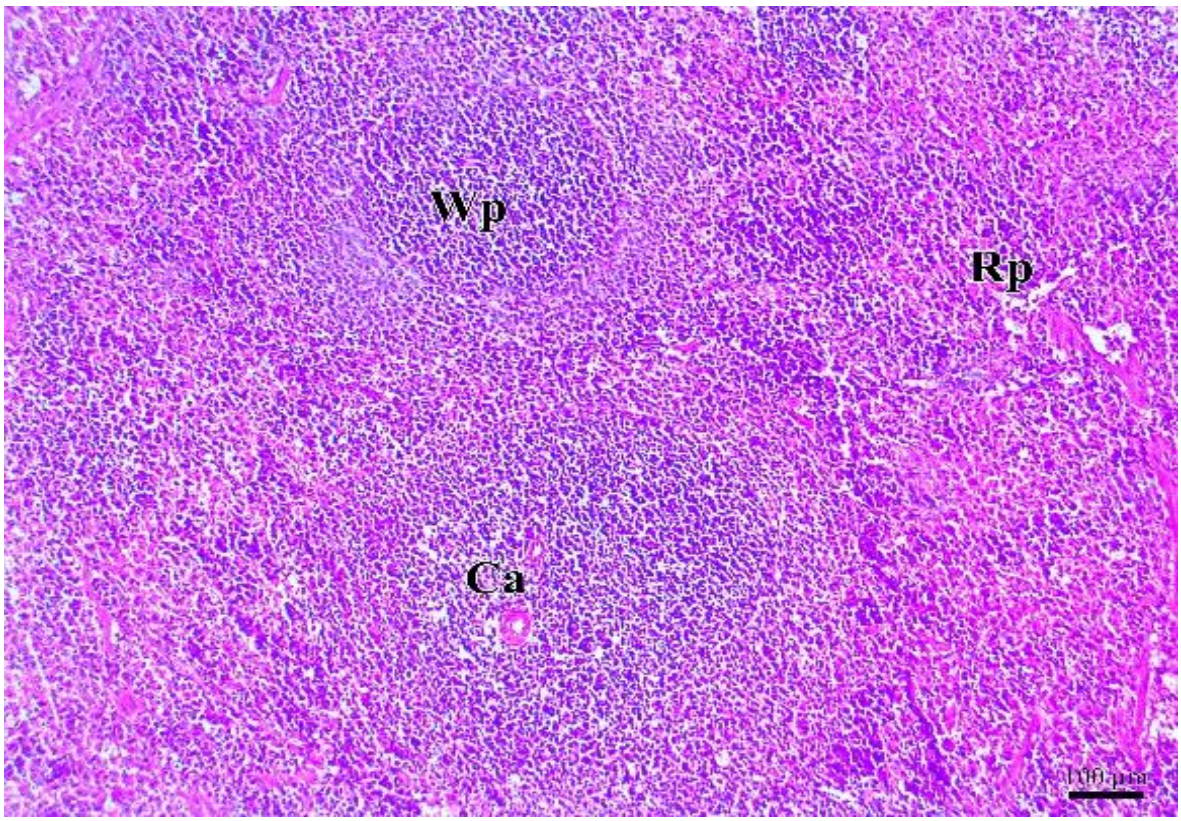

Fig (34): A photomicrograph of a section from a control rat spleen showed the normal structure of the spleen displayed as the parenchyma of the spleen (splenic pulp), which has two components the white pulp (WP) and red pulp (RP). Note central artery (Ca), (H&E X100).

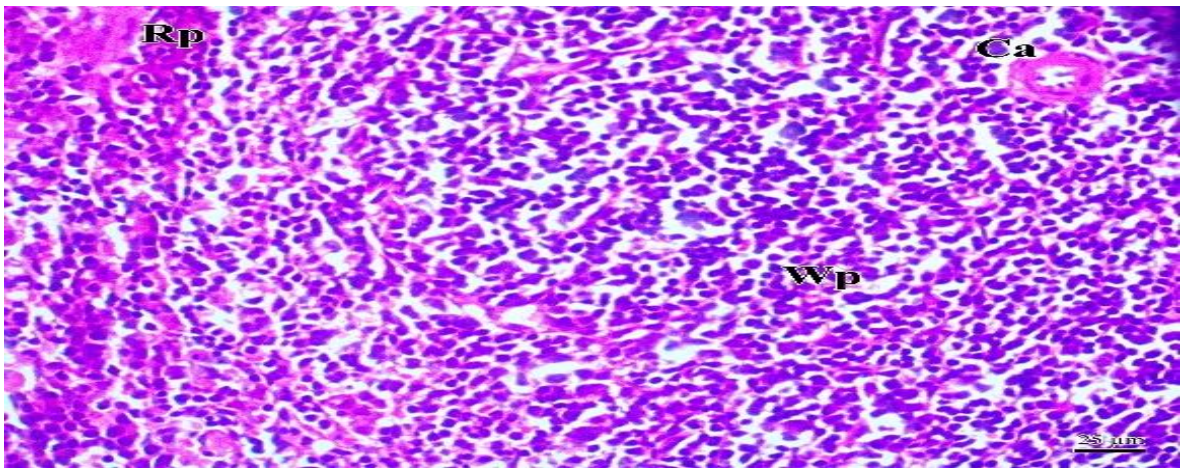

Fig (35): A photomicrograph of a section from a control rat spleen showing the white pulp (wp) formed from one lymphatic nodule which consists of the central artery surrounded by the periarterial lymphoid sheath with a normal distribution of lymphocytes in the lymphatic nodule. In between the WP is the RP which is formed of splenic cords and sinuses. Note: the marginal zone which demarcates the zone between the WP and the RP, (H&E X400).

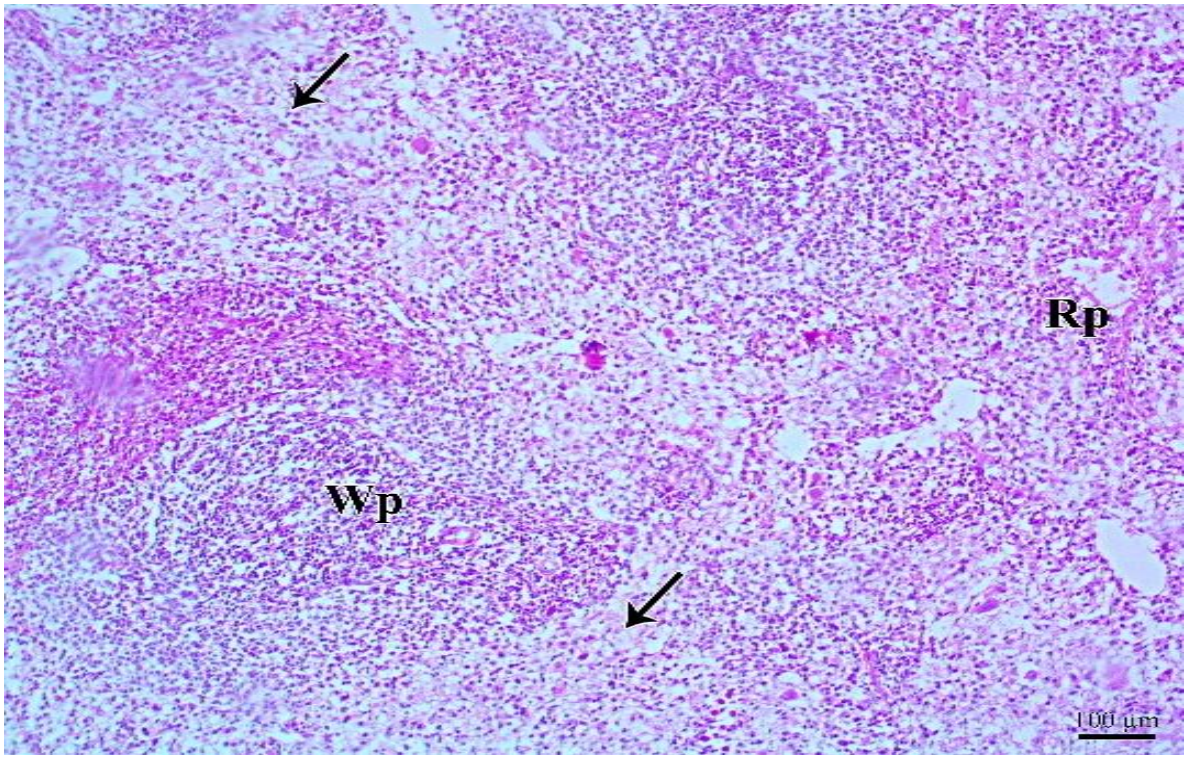

Fig (36): A photomicrograph of a section from an aflatoxin treated rat spleen showing distortion in the organization of the histological structure. Shrinkage of the lymphatic nodules of white pulp wp and decreased cellularity in the follicle and marginal zone could be detected. Note the degeneration of some cells (arrow), (H&Ex100)**.**

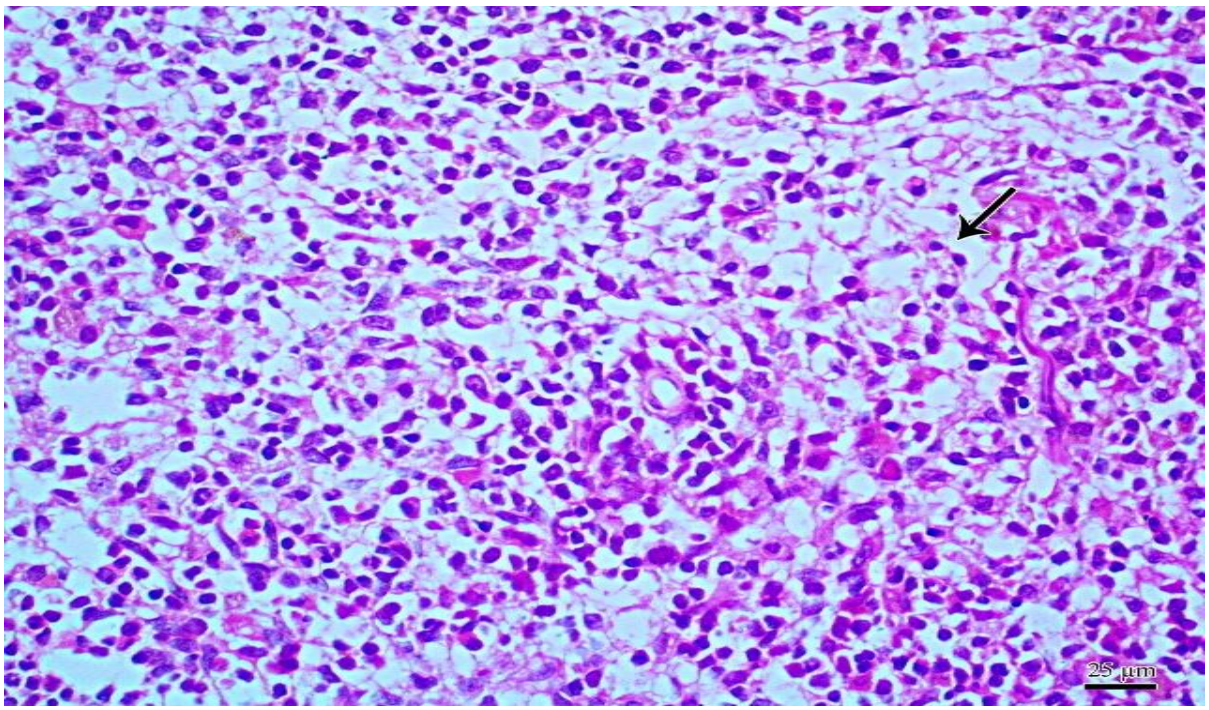

Fig (37): A photomicrograph of a section from aflatoxin treated rat spleen showing that a lot of cells appeared vacuolated and degenerated (arrow in RP & WP), (H&Ex400).

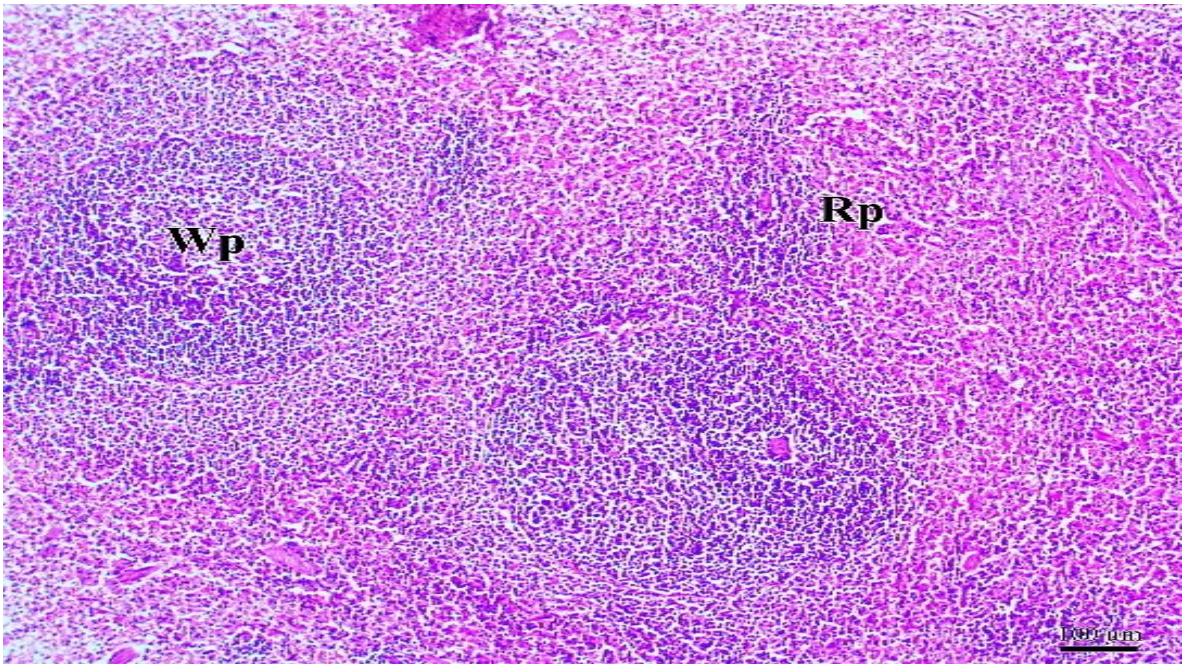

Fig (38): A photomicrograph of a section from an aflatoxin and camel way protein treated rat spleen **(CWP+AFB**) showing the normal organization of the structure of white pulp (WP) and red pulp (RP), (H&Ex100).

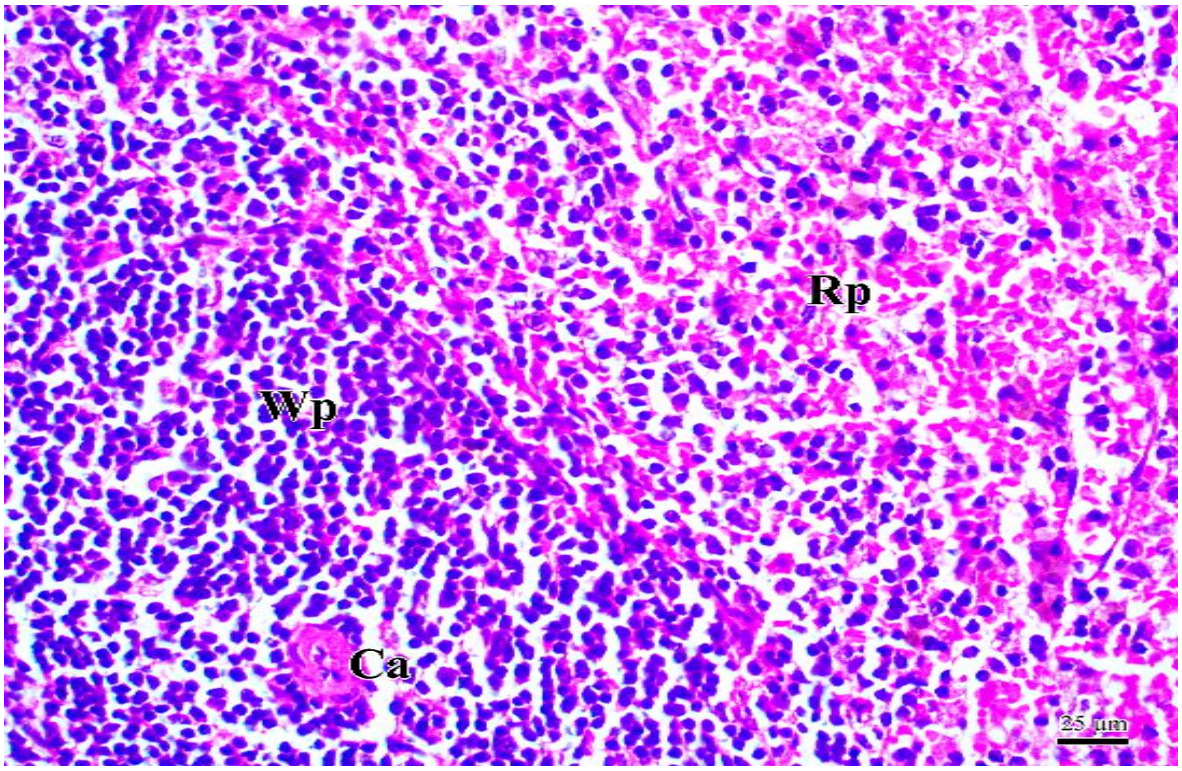

Fig (39): A photomicrograph of a section from an aflatoxin and camel way protein treated rat spleen **(CWP+AFB**) (magnified part) showing part of white pulp and red pulp with normal appearance of the cells, (H&Ex400)**.**

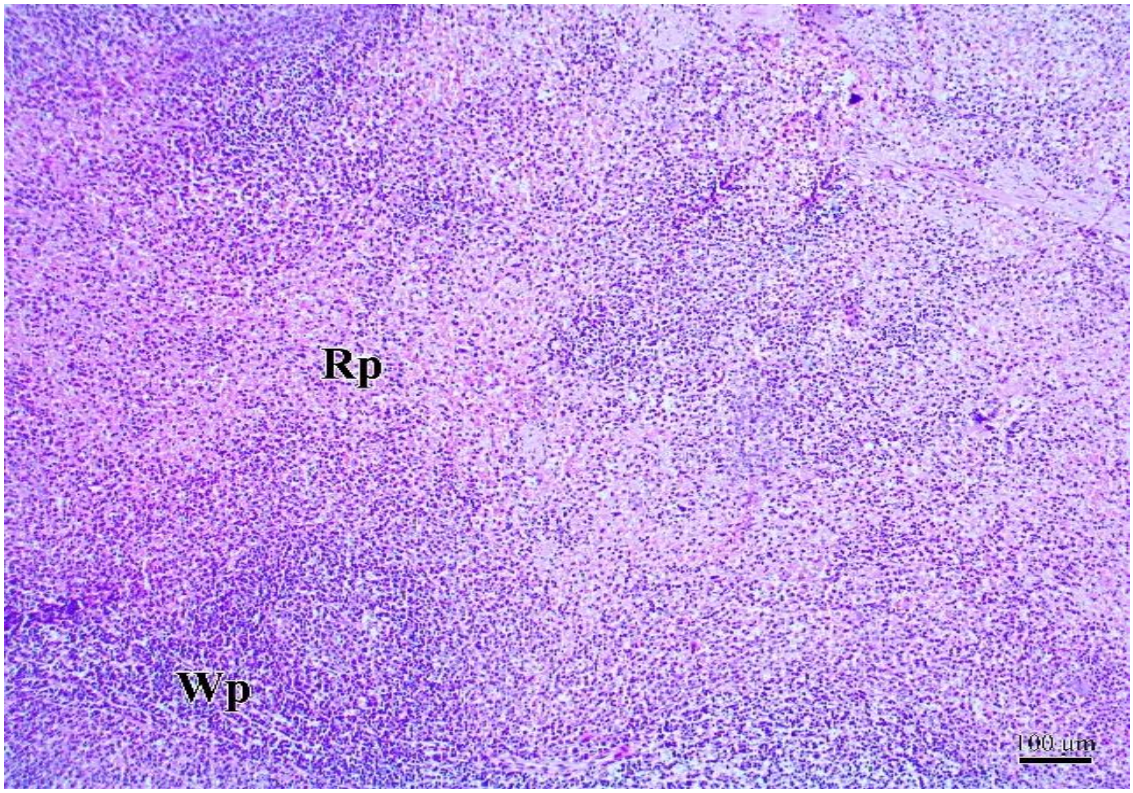

Fig (40): A photomicrograph of a section from aflatoxin and bovine whey protein treated rat spleen (**BWP+AFB**), vacuolation of splenic cells, degeneration of lymphocytes in the white pulp was also observed, (H&Ex100).

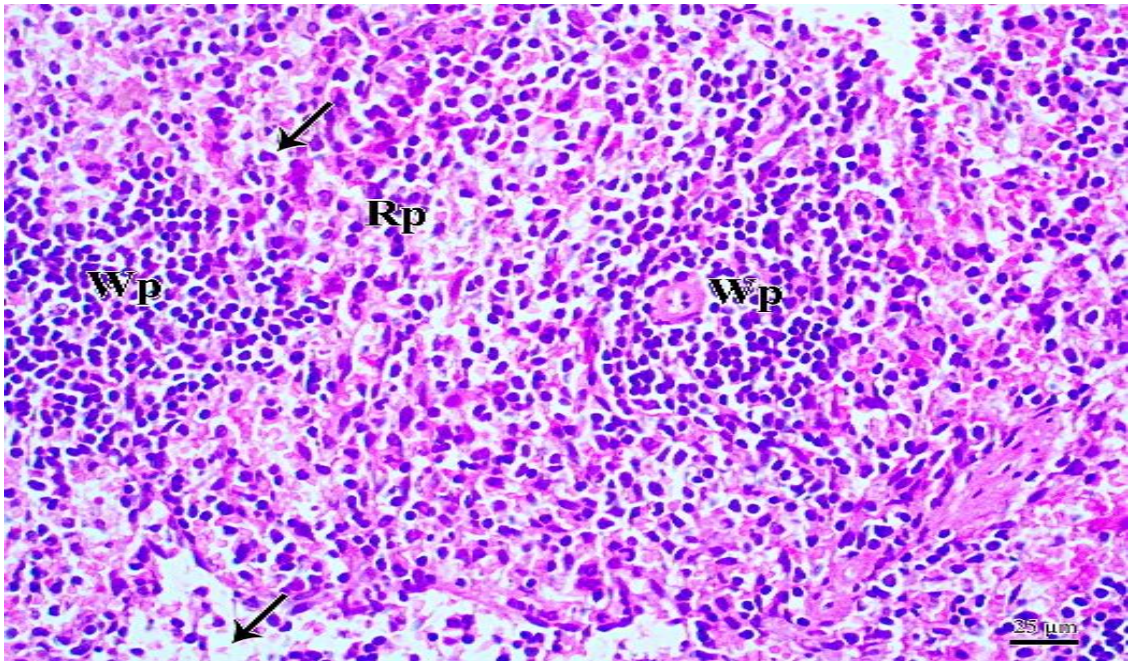

Fig (41): A photomicrograph of a section from aflatoxin and bovine way protein treated rat spleen (**BWP+AFB**) showing the number of lymphocytes was lightly vacuolated (arrows) in lymphatic nodule and periarterial lymphatic sheath, as well as in the red pulp, (H&Ex400).

B- Thymic tissue

Examination of thymus sections from the control group showed that the thymus consists of two distinct lobes connected by a connective tissue isthmus. A thin connective tissue capsule surrounds each lobe and, gives rise to septae, that partially subdivide the thymus into interconnecting lobules of variable size and orientation. Each thymic lobule consists of an outer darkly stain cortex and a slightly paler medulla **(figure 41).** the cortex is formed from densely packed small lymphocytes with few epithelial reticular cells while medulla is pale stained less densely cellular than cortex. It contained large lymphocytes and a lot of epithelial reticular cells **(figure 42).** In **Aflatoxin B group**, disfigurement in the organization with apparent decrease in the thickness of the cortex (cortical atrophy) with loss of corticomedullary demarcation **(figure 43)** and wide space appeared between cells with a lot of vacuolated cells **(figure 44). In camel whey protein treated group showed** slightly normal organization of the thymus lobules with increase of the cortical thickness (**figure 45**) and most of the cells retaining their normal appearance with slight vacuolated cells still present (**figure 46**)**.** In group treated with BWP**,** Cortex still showing atrophy slight demarcation appear between cortex and medulla (**figure 47**), and slight lymphoid depletion with spaced between cells which more apparent in medulla **(figure 48).**

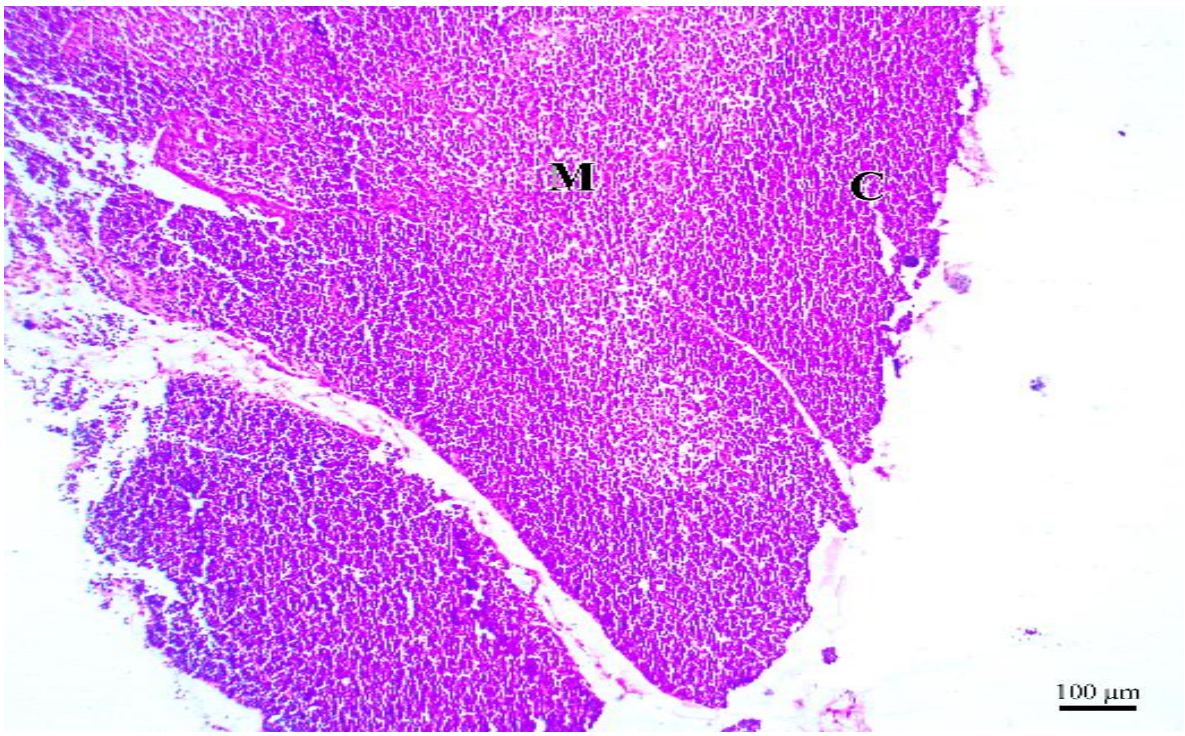

Fig (42): A photomicrograph of a section from a control rat thymus shows thymic lobules with normal architectures in form of outer darkly stained cortex and slightly paler medulla (H&E x100).

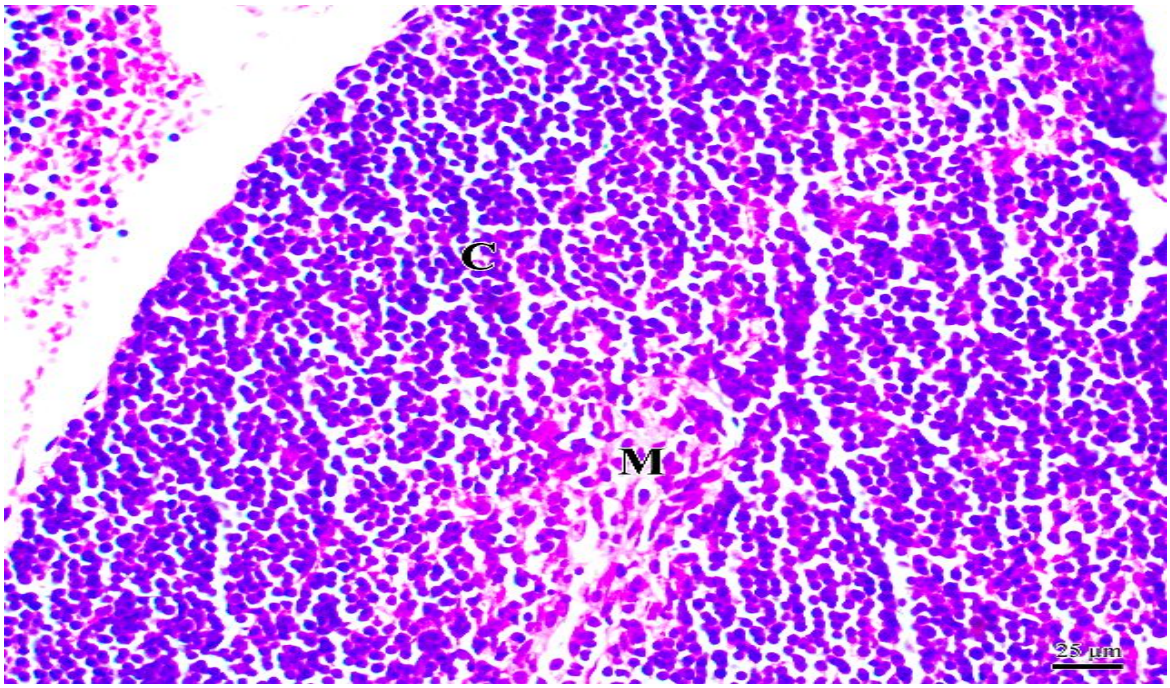

Fig (43): A photomicrograph of a section from a control rat thymus shows that the cortex formed from densely packed small lymphocytes with few epithelial reticular cells (C) while medulla is pale stained less densely cellular than cortex. It contained large lymphocytes and a lot of epithelial reticular cells (H&E x400).

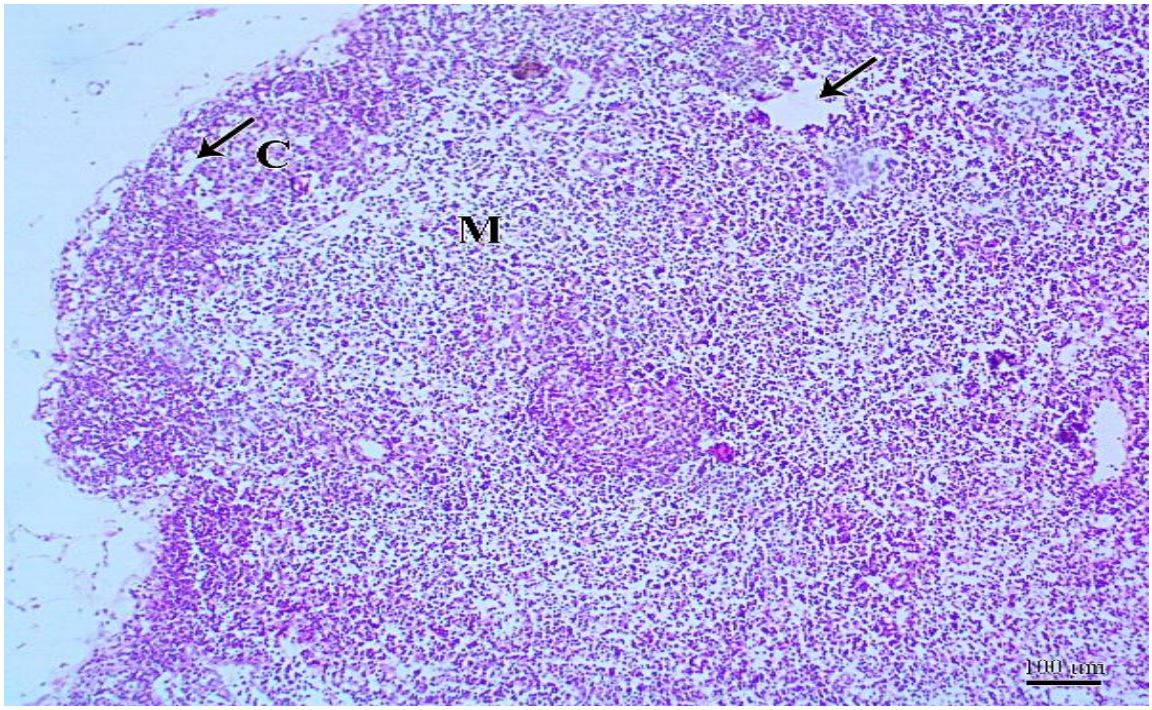

Fig (44): A photomicrograph of a section from an aflatoxin B rat thymus showed disfigurement in the organization with apparent decrease in the thickness of the cortex (cortical atrophy) with loss of corticomedullary demarcation (H&E x100).

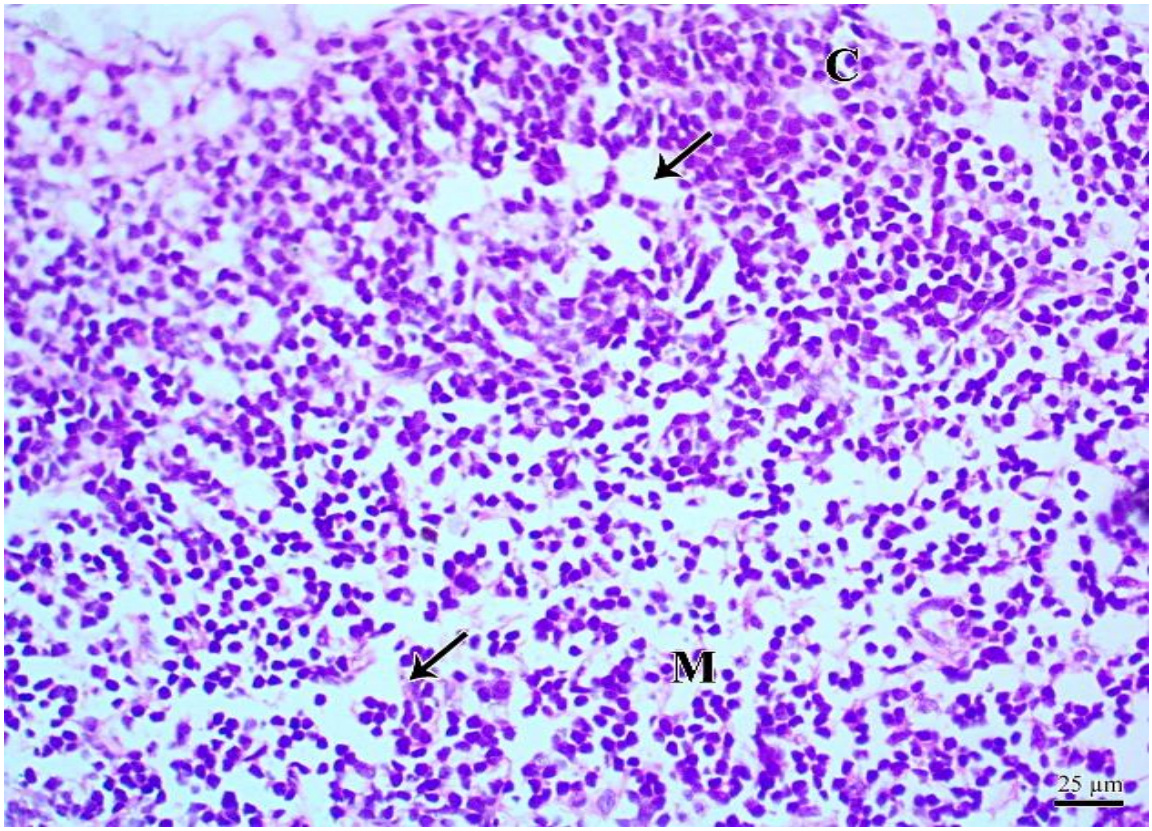

Fig (45): A photomicrograph of a section from an aflatoxin B rat thymus shows wide space appeared between cells with a lot of vacuolated cells (arrow) (H&E x400).

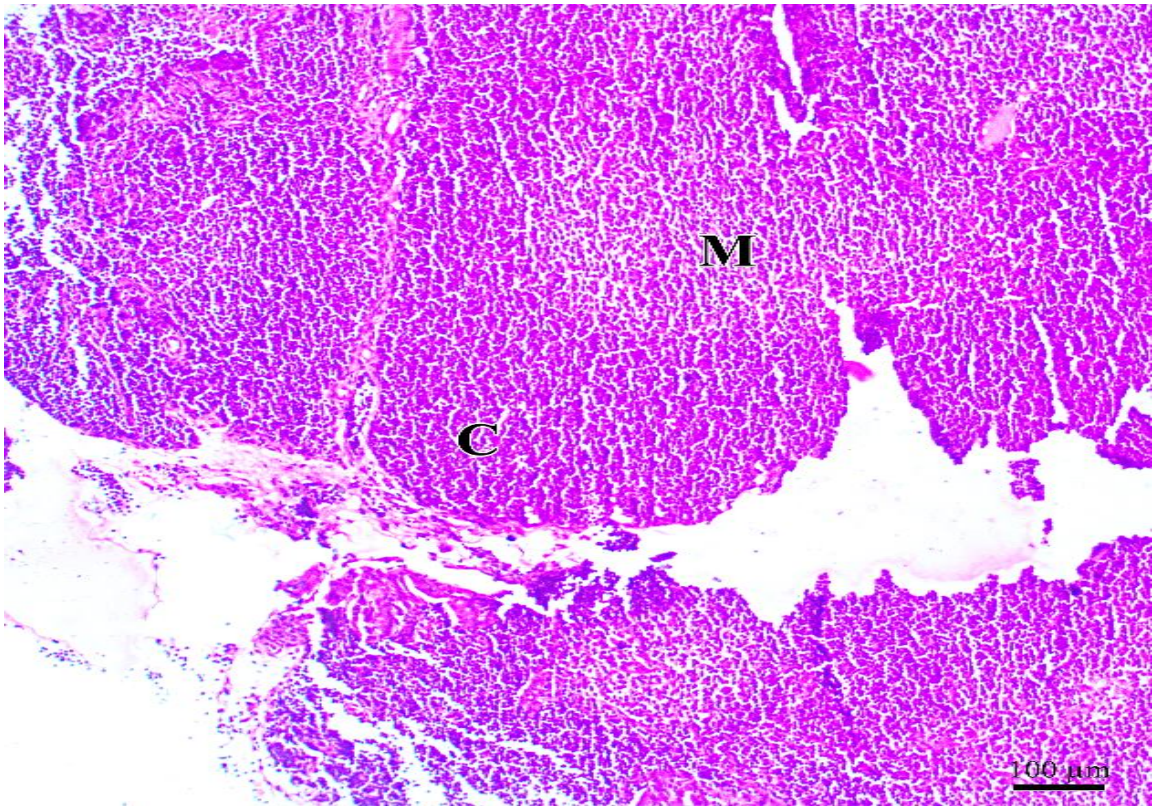

Fig (46): A photomicrograph of a section from aflatoxin rats treated with **CWP** thymus (CWP+AFB) shows slightly normal organization of the thymolobules with increase of the cortical thickness (H&E x100).

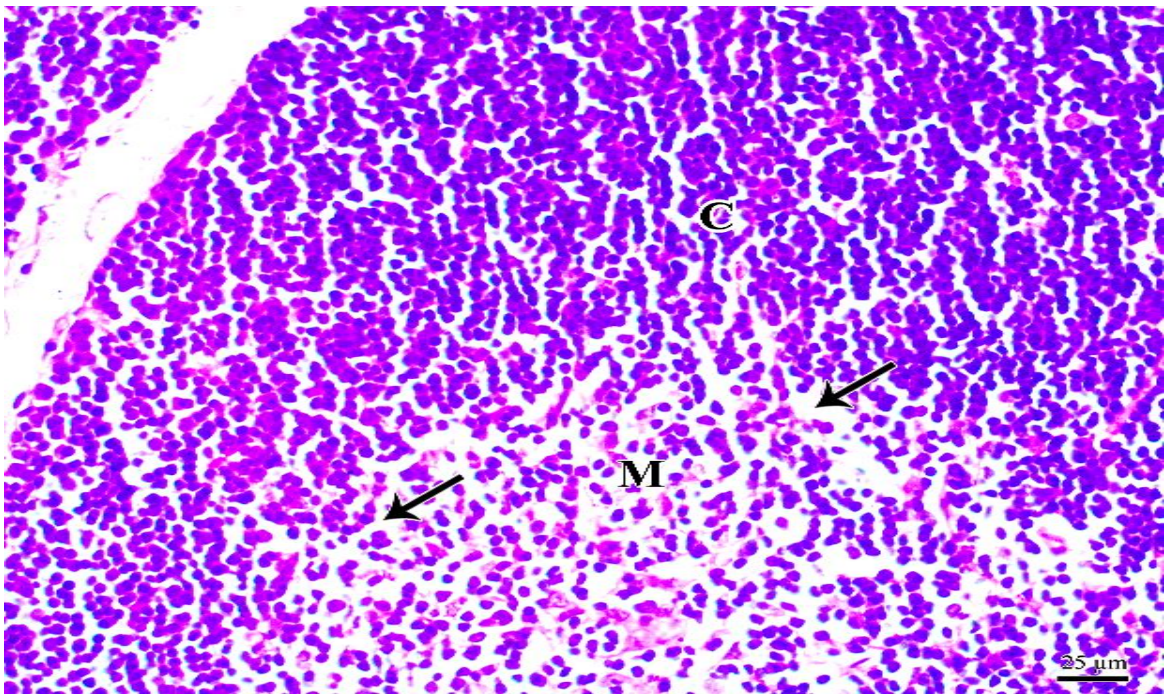

Fig (47): A photomicrograph of a section from aflatoxin rats treated with **CWP** rat thymus (CWP+AFB) shows most of the cells retaining their normal appearance with slight vacuolated cells still present (H&E x400).

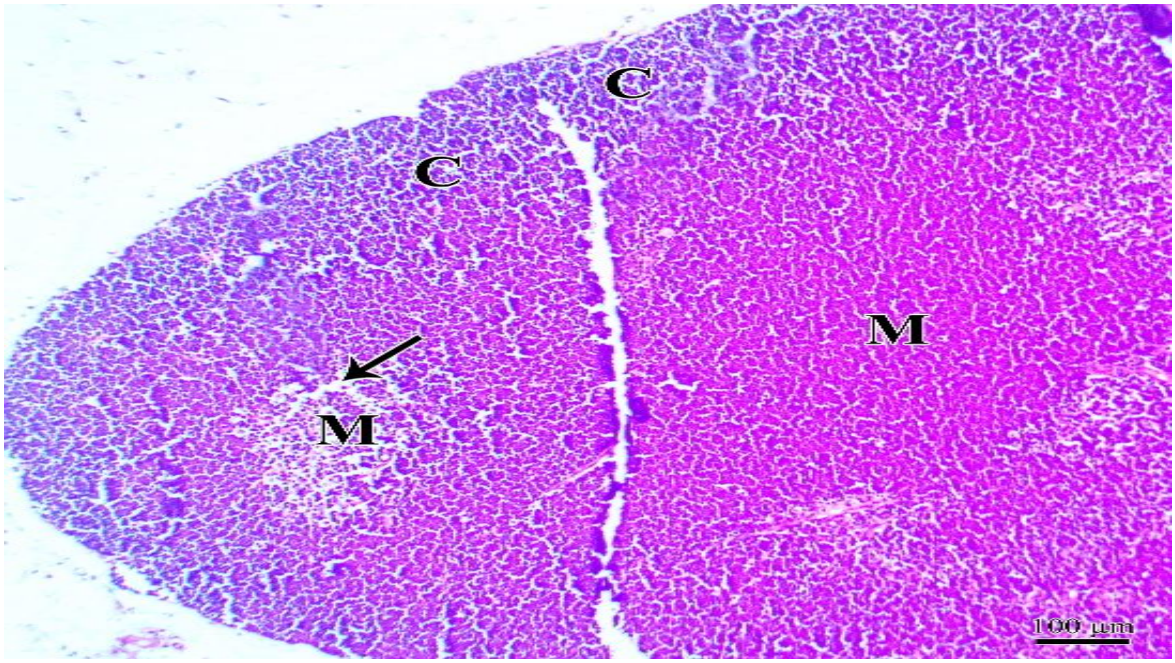

Fig (48): A photomicrograph of a section from an aflatoxicosis rat treated with **BWP** (**BWP+AFB**) thymus shows cortex (C) and medulla (M). Cortex still showing atrophy slight demarcation appear between cortex and medulla (H&E x100).

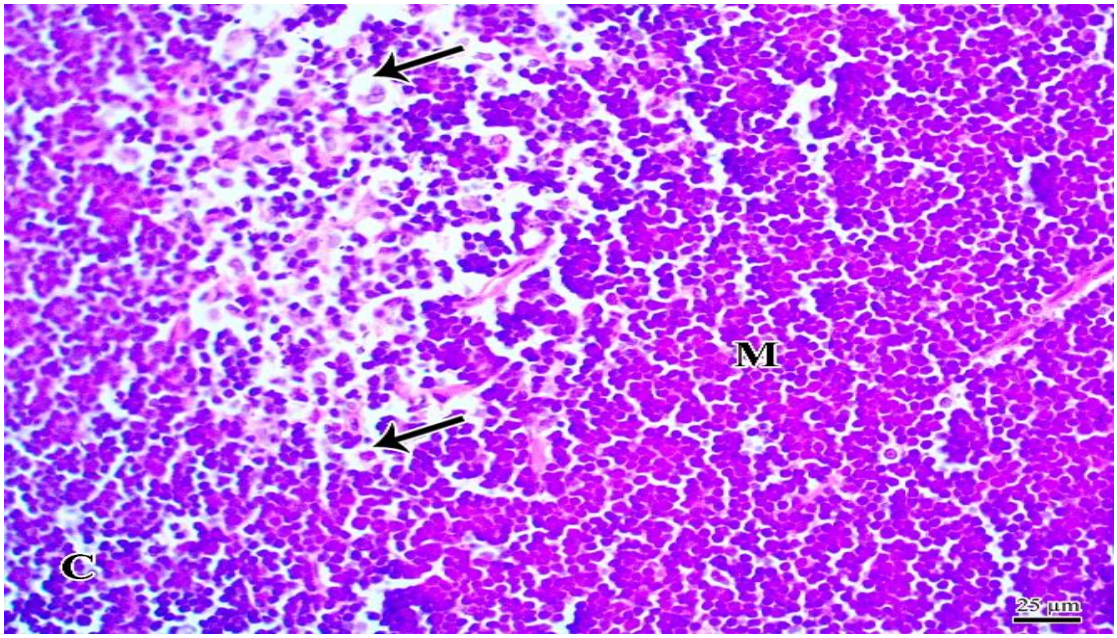

Fig (49): A photomicrograph of a section from an aflatoxicosis rat treated with **BWP** (**BWP+AFB**) thymus shows slight lymphoid depletion with spaced between cells which more apparent in medulla (arrow) (H&E x400).

Discussion

According to previous reports on aflatoxin cultivation, getting a sample of pure aflatoxin for biological use was done in laboratories as the aflatoxin sample was harvested from a nutritional medium for fungi species of *aspergillus flavus* as many scientists recommended **(Booth, 1971)**. The aflatoxicosis induction in lab animals was done according to the recommended dose of **Raisuddin *et al*., (1993)**, and signs of aflatoxicosis were detected on the targeted organs taken in this experiment; liver, thymus, and spleen.

After the purification of camel milk and bovine milk to obtain camel whey proteins and bovine whey proteins respectively, we made Native-PAGE electrophoresis for both camel whey proteins and bovine whey proteins to detect the protein bands and identified the dominant protein band in each type of whey protein. The most prominent electrophoretic zone in camel whey was lactoferrin content which constituted 33% of camel whey proteins so CWP considers a valuable source of this protein, this is compatible with the finding of **Hamers-Casterman *et al*., (1993)**.

The most prominent electrophoretic zone in bovine whey samples showed α-lactoalbumin which constitutes 32.7% of bovine whey proteins, these findings are compatible with **Ebaid *et al*., (2012)** and **Merin *et al*., (2001)**.

In the current study, we focused on the possible protective effects of camel whey proteins and bovine whey proteins against aflatoxicosis and we aimed to determine which one will be more powerful in protecting liver and immune organs (spleen and thymus) from the impact of aflatoxicosis, for this we investigate oxidants/antioxidants status in liver.

Aflatoxin B increases the production of free radicals, and lipid peroxidation in the liver causes liver oxidative damage and inflammation, and results in hepatic damage **(Rastogi *et al.*, 2001)**, these changes play a major role in liver carcinogenicity as reported by **Aziz *et al.*, (2005)**. A study by **Shen *et al.*, (1996)** stated the mechanism of liver damage in aflatoxicosis is due to AFB increase the production of ROS such as hydrogen peroxide (H2O2), hydroxyl radicals (•OH), and superoxide radicals (O2−) leading to an imbalance between the oxidants and the antioxidant systems causing oxidative stress, these ROS attack hepatocytes leading to the damage of the liver structure and function, which promote an inflammatory response in the liver.

One major consequence of oxidative stress/inflammation occurrence is the overproduction of NO that causes tissue damage by reacting with other oxygen radicals **(Sass *et al.*, 2001)**. A study by **New & Wong, (2007)** indicated that AFB1 increased the protein expression of NO and this agreed with our results that showed an increase in the level of NO in the liver homogenate of the aflatoxicosed group.

Free radicals are known to cause oxidative damage to lipids resulting in the formation of cytotoxic aldehyde and ketone derivatives, these lipid peroxidation products have a longer half-life, so they can diffuse into the bilayer and can cause oxidative damage away from their site of production. Malondialdehyde (MDA) is a significant final product, which alters membrane fluidity and enhances membrane fragility. Furthermore, MDA blocks particular enzyme reactions and causes mutagenicity and carcinogenicity by creating DNA adducts **(Marnett, 1999)**. The present study showed a significant increase in the MDA level in aflatoxicated liver tissues, these findings agreed with the results

reported by **Rotimi *et al*., (2019)**, who proved that subacute intoxication with AFB1 increases the mitochondrial MDA.

During the metabolization of AFB1 by CYP450 subfamilies, AFB1 passes through two phases of metabolism. In phase 2 reactions, the AFB metabolites had been detoxified by conjugation with reduced GSH by glutathione-S-transferases (GST), this reaction leads to the depletion of GSH and GST **(Wang *et al*., 1999)**. This explains our results as aflatoxicosis caused a decline in the level of GSH content and GST in the liver homogenate.

Mitochondrial antioxidant GSH-Px enzyme is the crucial antioxidant enzyme responsible for scavenging free radicals in cells and has a role in the electron transport chain and the generation of free radicals' reactive oxygen/ nitrogen species. This enzyme together with GST and GSH is responsible for maintaining the oxidant/antioxidant status of the mitochondria **(Mohajeri *et al*., 2018)**. Our result showed a decrease in the activity of GSH-Px and GST enzymes as well as a decline in the level of serum GSH but these results are in contrast with ***Rotimi et al*., (2019)** study that reported an increase in GST activity in rats intoxicated with aflatoxin.

Different therapeutic drugs became valid for the treatment of aflatoxicosis in humans and animals, it has also been shown that a high-protein dietary supplement (containing 41% whey proteins) had similar immunomodulatory, anti-inflammatory, and antioxidative functions in its undigested and digested form (**Kanwar & Kanwar, 2009**). Such intact whey proteins can be directly in contact with human leukocytes after their digestive absorption. It is thus important to understand how these proteins impact immunity. Camel whey protein (CWP) is considered a powerful natural antioxidant and has been reported to exhibit greater biological

activities than bovine and other whey proteins (WPs), including anti-heat stress (anti-HS), antioxidant, and anti-inflammatory activities **(Chen *et al*., 2014)**.

The most prominent protein in camel milk is LF which is well known to exert antioxidant activity through scavenging NO, LF has antiproliferative effects, LF also inhibited DNA damage most likely through binding catalytic iron, immunoglobulins especially IgG2, and IgG3 **(El-Hatmi *et al*., 2007).**

Our results showed that treatment with CWP significantly reduced the levels of NO and MDA in liver homogenate and increased GSH content, GSH-Px, and GST activities in liver homogenate, which agreed with what was reported by **(Du *et al*., 2021)** study, while BWP showed a significant improvement in oxidants/ antioxidants status of the body but not as effective as CWP.

This effect of CWP was caused by CLf which has been postulated to have hepatoprotective activity because LF can improve the imbalance in the levels of Th1/Th2 cytokines **(Saltanat *et al*., 2009)**. CWP provides cysteine (a substrate for GSH synthesis) to enhance GSH synthesis in numerous tissues and, consequently, the detoxification of free radicals during carcinogenesis **(Bounous *et al*., 1991).** Evidence from previous works revealed that CWP improved oxidative stress and repaired the damage in the immune organs by enhancing the chemotaxis of B and T cells towards the secondary lymphoid organs (site of antigen recognition) **(Sayed *et al*., 2017).**

Bovine α-LA is confirmed to have several beneficial effects on the prevention and treatment of some diseases through its anti-inflammatory actions. α-lactalbumin was possibly transported into the blood circulation from the intestine in the rat. Takeuchi and co-workers reported that intraduodenally infused, that is heterologous milk protein, is transported

into the blood circulation via the lymphatic pathway, not via portal circulation in adult rats **(Takeuchi *et al*., 2004)**.

Apha-Lactalbumin may be possibly transported into blood circulation via the lymphatic pathway. The observed decrease in NO levels in the liver of BWP treated group was due to the inhibition of the protein expression of iNOS by α-LA fraction. Another explanation is that the reduction in NO levels in hepatic cells might be due to the direct scavenging effect of α-LA **(Ho *et al*., 2007).**

Our results showed a reduction in MDA levels in the liver of the BWP+AFB group and this is agreed with (**Wang *et al*., 2008**) who reported that increasing dietary bovine lactoferrin levels decreased the MDA levels in serums and muscles of piglets, which indicated that bLf could protect from iron-induced lipid peroxidation. Bovine LF has been reported to lower lipid peroxidation by sequestering iron **(Shinmoto *et al*., 1992)** and may have an important role in binding pro-oxidative iron ions **(Lindmark-Månsson & Åkesson, 2000).**

A previous studies showed that bovine α-LA enhanced the antioxidant capability of GSH-Px enzymes and elevated the glutathione (GSH) content in the liver of broilers fed AFB-diets, The addition of α-LA into the AFB-diet increased the expression of the GSH-Px gene of those birds, compared with that of birds fed the AFB-diet alone **(Li *et al*., 2014)**, which agree with our results that showed that BWP could restore the level of glutathione (GSH) content in the liver of intoxicated rats and GSH-Px enzymes activity.

During liver cirrhosis, splenomegaly and hypersplenism are relatively sub-fatal complications. Splenic enlargement is one of the most palpable abnormalities accompanying liver cirrhosis and frequently occurs in parallel with hypersplenism **(Bashour *et al*., 2000)**. We investigated the effects of AFB on the spleen as it considers the

secondary lymphoid organ containing specialized subsets of lymphocytes and myeloid cells. The spleen was chosen because this secondary lymphoid organ plays a central role in the inflammatory response and the development of acquired immunity.

Histological examination of normal control spleen tissues showed that spleen is composed of two major parts: white pulp and red pulp, the white pulp has three characteristic components: periarteriolar lymphoid sheath follicle and marginal zone. Histological examination of spleen of aflatoxin B group showed the number of lymphocytes was lightly decreased and vacuoles appeared in lymphatic nodule and periarterial lymphatic sheath, as well as congestion in the red pulp. CWP supplementation improved spleen structure in rats with aflatoxicosis while BWP was unable to restrain the normal structure of spleen architecture.

Immune cells passively enter the spleen via arterioles. Native and central memory T-cells migrate from the red pulp to the white pulp by following chemotactic gradients. Once they reach the T zone of the white pulp, they search for dendritic cells that can potentially present cognate antigens originating from blood **(Lewis *et al*., 2019)**. T-cells that migrate out of the arterioles in the red pulp and marginal zone (MZ) move to the white pulp under the guidance of the chemotactic signals created by the periarteriolar stroma cells. Thus, T-cells undergo directional migration from the red to the white pulp through a route created by specialized stromal cells **(Chauveau *et al*., 2020).**

Since CXCL12 participates in native T- and B-cell recruitment to the extra-follicular area in secondary lymphoid organs via their lymphocyte receptors. Chemotaxis is an essential phenomenon for evaluating immune responses **(Gunn *et al*., 1999).**

CXCL12 was constitutively expressed by vascular, stromal, and hemato-poetic cells. It is a key regulator of hematopoiesis and myelopoiesis **(Sallusto & Baggiolini, 2008)**. In addition, CXCL12 regulates the recruitment and migration of hemato-poetic progenitors, monocytes, and lymphocytes and plays important role in the development of chronic inflammation, tumorigenesis, and metastasis of distinct solid tumors. Several extracellular stimuli including oxidative stress resulted from aflatoxicosis, hypoxia, and various growth factors and cytokines up-regulate CXCL12 expression according to **(Vandercappellen *et al*., 2008)** study and this contrast to our results.

Al Ghamdi *et al*., (2015) proved that the increased ROS in the spleen in diabetic rats altered the proliferative capacity of B and T lymphocytes and decreases the migration of B and T lymphocytes which migrate in response to CXCL12. Our data demonstrated that a decline in CXCL12 level in aflatoxicosed rats means a decline in the migration of T and B lymphocytes in splenocytes. As previous scientists reported that ingestion of AFB-contaminated feed by piglets resulted in reduced T-cell proliferation, presumably due to a direct effect of AFB on DCs **(Meissonnier *et al*., 2008)**.

NF-κB is a major transcription factor in the regulation of immune molecules, Furthermore, it is a key element in the regulation, development, maturation, and function of DCs. NF-κB is an essential transcription factor not only activated by oxidative stress but also involved in inflammation, immunity, cell proliferation, differentiation, and survival **(Lawler, 2011)**. NF-κB gene expression was increased after 2 h of AFB exposure, the stimulatory effect of AFB on NF-κB could be important for the modulation of DCs differentiation and function. Thus, it seems that AFB1 potentiates DCs migration toward tissues and secondary

lymphoid organs **(Ckless *et al.*, 2007),** these findings agreed with our results, as we reported that expression of NF-κB protein in the spleen was significantly up-regulated in aflatoxicosis rats in comparison to the control group.

This significant increase in NF-κB accompanied by a significant decrease in CXCL12 in the spleen of aflatoxicosis animals. These results agreed with **(Madge & May, 2010)** study that reveal, that classical NF-κB activation negatively regulated NF-κB-dependent CXCL12 expression.

Rajput *et al.*, (2019) explain the immunotoxicity induced by AFB1 promoted the phosphorylation of the NF-κB and the degradation of the nuclear factor of the kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) protein. The phosphorylation and degradation of the IκBα proteins showed to be essential for the activation of the NF-κB, hence leading to a rapid translocation of NF-κB from the cytoplasm to the nucleus. **DiDonato *et al.*, (1995)** revealed that the presence of AFB could aggravate immunotoxicity through the NF-κB signaling pathway. Many studies have demonstrated that NF-κB could be activated directly by H_2O_2 which can induce strong oxidative stress in cells **(Schreck *et al.*, 1992).**

Activation of NF-κB results in the upregulation of pro-inflammatory cytokines in the spleen. These early molecular events could ultimately lead to splenic fibrosis and/or fibro-sarcomas, activation of upstream pathways such as NF-κB is associated with simultaneous increases in gene transcription of cytokine TNF-α, which are not only important pro-inflammatory cytokines but also known to stimulate fibroblast proliferation and extracellular matrix production **(Chen *et al.*, 1998)**.

Our results recorded an increase in the gene transcription of pro-inflammatory cytokine TNF-α in the spleen of aflatoxicosed group which agreed with **(Helmy *et al.*, 2018)** who reported that aflatoxins had a serious effect on the immune system in albino female mice as they promoted pro-inflammatory cytokine TNF-α through the oxidative stress-mediated mechanism. **Long *et al.*, (2016)** recorded that increased expressions of TNF-α mRNA were observed in mice exposed to AFB and these results also approved by **Qian *et al.*, (2014).**

While this is contrasted with the findings of **Abbès *et al.*, (2016)** study that reported a decrease in TNF-α within-host splenocytes. **Dugyala & Sharma, (1996)** observed that the high-dose-treated animals with AFB (0.7 mg AFB~/kg body weight orally every other day for 2 weeks) showed a significant decrease in mRNA and protein levels of TNF-α, these results suggested that AFB1 suppressed TNF-α gene expression and transcriptional regulation in the high-dose group, and more strongly at the post-transcriptional level in all dose groups which isn't compatible with our results.

The IL-6 is involved in several immune responses and inflammatory reaction cascades, AFBI exerts its immune-toxic effects by altering IL-6 production, through the differential regulation of the factors which are responsible for the transcription or mRNA stability by AFB1 **(Debruyne & Delanghe, 2008).**

Our results as we recorded a decline in IL-6 level in the spleen of aflatoxicosed rat, these findings agreed with **(He *et al.*, 2014)** who recorded that mRNA contents of IL-6 in the AFB1 group in the ileum was decreased, as a consequence of the decline in mature T lymphocytes and secreted fewer cytokines, also the decreased content of IL-6 could reduce the proliferation of T-lymphocyte.

Another explanation of the depression in IL-6 expression in the spleen of aflatoxicosed group, peroxisomes are subcellular organelles found in the cytoplasm of mammalian cells. Peroxisomes are known to proliferate under a variety of altered physiological and metabolic states. Peroxisome proliferation was generated when extensive peroxisome induction was noted in rodent hepatocytes in response to the administration of certain xenobiotics **(Reddy & Chu, 1996)**. The peroxisome proliferator-activated receptor (PPAR) opened important avenues of investigation that subsequently have provided important information on the mechanism of the cellular responses to peroxisome proliferators **(Issemann & Greenm, 1990)**. Among three PPARs, fibrates-activated PPARα interacts with c-Jun and p65 NF-κB subunits, which negatively regulate IL-6 transcription **(Delerive *et al*., 1999)**.

Mehrzad *et al*., (2018) recorded that AFB changes cytokines secretions due to affecting the secretory properties of DCs, AFB changes the maintenance of homeostasis of human DCs by inducing apoptosis, diminishes phagocytosis capacity and its related molecules, and by producing pro-inflammatory cytokines like TNF-α, this phenomenon potentially breaks immune tolerance and causes autoimmune and allergic problems.

Apoptosis has an important role in the development, differentiation, proliferation, and homeostasis of cells, tissue, and organ **(Melnikova *et al*., 2006**) AFB1 directly or indirectly activated the apoptotic process **(Ribeiro *et al*., 2010)**, inducing apoptosis of several poultry and mammal cells **(Peng *et al*., 2016)**. **Zhu *et al*., (2017)** study revealed increased apoptosis in the AFB group and suggested that AFB could lead to excess apoptosis in the chickens' splenocytes**.**

Zhu *et al*., (2017) study demonstrated that the AFB diet led to the elevated expression of TNF receptor-1 (TNF-R1), and caspase-3 mRNA expression in the spleen, this agreed with our results which showed an elevation in the level of cleaved caspase 3 in spleen tissues of aflatoxicosed groups.

Lymphocytes are the main components within the lymphoid organs, and lymphocyte depletion in the lymphoid organs was due to apoptosis **(Solcan *et al*., 2014)**, Therefore, it is tempting to speculate that the increased apoptosis of splenocytes provoked by AFB1 might lead to lymphocyte depletion, which may partly responsible for immunosuppression in various circumstances **(Rathmell & Thompson, 2002)**. After TNF-α activation, then caspase-3 was activated, leading the cell apoptosis **(Park *et al*., 2001)**.

Clear evidence for the immunomodulatory roles of CWP in enhancing the efficiency of chemotaxis of different immune cells towards different chemokines in a mouse model has been described (**Badr *et al*., 2012**). Therefore, recent studies have focused on the importance of food antioxidants with a special focus on milk-derived peptides **(Power *et al*., 2013).**

Camel whey protein treatment in diabetic mice enhanced innate immunity by improving B and T cell chemotaxis efficiency **(Mohany *et al*., 2012)**. Our research recorded that CWP increased CXCL12 in spleen tissues which means improvement in the chemotaxis of B-cell and T-cell migration in the spleen and thymus. **Badr *et al*., (2017)** study recorded that CWP-treated diabetic mice showed an increase in the number of T-cells in the red and white pulp of the spleen, CWP stimulates the proliferation of B- lymphocytes rather than T- lymphocytes in the spleen.

Our investigations of NF-κB in spleen tissue of CWP treated group agreed with the results recorded by **(Ibrahim *et al*., 2019)** study that

observed the anti-inflammatory effect of camel milk through down-regulation of NF-κB in rat mammary tumor tissues **(Badawy *et al.*, 2018)**.

CWP down-regulated the levels of TNF-α in lymphocytes of heat stress (HS) mice by inhibiting the activation of NF-κB **(Badr *et al.*, 2017)**, this agreed with our results as we reported a reduction in NF-κB accompanied by a reduction in TNF-α of rats treated with CWP in spleen.

Arab *et al.*, (2018) study reported that camel milk suppressed renal inflammation induced by 5-fluorouracil by the inhibition of TNF-α, and inhibited NF-κB activation in wistar rats which agreed with our results that showed a decrease in NF-κB and TNF-α in the spleen as CWP significantly decreased early changes in the inflammatory cytokines TNF-α, during the acute phase of the inflammatory response in diabetes **(Ebaid *et al.*, 2011)**.

Our results recorded an increase in IL-6 expression in CWP-treated group and this agreed with **(Rusu *et al.*, 2010)**, we explain this result according to the records of **El-Shinnawy *et al.*, (2018)** who proved that camel whey up regulate PPAR-α which in turn interacts with c-Jun and p65 NF-κB subunits, which negatively regulate IL-6 transcription **(Delerive *et al.*, 1999)** leading to decrease the expression of IL-6.

Badr *et al.*, (2017) reported that CWP resulted in a significant reduction in lymphocyte apoptosis in heat stress (HS) animals, these findings consistent with our findings we recorded a significant reduction in cleaved caspase-3 in aflatoxicated rats treated with CWP.

Bovine whey protein failed to restore the levels of CXCL12, NF-κB, IL-6 in spleen as CWP treated group while bLF exerts anti-inflammatory effects by inhibiting the production of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) **(Godínez-Victoria *et al.*,2017)** which agree with our results in spleen.

As a master gland of immunity, the thymus plays many important regulatory roles in the immune system. The thymus is an organ for T-cells differentiation and selection. Developing thymocytes travel within the thymus through appropriate migration to undergo positive and negative selection in the cortex and medulla of the thymus, respectively.

Histological examination from a control rat thymus showed that the cortex formed from densely packed small lymphocytes with few epithelial reticular cells while medulla is pale stained less densely cellular than cortex. It contained large lymphocytes and a lot of epithelial reticular cells. Histological examination of aflatoxin B rat thymus showed disfigurement in the organization with apparent decrease in the thickness of the cortex (cortical atrophy) with loss of corticomedullary demarcation, this agreed with **Karaman *et al*., (2010)** who recorded an atrophy characterized by widening in the medulla, thinning in the cortex and depletion of the lymphoid cells in the AF1 and AF2 treated groups. Histological examination of CWP treated thymus showed slightly normal organization of the thymus-lobules with increase of the cortical thickness, while BWP was unable to restrain the normal structure of thymus.

The microscopic studies proved that aflatoxin affected the two major organs responsible for immunity, B-cells in the spleen and T-cells in the thymus, this toxicity occurred as a result of aflatoxin metabolism in the liver, the main organ responsible for aflatoxin metabolism, to give the toxic metabolites that cause an increase in the ROS production, as a consequence ROS cause the previous alterations that we measured to detect the impact of aflatoxin on the animal model immunity.

The role of chemokines in homing to or migration within the thymus has been indicated by several earlier. Thymic DCs are also able to produce CXCL12. In addition, thymic DCs mostly express its receptor

CXCR4, indicating that CXCL12/CXCR4 axis may influence DC functionality. CXCL12 seems to exert pleiotropic effects regulating critical processes in thymocyte development **(Hernández-López *et al.*, 2002)**, The movement of some populations of immature CD4+CD8+ thymocytes in the cortex **(Swainson *et al.*, 2005)**, and the emigration of mature thymocytes from the thymus are the result of a huge tactic migratory response to CXCL12 **(Poznansky *et al.*, 2002).**

Thymocyte depletion is seen in several infectious diseases, and changes in the migratory responses have also been observed. As mentioned earlier, thymocyte depletion is accompanied by a suppression of chemokines CXCL12 **(Mendes-da-Cruz *et al.*, 2006)** which agreed with our results as we recorded a decrease in CXCL12 level in the thymus of aflatoxicosed thymus rats.

Our results about IL-6 and TNF-α expression in the thymus of aflatoxicosis rats were explained by **(Hinton *et al.*, 2003)** study that suggested that the elevation of IL-6 and TNF-α may be attributed to the increased oxidative stress that depleted GSH, GST, and GSH-PX and increased NO and MDA, this finding was also proved by **(Lawrence, 2009)** study.

NF-κB, as an important activator of inflammatory processes, is capable of regulating the expression levels of inflammatory cytokines, chemokines, and mediators of various cell types **(Liu *et al.*, 2017).** It is well recognized that NF-κB/Rel plays a crucial role in the immune system by controlling many cytokine genes and responding to various signals required for immune cell survival **(Dijkstra *et al.*, 2002)**.

Induced oxidative stress by aflatoxicosis increased cytosolic IκBα retention and increased NF-κB nuclear translocation in T cells **(Pestka, 2008),** and this consistent with our results that showed an increase in the transcription of NF-κB in thymus tissue of aflatoxicosed rats.

Perturbation in NF-κB distribution promoted TNF-α–mediated T-cell apoptosis through TRADD-associated caspase-8 activation resulting in thymic atrophy. Caspase-3 activation is mediated by caspase-8 or -10, caspase-8 may be involved in the activation of caspase-3 by singlet oxygen **(Alnemri, 1997).**

Interestingly, two cellular responses to TNF-α have been well documented, the induction of cell death through caspase cascade and the activation of gene transcription for cell survival via activation of NF-κB **(Ling et al., 1998)**, although the apoptogenic activity of this cytokine also associates with a block in NF-κB–mediated cell survival signals **(Butt *et al.*, 2005)**.

A previous study reported increased apoptosis in lymphocytes attributed to increased inflammatory response during diabetes **(Khaskheli *et al.*, 2005)**. Inflammatory cytokines stimulate many signaling cascades that lead to B cell apoptosis **(Faloon *et al.*, 2011)**.

Camel whey protein treatment in diabetic mice enhanced innate immunity by improving B and T cell chemotaxis efficiency **(Mohany *et al.*, 2012)**. Our research recorded that CWP increased CXCL12 in thymus tissues which means improvement in the chemotaxis of T-cell migration in the thymus. **Badr *et al.*, (2017)** study agreed with our results and recorded that CWP-treated diabetic mice showed an increase in the number of T-cells in cortex of the thymus.

Our results also agreed with **Ebaid *et al.*, (2013)** study that reported the effect of camel whey protein in limiting the prolonged inflammation in diabetic rats through the enhancement of reduction in the levels of serum TNF-α and IL-6 in the thymus.

Previous data indicated that bovine proteins have functional effects on human cells. Although most of the proteins are degraded during digestion, certain whey proteins, such as β-LG, and α-LA, are resistant to

gastric digestion and remain intact after absorption in vivo **(Yamaguchi & Uchida, 2007)**. Bovine WPE could enhance the body's defense by priming human neutrophil chemotaxis, degranulation, and superoxide production **(Rusu *et al.*, 2009).** Alpha-lactalbumin has the capacity of modulating both innate and adaptive immunity. Activation and priming of neutrophils are associated with intracellular signaling that regulates their effector functions **(Downey *et al.*, 1995)**.

Our results showed the inhibitory effects of BWP on the level of NO in the liver of intoxicated rats, these results were confirmed by **(Ma *et al.*, 2015)** study that proved the inhibitory effects of α-LA on the release of NO, and the expression of iNOS might be at least partly related to its ability to modulate the NF-κB signaling pathway. However, this does not exclude the possibility that α-LA could regulate the expression of iNOS by other mechanisms, including the stimulation of pro-inflammatory cytokines, AFB1-induced chronic liver injury is alleviated when the NF-κB / iNOS/ NO pathway is significantly depressed by α-LA supplementation, which may be linked to both the suppression of inflammatory responses and the prevention of oxidative stress and that agreed with our results as we recorded significantly inhibition of NF-κB gene expression in thymus and spleen correlated with the inhibition of NO in the liver.

It is well known that NF-κB binding activity was induced only following an increase in the formation of reactive oxygen species (ROS), blockade of endogenous nitric oxide (NO) synthesis, and suppressed the generation of ROS blocking the activation of NF-κB **(Kupatt *et al.*, 1997)**. NF-κB is a DNA binding factor that is essential for the activation of several inflammatory mediators, e.g., TNF-α, and IL-6. NF-κB bind to the respective DNA promoter region and subsequently stimulated the expression of the pro-inflammatory cytokines, such as IL-6 and TNF-α.

Two heterodimers of NF-κB are termed p50 (NF-κB1) and p65 (RelA). Upon its activation, in most types of cells, IkBa NF-kB-inhibitor, is phosphorylated and proteolytically degraded. NO inhibits the dissociation of the NF-kB/IkBa complex, as a result, suppresses IL-6 and TNF-α release. These findings suggest that α-LA maybe suppresses ischemia/reperfusion-induced IL-6 release by inhibition of NF-κB transcriptional activation via NO generation. NO derived from eNOS inhibits the transcription factor NF-κB whereas NOS inhibitors increase basal NF-κB activation **(Tamion *et al.*, 1997)**. This explanation cleared our results which showed suppression of NO in the liver of intoxicated rats treated with BWP with suppression of NF-κB in thymus correlated with a reduction of IL-6 in thymus as well as a reduction in TNF-α in thymus.

Yamaguchi & Uchida, (2007) study reported that α-LA affects mouse monocyte cell line (RAW 264) and suppressed LPS-induced IL-6 production by macrophages in vitro. The interaction between bovine α-LA and macrophages resulted in the suppression of inflammation, this study agreed with our results as we proved the effect of BWP in suppressing the gene expression of IL-6 in the thymus due to its anti-inflammatory characteristics. These results are consistent with those of **(Park *et al.*, 2010)** study that demonstrated various anti-inflammatory effects attributed to α-LA. The mRNA modulation simply suggests a possible modulation of cytokine secretion.

Mohammed *et al.*, (2019) reported that high doses of bovine whey proteins and lactoferrin reduced significantly the alteration in hepatic architecture and hepatocyte apoptosis, inhibiting the release of TNF-α and improving the hepatic antioxidant defense system. Cytosol GSH inhibited the activity of caspase-8 and activated the NF-κB-dependent survival pathways of hepatocytes to regulate the TNF-α-induced extrinsic

apoptotic pathway. In addition, the mitochondrial GSH reduced the hepatocyte's response to the TNF-α-induced intrinsic apoptotic pathway by scavenging ROS. Therefore, hepatocyte GSH protected the liver from TNF-α-induced apoptosis **(Ramadan *et al.*, 2017),** BWP decreased significantly the activated caspase-3 as a result of increased GSH in the rat model of mammary tumors **(Cheng *et al.*, 2017)**, This result was consistent with our findings. These data revealed that camel whey protein is more significant in protecting liver, spleen and thymus from the hazard effects of aflatoxins more than bovine whey protein.

Summary and conclusion

The study was performed on forty adult albino rats weighing about 110-130g. The study continued for 4 weeks. Rats were divided into four groups; the control group (n = 10): received the basal diet (ad libitum). Aflatoxicosis group (n = 10): it was individually intoxicated by oral administration (500 µg of AFB suspended in corn oil/ kg b.wt) a day after day for successive 4 weeks. CWP+AFB treated group (n = 10): this group individually intoxicated by oral administration (500 µg of AFB suspended in corn oil/ kg b.wt) a day after day for successive 4 weeks and were orally supplemented with CWP (200 mg/kg body weight dissolved in 250 µl distilled water) a day after day. BWP-treated group (n = 10): this group was individually intoxicated by oral administration (500 µg of AFB suspended in corn oil/ kg b.wt) a day after day for successive 4 weeks and were orally supplemented with BWP (200 mg/kg body weight dissolved in 250 µl distilled water) day after day.

After 4 weeks rats were sacrificed by cervical dislocation, the spleen and thymus were quickly removed, washed with saline solution, and cut into three equal parts, one part was fixed in formalin for histological examination and the second part was imbedded in liquid nitrogen and kept frozen at -80 ℃ until RNA extraction, the third part was preserved in RIPA lysis buffer for western blots analysis. The liver samples were kept at -20 ℃ until the measurement of oxidant/ antioxidant biomarkers.

Oxidative stress biomarkers including NO, MDA, GSH, GST, and GSH-PX were measured by colorimetric methods in liver homogenate. CXCL-12, NF-κB, TNF-α, and IL-6 gene expressions were determined in the spleen and thymus by qRT-PCR. Cleaved caspase-3 was determined

by western blot in the spleen and thymus. Histopathological examinations of the spleen and thymus by light microscopy were also performed.

A growing body of evidence suggests that the CXCL12/CXCR4 axis is essential for the migration of progenitor cells during embryonic hematopoiesis and organogenesis as well as for organ homeostasis, vascularization, and tissue regeneration.

The present study concluded that CWP can improve the immuno-suppression caused by AFB through enhancing CXCL12 gene expression, modulating TNF-α, IL-6, and NF-κB gene expression, and modulation of Cleaved caspase-3 for the treatment of the toxicity caused by AFB.

- The aflatoxin intoxicated group showed the following changes:

An increase in the level of NO and MDA, a decrease in the levels of reduced glutathione, glutathione transferase enzyme, and glutathione peroxidase (GSH, GST, GSH-PX) in the liver compared to the control group.

- The spleen of the aflatoxicosis group showed an increase in TNF-α, Cleaved caspase-3, and NF-κB. In addition to a decrease in the level of immune system promoter chemokine (CXCL12) and interleukin-6 (IL-6).
- In the thymus gland, AFB group was characterized by an increase in the gene expression of interleukin-6 (IL-6), tumor necrosis factor (TNF-α), the nuclear stimulus for oxidative activation-kb (NF-κB), and a decrease in the expression of (CXCL12) and Cleaved caspase-3.
- Treatment of rats exposed to AFB with camel's milk whey proteins and cow's milk whey proteins led to an improvement in all

biochemical changes in the liver, spleen, and thymus to varying degrees between the whey of the two species.

- The results of the study concluded that CWP protein was able to reduce inflammation and oxidative stress and improve immune weakness resulting from aflatoxin poisoning in a significant way compared to what happened when treated with CWP, we recorded the effect of CWP in treating aflatoxin poisoning as follows
- Camel whey proteins had a remarkable effect on reducing the level of toxic nitric oxide and aldehyde in the liver, raising the stores of reduced glutathione, and raising the efficiency of the enzymes; glutathione peroxidase and glutathione s transferase (GSH-PX, GST).
- Camel's whey proteins had a noticeable effect on raising the efficiency of the immune system booster CXCL12 in the spleen and thymus gland, reducing the nuclear stimulus for oxidative- κB activation (NF-κB) in the spleen and thymus gland, inhibiting the inflammatory cytokine-6 (IL-6) in the thymus gland and increasing its gene expression in the spleen, inhibiting of tumor necrosis factor-α (TNF-α) in both spleen and thymus, and Cleaved Caspase-3 deficiency in both spleen and thymus.
- The signs of improvement mentioned above in the previous assessment sites (NO, MDA, GST, GSH-PX, GSH, CXCL12, IL-6, TNF-α, NF-κB, cleaved caspase-3 were due to the activity of lactoferrin protein in the camel whey as stated and it was proven in the previous studies that the fewer improvements less when treated with cow’s milk whey, and this improvement may be attributed to the higher percentage of lactoferrin protein in camel milk whey than in cows.

- Histopathological examination of aflatoxicosis spleen showing distortion in the organization of the histological structure, Shrinkage of the lymphatic nodules of white pulp, and decreased cellularity in the follicle and marginal zone. Treatment of aflatoxicosis spleen with BWP showing vacuolation of splenic cells, and degeneration of lymphocytes in the white pulp. histopathological examination of aflatoxicosis spleen treated with CWP shows the normal organization of the structure of white pulp and red pulp.
- Histopathological examination of aflatoxicosis thymus showing disfigurement in the organization with an apparent decrease in the thickness of the cortex (cortical atrophy) with loss of corticomedullary demarcation. Histopathological examination of aflatoxicosis rat treated with BWP thymus shows atrophy and slight demarcation appears between cortex and medulla histopathological examination of aflatoxicosis rat treated with CWP thymus shows the normal organization of the thymus lobules with an increase of the cortical thickness.

• Recommendations

The current study confirmed and declared the beneficial role of various whey proteins; Camel whey proteins and Bovine whey proteins in aflatoxicosis and related complications including liver injury, and immunity suppression. In addition, dominant proteins in camel whey proteins (Lactoferrin) and bovine whey proteins (α-lactalbumin) exert their effects at cellular and molecular levels. The relatively low cost, safety, and efficacy via multiple molecular targets of camel whey protein and bovine whey proteins offer advantages of them over the chemical treatments. Accordingly, we recommend the use of this natural supplement in a daily diet. Moreover, these proteins deserve further investigations as adjuvant therapies in animals and man**.**

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الملخص العربي

مشكلة التسمم الفطري بالأفلاتوكسين تتصدر أخطر المشاكل التي تواجه الإنسان والحيوان وتؤثر على الإقتصاد والإنتاج الحيواني والصحة العامة للإنسان وقد أجريت العديد من الأبحاث سابقاً لتقييم هذه المشكله ومحاولة العلاج بنجاح لتخطي هذه المشكلة.

تم عمل الدراسة الحالية والتي تُمثل نموذج للتسمم الفطري بالأفلاتوكسين لمعرفة الأثار المترتبه للتسمم بلأفلاتوكسين على الأعضاء المناعية والكبد , بالإضافة لإيجاد علاج يقلل من هذه الأثار لذلك فإن الهدف من هذه الدراسة هو معرفة دور بروتين شرش لبن الإبل ودور بروتين شرش لبن الابقار في مقاومة الأثار الضارة التي يحدثها التسمم الفطري في الفئران والمقارنة بين فِعليهما المقاوم وأيهما أقدر على ذلك.

وللوفاء بالحصول على المسمم والمعالج إعتمدنا على تخليق الاول وإستخلاص الثاني فكان تخليق الأول معملياً ناجحاً في الحصول على عينات نقية من الأفلاتوكسين المسبب الرئيسي للتسمم الفطري للأفلاتوكسين.

وبناء على خبرة سابقة للعاملين في هذا المجال تم إستزراع عينة نقية من الفطر المُخلق للأفلاتوكسين في الوسط الغذائي الذي تم تربية الفطر علية والذي نتج عنه الأفلاتوكسين كمحصول لهذا الاستقلاب الفطري, وتم استخلاصة طبقا لما جاء في التعامل مع التخليق المعملي لهذا السم بواسطة تقنية الكروماتوجرافيا على السطح والمحاليل الفاصلة.

وقد ذَكرت المراجع بما يفيد أن المحفز المناعي في مكونات اللبن قد تكون متباينه من حيوان لآخر وكَثُر الحديث عن تقييم هذه المحفزات من نوع لأخر بين تلك المواشي وهذا البحث واحد منها حيثُ مناط الإهتمام يتركز على مقارنة بين محتويات هذا المحفز المناعي في شرش لبن الجمال وأخر من شرش لبن الأبقار في حيوانات تجريبية قد تعرضت للسم الفطري الذي يهدد حياة الإنسان والحيوان على السواء بإعتباره ملوثا غذائيا يبحث العلماء عن أقدر التعاملات البيولوجية والكيميائية للخلاص من سمومها.

لذلك أجريت هذه الدراسه لتقييم تأثير التسمم الفطري بالأفلاتوكسين ب1 على الكبد والطحال والغدة التيموسية والمقارنه بين تأثير شرش لبن الجمال وشرش لبن الأبقار على مقاومة التسمم بالأفلاتوكسين في تلك الأعضاء وإرتباطها بالمناعة.

أُستخدمت هذه الدراسة عدد 40 فأراً ذكر سليماً, تم شراؤها من بيت الحيوان الملحق بكلية الطب البيطري في أسيوط وتم تحديد الفترة الزمنية للتجربة بمدة شهر كامل (أربعة أسابيع), وقد قُسمت هذه الحيوانات إلى أربعة مجموعات كُلاً منها 10 فئران كالأتي:

1. **المجموعة الأولى** (المجموعة الضابطة): أُستخدمت كمجموعة سليمة مرجعية.
2. **المجموعة الثانية** (مجموعة التسمم بالأفلاتوكسين دون معالجة): تم تجريعها فقط يوم بعد يوم بجرعات من الأفلاتوكسين 500 ميكروغرام/كجم من الوزن مُعلقه في زيت الذرة عن طريق الفم لمدة أربعة أسابيع وهي النهاية الزمنية للتجربة.

3. **المجموعة الثالثه** (المجموعه المُعالجه بشرش لبن الجمال ثم التسمم بالتواتر): تم تجريعها بيوم قبل التسمم بجرعة وقائية من شرش لبن الجمال عن طريق الفم بجرعة 200 مجم/كجم من شرش لبن الجمال مُعلقة في الماء النقي.
4. **المجموعة الرابعة** (المجموعه المُعالجه بشرش لبن الأبقار ثم التسمم بالتواتر): تم تجريعها بيوم قبل التسمم بجرعة وقائية من شرش لبن الأبقار عن طريق الفم بجرعة 200 مجم/كجم مُعلقة في الماء النقي.

عند نهاية التجربة تم تخدير جميع الفئران بمادة فينوباربيتال وتشريحها وبعد أخذ عينات الدم من كل مجموعة تذبح الفئران لأخذ عينات من الكبد – االطحال – الغدة التيموسية لإجراء التحاليل البيوكيميائية والبيولوجيا الجزيئية وتسجيل الملاحظات الهستوباثولوجية.

وجاءت النتائج المعملية كالأتي:

❖ **الفحص الضوئي الميكروسكوبي:**

في قطاعات نسيج الطحال وكما هو معروف بأنه يتألف من قطاع البلاعم البيضاء ومناطق البلاعم الحمراء وكليهُما مُكون من خلايا البلازما وخلايا الدم البيضاء والخلايا الليمفاوية وفي حالة التسمم بالأفلاتوكسين أدي إلى تغير الشكل الطبيعي للطحال وفقد قطاع البلاعم الحمراء وقطاع البلاعم البيضاء بنيتُه المنتظمة وأضمحلال في عدد الخلايا داخل القطاعين مع ظهور فجوات دهنيه بين الخلايا وإنخفاض نسبة السيتوبلازم داخل الخلايا وجميعها تُشير إلى تسمم هذا النسيج بالأفلاتوكسين.

أظهر الفصح الميكروسكوبي للطحال في المجموعة المعالجة ببروتين مصل لبن الإبل, نجاح مصل لبن الإبل في الحفاظ على التنظيم الطبيعي لبنية خلايا اللب الأبيض واللب الأحمر بينما لم ينجح مصل لبن الأبقار في الحفاظ على التنظيم الطبيعي لشكل الطحال وظهور الفجوات الدهنية بين الخلايا مع الإنخفاض الملحوظ في عدد الخلايا.

وبما أن الغدة التيموسية في حالتها الطبيعية تتكون من فصين متصلين بنسيج ضام حيث يتكون كل فصيص من قشرة داكنة اللون تحتوي على خلايا ليمفاوية صغيرة ونخاع أكثر شحوبا وفي حالة التسمم الفطري بالأفلاتوكسين ظهر تشوه في تنظيم الخلايا مع إنخفاض في سمك القشرة وظهور مساحات واسعه بين الخلايا مما يوحي بأن وظيفة النسيج قد تضررت في إنتاج الخلايا التيموسية في هيئتها الطبيعية.

كما أظهر الفحص الميكروسكوبي للغدة التيمسويه في المجموعة المعالجة ببروتين مصل الإبل بإحتفاظ الخلايا بمظهرها الطبيعي مع وجود خلايا مفرغة طفيفة بينما فحص الغدة التيموسية في المجموعة المعالجة ببروتين مصل لبن الأبقار أظهرت ضمور في القشرة مع تباعد بين الخلايا في منطقة النخاع أي أن الخلل في النسيج مازال باقياً.

❖ **المؤشرات الحيوية للإجهاد التأكسدي:**

- معايير الإجهاد الأكسدي المتمثلة في أكسيد النيتريك الذي تضاعف عشر مرات بالتسمم أنخفض إلى النصف تقريباً بفعل شرش لبن الجمال بينما هذا الإنخفاض كان أقل بالضعف للبن الأبقار أما المالوندهيدات (MDA) الذي تضاعف بالتسمم سبعة أضعاف وعاد مرة أُخري إلى المستوى الطبيعي بلبن الجمال وإن هذا الإنخفاض لم يتعدى ثُلث هذه القدرة أما الجلوتاثيون المُختزل تناقص إلى

الثُلث بالتسمم وعاد مرة أخري بلبن الجمال و زاد عليه وكذلك لبن الأبقار أما إنزيم الجلوتاثيون الفوق أكسيدي (GSH-PX) الذي تناقص عشرات المرات عن نشاطه الطبيعي بالتسمُم عاد مرة أخرى إلى مستواه بلبن الجمال ولكن تعذر قُدرة هذا الإنزيم على العودة وبقى عند ضعف القدر الطبيعي مع لبن الأبقار. أما إنزيم ناقل الجلوتاثيون المختزل (GST) الذي تناقص عشرة مرات بالتسمم وإقترب من الطبيعي بلبن الجمال لم تكن قدرة لبن الأبقار أن تجعله يصل إلى النصف.

- أما مستويات نشاط التعبير الجيني في نسيج الغدة التيموسية (النسيج الليمفاوي التائي) حيث الكيموكين (داعي الجهاز المناعي الكيماوي CXCL12) والذي إنخفض إلى أكثر من عشرة أضعاف بالتسمم الفطري قد أصبح زائداً ضعف الطبيعي بلبن الجمال أما لبن الأبقار فالبكاد كان في الموقع الطبيعي دلالة على أن شرش لبن الجمال يحفز إستدعاء الجهاز المناعي بما يقرب من عشرة أضعاف ما يستدعية لبن الأبقار.
- أما الجين المعبر عن تحفز النسيج المُمثل بالعامل NF-kB لكي يقوم بالإجهاد الأكسيدي والذي أرتفع خمسين ضعفا بالتسمم إنخفض إلى عُشر قيمته الطبيعية بلبن الجمال مما يدل على أن الأخير لة قدرة عالية على إسكات التعبير الجيني للإجهاد الأكسيدي المناعي في الجهاز الليمفاوي التائي وهذا القدر الذي لم يعد يصل إلية فعل شرش لبن الأبقار.
- أما عامل الإلتهاب المبكر الإنترولكين-6 (IL-6) والذي أرتفع بالتسمم عشرة أضعاف مستواه الطبيعي قد إنخفض إلى ضعف واحد بشرش لبن الجمال بينما ظل عند مستوي سبعة أضعاف في لبن الأبقار مما يدل على أن علامة تميز الإلتهاب المبكر قد أشتعلت إلى أقصى مدى بالتسمم كما كان شرش لبن الجمال هو الأكثر قدرة على تهدئته من لبن الأبقار وكذلك عامل الإلتهاب الورمي (TNF-α) الذي تضاعف مرتين ونصف بالتسمم قد عاد أدراجه إلى المستوي الطبيعي بشرش لبن الجمال وإلى حد ما بذات القدر بشرش لبن الأبقار ومما سبق فإن شرش لبن الجمال كان هو الأكثر قبولاً لدى خلايا نسيج الجهاز اليمفاوي التائي لظبط إيقاع الجهاز المناعي لمواجهة التسمم الفطري بقدرة عالية وإن لبن الأبقار كان عاجزاً أن يُماثل هذا القدر من تدعيم الجهاز المناعي أما ماحدث في الطحال (النسيج المناعي الليمفاوي البائي) كان بصورة مُماثلة لذات

الأبعاد التى قصدناها في الجهاز الليمفاوي التائي مما يعني أن الجهاز المناعي لدية القبول لحفظ توازنة أمام التسمم الفطري بقدر عال بشرش لبن الجمال الذي لم يعُد يجارية شرش لبن الأبقار عند قياس النشاط التعبير الجيني في النسيج الليمفاوي التائي او البائي.

وهكذا أدت معالجة الفئران المعرضة للتسمم الفطري ببروتينات شرش لبن الجمال وبروتينات شرش لبن الأبقار إلي تحسن في جميع التغيرات البيوكيميائية في الكبد والطحال والغدة التيموسية بدرجات متفاوتة بين شرش لبن الجمال وشرش لبن الأبقار حيث أن خَلُصت نتائج الدراسة إلى قدرة بروتين شرش لبن الإبل على تقليل الإلتهابات وضغط الأكسدة وتحسين الضعف المناعي الناتج من التسمم بالافلاتوكسين بطريقة معنوية أكبر من تأثير العلاج بشرش لبن الابقار.

مقارنة تأثيرات بيوكيميائية لبروتينات مصل ألبان الأبقار والجمال في مقاومة التسمم بالأفلاتوكسين في الحيوانات

رسالة مقدمة من

طبيب بيطري/ هند محمد عمر حمدين

بكالوريوس الطب البيطري

ماجستير الكيمياء الحيوية

طبيب بيطري بمعهد بحوث صحة الحيوان

توطئة للحصول على درجة الدكتوراة في الكيمياء الحيوية

تحت إشراف

الأستاذ الدكتور/ أحمد يس نصار

أستاذ الكيمياء الحيوية الطبية

كلية الطب- جامعة أسيوط

الأستاذ الدكتور/ عبد الرحيم محمد عبدالحفيظ مكي

أستاذ الكيمياء الحيوية الطبية

كلية الطب- جامعة أسيوط

الدكتورة/ غادة محمد عزت محمد

أستاذ مساعد الكيمياء الحيوية الطبية

كلية الطب- جامعة أسيوط

الأستاذ الدكتور/ محمد عبدالسلام محمد

أستاذ الكيمياء الحيوية وأمراض النقص الغذائي

معهد بحوث صحة الحيوان، الجيزة

ورئيس معمل أبحاث الصحة الحيوانية بأسيوط

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