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Intitulé :

Biological activities of medicinal plant extracts on arthritis induced by formalin and on tumoral process

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This thesis is dedicated to my husband YASSINE, who always supported me with wonderful advice and helped me overcoming the difficulties without complaining.

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International publications

1. **Slimani W, Zerizer S and Kabouche Z. (2020).** Immunomodulatory and anti-arthritic activities of *Stachys circinata*. *Jordan Journal of Biological Sciences*, 13 (2): 183-189.

International communications

1. **Slimani W, Kehili H E, Baghriche I, Messaoudi S, Zerizer S. (2017).** Toxic and immunomodulatory effects of the dichloromethane extract of *Stachys circinata.* Les 4èmes Journées Internationales de Nutrition. El Eulma (Algérie), 23/24 Février 2017

2. **Kehili H E, Slimani W, Messaoudi S, Beghriche I, Zerizer S. (2017).** Effet anti-inflammatoire de la datte Algérienne. Les 4emes Journées Internationales de Nutrition. Eulma, 23_24 Février 2017.

3. **Kehili H E, Slimani W, Zerizer S, Kabouche Z. (2019).** Immunomodulatory, Antioxidant, Anti-inflammatory and toxic effects of Algerian dates *Phoenix dactylifera.* Séminaire International Environnement et Toxicologie. Constantine, 18_19 Février 2019.

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ACPA: Anti-citrullinated protein antibodies

ANOVA: Analysis of variance

Anti CCP: Anti-cyclic citrullinated peptide

APC: Antigen presenting cells

As: Arsenic

ATP: Adenosine triphosphate

BBE: Bovine brain extract

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

CAT: Catalase

Cd: Cadmium

COX-2: Cyclooxygenase-2

CRP: C-reactive protein

DAMPs: Damage-associated molecular patterns

DMARDs: Disease modifying anti-rheumatic drugs

DMEM: Dubelcco's modified eagle's medium

DMSO: Dimethyl sulfoxid

DNA: Deoxyribonucleic acid

DPBS: Dulbecco's phosphate-buffered saline

DTNB: 5,5′-dithiobis-(2 nitrobenzoic acid)

EBM: Endothelial cell basal medium

EDTA: Ethylendiaminetetracetic acid

FBS: Fetal bovine serum

Fe: Iron

FIA: Formalin-induced arthritis

GPx: Glutathione peroxidase

GR: Glutathione reductase

GSH: Glutathione reduced

GSSG: Glutathione disulfide

HCC: Hepatocellular carcinoma

HepG2: Hepatocarcinoma cell line

Hg: Mercury

HS: Horse serum

HUVEC: Human umbilical vein endothelial cell

H_2O_2: Hydrogen peroxide

H+: Hydrogen ions

IC: Immun complex

i.e.: id est ”that is”

Ig: Immunoglobulin

IL: Interleukins

i.p: Intraperitoneal

IRs: Ionizing radiations

LD50: Medial lethal dose

LTB4: Leukotriene B4

(MEM, NEAA): Minimum essential medium and non-essential amino acids

MeOH: Methanol

MHC: Major histocompatibility complex

MMP-9 : Matrix metalloproteinase- 9

MTT: Microculture tetrazolium

NADPH : Nicotinamide adenine dinucleotide phosphate

NF-κB : Nuclear factor κB

NO· : Nitric oxide

$NSAID_S$: Non steroidal anti-inflammatory drugs

OD: Optical density

OECD: Organisation for economic cooperation and development

· OH: Hydroxyl radical

ONOO−: Peroxynitrite

O_2: Oxygen

O_2 ·− : Superoxide anion

PAD_S: Peptidyl arginine deiminases

PAMPs: pathogen-associated molecular patterns

Pb: Lead

PBS: Phosphate-buffered saline

PC12: Phaeochromocytoma cell line

PGE2: Prostaglandin E2

PMN: Polymorphonuclear neutrophils

RA: Rhumatoid arthritis

RANKL: Receptor activator of nuclear factor-κB ligand

RES: Reticuloendothelial system

RF: Rheumatoid factor

rhEGF: Epidermal growth factor

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

SAIDs: Steroidal anti-inflammatory-immunity drugs

SC: *Stachys circinata*

SD: Standard deviation

SM: *Stachys mialhesi*

SOD: Superoxide dismutase

SPSS: Statistical package for social science

STAT3: Signal transducer and activator of transcription 3

TBS: Tampon buffer saline

TH cell: T-helper cell

TLR_S: Toll-like receptors

TNFα: Tumor necrosis factor α

Tris-HCl: Tris-Hydrochloride

UVR: Ultraviolet radiation

WHO: World health organization

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Introduction

Introduction

The immune system is a highly sophisticated defensive mechanism in living organisms that protects the host from foreign pathogens (Xu and Larbi, 2017). Malfunction of the immune system is thought to be one of the major etiologies responsible for the development of many abnormal conditions like autoimmune diseases, organ transplant rejection, infectious diseases and cancer. However, modulatory response of the immune system to alleviate such diseases remained of great interest since long (Rasheed *et al.*, 2016).

Inflammation is characterized by a sequence of events comprising an induction phase, which leads to the peak of inflammation and is gradually followed by a resolution phase. The induction phase of inflammation is designed to allow fast and robust immune activation that is required for effective host defense. It is initiated by the sensing of exogenous and endogenous danger signals resulting from mechanically, chemically, or biologically induced tissue damage followed by the recruitment of effector cells, which orchestrate an inflammatory response characterized by the release of lipid and protein mediators of inflammation (Schett and Neurath, 2018).

Acute inflammation is a part of innate immunity initiated by the immune cells that persists only for a short time. However, if the inflammation continues, the second stage of inflammation called chronic inflammation commences which leads to many chronic diseases, including arthritis, cancer, respiratory cardiovascular diseases, diabetes, and neurodegenerative diseases (Kunnumakkara *et al.*, 2018).

Rheumatoid arthritis (RA) is a chronic inflammatory and systemic disease that is characterized by extensive synovitis resulting in erosions of articular cartilage and marginal bone that lead to joint destruction (Chimenti *et al.*, 2015). The release of cytokines, especially tumor necrosis factor α (TNF-α), interleukine-6 (IL-6) and IL-1, causes synovial inflammation. In addition to their articular effects, pro-inflammatory cytokines promote the development of systemic effects, including production of acute-phase proteins (Zampeli *et al.*, 2015). Rheumatoid arthritis is considered an autoimmune disease since the production of the rheumatoid factor (RF), an autoantibody directed against determinants on the Fc fragment of immunoglobulin (Ig) G molecules, was first observed. The most relevant autoantibodies appear to be the anti-citrullinated protein antibodies (ACPA). Citrullination is the critical step for the

recognition of several proteins (fibrin, vimentin, fibronectin, collagen type II), highly expressed in the synovial membrane during inflammation, by anti-citrullinated protein antibodies. The pathogenesis of RA is a multistep process that starts with the development of autoimmunity, continues with local inflammation and finally induces bone destruction (Chimenti *et al.,* 2015).

Inflammatory process induces oxidative stress (Khansari *et al.,* 2009) which constitutes a disturbance caused by, an imbalance between generation and accumulation of oxygen reactive species (ROS) in cells and the ability of a biological system, to detoxify these reactive products (pizzino *et al.,* 2017). These ROS are generated under the stimulus of pro-inflammatory cytokines in phagocytic and nonphagocytic cells by, the activation of protein-kinases signaling. Thus, TNF-α increases the formation of ROS by neutrophils and other cells, while interleukin-1-β (IL-1-β), TNF-α and interferon (IFN)-γ stimulate the expression of inducible nitric oxide synthase in inflammatory and epithelial cells (Federico *et al.,* 2007).

In a healthy human being generation of ROS is kept in check by cellular antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH) and glutathione reductase (GR) (Salla *et al*., 2016). Superoxide dismutase catalyzes the dismutation of superoxide radical into oxygen (O_2) and hydrogen peroxide (H_2O_2), and CAT catalyzes the breakdown of the harmful H_2O_2 to water and O_2. Glutathione disulfide (GSSG) reduced to GSH catalyzed by glutathione reductase and provide hydrogen ions (H+) by nicotinamide adenine dinucleotide phosphate (NADPH) with a decrease of NADPH levels (Zhao *et al.,* 2014). However, free radical over production can cause an imbalance in cellular redox status producing oxidative damage to biomolecules, (lipids, proteins, deoxyribonucleic acid (DNA) (Rouabhi *et al.,* 2015), which has major implications in the etiology of chronic diseases such as diabetes, cardiovascular conditions and cancer (Choi and Kim. 2013).

Cancer is one of the major causes of dead worldwide associated with 8.2 million deaths in the world in 2012 (Meneses-Sagrero *et al.,* 2017). It can be described as a set of complex processes involving impaired cells death, unlimited cell proliferation and temporal–spatial changes in cell physiology that often leads to malignant tumor formation resulting in invasion of distant tissues to form metastasis (George *et al.,* 2017). Carcinogenesis is a multistage process that involves a series of events comprising of genetic and epigenetic changes leading to the initiation, promotion and progression of cancer. Carcinogenesis may result from extensive DNA damage, often caused by exposure to a variety of exogenous and endogenous agents including

ultraviolet radiation (UVR), ionizing radiations (IRs), mutagenic chemicals, environmental agents, therapeutic agents or diagnostic imaging (George *et al.,* 2017).

The natural products from plants and biological sources still remain an unlimited and uncondensed source of new phytochemicals and nutraceuticals (Ahmad *et al.,* 2017). Medicinal plants have been prescribed and used widely for thousands of years to treat several disorders and ailments in folk herbal medicine systems all around the world and have been considered as an effective approach (Hiwa, 2016) to control various disorders such as autoimmune diseases (Singh *et al.,* 2020), inflammation and cancers (Panahi Kokhodan *et al.,* 2018).

In addition, there is a great deal of attention on natural products, due to carcinogenic effects of synthetic antioxidants used in food industry (Demiroz *et al.,* 2020) and side effects of anti-inflammatory drugs including gastrointestinal injury and hepatotoxicity (Ou *et al.,* 2019). Natural antioxidant plays a vital role in prevention of oxidative damage, which can lead to many diseases (Xia *et al.,* 2017). Furthermore, traditional knowledge and scientific reports demonstrate that medicinal plants are rich sources of biologically active compounds that can be used for the prevention or treatment of various diseases including some type of cancers (George *et al.,* 2017).

This study investigated the mode of action of two endemic species, belonging to the genus *Stachys* (Lamiaceae) and we have attempted to focus these objectives:

- Testing the safety use of *S. mialhesi* and *S. circinata* extracts and determining the lethal doses using toxicity tests in animals.

- Evaluation of immunomodulatory effect of *S. mialhesi* and *S. circinata* extracts using carbon clearance assay.

- Evaluation of antioxidant effect of *S. mialhesi* and *S. circinata* extracts by GSH and CAT assay from mice liver.

- Examination of the effect of *S. mialhesi* and *S. circinata* extracts on inflammation and arthritis through the measurement of plasma hs-CRP and Anti-CCP.

- Confirmation of the action of formalin induced arthritis and plant extract on joint inflammation by histological study.

- Evaluation of the anti-proliferative activity of *S. mialhesi* and *S. circinata* extracts on liver cancer (hepatocarcinoma cell line HepG2), breast cancer cell line MCF7 and Pheochromocitoma cells (PC12) using cell culture techniques.

- Study the effect of extracts *S. mialhesi* and *S. circinata* on cell viability of healthy human cells: endothelial cells of blood vessels HUVEC.

- Evaluation of *in-vitro* antioxidant activities (CAT and GSH) of *S. mialhesi* and *S. circinata* extracts on liver cancer (hepatocarcinoma cell line HepG2).

Chapter I

Literature Review

I.1. Medicinal plants and phytotherapy

Throughout the centuries, humans have depended on plants for basic needs such as food, clothing, and shelter. Plants have also been employed as arrow and dart poisons for hunting, poisons for murder, hallucinogens used for ritualistic purposes, stimulants for endurance, and hunger suppression, as well as inebriants and medicines (Salim *et al.,* 2008). Taking account that at the same time there was not enough information either concerning the reasons for the illnesses or concerning which plant and how it could be used as a cure, everything was depended on experience (Petrovoska *et al.,* 2012). Over the development of human culture, utilization of medicinal plants has had magical-religious significance and different points of view regarding the concepts of health and disease which existed within each culture (Akinyemi *et al.,* 2018).

Actually, 80% of people in non-industrialized societies and developing countries in the world, as a way of guaranteeing this primary health care, essentially depend on folk medicine. This later involves the use of medicinal plants up to 85% (Rezende, 2020), because they are thought to be very effective, inexpensive and extensively available (Awuchi, 2019).

According to the World Health Organization (WHO), medicinal plants are those which in any case, introduced or put in contact with a human or animal organism, produce pharmacological activity (Miraldi and Baini, 2018).

Modern pharmacology relies widely on the diversity of plant secondary metabolites to discover new molecules with novel biological properties. This source seems inexhaustible since each species of the 400,000 known, can contain various thousands of different components whose several uses aim to repress suffering and ameliorate the health of men (Sanogo *et al.,* 2018). In this context, some researchers have performed several biological and biochemical assay with numerous of medicinal plants and have reported considerable immunomodulatory, anti-inflammatory (Gollo *et al.,* 2020), antioxidant, antimutagenic and/or anticarcinogenic effects (Demiroz *et al.,* 2020).

I.1.1. *Stachys* species medicinal plants

The genus *Stachys* (Lamiaceae), widely known in folk medicine contains 300 species with a worldwide distribution and in Algeria this genus is represented by 14 species including the endemic species *Stachys mialhesi* and *Stachys circinata* (Quezel and Santa 1963).

I.1.1.1. *Stachys mialhesi*

a. botanical description

Stachys mialhesi de Noé, is an endemic plant of the North, with soft leaves loosely ruffled with whitish steep bristles. Flowering epis very loose, with disjointed verticillaster into the top (figure 6). Whitish corolla spotted with pink, with internal ring of hairs (Quezel and Santa, 1963). It is located in north eastern Algeria, mainly in the wilaya of Constantine (Laggoune et *al.*, 2011).

Figure 6. *Stachys mialhesi* (Laggoune, 2011).

b. Botanical classification

Kingdom	Plantae
Sub- kingdom	Tracheobiontae
Division	Spermatophytae
Sub-division	Angiospermae
Class	Dicotyledonae
Sub-class	Asteropsidae
Order	Lamiales
Family	Lamiaceae
Genus	*Stachys*
Species	*Stachys mialhesi*

I.1.1.2. *Stachys circinata*

a. botanical description

Stachys circinata is an endemic plant of the North Algerian, with cross-linked embossed leaves, covered with a thick tomentum of hairs short. Flowering spikes disjointed at the bottom, very dense at the top (figure 7). Corolla dew ring oblique and incomplete hair (Quezel and Santa, 1963). It's common in Algeria and especially in the mountains.

Figure 7. *Stachys circinata*
*(*http://species.wikimedia.org/wiki/*Stachys_circinata)*

Pharmacological properties

The presence of flavonoids phenylethanoid glycosides, diterpenes, saponins, terpenoids, and steroids have been reported in *Stachys* species (Panahi Kokhdan *et al.*, 2018). Nassar *et al.* (2015) has revealed that three plant extracts, belonging to the Lamiaceae family, exert antioxidant and immunostimulant activity. In addition, beside the antioxidant activity, the study of Laggoune *et al.* (2016) has shown that the n-butanolic extract of the aerial parts of *Stachys mialhesi* exhibited a significant antioxidant and antinociceptive and anti-inflammatory activity in laboratory animals. *In vivo* studies on *Stachys pilifera* extracts have revealed significant anti-inflammatory effect (Sadeghi *et al.*, 2014), and considerable cytotoxic and anti-proliferative properties on HT-29 colorectal cell line (Panahi Kokhdan *et al.*, 2018).

I.2. Immunomodulation

Immunomodulation is a multifaceted mechanism implicated in the pathophysiology and etiology of many diseases by the modulation of the immune system (Raj and Godhandam, 2015). In recent years, the field immunomodulation gained attention from the scientific experts in developing awareness regarding the necessity to modulate immune system to achieve desirable effects on diseases prevention (Rasheed *et al.*, 2016). The immunomodulators are biological or synthetic substances able to stimulate or suppress the immune response and can be classified into three types (Shantilal *et al.*, 2018).

1.2.1. Classification of immunomodulators

1.2.1.1. Immunostimulants

Immunostimulants enhance body's resistance against infection. They can be exploited as prophylactic agents in healthy individuals; however, they can be used as immunotherapeutic agents by individuals with immunocompromise diseases (Shantilal *et al.*, 2018).

1.2.1.2. Immunoadjuvants

Are used to enhance immune response to vaccines without generating specific antigenic effects, and approached in recent times as adjuvant pharmacological treatments, particularly for viral infections and cancers (Di sotto *et al.*, 2020).

1.2.1.3. Immunosuppressants

Immunosuppressants are drugs employed to inhibit the immune response and widely used in the treatment and control of various autoimmune conditions and organ transplantation (Kumar *et al.*, 2012).

1.3. Oxidative stress

Oxidative stress occurs from the imbalance between pro- and antioxidant species, which results in molecular and cellular damage (Tan *et al.*, 2018). Oxidative stress is related to several chronic diseases such as atherosclerosis, neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, cancer, diabetes mellitus, inflammatory diseases, as well as psychological diseases (Al-Dalaen and Al-Qtaitat, 2014) and it is also involved in aging theory which is based

on the hypothesis that age-associated functional losses are due to the accumulation of reactive oxygen and nitrogen species induced damages (Liguori *et al.*, 2018). Indeed, oxidative stress plays a crucial role in the development of age-related diseases including arthritis, dementia, vascular diseases, obesity, osteoporosis, and metabolic syndromes (Tan *et al.*, 2018). However oxidative stress perform various useful functions such signal transduction, influencing synthesis of antioxidant enzymes, repair processes, immune system, as a way to attack and kill pathogens (Al dalaen and Al-Qtaitat, 2014).

I.3.1. Free radicals

Free radicals are essentials to any biochemical progression and signify a fundamental part of metabolism and energy production. It can be defined as any molecular variety containing an unpaired electron in an atomic orbital and the presence of such unpaired electron may result in certain common properties shared by most radicals. Many radicals are unstable and extremely reactive that can either donate or accept an electron from other molecules, therefore behaving as oxidants or reductants (Ayoub *et al.*, 2017).

In general, free radicals are very short lived, with half-lives in milli-, micro- or nanoseconds (Ayoub *et al.*, 2017). Free radicals generated in aerobic metabolism are involved in various physiological functions such as in immune function (i.e. defense against pathogenic microorganisms) (Phaniendra et al., 2015) and in a series of regulatory processes such as cell proliferation, apoptosis, and gene expression. However, when generated in excess, free radicals can counteract the defense capability of the antioxidant system, impairing the essential biomolecules in the cell by oxidizing membrane lipids, cell proteins, carbohydrates, DNA, and enzymes (Pisoschi *et al.*, 2016).

There are two major free radical groups; nitrogen-free radicals and oxygen-free radicals or, more generally, ROS, which are the most important class of radical species generated in living systems (Husain and Kumar, 2012).

Most common type of ROS are hydroxyl radical (·OH), whose reactivity is so high that it reacts very close to its site of formation, while other types like, superoxide anion ($O_2{\cdot}^-$) and H_2O_2 are less reactive. Thus, nitrogen containing species, that are now indicated as reactive nitrogen species (RNS), include nitric oxide (NO·), which is relatively unreactive and its derivative the peroxynitrite ($ONOO^-$), a powerful oxidant, able to damage many biological molecules (Di Meo *et al* ., 2016).

I.3.1.1. ROS sources

The reactive oxygen species can be produced from either endogenous or exogenous sources.

a. Endogenous source

The endogenous sources of ROS include different cellular organs such as mitochondria, peroxisomes and endoplasmic reticulum, where the oxygen consumption is high (Phaniendra *et al.*, 2015).

Mitochondria are major organelles that are accountable for generation of energy through oxidative phosphorylation to produce adenosine triphosphate (ATP), a molecule which is crucial for cellular actions (Tan *et al.*, 2016). The mitochondrial electron chain transport consumes nearly 90% of total oxygen content in the cell. In normal healthy state oxygen is reduced to produce water. However, it is estimated that a small percentage of O_2 consumption undergoes transformation to O_2•- (Bolisetty and Jaimes, 2013).

Another source of ROS generation is NADPH oxidase a multi-subunit enzyme that catalyzes the production of superoxide by transferring one electron from NADPH to molecular oxygen (Tarafdar and Pula, 2018). NADPH oxidase originally found in neutrophils, exists also in other tissues and various cell type especially in colon epithelial cells and vascular smooth muscle cells (Fu *et al.*, 2014). The leucocyte NADPH oxidase which is predominantly expressed in PMNs has a crucial role in antimicrobial host defense (Kobayashi *et al.*, 2018).

b. Exogenous sources

Exogenous ROS derive mainly from exposure to environmental pollution, heavy metals such us Cd, Hg, Pb, Fe, and As, industrial solvents, smoking, certain drugs like cyclosporine, tacrolimus, bleomycin, and gentamycin, cooking (smoked meat, waste oil and fat), alcohol and radiations (Pizzino *et al.*, 2017).

I.3.2. Antioxidants

Antioxidants are molecules that inhibit or quench free radical reactions and delay or inhibit cellular damage (Nimse and Pal, 2015) and include both endogenous and exogenous molecules (Pisoschi *et al.*, 2016).

Endogenous antioxidant can be enzymatic and non-enzymatic pathways. Enzymatic antioxidants work by breaking down and removing free radicals (Nimse and Pal, 2015). The primary antioxidant enzymes are SOD, CAT, and GSH-Px. Di-oxygen is converted by SOD to H_2O_2, which is decomposed to water and oxygen by CAT, preventing hydroxyl radicals production. Additionally, GSH-Px converts peroxides and hydroxyl radicals into nontoxic forms by the oxidation of GSH into GSSG and then reduced to GSH by GR. Other antioxidant enzymes are glutathione-S-transferase and glucose-6-phosphate dehydrogenase (Liguori *et al.*, 2018).

Non-enzymatic antioxidants work by interrupting free chain reactions. Few examples of the non-enzymatic antioxidants are: Glutathione (Nimse and Pal, 2015), alpha-lipoic acid, coenzyme Q, ferritin, uric acid, bilirubin, metallothionein, l-carnitine, melatonin, albumin (Pisoschi *et al.*, 2016), lactoferrin (Zlutski *et al.*, 2017), transferrin and estrogen (Mancini *et al.*, 2013).

Exogenous antioxidant represented by carotenoids, vitamin E, vitamin D, phenolic acids, flavonoids, or ascorbic acid, as well as high-molecular weight metabolites such as tannins (Pisoschi *et al.*, 2016) and synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Husain and Kumar, 2012).

Table 1: Types of endogenous and exogenous dietary antioxidants.

Antioxidants	Types	Examples
Endogenous	Enzymes	Superoxide dimustase Glutathione peroxidase Catalase
	Hormones	Melatonin Estrogen
	Other molecules in blood	Albumin Transferrin Lactoferrin Uric acid
Exogenous	Vitamins	Vitamin E Vitamin C
	Polyphenols	Flavonoids Phenolic acids Tannins
	Synthetic compounds	Butylated hydroxyanisole (BHA) Butylated hydroxytoluene (BHT)

I.4. Inflammation

Inflammation is a protective strategy evolved in higher organisms in response to external and internal aggressions, such as microbial infection, tissue injury and other noxious conditions. It is an essential immune response by the host that enables the removal of harmful stimuli as well as the healing of damaged tissue (Afsar, 2011). At the tissue level, inflammation is characterized by redness, swelling, heat, pain, and loss of tissue function, which result from local immune, vascular and inflammatory cell responses to infection or injury (Chen *et al.*, 2018) (figure 3).

Mechanism of inflammation represents a chain of organized, dynamic responses including both cellular and vascular events with specific humoral secretions. These pathways involve changing physical location of white blood cells (monocytes, basophils, eosinophils, and neutrophils), plasma, and fluids at inflamed site. A group of secreted mediators and other signaling molecules (e.g., histamine, prostaglandins, leukotrienes, oxygen- and nitrogen-derived free radicals, and serotonin) are released by immune defense cells principally in the mechanism which can contribute in the event of inflammation (Abdulkhaleq *et al.*, 2018) Inflammation response occurs in two stages namely, acute and chronic inflammation.

I.4.1. Acute inflammation

Acute inflammation is a part of innate immunity initiated by the immune cells that persists only for a short time (Kunnumakkara *et al.*, 2018). The course of acute inflammatory process evolves in two general phases: initiation and resolution (figure 4). Initiation is characterized by tissue edema issue from increased blood flow and permeability of the microvasculature; processes that are mediated by lipid mediators such as cysteinyl leukotrienes and prostaglandins and other vasoactive products such as histamine, bradykinin. Subsequently, polymorphonuclear neutrophils (PMNs) migrate to the zone to defend against microbial invasion (Sansbury and Spite, 2016).

Drawn to the site of injury by exuded chemical signals including pro-inflammatory lipid mediators such as leukotriene B4 (LTB4) and chemokines, PMN traverse the vasculature through precise interactions with endothelial adhesion receptors and subsequently engulf and degrade pathogens within phagolysosomes (Sansbury and Spite, 2016). In the resolution phase, neutrophils undergo apoptosis after performing their action at the inflamed site and macrophages ingest apoptotic neutrophils. Clearance of apoptotic neutrophils prompts a switch from a pro- to an anti-inflammatory macrophage phenotype, which is a prerequisite for macrophage egress via the lymphatic vessels favoring return to tissue homeostasis (Ortega-Go´mez *et al.*, 2013).

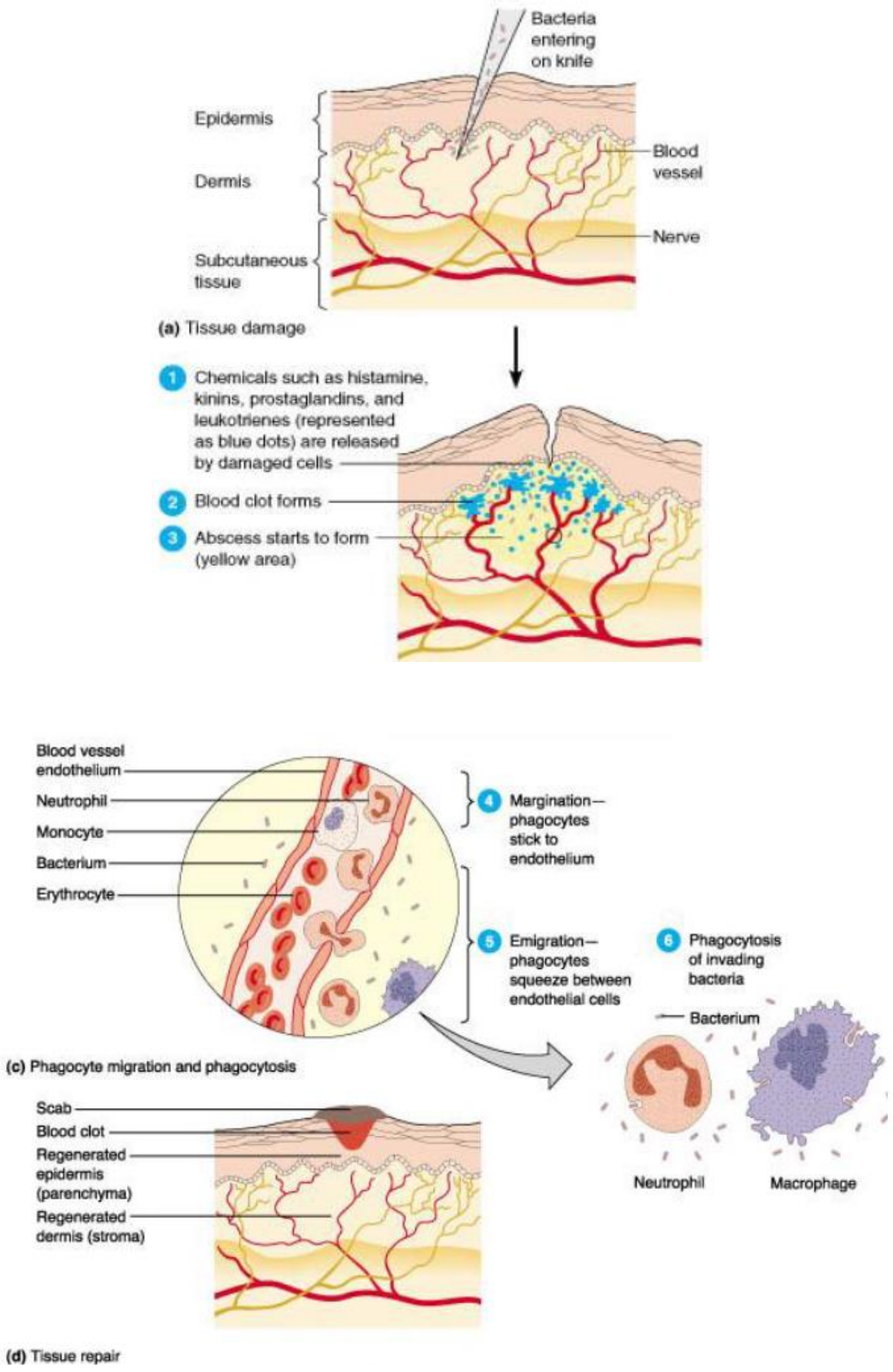

Figure 3. Inflammation phases (Bounihi, 2016).

I.4.2. Chronic inflammation

Chronic inflammation occurs when acute inflammatory processes fail to eliminate tissue injury (Chen *et al.*, 2018). Chronic inflammation is also referred to as a low-grade, persistent inflammatory response lasting for several months to years (Rahtes *et al.*, 2018), it's characterized by the presence of monocytes / macrophages and lymphocytes with the proliferation of fibroblasts, collagen fibers, and the formation of the connective tissue, which ultimately result in 2-mm granuloma. With chronic inflammation, the tissue degeneration is normally mediated by nitrogen species, proteases, and other reactive oxygen species released from infiltrated inflammatory cells (Antonelli and Kushner, 2017; Abdulkhaleq *et al.*, 2018).

Chronic inflammation can result from the following:

- ✓ Failure of eliminating an irritant causing an acute inflammatory response or resistance of the irritant to the host defenses and continues to be generated locally.
- ✓ An autoimmune disorder, which involves your immune system mistakenly attacking healthy tissue.
- ✓ Long persistence to irritants with low intensity, which does not evoke a significant acute inflammatory reaction (Wakefield and Kumar, 2003).

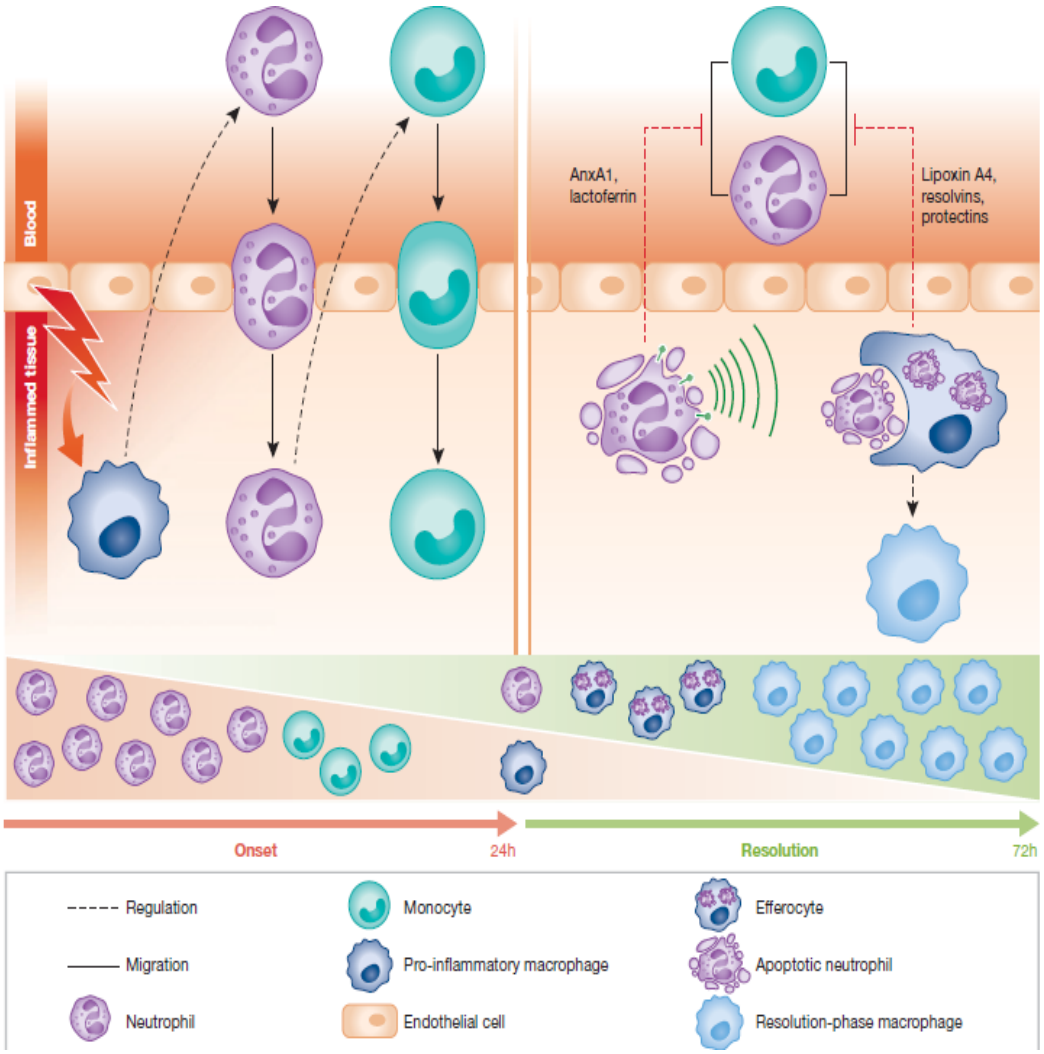

Figure 4. Cellular interplay during resolution of inflammation (Ortega-Go´mez *et al.*, 2013).

I.4.3. Cells and mediators of the inflammation

Inflammation begins when the body senses "danger," in the form of infective, traumatic, ischemic, physical, chemical, or other challenges. Exposure to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) leads to activation of cells from the monocyte macrophage lineage, resulting in expression of pro inflammatory and suppression of anti-inflammatory genes (Bennett *et al.*, 2018), it was discovered that transcription factors such as nuclear factor κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3), inflammatory enzymes such as cyclooxygenase-2 (COX-2), matrix metalloproteinase- 9 (MMP-9), and inflammatory cytokines such as TNF-α, interleukins such as IL-1, -6, -8, and chemokines are the main molecular mediators of the inflammation (Kunnumakara *et al.*, 2018) (Figure 5).

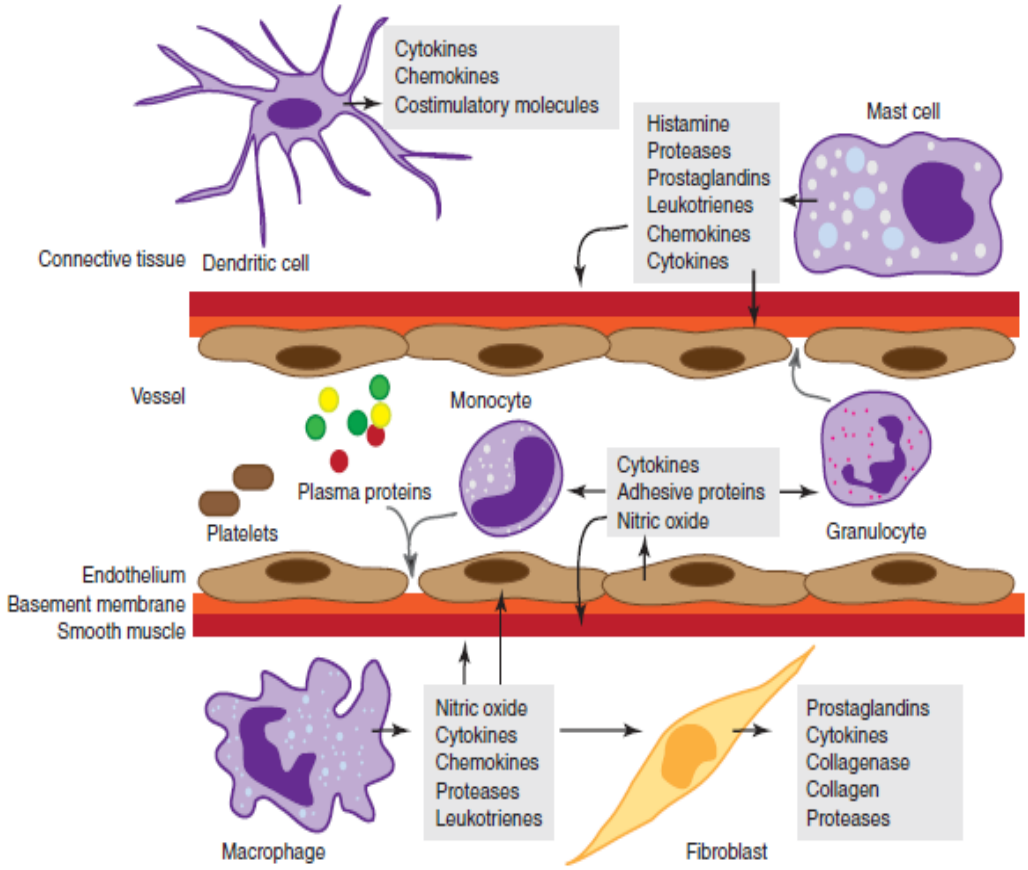

Figure 5. Cells and mediators of the inflammatory response (Newton and Dixit, 2012).

1.4.4. Anti-inflammatory drugs

1.4.4.1. Non-steroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) belong to a wide group of therapeutic agents with anti-inflammatory and analgesic effects (Fokunang *et al.*, 2018) that inhibit cyclo-oxygenase (COX), the enzyme responsible for biosynthesing the prostaglandins and thromboxane (Bacchi *et al.*, 2012), however, NSAIDs are associated with an increased risk of adverse gastrointestinal bleeding, renal and cardiovascular effects (Wongrakpanich *et al.*, 2017).

1.4.4.2. Steroidal anti-inflammatory drugs (glucocorticoids)

Glucocorticoids are steroid hormones essential for the daily functioning of mammals and mainly produced by the cortex of adrenal glands (Timmermans *et al.*, 2019). Synthetic glucocorticoids are widely employed and have an important clinical utility as anti-inflammatory, immunosuppressive (Liberman *et al.*, 2018) and anti- proliferative agents (Tmmermans *et al.*, 2019). However, as with any potent medication, they are not without side effects. Glucocorticoids can lead to various serious musculoskeletal, gastrointestinal, cardiovascular,

endocrine, neuropsychiatric, dermatologic, ocular, and immunologic side effects (Oray *et al.*, 2016).

I.5. Rheumatoid arthritis

Rheumatoid arthritis is a systemic autoimmune disease that clinically manifests as joint pain, stiffness and swelling (Hussein *et al.*, 2016). RA is the commonest form of chronic inflammatory arthritis, characterized by synovial inflammation (Shrivastava and Pandey, 2013) and hyperplasia, autoantibody production (RF and ACPA), cartilage and bone destruction (Mclness and Shett, 2011) (Figure 6).

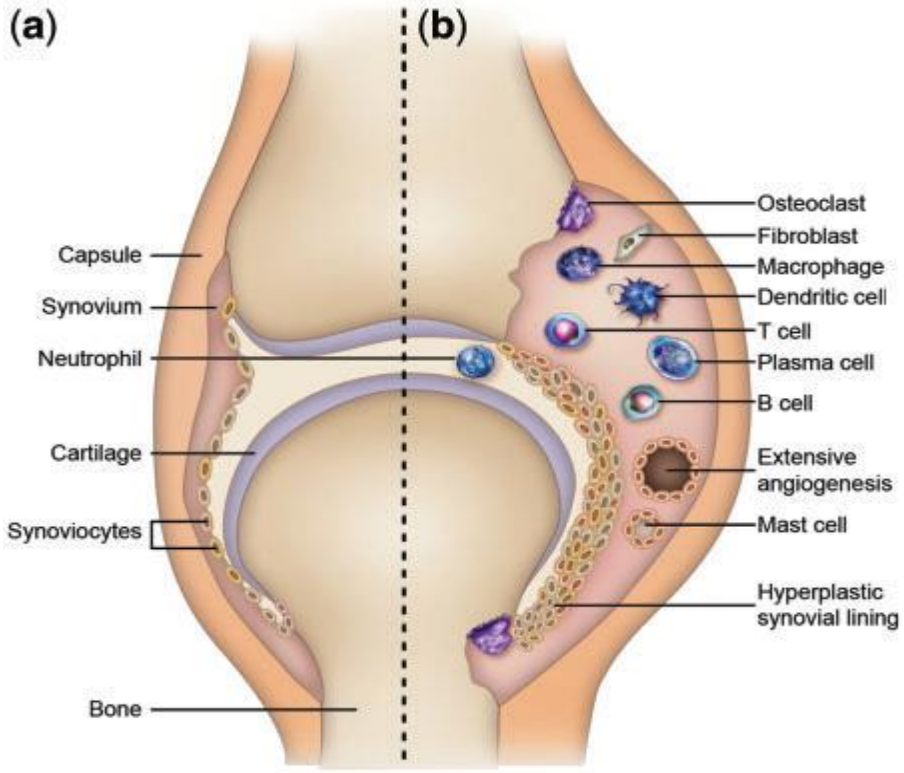

Figure 6. Schematic view of a normal joint (a) and a joint affected by RA (b) (Choy, 2012).

I.5.1.Pathogenesis

The pathogenesis of RA is a multistep process that starts with the development of autoimmunity, continues with local inflammation and finally induces bone destruction (Chimenti *et al.*, 2015). The pathophysiology of RA involves numerous different cell-types, including macrophages, B-cells, T-cells, chondrocytes and osteoclasts and synovial cells, all of which contribute to a local articular destructive process (Zampeli *et al.*, 2015).

Genetic and environmental factors appear to play a significant role in activating the immune system and eventually producing aberrant and sustained inflammatory responses (Zampeli *et al.,* 2015). Environment-gene interactions can lead to immunological dysfunction and initiate the production of auto-antibodies, such as anti-cyclic citrullinated peptide (anti-CCP) or rheumatoid factor antibodies (Smolen *et al.,* 2016). ACPAs recognize citrullinated peptides found in many matrix proteins such as fibrinogen, vimentin, alpha-enolase (Boissier *et al.*, 2012) and collagen II (Klareskog *et al*., 2014) in which the amino acid arginine has been converted into a citrulline by post-translational modification (Sakkas *et al*., 2014). Antigen-presenting cell (APCs) present self or nonself antigens to T cells, which differentiate into T-helper cell (TH1) or TH17 cells that induce macrophages to secrete pro-inflammatory cytokines and help B cells to produce (auto)antibodies that bind to target antigens forming immune complexes. These immune complexes can bind complement and engage complement receptors and/or Fc receptors on macrophages, augmenting secretion of TNF, IL-1 and IL-6 (Smolen *et al.,* 2012).

C reactive protein (CRP) an acute phase protein is synthesized by hepatocytes in response to pro-inflammatory cytokines, in particular IL-6. It has been shown to be of great value as an inflammatory marker in RA and has been suggested to mediate part of the complement activation in RA (Singh *et al.,* 2013).The pro-inflammatory cytokines induce the receptor activator of NF-κB ligand (RANKL) expression on T cells, promote the differentiation of B cells and stimulate the release of matrix metalloproteases (MMPs) provoking the degradation of the cartilage and the activation of osteoclasts leading to the bone resorption (Chimenti *et al.,* 2015).

I.6. Carcinogenesis

Cancer is a common most fatal diseases, that affects the majority of the population, annually, cancer results in the death of >3500 million people all over the world (Balabhaskar *et al.,* 2019). It engages morphological, cellular transformation, uncontrolled cellular proliferation, deregulation of cell death, invasion, blood vessel formation, and metastasis with substantial economic, physiological, and pathological impacts (Wang *et al.,* 2020).

Carcinogenesis my result from the accumulation of genetic and epigenetic alterations in the genome caused by errors in the replication of DNA or interactions with exogenous agents, such as radiation and carcinogenic chemicals. The accumulation of such tumor‑promoting modulations in the DNA of cells endows them with the ability to evade programmed cell death, a

process known as apoptosis, which is a crucial homeostatic mechanism that regulates cell turnover and maintains cell populations in tissues (Raina *et al*., 2020). Multistage carcinogenesis is a widely accepted hypothesis in the development of cancers and is operationally divided into three stages, namely, initiation, promotion and progression (George *et al.,* 2017).

Initiation involves the formation of an irreversibly mutated, preneoplastic cell from a genotoxic event. Promotion, the second stage involves the selective clonal expansion of the initiated cell through an increase in cell growth through either an increase in cell division and/or a decrease in apoptotic cell death. The events of this stage are dose dependent and reversible upon removal of the tumor promotion stimulus. Progression, involves cellular and molecular changes that occur from the preneoplastic to the neoplastic state (Klaunig *et al.,* 2018). It's characterized by irreversibility, faster growth, genetic instability, invasion, metastization and changes in the metabolical, morphological and biochemical characteristics of cells (Oliveira et al., 2007) (figure 7).

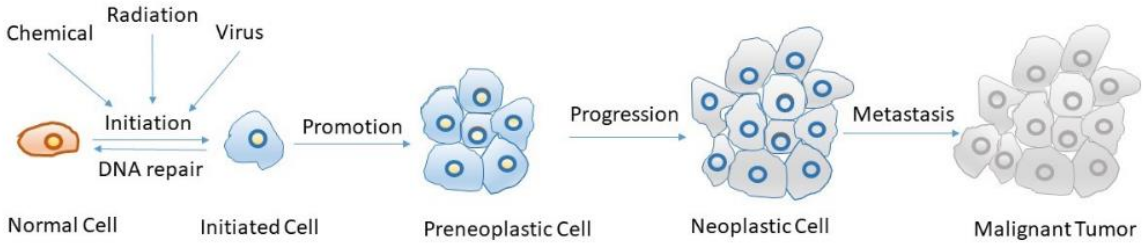

Figure 7. Multistep process involved in carcinogenesis that transforms a normal cell into a malignant tumor (Klaunig *et al.,* 2018).

Chapter II

Materials and Methods

II.1. Materials

II.1.1. Plant material

II.1.1.1. Collection

Aerial parts of *Stachys circinata* and *Stachys mialhesi* were collected from Djebel El-Ouahch-Constantine (North Eastern Algeria) in April 2013 during the flowering stage. A voucher specimen (LOST SC04/13) has been deposited in the Herbarium of the Department of Chemistry, University Mentouri-Constantine, and authenticated by Prof. G. De Belair (University of Annaba, Algeria).

II.1.1.2. Preparation of the extracts

Air-dried and powdered aerial parts (1kg) of *S. circinata* and *S. mialhesi* were macerated separately three times at room temperature with MeOH-H2O (70:30, v/v) for 24h. After filtration, the filtrate was concentrated and dissolved in water (300 mL). The resulting solution was extracted successively with petroleum ether, dichloromethane (CH_2CL_2), ethyl acetate (EtOAc) and *n*-butanol and concentrated in vacuo at room. The resulting dichloromethane extract of *S. circinata* and the butanolic extract of *S. mialhesi* were then used in all experiments.

II.1.2. Animals

Adult male *Mus Musculus* Albinos mice (2-2.5 months old) were obtained from central pharmacy Institute, Constantine, Algeria. The animals used in all experiments had a weight range between 26 and 35 g. All the mice were kept under standard laboratory conditions at 24 ± 1°C relative humidity 55% with a 12 h light/dark cycle. They were fed with a stock rodent diet and tap water. The animals were acclimatized under the laboratory conditions for one week before the commencement of experiment.

II.2. Methods

II.2.1. Acute Oral Toxicity

The present study was conducted according to the guideline proposed by the Organisation for Economic Cooperation and Development (OECD) revised up-and-down procedure for acute toxicity testing. This guideline is based on the procedure of (Bruce, 1985).

A 2000 mg/kg dose of *S. circinata* and *S. mialhesi* extracts was used in five adult male mice, the dose was given orally to a sole mouse, with the aim to monitor mortality and clinical

signs (behaviors recorded: unusual aggressiveness, unusual vocalization, restlessness, sedation and somnolence movements, paralysis, convulsion, fasciculation, prostration and unusual locomotion). Observations lasted 48 h and were performed during the first hour and then each 3 h until the end. Upon survival of this mouse, four additional mice were given the same dose sequentially at 48 h intervals and again, clinical signs were monitored. All of the experimental animals were maintained under close observation for 14 d following extracts administration, and the number of mice that died within the experimental period was noted. The lethal dose 50 (LD50) was established to be above 2000 mg/kg, if no health disorders nor death was registered in three or more mice.

II.2.2. Evaluation of the immunomodulatory activity of the plant extracts

Many plant extracts have long been described as possessing anti-inflammatory and immunomodulatory actions. The first line of human body defense against invading pathogens is the innate immune system, through macrophage cells. In the present study phagocytic activity of reticuloendothelial system (RES) was assayed by carbon clearance test. Phagocytic index was calculated as a rate of carbon elimination of RES by carbon clearance test determined by a reported method (Halpern *et al.*, 1953).

Mice were divided into four groups each of 7 animals: group I (Control) received 0.5 mL of a 0.9% NaCl saline solution via intraperitoneal (i.p.) injection; groups II, III and IV were administred by i.p injection with 50, 150 and 200 mg/Kg of each species, respectively (Table 2 and 3).

Forty-eight hours after the i.p. injection of the treatment, a colloidal carbon ink suspension was injected via tail vein to all groups at a dose of 0.1 mL/10g. The ink suspension consisted of black carbon ink 3ml, saline solution 4ml and 3% gelatin solution 4ml. Then, blood samples (≈14 drops or 25µL) were withdrawn from the retro-orbital plexus via heparin glass capillaries at interval of 5 and 15 min after carbon ink injection. Collected blood samples were lysed in a 0.1% sodium carbonate solution (4mL) and optical density (OD) measured spectrophotometrically at 675nm. At the end of the experiment, liver and spleen were removed from each mice, weighted and values used to calculate the phagocytic index K.

Table 2: Treatment of mice in carbon clearance rate test *S. circinata* n=7

Experimental groups	Treatment	Dose
GI	NaCl 0.9 %	0.5ml/mouse
GII	*S. circinata*	50mg/kg
GIII	*S. circinata*	150mg/kg
GIV	*S. circinata*	200mg/kg

Table 3: Treatment of mice in carbon clearance rate test *S. mialhesi* n=7

Experimental groups	Treatment	Dose
GI	NaCl 0.9 %	0.5ml/mouse
GII	*S. mialhesi*	50mg/kg
GIII	*S. mialhesi*	150mg/kg
GIV	*S. mialhesi*	200mg/kg

Clearance kinetic was expressed by:

The phagocytic index K, which follows an exponential function of concentration to time and measures all the RES activity in contact with the circulating blood, and the corrected phagocytic index α, which expresses this activity by, unit of active weight organs (liver and spleen).

Finally, the clearance rate was expressed as the half-life period (t½, min) of the carbon ink in the blood. Parameters have been calculated using the following formulas according to Biozzi *et al*. (1970).

$$\mathbf{K} = \frac{\mathbf{Log\ OD1 - Log\ OD2}}{\mathbf{t2 - t1}} \qquad \mathbf{t½} = \frac{\mathbf{0.693}}{\mathbf{K}}$$

$$\boldsymbol{\alpha} = \sqrt[3]{\mathbf{K}}\ \frac{\mathbf{Body\ weight\ of\ animal}}{\mathbf{Lever\ weight + spleen\ weight}}$$

OD1 and OD2 are the optical densities (at 675nm) recorded at time t1 (5 min) and t2 (15 min), respectively.

II.2.3. Evaluation of the anti-oxidant activity of the plant extracts

The reduced glutathione was estimated using a colorimetric technique, as mentioned by Weckbeker and Corey. (1988), while the activity of catalase was measured according to the method of Aebi (1983) .The total protein concentrations of liver were measured by Bradford's method (1976) (see appendix I).

II.2.3.1. Preparation of the homogenate

The mice were sacrificed, and the livers were quickly removed, placed in a buffer solution of TBS (Tampon buffer saline, 50 mM Tris, 150 mM NaCl, pH = 7.4).

The weight of 0.5 gr of the mice livers were homogenized in 2 ml of TBS in an ultrasound homogenizer. Homogenates were centrifuged at 9000 rpm for 15 min at 4°C.

The supernatant fraction was used for determination of the reduced glutathione (GSH) and catalase activity.

II.2.3.2. Estimation of reduced glutathione (GSH) level

The measurement of the intracellular GSH concentration is based on the optical absorbance of 2-nitro 5-mercapturic acid, the latter results from the reduction of 5,5'-dithio-bis-2- acid nitriobenzoic acid (DTNB) by the thiol groups (-SH) of glutathione.

The experimental procedure for the glutathione dosage is the following:

- 0.8 ml of the homogenate was added to 0.2 ml of the sulfo-salicylic acid (0.25%) solution, then the acidified suspension was homogenized, left on ice for 15 min and centrifuged for 5 min at 10,000 rpm at 0°C.

- 0,5 ml of the supernatant was added to 1.0 ml Tris-HCl buffer (0.4 M, pH 9.6) containing 20 mM EDTA.

- DTNB (25µl of 10 mM solution in MeOH) was added and the absorbance was read within 5 min at 412 nm.

The glutathione concentration was calculated by the formula:

$$GSH\left(\frac{nmol}{mg\ protein}\right) = \frac{DO\ X\ 1\ X\ 1.525}{13100\ X\ 0.8\ X\ 0.5\ X\ mgprotein}$$

DO: Optical Density.

1: total volume of the protein decomposition solutions (0.8 ml the homogenate + 0.2 sulfo-salicylique acid).

1.525: volume of the total solutions used for the GSH dosage at the supernatant level (0.5 ml supernatant + 1 ml Tris-EDTA + 0.025 ml DTNB).

13100: group –SH absorbance coefficient at 412 nm.

0.8: homogenate volume.

0.5: supernatant volume.

II.2.3.3. Estimation of catalase activity

The activity of catalase (CAT) is measured according to Aebi, (1983) using a spectrophotometer by the variation of the optical density following the decomposition of hydrogen peroxide (H_2O_2) by reacting in 100 mM of phosphate buffer for 1 min at pH 7.5, 100 µl of H_2O_2, 500 mM on 20 µl of the homogenate, at an incubation temperature of 25 ° C.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

The units of catalase were expressed as the quantity of enzyme which breaks down 1 µM of H_2O_2 per minute at 25 ° C

The results were expressed in µmole of H2O2 per minute and per mg of protein (Table 4). The decrease in optical density is due to the decomposition of hydrogen peroxide which was measured against a blank at 240 nm (see Appendix I).

Table 4: Protocol used for the determination of catalase activity.

Reagent	Test	Blank
Phosphate buffer 100 mM; pH= 7.5	**780 µl**	**800 µl**
H_2O_2 500 mM	**200 µl**	**200µl**
Homogenate	**20 µl**	**0 µl**

It should be noted that the amount of homogenate must be determined as a function of the amount of proteins which is between 8 and 12 g/dl, i.e. a quantity of 10 to 20 µl of homogenate. The activity decreases rapidly, it is important to always put the same time between pipetting and the moment when it is placed in the quartz tank during the 60 seconds of measurement; the extinction coefficient being 0.040Mm.cm. The specific activity was expressed in terms of units per gram of tissue according to following formula:

$$\boldsymbol{AT\ (\mu mol/\ mn/\ mg\ Hb) = \frac{(\Delta\ Do\ \times 10)}{\varepsilon \times L \times Y\ mg\ of\ proteins}}$$

ε: Molar linear extinction coefficient in Mm.cm

L: Width of the measuring tank in cm = 1cm

Y: Protein content in mg.

II.2.4. Evaluation of the anti-arthritis activity of the plant extracts

II.2.4.1. Formalin induced arthritis

To perform this test, mice (20-30 g) were divided into four groups (F, FF, SC/ SM, D) of five animals each. Group ‘F’ remained untreated (negative control); group ‘FF’ (positive control) was subjected to the sole formalin treatment; group ‘SC’, ‘SM’ to formalin + *S.circinata* and *S.mialhesi* respectively at 150 mg/kg and group ‘D’ to formalin + the anti-inflammatory standard drug (diclofenac of sodium) at 10 mg/kg (Table 5).

The administration was done orally by mixing the plant extracts or diclofenac of the treated groups into a flour balls. The delivery of extracts or diclofenac of sodium was carried out while maintaining the standard diet. In this experiment, the lowest concentration with the highest

efficacy of extracts (150 mg/kg) was employed according to the results attained in the phagocytosis experiment.

Table 5: Treatment of mice in formalin induced arthritis test

Experimental groups	Flour (g)	Formalin (µl)	Dose
F	**0,1**	**0**	
FF	**0,1**	**100**	
SC	**0,1**	**100**	**150mg/kg**
SM	**0,1**	**100**	**150mg/kg**
D	**0,1**	**100**	**10mg/kg**

According to the protocol of Mazumder *et al.* (2012), Formalin treatments were performed by injecting into the sub-plantar of the right hind paw 100 µL of formalin (2%) on the 1st and 3rd day of the experiment. Then, diclofenac and the extracts (SC, SM) were daily administered until the end of the experiment. During the 10 d experiment, a daily measurement of the edema size was realized with a digital caliper.

II.2.5. Blood investigation

At the end of the experiments, animals from the entire groups were fasted overnight before collecting the blood samples from retro-orbital vein into heparin tubes by using glass capillaries. The separated plasma was assayed for hs-C-reactive protein by animmunoturbidimetric method on a Cobas integra 400 plus analyser (Roche) and anti-CCP was measured by Stratec Biomedical Systems Gemini 6280 Automated Compact Microplate Processor at Iben Sina laboratory.

II.2.6. Histological study of the joints

At the end of the experiment, the animals were sacrificed with chloroform and hind paws were cut off and fixed in 10% formalin for 24 hr. Then the hind paws decalcified in 0.3% formic acid. After the tissues were embedded in paraffin and cut in 5 µm thick sections after alcohol dehydratation (see appendix I).

II.2.7. Evaluation of the anti-proliferative activity of the plant extracts

II.2.7.1.Used material

a. Plant material

The anti-proliferative effect was evaluated using the extracts of dichloromethane of *S. circinata* and the n-butanolic of *S. mialhesi*. The extracts were dissolved in DMEM.

b. Reagent and cells

Dulbecco's phosphate buffered saline (DPBS) (Euroclone, Milano, Italy); Dubelcco's modified Eagle's medium (DMEM) (LONZA, BioWhittaker®); minimum essential medium and non-essential amino acids (MEM, NEAA) (gibco®, Life Technologies™. Grand Island, NY, USA); DMEM/F-12, endothelial cell basal medium (EBM) (LONZA, Clonetics®); fetal bovine serum (FBS) (Life Technologies. Grand Island, NY, USA); L-glutamine, penicillin, streptomycin, bovine brain extract (BBE), epidermal growth factor (rhEGF), gentamicin sulfate, ascorbic acid and hydrocortisone (Euroclone, Milano, Italy); (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction (MTT) (Cell Proliferation Assay ATCC® 30-1010K kit Invitrogen Co), dimethylsufoxide (DMSO) (Sigma Aldrich).

The hepatocarcinoma cell line (HepG2) were obtained from ATCC (American Cell Culture Collection), breast cancer cell line (MCF7) from the anatomia patologica (Civil hospital of Cagliari, Italy), rat pheochromocytoma cell line (PC12) from pharmacology laboratory (Department of clinical and experimental medicine, university of Sassari, Italy) and normal human umbilical vein endothelial cell (HUVEC CC-2519) from LONZA (Clonetics®).

c. Cell culture

Cell cancer of (MCF7 and HepG2) were kept in DMEM amended with (v/v) 10% of FBS, 1% of antibiotic P/S (penicillin- streptomycin), 1% of glutamine and 1% of MEM and NEAA. HUVEC cells were cultured in EBM containing 2% FBS, 1% P/S, 0.4% BBE, 0.1% of EGF, 0.1% Gentamicin Sulfate, 0.1% Ascorbic Acid, and 0.1% Hydrocortisone. PC12 was maintained in DMEM/F-12 supplemented with 10% of HS, 5% of FBS and 1% of P/S. Cells

were grown in 75-cm^2 tissue culture flasks in a culture incubator at 37°C with 5%CO_2 and saturated humidity.

II.2.7.2. Experimental procedure

a. Trypsinization and cell counting

The first step in subculturing adherent cells is to detach them from the surface of the culture vessel. The enzymatic mean was used in this study.

MCF7, HepG2, PC12 and HUVEC from one T75 flask were used. First, the medium was removed and the flask was washed by 3 ml of Phosphate-Buffered Saline (PBS) .After removing the PBS, 1.5 ml of trypsin-EDTA was added to the flask and incubated for 5 min in CO2 incubator (Thermo Electron Corporation, Steri-Cycle CO2 Incubator HEPA Class100).

After that, 8.5 ml of medium was added to flask (the trypsin was inhibited by the FBS containing in the medium) and with a smooth pipetting the cells were dissociated and the solution was then transferred to a conical tube. The last step in this part of the study was to determinate the total number of cells in 1 ml of cell suspension. 20 µl of the cell suspension was mixed with 20 µl of trypan blue and 20 µl of the mix was transferred to each side of Countess™ cell counting chamber slides. The last step was the cell counting using Invitrogen™ "Contess automated cell counter".

II.2.7.3. Cell viability MTT assay

The anti-proliferative activity of the *S. circinata* and *S. mialhesi* extracts on HepG2, MCF-7, PC-12 and HUVEC cells were determined using the MTT reduction assay. This rapid colorimetric method is based on the OD shift following the yellow tetrazolium salt cleavage to the purple formazan crystals by the succinate dehydrogenase of functional mitochondria (Mosmann, 1983).

In short, once HepG2, MCF-7, PC-12 and HUVEC cells reached the exponential growth phase, cells were seeded on a 96-well plate at a concentration of 5,000 cells/well in 100 µL of medium and incubated at 37°C in a 5% CO2 atmosphere incubator (Thermo Electron Corporation, Steri-Cycle CO2 Incubator HEPA Class100).

After 24 hr incubation, the medium was replaced with a fresh one containing 17,87, 170, 350, 500 and 570 µg/100 µL of the extracts and cultures were returned at 37°C with 5% CO2 for additional 24 hr. For the negative control, the medium was only refreshed (0.0 µg/100 µL of

extracts). Finally, the medium was removed and substituted with 100 µL of MTT prepared in sterile DPBS(0.65 mg/mL) and then, cells in culture were incubated for additional 2h at 37 °C with 5% CO2 and saturated humidity.

After incubation, the MTT-DPBS was removed and replaced by 200 µL/well of DMSO in order to solubilize the formazan crystals. The solubilized purple formazan was quantified with a spectrophotometer at 578nm with background subtraction at 630-690nm (Gemini EMMicroplate Reader -Molecular devices). Experiments were performed in triplicate. Optical densities were used to determine the % of cell proliferation using the formula of Patel et al. (2009).

$$\textbf{\% Cell proliferation} = (\boldsymbol{At - Ab / Ac - Ab}) \times \mathbf{100}$$

Where,

At= Absorbance value of test compound (*S.mialhesi / S.circinata*).
Ab= Absorbance value of blank (Medium alone).
Ac= Absorbance value of control (Medium+Cells).

II.2.8. Evaluation of *in vitro* anti-oxidant activities

To evaluate the antioxidant activity of *S. circinata* and *S. mialhesi* extracts, glutathione and catalase were investigated in HepG2 cells exposed to the extract at a concentration of 17, 87, 170, 350, 500, and 570 µg/100 µL for 24 hr.

II.2.8.1. Catalase activity

Catalase activity was evaluated using Catalase Assay Kit CAT100 (SIGMA-ALDRICH®). This assay method is based on the measurement of the hydrogen peroxide remaining after the action of catalase. First, the catalase converts hydrogen peroxide to water and oxygen and then this enzymatic reaction is stopped with sodium azide. An aliquot of the reaction mix is then assayed for the amount of hydrogen peroxide remaining by a colorimetric method. Therefore, the catalase activity present in the sample is reversely proportional to the signal obtained.

Cells of HepG2 (250.000 per well, 500µl) were seeded in a 24 well plate and then incubated at 37°C for 24 hours. The medium was removed and 500 µl of medium containing different concentrations of each extracts were added and incubated at 37°C for 24 hours.

After that, the extracts were removed and the wells were washed with 500µl of DPBS. Then 300µl of trypsin was added to each well and incubated at 37°C for 5min. The cells were observed under the microscope for detachment and washed by 900µl of medium/1ml of DPBS.

The cell suspension was transferred in a conical tube for centrifugation (5min, 300g) and the supernatant was decanted. The cell pellet was washed with 500µL of DPBS, centrifuged for 5min at 500g and resuspended in 300µl of the enzyme dilution buffer and centrifuged for 10min at 1200g. Then the supernatant was transferred to an eppendorf tube and the protein concentration (mg/ml) of each sample was measured by spectrophotometer (Thermo scientific, NANODROP 2000).

The catalase enzymatic reaction was started by adding 25µl of the colorimetric assay substrate solution (200 Mm H_2O_2), the solution was mixed and incubated at room temperature for 5mn, then 900µl of the stop solution was added. A 10µl aliquot of the catalase enzymatic reaction mixture was removed and added to another microcentrifuge tube and 1ml of the Color Reagent was added and mixed, after 15mn at room temperature for color development, the absorbance was read at 490nm.

II.2.8.2. Reduced glutathione (GSH)

The measurement of GSH was performed using Glutathione Assay Kit CS0260 (SIGMA-ALDRICH®).

HepG2 cells were seeded in Petri dishes (5.000.000 Cells/6ml for dish) and incubated for 24h at 37°C. Then, the growth medium was removed and the plant extracts were added at a concentration of 17, 87, 170, 350, 500, and 570 µg/100 µL and incubated at 37° C for 24h.

After that, the extracts were removed and the cells were washed with 2ml of DPBS. Then the DPBS was removed and 1.5ml of Trypsin-EDTA was added to each dish and incubated for 5mn at 37°C. 4.5ml of medium was added and by pipetting the cells were dissociated and transferred to a Falcon tubes, the dishes were washed with 4ml of DPBS and the solution was added to the same tubes for centrifugation (600g/5mn).

The obtained cell pellet was resuspended in 500µl of DPBS, mixed and transferred in eppendorf tubes for a second centrifugation (600g/5mn). The supernatant was removed and 180µl of the 5% SSA solution was added to the packet cell pellet, then, the suspension was mixed using Multi Speed Vortex (MSV-3500 biosan) and was frozen with liquid nitrogen, thawed at 37°C bath twice, then leaved for 5mn at 2-8°C.

After, the suspension was centrifuged for 10mn at 10000g and the supernatant volume was measured and transferred into eppendorf tubes. 150µl of the working mixture solution was added in each well (96 well plates) and 10µl of each sample was added to wells, the solution was mixed by pipetting up and down and incubated for 5mn at room temperature, then 50µl of diluted NADPH solution was added. The absorbance in each well was measured using plate reader at 405nm and read at 1 mn intervals for 5mn.

Statistical analysis

Data from *in vivo* and *in vitro* studies were analyzed using Statistical Package for Social Science (SPSS) program, version 20.

All data were showed as mean ± SD and the number of assays (n) is mentioned under every result.

Analysis of variance was performed using one-way ANOVA procedures followed by Tukey-test for multiple comparisons.

Significant differences ($P<0.05$) between means were considered.

Chapter III

Results

III. Results

III.1. Acute oral toxicity

According to the preliminary toxicity test, the extracts of *S. circinata* and *S. mialhesi* were found to be safe up to 2000 mg/kg. A total of five male adult mice were treated orally with the same extract at the same dose and observed for 14 days, remained healthy and with no visible signs of toxicity nor mortality. This result stand up for an LD50 higher than 2000 mg/kg.

III.2. Evaluation of the immunomodulatory activity of medicinal plants extracts

III.2.1. Immunomodulatory activity of *S. circinata*

The results show a significant increase of K index mean values in mice belonging to *S. circinata* extract supplied groups if compared to the control (NaCl group) with P= 0.001 **(**Figure 8). Index values for the *S. circinata* administered groups were: 0.031 ± 0.004; 0.038 ± 0.005 and 0.035 ± 0.007 with 50, 150 and 200 mg/kg, respectively. The NaCl group attained a mean index value of 0.017 ± 0.005. The highest activity was monitored in the group of mice fed with 150 mg SC /kg (65.3% increase), but difference among *S. circinata* doses were not significant. This indicates that *S. circinata* enhanced the phagocytic activity by stimulating the RES and according to the results, it seems that the tested concentrations lower or higher than 150 mg/kg do not improve the phagocytic index value.

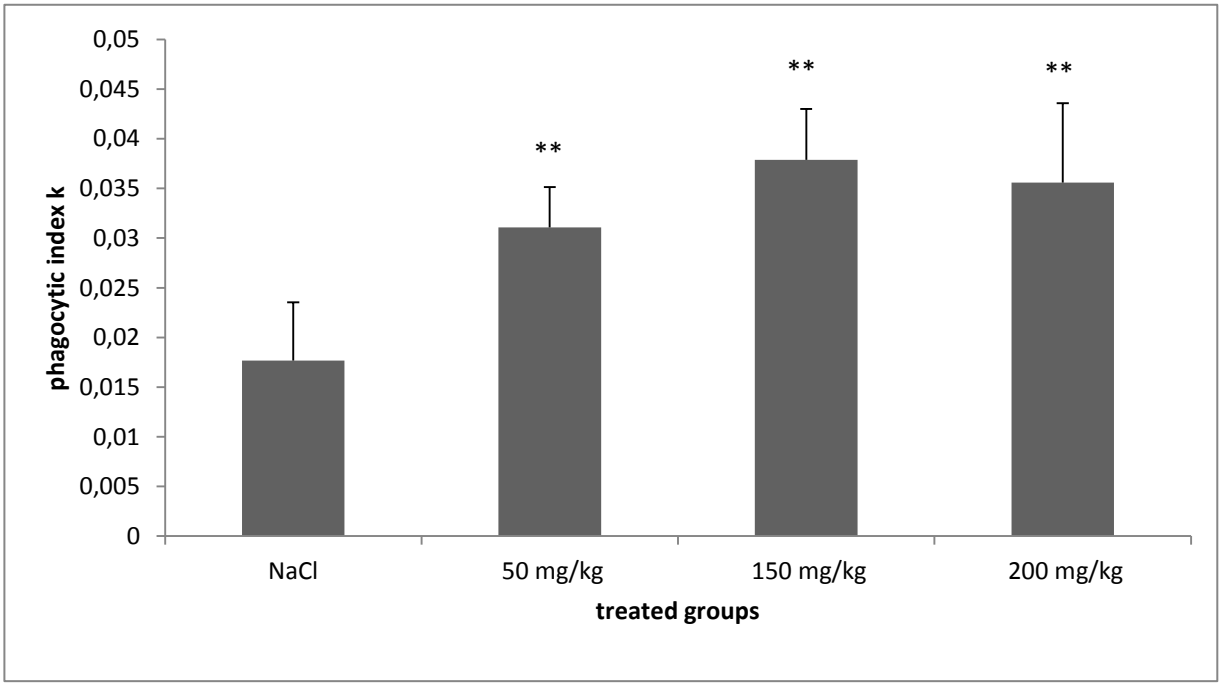

Figure 8: Effect of *S. circinata* extract on phagocytic activity. Values are mean ± SD (n=7) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001 expressed as index phagocytic.

GI: Control group received saline; **GII:** group received dichloromethane extract of *Stachys circinata* at dose 50mg/kg; **GIII:** group received dichloromethane extract of *Stachys circinata* at dose 150mg/kg; **GIV:** group received dichloromethane extract of *Stachys circinata* at dose 200mg/kg

S. circinata extract supply to mice influenced significantly the calculated half time (t½) of colloidal carbon clearance which decreased by nearly 50% compared to the control (NaCl). Among *S. circinata* supplied groups the probability was *P*= 0.01 with a t½ of 23.01 ± 3.14 min; 18.65 ± 2.52 min and 20.56 ± 2.83 min with 50, 150 and 200 mg/kg, respectively (Figure 9). The NaCl group owned a t½ of 40.62 ± 20.35 min. Compared to control, the clearance rate with150 mg *S.circinata*/kg was lowered more than twice.

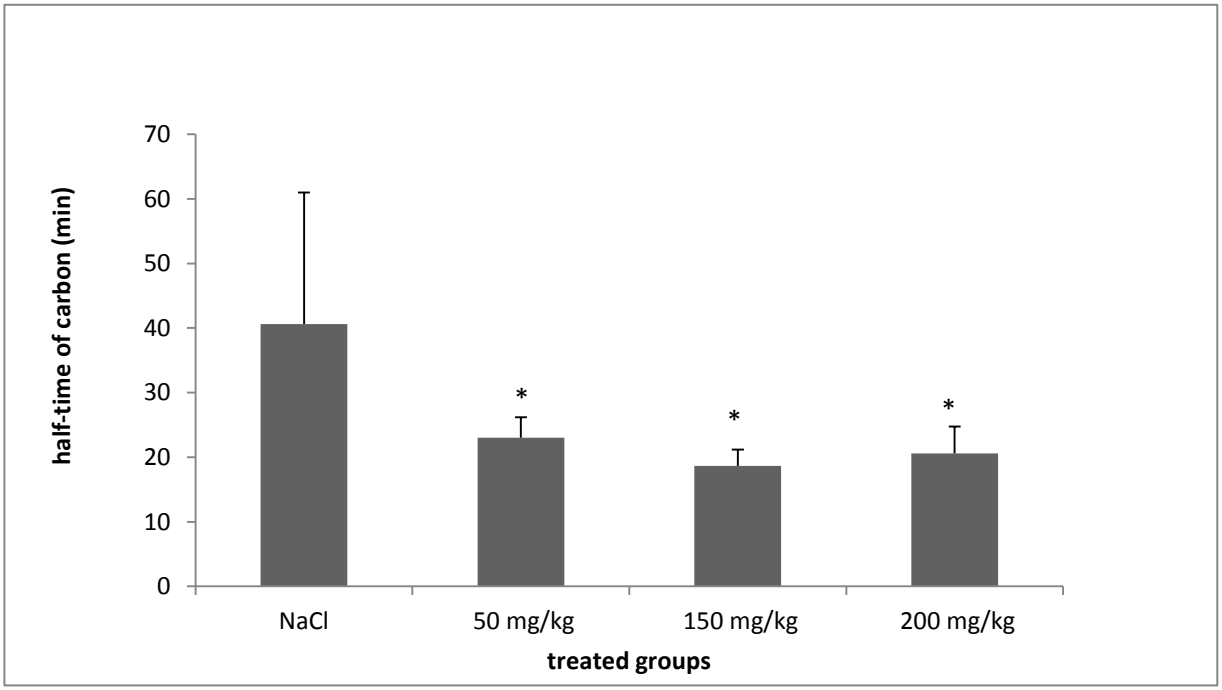

Figure 9: Effect of *S. circinata* extract on phagocytic activity expressed as half-time t1/2 of carbon in blood. Values are mean ± SD (n=7) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001.
GI: Control group received saline; **GII:** group received dichloromethane extract of *Stachys circinata* at dose 50mg/kg; **GIII:** group received dichloromethane extract of *Stachys circinata* at dose 150mg/kg; **GIV:** group received dichloromethane extract of *Stachys circinata* at dose 200mg/kg

In addition to the results reported in figures 6 and 7, a significant increase in the corrected phagocytic index α occurred between *S. circinata* group *P*=0.000 (α= 4.26 ± 0.74; 5.05 ± 0.57; 4.78 ± 0.60 with 50, 150 and 200 mg/kg, respectively) and the control group (α= 2.97 ± 0.50) (Figure 10).

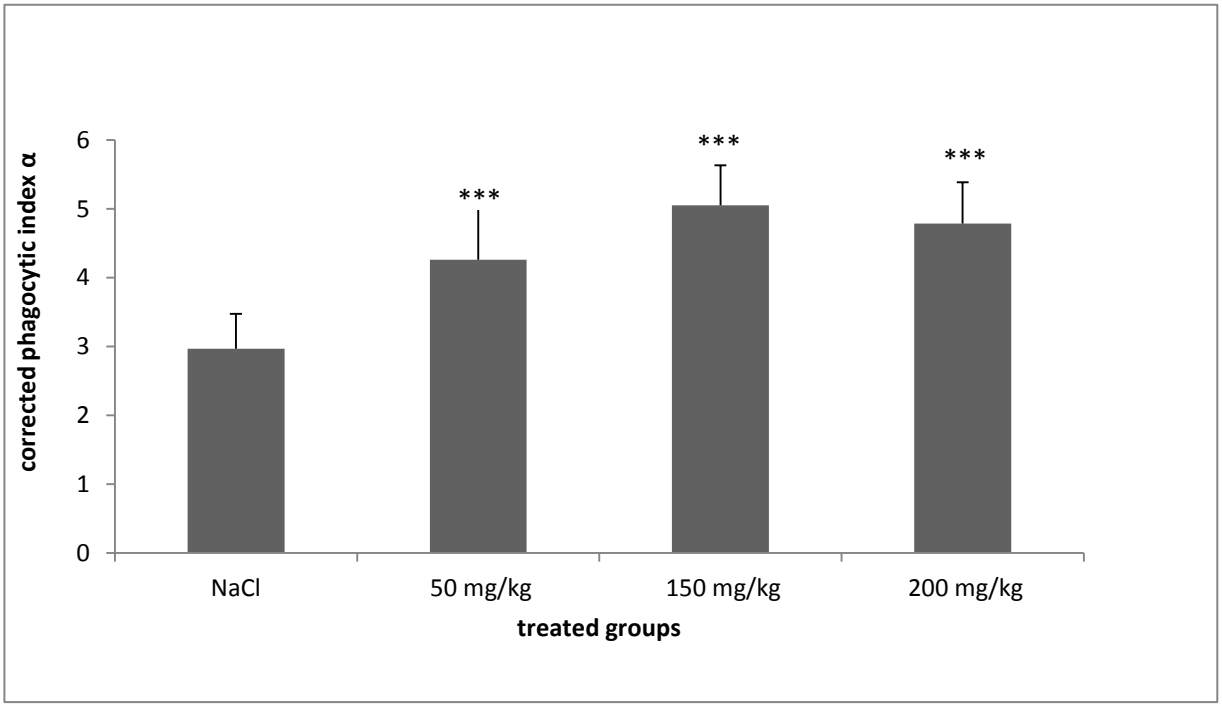

Figure 10: Effect of *S. circinata* extract on phagocytic activity expressed as corrected phagocytic index α. Values are mean ± SD (n=7) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001.

GI: Control group received saline; **GII:** group received dichloromethane extract of *Stachys circinata* at dose 50mg/kg; **GIII**: group received dichloromethane extract of *Stachys circinata* at dose 150mg/kg; **GIV:** group received dichloromethane extract of *Stachys circinata* at dose 200mg/kg

III.2.2. Immunomodulatory activity of *S. mialhesi*

The following data shows that there is a very high significant difference in the means for the phagocytic index K among groups (GI, GII, GIII and GIV) with P= 0.000 (figure 11).

Index K values for the *S. mialhesi* administered groups were: 0.029 ± 0.0025; 0.040 ± 0.006 and 0.033 ± 0.007 with 50, 150 and 200 mg/kg, respectively. The NaCl group attained a mean index value of 0.017 ± 0.005.

The highest activity was monitored in the group of mice fed with 150 mg *S. mialhesi* /kg P<0.05. This indicates that *S. mialhesi* enhanced the phagocytic activity by stimulating the RES and according to the results, it seems that the tested concentrations lower or higher than 150 mg/kg do not improve the phagocytic index value.

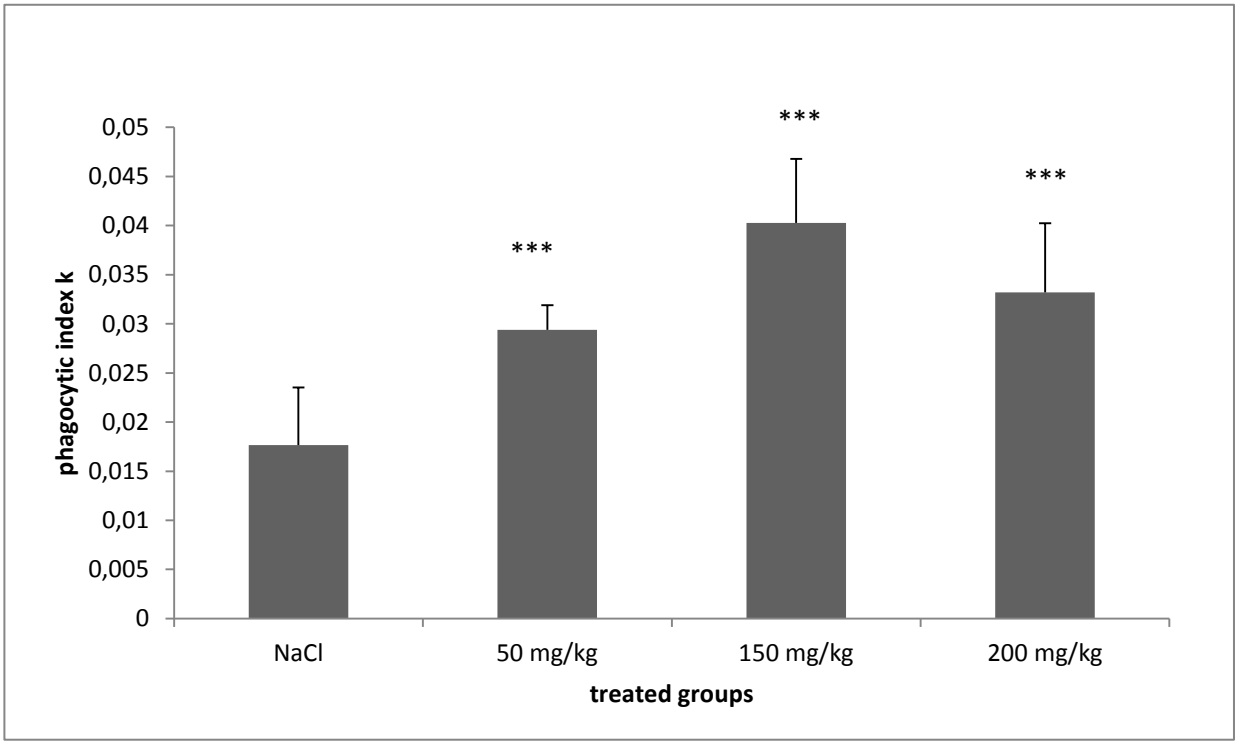

Figure 11: Effect of *S. mialhesi* extract on phagocytic activity. Values are mean ± SD (n=7) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001 expressed as index phagocytic.
GI: Control group received saline; **GII:** group received butanolic extract of *Stachys mialhesi* at dose 50mg/kg; **GIII:** group received butanolic extract of *Stachys mialhesi* at dose 150mg/kg; **GIV:** group received butanolic extract of *Stachys mialhesi* at dose 200mg/kg

As seen in the figure 12, the calculated half-time of colloidal carbon clearance was significantly faster at 150 mg *S. mialhesi*/kg in GIII (17.94±1.71), when it is compared to the control group GI (40.62± 20.35) where P= 0,01. Then, the clearance of carbon was slow in GII (24.21 ± 2.09) and significantly slow in GIV (21.76±2.59), when compared with GIII.

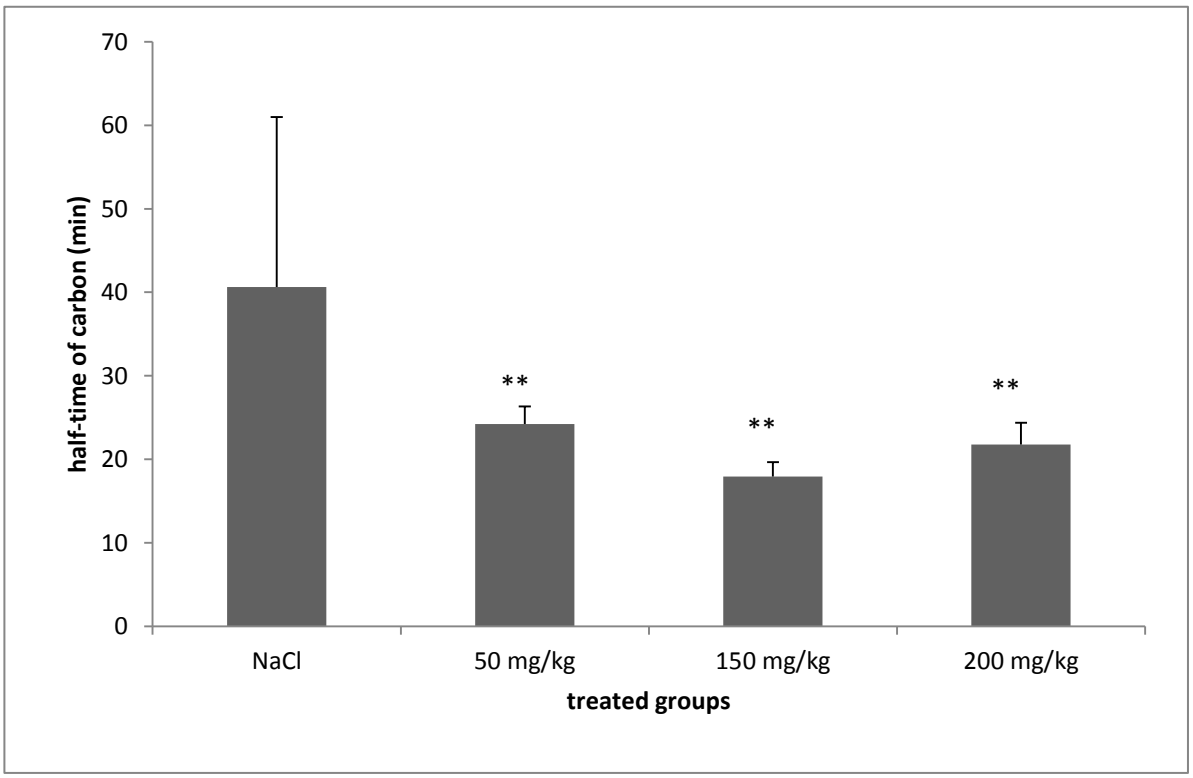

Figure 12: Effect of *S. mialhesi* extract on phagocytic activity expressed as half-time t1/2 of carbon in blood. Values are mean ± SD (n=7) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001.
GI: Control group received saline; **GII:** group received butanolic extract of *Stachys mialhesi* at dose 50mg/kg; **GIII:** group received butanolic extract of *Stachys mialhesi* at dose 150mg/kg; **GIV:** group received butanolic extract of *Stachys mialhesi* at dose 200mg/kg

The following data shows that there is a high significant difference in the means for the corrected phagocytic index α among groups (GI, GII, GIII and GIV) P= 0.000.

Figure 13 demonstrated that the corrected phagocytic index α increased in group GIII (5.13 ± 0.54) when it's compared with the control group GI (2.97 ± 0.50). The corrected phagocytic index α decreased significantly in GII (3.86 ± 0.57) and decreased in GIV (4.38 ±0.38), when it is compared with GIII.

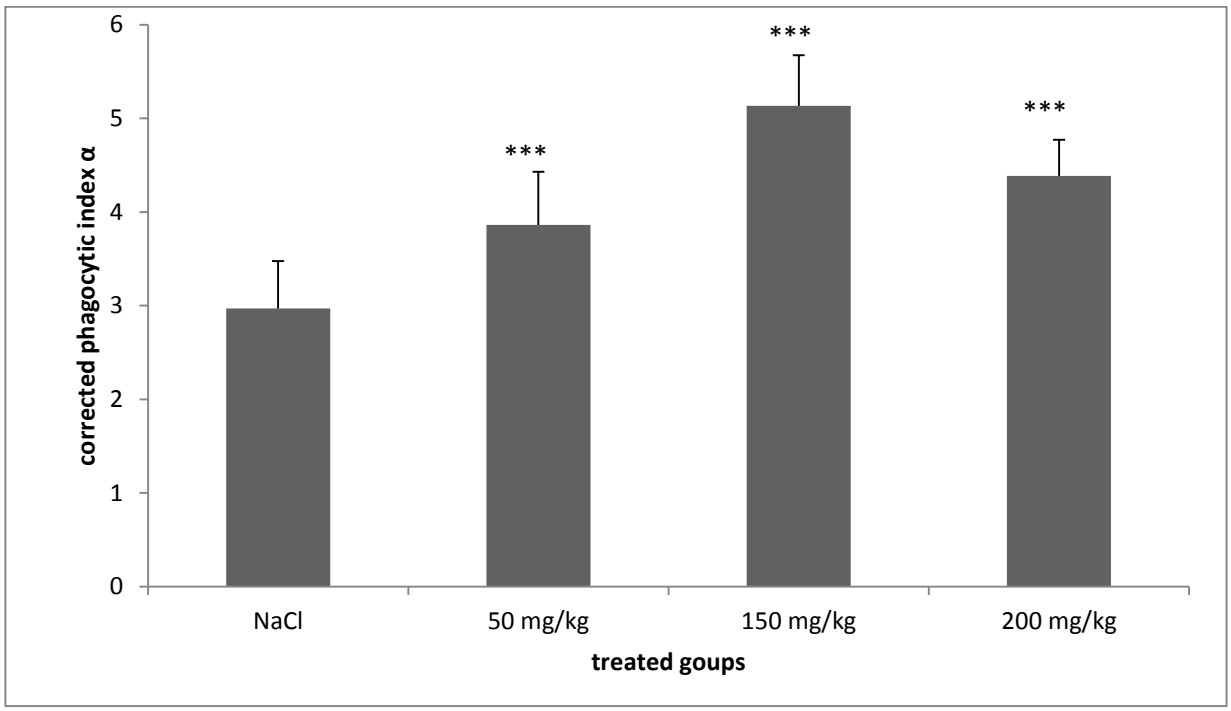

Figure 13: Effect of *S. mialhesi* extract on phagocytic activity expressed as corrected phagocytic index α. Values are mean ± SD (n=7) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001.

GI: Control group received saline; **GII:** group received butanolic extract of *Stachys mialhesi* at dose 50mg/kg; **GIII:** group received butanolic extract of *Stachys mialhesi* at dose 150mg/kg; **GIV:** group received butanolic extract of *Stachys mialhesi* at dose 200mg/kg

III.3. Evaluation of *in vivo* antioxidant activities of medicinal plants extracts

III.3.1. Evaluation of the antioxidant activity of *S. circinata*

III.3.1.1.GSH liver levels

As shown in the figure 14, there is a high significant difference in the means for the Glutathione values among groups (GI, GII, GIII and GIV) P= 0.000. Liver GSH levels of treated groups decreased were: 0.25 ± 0.09; 0.19±0.43; 0.21 ± 0.02 with 50, 150 and 200 mg/kg, respectively compared to control group (0.54± 0.054) with p<0.01.

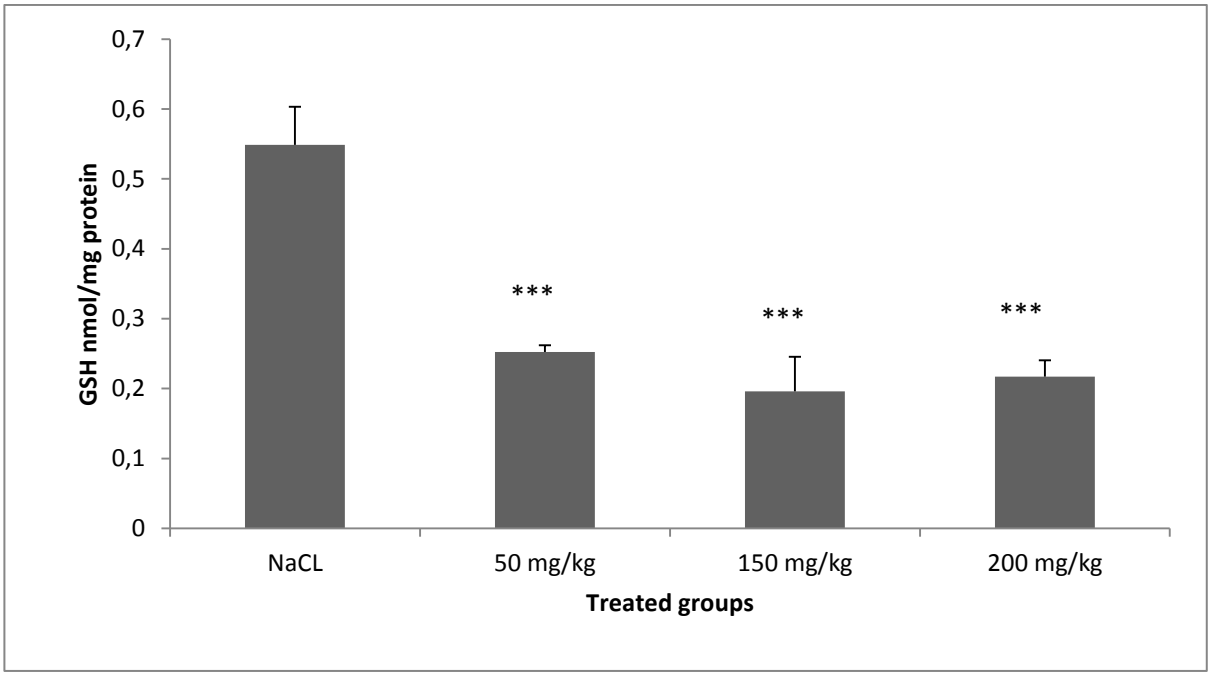

Figure 14: Effect of *S. circinata* extract on Glutathione liver of mice. Values are mean ± SD (n=7) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001.
GI: Control group received saline; **GII**: group received dichloromethane extract of *Stachys circinata* at dose 50mg/kg; **GIII:** group received dichloromethane extract of *Stachys circinata* at dose 150mg/kg; **GIV:** group received dichloromethane extract of *Stachys circinata* at dose 200mg/kg

III.3.1.2.Catalase activity

The results of this study showed that there is a very high significant difference in the means for the catalase values among groups GI, GII, GIII and GIV, where P= 0.000. The catalase values were decreased highly and significantly in groups GII, GIII and GIV (0.50 ± 0.15; 0.43 ± 0.14; 0.68 ± 0.15) when it is compared to the control group GI (0.89 ± 0.092) with P<0.05 (figure 15).

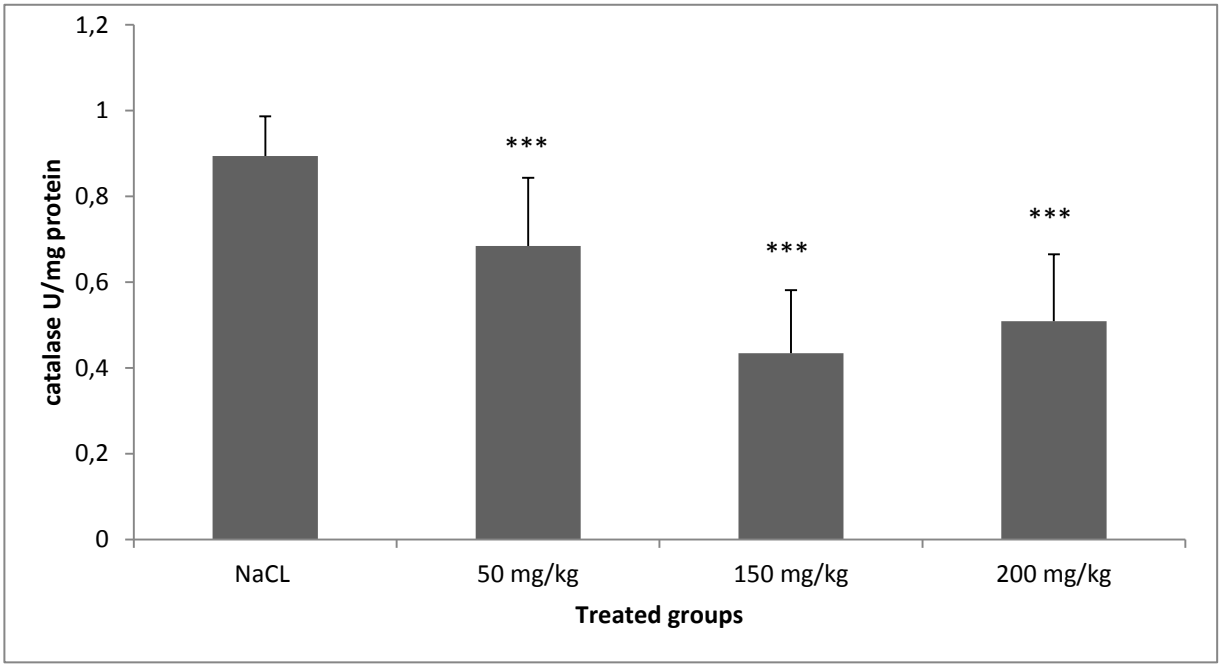

Figure 15: Effect of *Stachys circinata* extract on catalase activity. Values are mean ± SD (n=7) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001.
GI: Control group received saline; **GII:** group received dichloromethane extract of *Stachys circinata* at dose 50mg/kg; **GIII:** group received dichloromethane extract of *Stachys circinata* at dose 150mg/kg; **GIV:** group received dichloromethane extract of *Stachys circinata* at dose 200mg/kg

III.3.2. Evaluation of *in vivo* antioxidant activity of *Stachys mialhesi*

III.3.2.1. GSH liver values

The figure 16 showed that there is a very high significant difference in the means for the Glutathione values between groups GI, GII, GIII and GIV with P= 0.000. The Glutathione values were decreased significantly in groups GII, GIII, GIV (0.19 ± 2.95; 0.21 ± 4.43; 0. 24 ± 6.48) when it is compared to the control GI (0.54 ± 0.054).

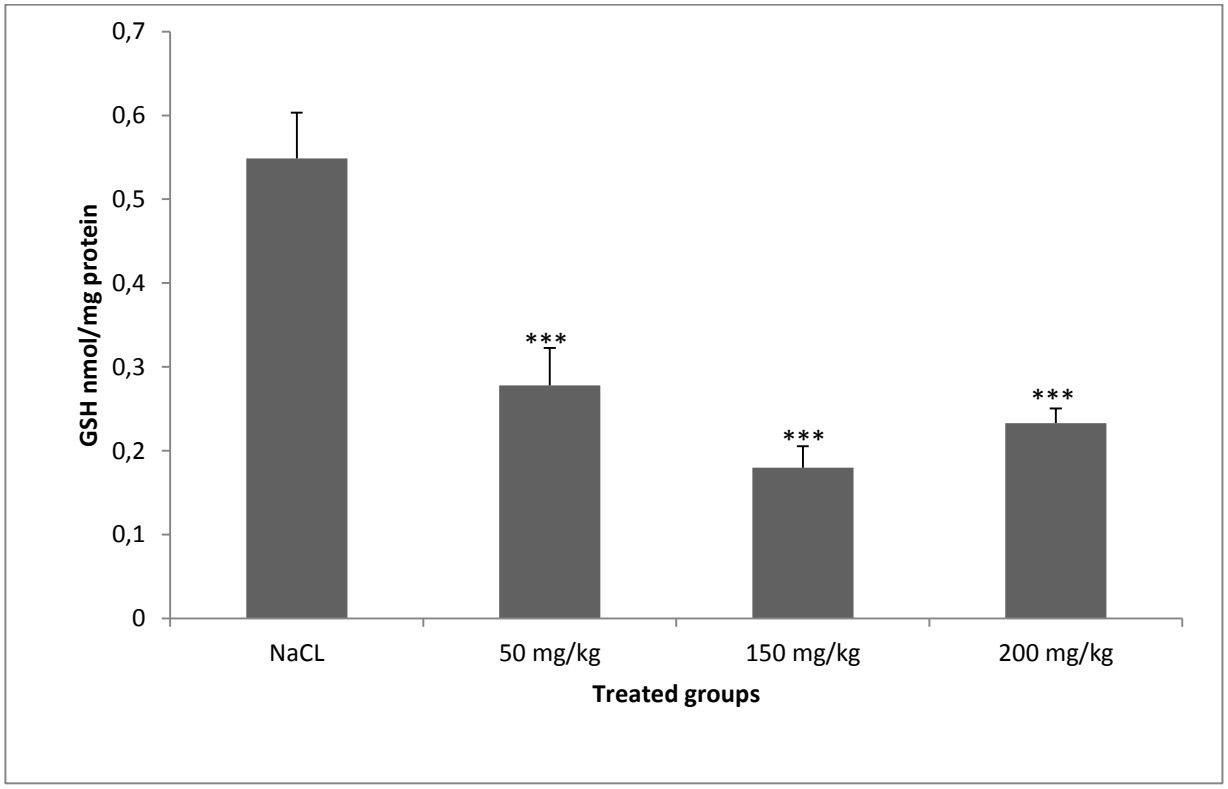

Figure 16: Effect of *S. mialhesi* extract on Glutathione liver of mice. Values are mean ± SD (n=7) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001.
GI: Control group received saline; **GII**: group received butanolic extract of *Stachys mialhesi* at dose 50mg/kg; **GIII**: group received butanolic extract of *Stachys mialhesi* at dose 150mg/kg; **GIV**: group received butnolic extract of *Stachys mialhesi* at dose 200mg/kg

III.3.2.2. Catalase activity

The present data showed that there is a very high significant difference in the means for the catalase values among groups GI, GII, GIII and GIV P= 0.000. The catalase values were decreased highly and significantly in groups GII, GIII and GIV (0.111 ± 0.62; 0.4 ± 0.31; 0.28 ± 0.48) when it is compared to the control GI (0.89 ± 0.092) P<0.05 (figure 17).

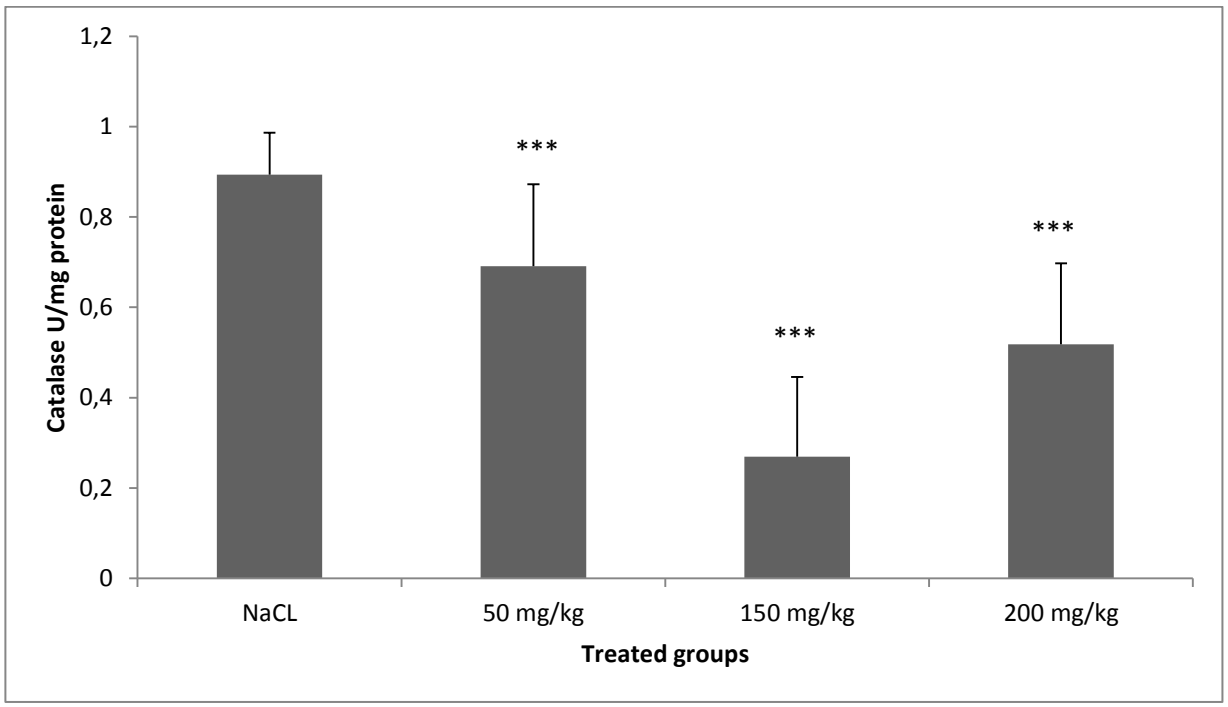

Figure 17: Effect of *Stachys mialhesi* extract on catalase activity. Values are mean ± SD (n=7) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001.
GI: Control group received saline; **GII:** group received butanolic extract of *Stachys mialhesi* at dose 50mg/kg; **GIII:** group received butanolic extract of *Stachys mialhesi* at dose 150mg/kg; **GIV:** group received butnolic extract of *Stachys mialhesi* at dose 200mg/kg

III.4. Evaluation of the anti-arthritis activity of the medicinal plants extracts

III.4.1. Evaluation of the anti-arthritis activity of *Stachys circinata*

The results evidenced a very high significant inhibition in the edema size in group SC and D (P=0.000) while, on the other hand a significant increase of the size occurred in group FF (P=0.000) in comparison to the negative control, group F (P=0.000) (Figure 18).

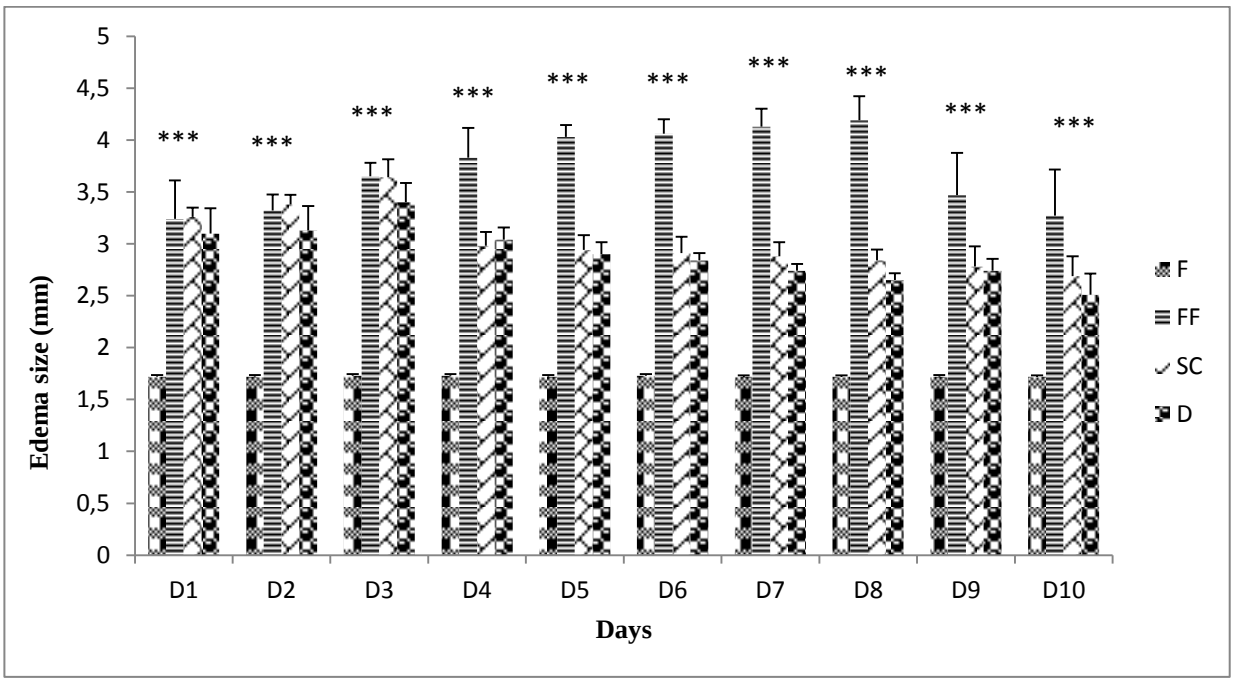

Figure 18: Anti-inflammatory effect of *S. circinata* dichloromethane extract on the Formalin induced mice hind paw edema during a 10 day experimental period. Values are mean ± SD (n=8); and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001.
Treatments: **F=** untreated (negative control); **FF=** (positive control) sole formalin treatment; **SC=** formalin + DMESC (150 mg/kg); **D=** formalin + diclofenac of sodium (10 mg/kg).

As shown in figure 18, on day following the first injection of formalin, the edema size enlarged in groups FF, SC and D (3.24 ± 0.37; 3.26 ± 0.08 and 3.10 ± 0.24 mm, respectively) and resulted significantly larger in comparison to the negative control group F (1.71 ± 0.016 mm), while, after the second injection of formalin, size increase on the 3rd day was negligible.

On the 4th d, size decreased significantly in group SC and D (2.98 ± 0.13 and 3.04 ± 0.11 mm, respectively) compared to the group FF, positive control, where the edema size increased until 5 days after the 2nd formalin injection (3.83 ± 0.28 mm).

From the 5th d until the end of the experiment, the sizes in group SC and D decreased slightly and mean values were almost identical, whereas a significant decrease occurred in the positive control group FF'

By comparing the edema size of each group during the experimental period, the size of the negative control group 'F' remained nearly stable throughout the experiment with an average of 1.7 mm. While, compared to the edema size in group FF, a decrease of about 77.6 and 79.2% was attained in treated groups SC and D, respectively.

It is noteworthy to evidence that the edema development in group FF progressed differently from group SC and D following the 2nd injection of formalin at the 3rd day. Indeed, in group FF size continue to enlarge until day 8 and then decreased till 10th d (3.27 ± 0.44 mm). On the other hand, starting from day 4, edema in group SC and D slowly underwent a similar decease evidencing clearly a comparable anti-inflammatory effect of SC extract and diclofenac of sodium.

The C-reactive protein concentration in mice blood following formalin injection decreased by supplying mice with 150 mg/L *S. circinata* (group SC) or with 10 mg/kg diclofenac of sodium (group D) and values were about 1.03 ± 0.74 and 0.68 ± 0.26 mg/L, respectively with P=0.02. Whereas, in mice belonging to the positive control (group 'FF') the mean value resulted higher (1.62 ± 0.82 mg/L) (Figure 19).

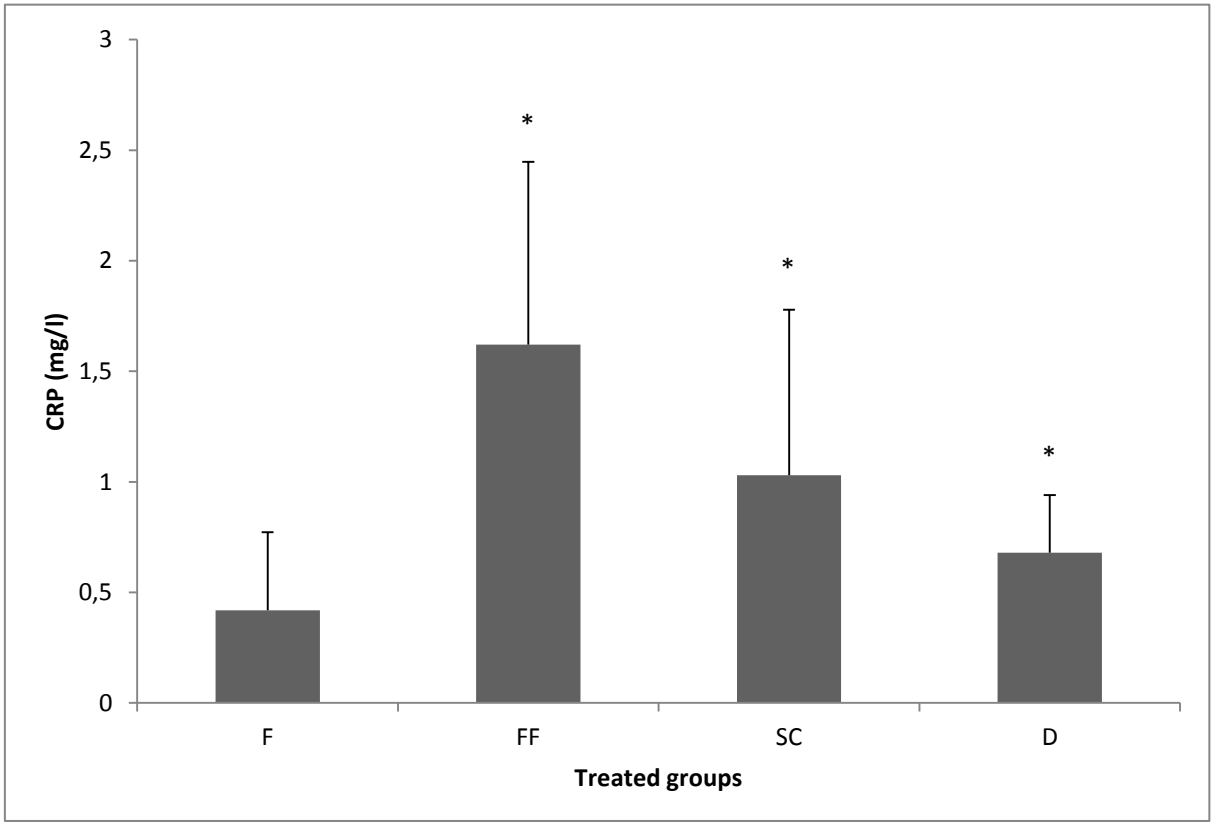

Figure 19: Effect of dichloromethane extract *Stachys circinata* on inflammatory marker (CRP blood level) during formalin induced arthritis in mice. Values are mean ± SD (n=7) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001. Group **F:** untreated (negative control); Group **FF:** (positive control) formalin inflammation; Group **SC:** formalin inflammtion+ DMESC (150 mg/kg); Group **D:** formalin inflammation+ diclofenac of sodium (10 mg/kg).

The ACCP values were also influenced by treatments and had a similar trend to those of CRP but differences resulted a significantly lower (P = 0.000) in blood of mice supplied with DMESC or diclofenac of sodium. Blood values of group SC and D were close to each other and resulted 2.14 ± 0.38 and 1.57 ± 0.56 UI/mL, respectively. The group FF (positive control) had the highest ACCP values (3.13 ± 0.96 UI/mL) (P = 0.000) while, the group F (negative control) had the lowest (0.62 ± 0.32 UI/mL) values as shown in figure 20.

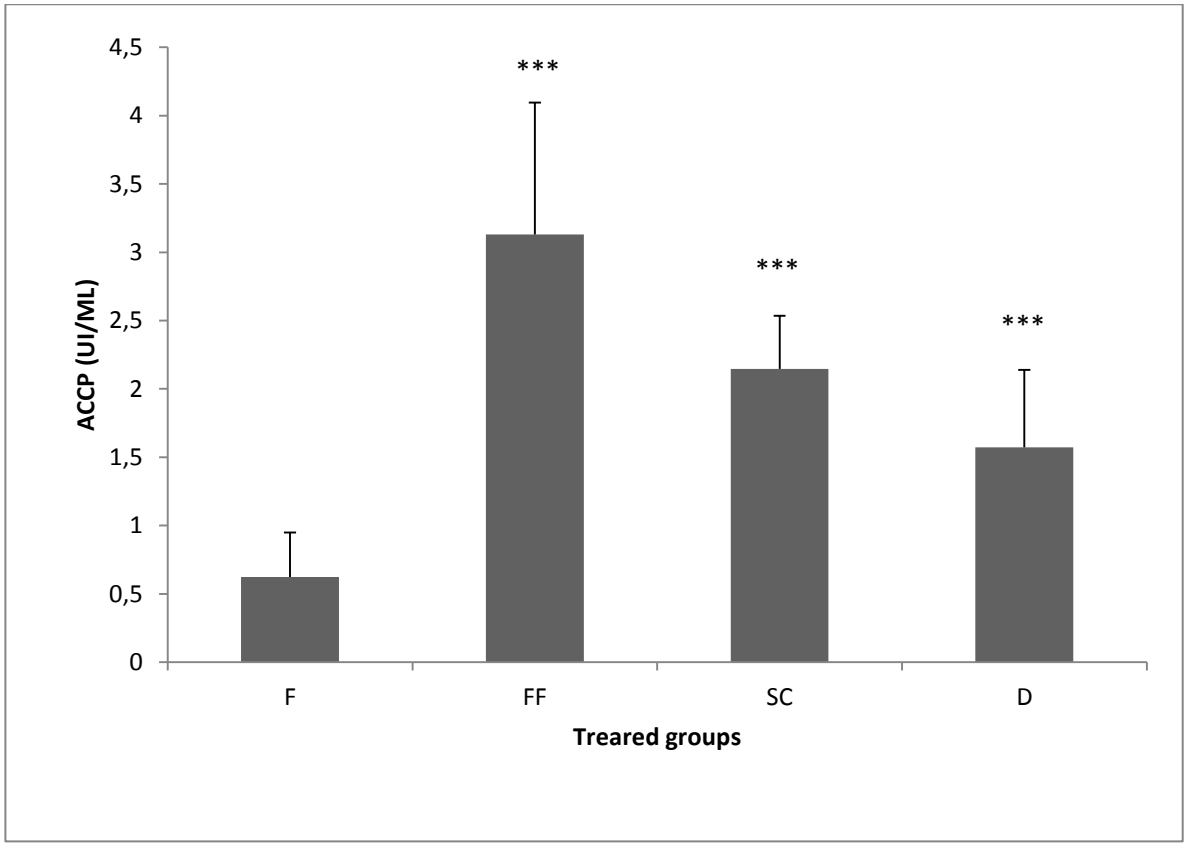

Figure 20: Effect of dichloromethane extract of *Stachys circinata* on ACCP blood level during formalin induced arthritis in mice. Values are mean ± SD (n=7) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001.

Group **F:** untreated (negative control); Group **FF:** (positive control) formalin inflammation; Group **SC:** formalin inflammtion+ DMESC (150 mg/kg); Group **D:** formalin inflammation+ diclofenac of sodium (10 mg/kg).

III.4.2. Evaluation of the anti-arthritis activity of *Stachys mialhesi*

The figure 21, showed a highly significant decrease of the edema size (P = 0.000) in the groups treated with SM and D, with a highly significant increase of the edema size (P = 0.000) in the group FF comparing to the negative control group F.

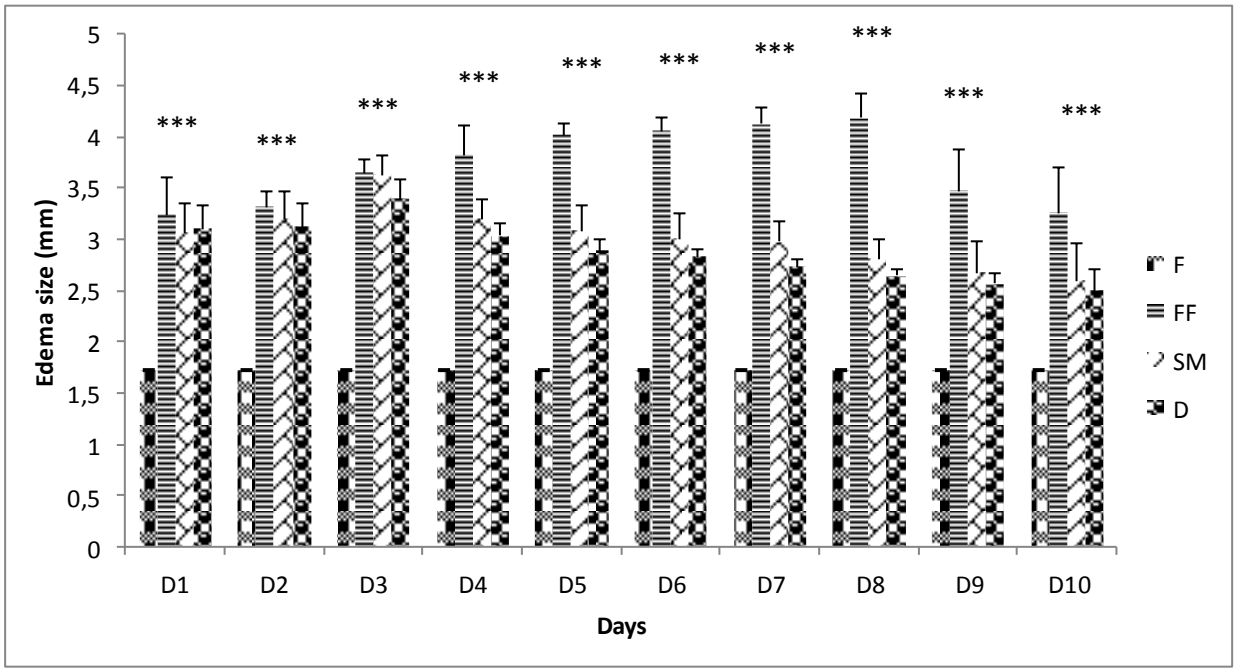

Figure 21: Anti-inflammatory effect of *S. mialhesi* butanolic extract on the Formalin induced mice hind paw edema during a 10 day experimental period. Values are mean ± SD (n=8); and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001. Treatments: **F:** untreated (negative control); **FF:** (positive control) sole formalin treatment; **SM:** formalin + BESM (150 mg/kg); **D:** formalin + diclofenac of sodium (10 mg/kg).

As shown in figure 21, there was a highly significant decrease in the edema size among groups (P = 0.000) during the experiment period of 10 days. Also a highly significant decrease of edema size (P = 0.000) in the groups treated with SM and D (Diclofenac) from the 3rd day of inflammation until the end of the study (10th day) where the values were almost identical (day 3: SM= 3.63mm, D= 3.40mm), (day 6: SM= 3.01mm, D= 2.84mm), (day 8: SM= 2.81mm, D= 2.65mm), (day 10: SM= 2.60mm, D= 2.51 mm).

Also a highly significant increase in the edema size of FF group (P = 0.000) comparing to the negative control group F from the 1st day of the treatment (1st day= 3.24 mm, 3rd day= 3.65, 6th day= 4.06mm) then it started to decrease in the 9th day (3.47mm) and the 10th day (3.27mm).

As seen in the figure 22, the results showed a high significant decrease of the CRP concentration in mice blood in *S. mialhesi* group (SM) and diclofenac of sodium group (D) (P=0.005). The mean value of CRP in group treated with SM is the lowest and the best result 0.498 ± 0.33 mg/L when it's compared to group D 1.03 ± 0.74 mg/L. However the results

showed a high significant increase of the CRP values in the group FF (positive control) (1.62 ± 0.82 mg/L) with P = 0.007.

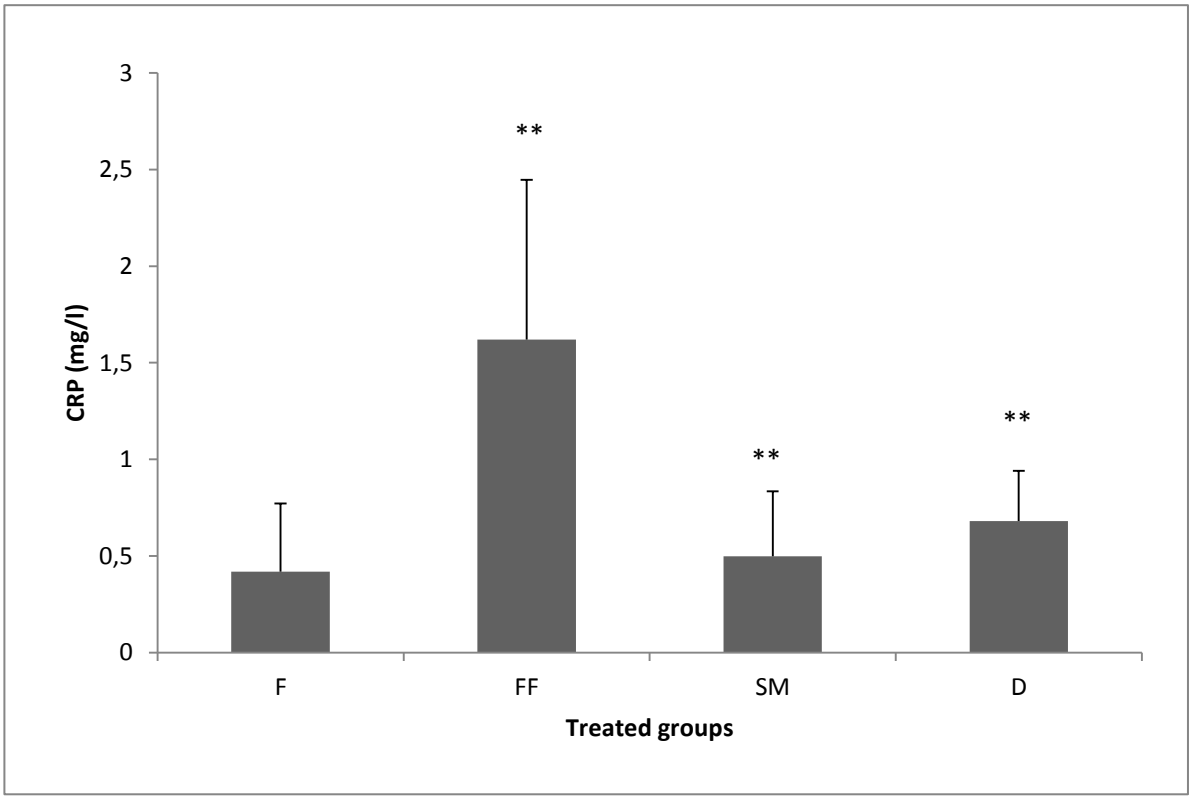

Figure 22: Effect of *Stachys mialhesi* butanolic extract on inflammatory marker (CRP blood level) during formalin induced arthritis in mice. Values are mean ± SD (n=7) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001. Group **F:** untreated (negative control); Group **FF:** (positive control) formalin inflammation; Group **SM:** formalin inflammtion+ BESM (150 mg/kg); Group **D:** formalin inflammation+ diclofenac of sodium (10 mg/kg).

As shown in figure 23, there was a highly significant decrease of the anti-CCP antibodies values in the groups treated SM and D (P = 0.000) with approximately similar results between the two groups (SM= 1.96±0.382 UI/ML), (D= 1.57±0.566 UI/ML) and a highly significant increase of the Anti-CCP antibodies values (P = 0.000) in the group FF (3.13±0.964 UI/ML) in comparison with the negative control group F (0.624±0.324 UI/ML).

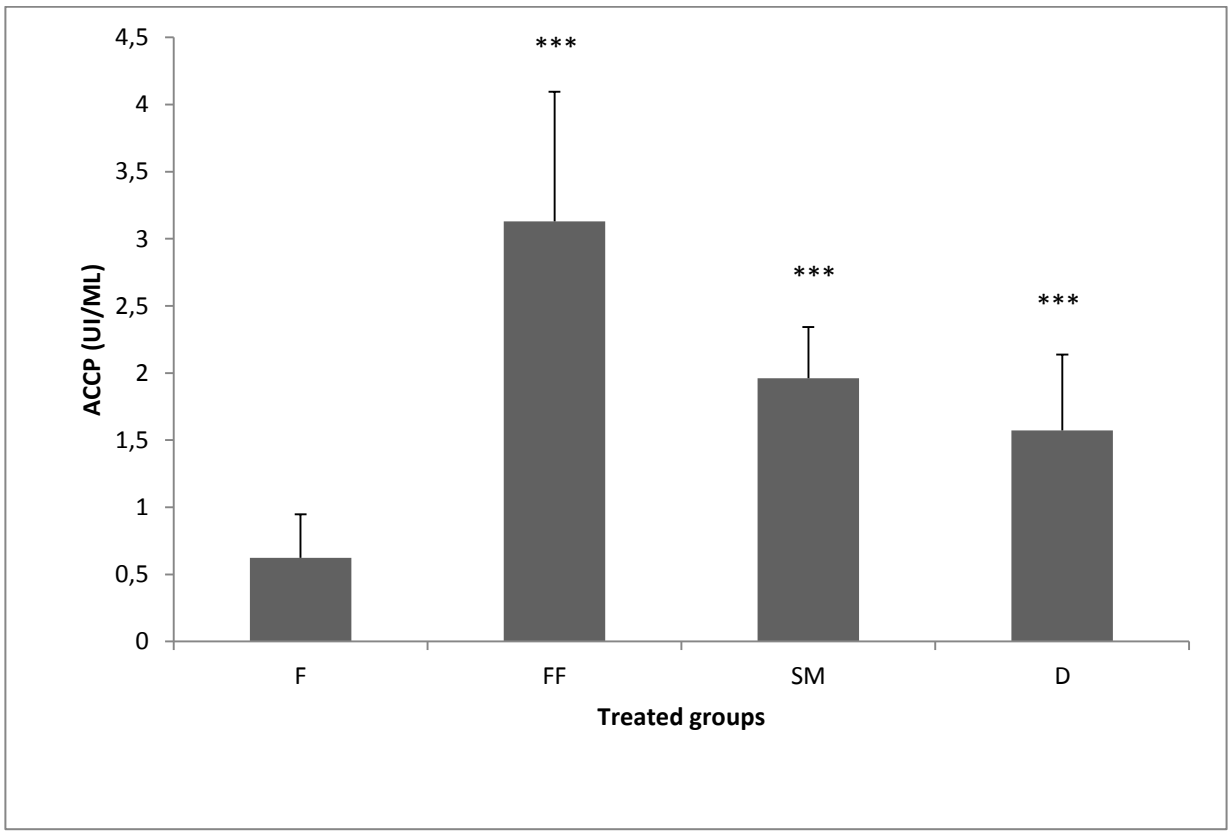

Figure 23: Effect of *Stachys mialhesi* butanolic extract on ACCP blood level during formalin induced arthritis in mice. Values are mean ± SD (n=7) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001.

Group **F:** untreated (negative control); Group **FF:** (positive control) formalin inflammation; Group **SM:** formalin inflammtion+BESM (150 mg/kg); Group **D:** formalin inflammation+ diclofenac of sodium (10 mg/kg).

III.5. Histological study of mice ankle joint

Histological assessment of the ankle joint showed that subplantar injection of formalin has induced cartilage damage and bone erosion, however group F showed intact hyaline cartilage, absence of infiltrate in the synovium and normal joint space (figure 24, 25).

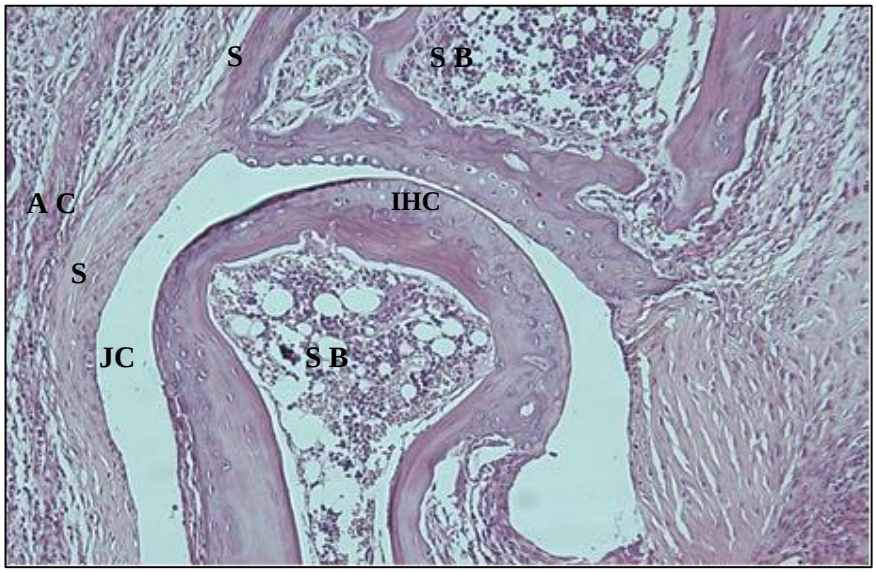

Figure 24: Histology of mouse normal joint (F), H.E staining (X100).

AC. Articular capsule **S.** Synovium **JC. Joint cavity**

IHC. Intact hyaline cartilage **SB.** spongy Bone

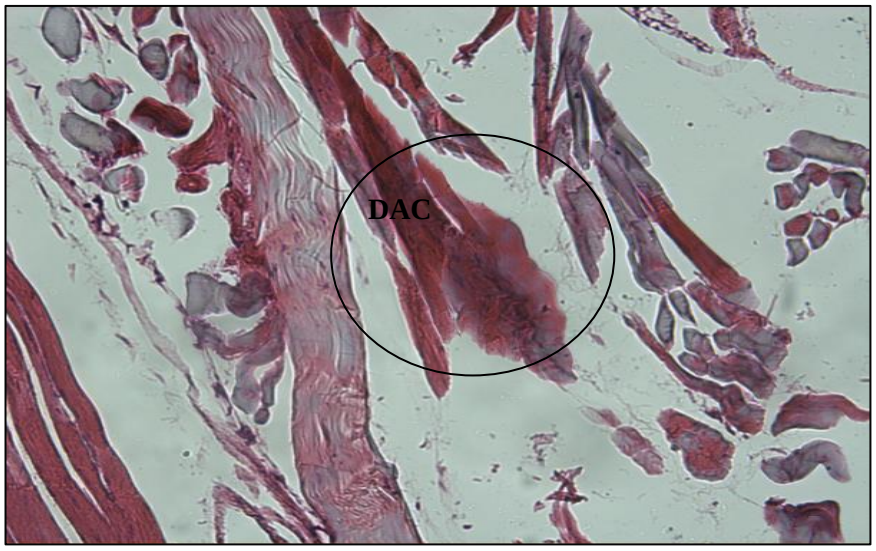

Figure 25: Histology of ankle joint of formalin induced arthritis mouse (FF), H.E staining (X100)**.**

DAC Degeneration of articular cartilage.

Histological investigation of the ankle joint of mice treated with *S. mialhesi* and *S. circinata* (150 mg/kg), showed intact hyaline cartilage and normal synovium, nevertheless we have observed in the group treated with *S. circinata* degeneration on superficial zone of articular cartilage in just one position of the articular joint (figures 27 and 28), while treatment with diclofenac (10 mg/kg) in the group (D) showed normal connective tissue of ankle joint with the presence of lower degree of edema (figure 26).

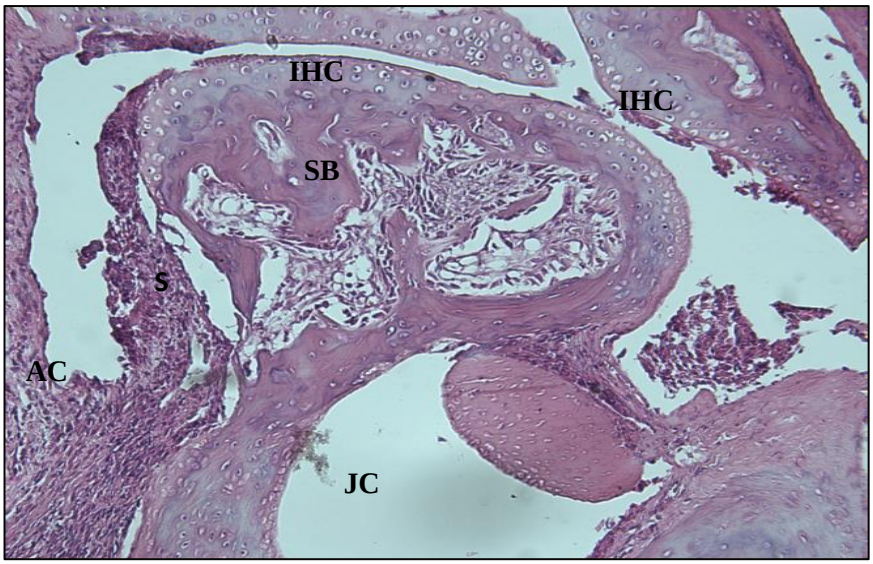

Figure 26: Histology of ankle joint of diclofenac treated group (D), H.E staining(X100)

AC. Articular capsule **S.** Synovium **JC**. Joint cavity

IHC. Intact hyaline cartilage **SB.** spongy Bone

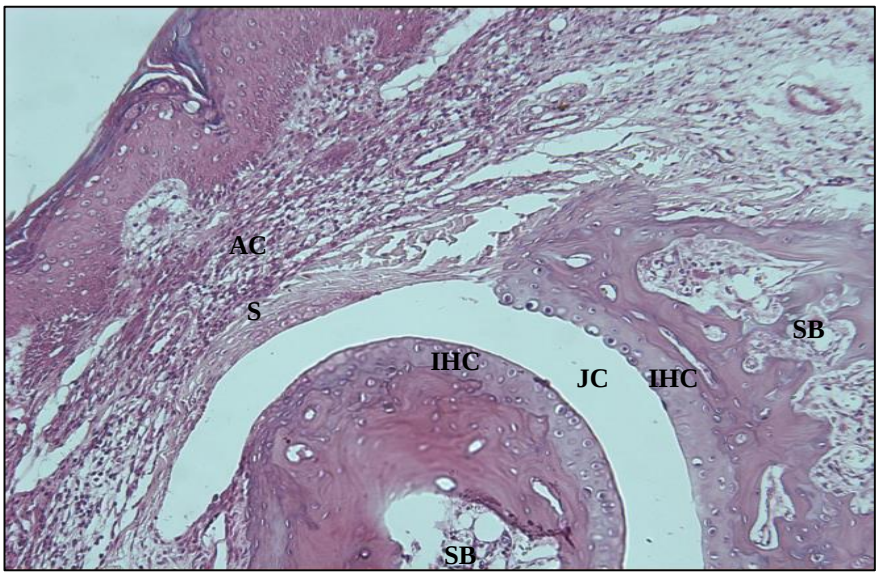

Figure 27: Histology of ankle joint of *S .mialhesi* treated group (SM),H.E staining(X100). **IHC.** Intact hyaline cartilage **SB.** spongy Bone **AC.** Articular capsule **S.** Synovium **JC.** Joint cavity

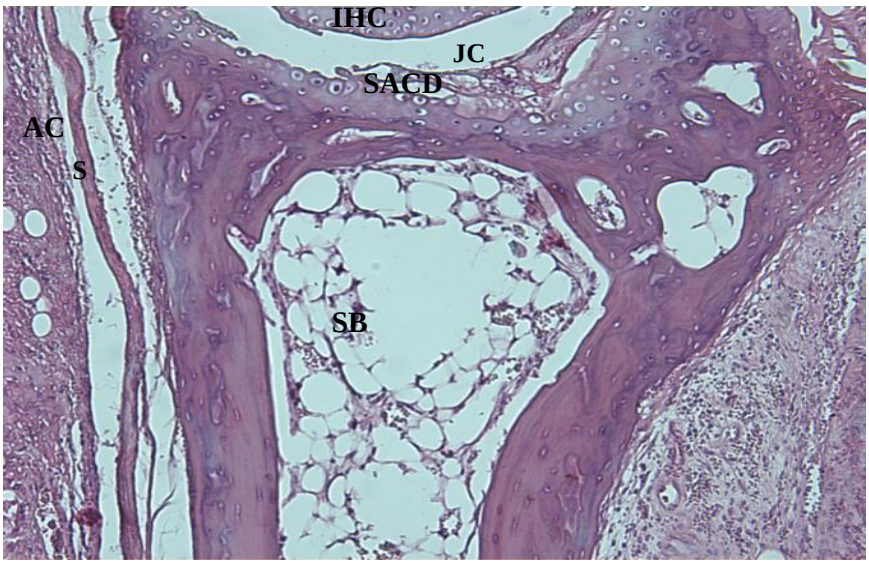

Figure 28: Histology of ankle joint of *S. circinata* treated group (SC), H.E staining(X100) **AC.** Articular capsule **S.** Synovium **JC.** Joint cavity **IHC.** Intact hyaline cartilage **SACD.** Superficial zone of articular cartilage degeneration **SB.** spongy Bone

III.6. Evaluation of anti-proliferative activity of the plants extracts

III.6.1. Effect of *Stachys mialhesi* on Hepatocarcinoma cell line (HepG2)

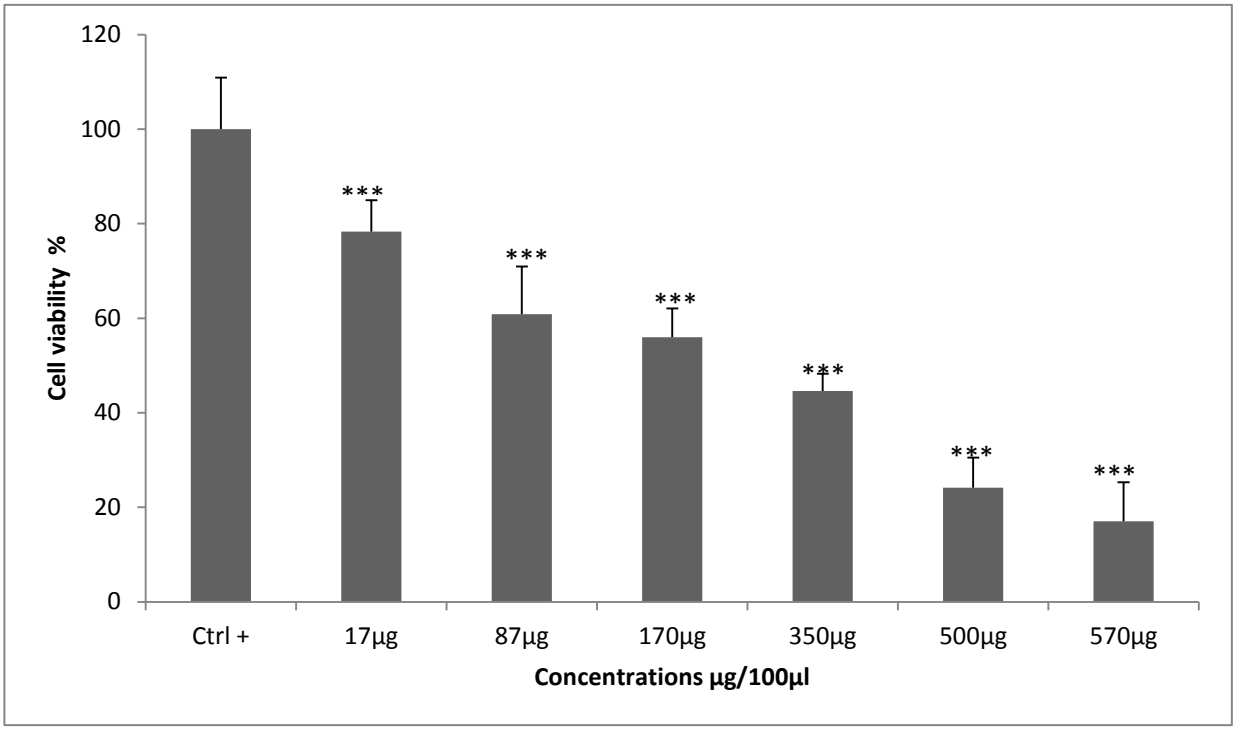

Figure 29: Dose-dependent cytotoxic activity of S. *mialhesi* extract on Hepatocarcinoma cell line (HepG2). Cells were treated with increasing doses of the extract."Ctrl" [cell +medium]. Cells were incubated with the extracts for 24 h at 37 °C, and subjected to MTT assays to measure % cell viability. The data were obtained from three independent assays using three wells for each assay.

The figure 29 showed a strong reduction of cell viability compared to the control (P=0.000). The results showed that when the *Stachys mialhesi* extract concentration added to the cell culture was increased (17, 87, 350 and 570 µg/100µl), the HepG2 cell viability decreased (77.98, 61.46, 44.72, 17.06%, respectively).

III.6.2. Effect of *Stachys circinata* on Hepatocarcinoma cell line (HepG2)

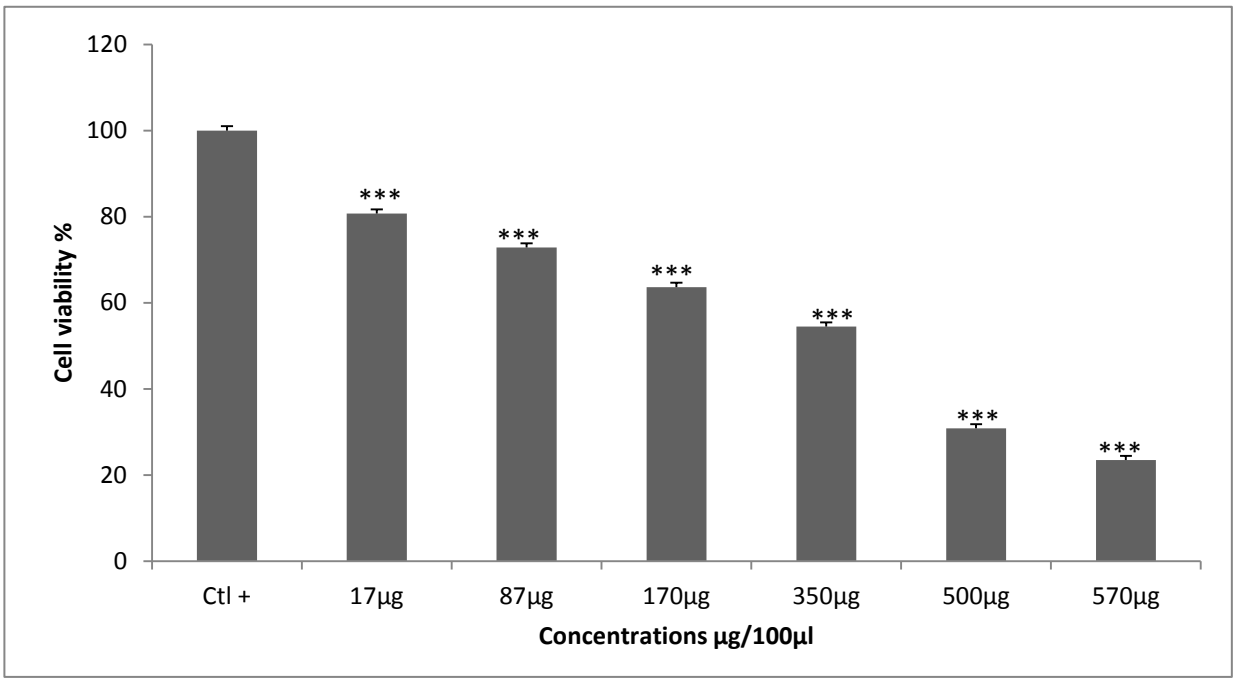

Figure 30: Dose-dependent cytotoxic activity of S. *circinata* extract on Hepatocarcinoma cell line (HepG2). Cells were treated with increasing doses of the extract."Ctrl" [cell +medium]. Cells were incubated with the extracts for 24 h at 37 °C, and subjected to MTT assays to measure % cell viability. The data were obtained from three independent assays using three wells for each assay.

The results in figure 30 concerning the effect of *Stachys circinata* extract on the proliferation of HepG2, showed a very high significant decrease in the cancer cells viability (P=0.000) with the high concentration of the extract of *S. circinata* µg/100µl 570 with the percentage of 23.48%. The decrease in cells viability was dose or concentration dependent more the concentration increased (17, 170, 350 and 570 µg/100µl) more the viability decreased (80.72, 63.66, 54.49 and 23.48 %, respectively).

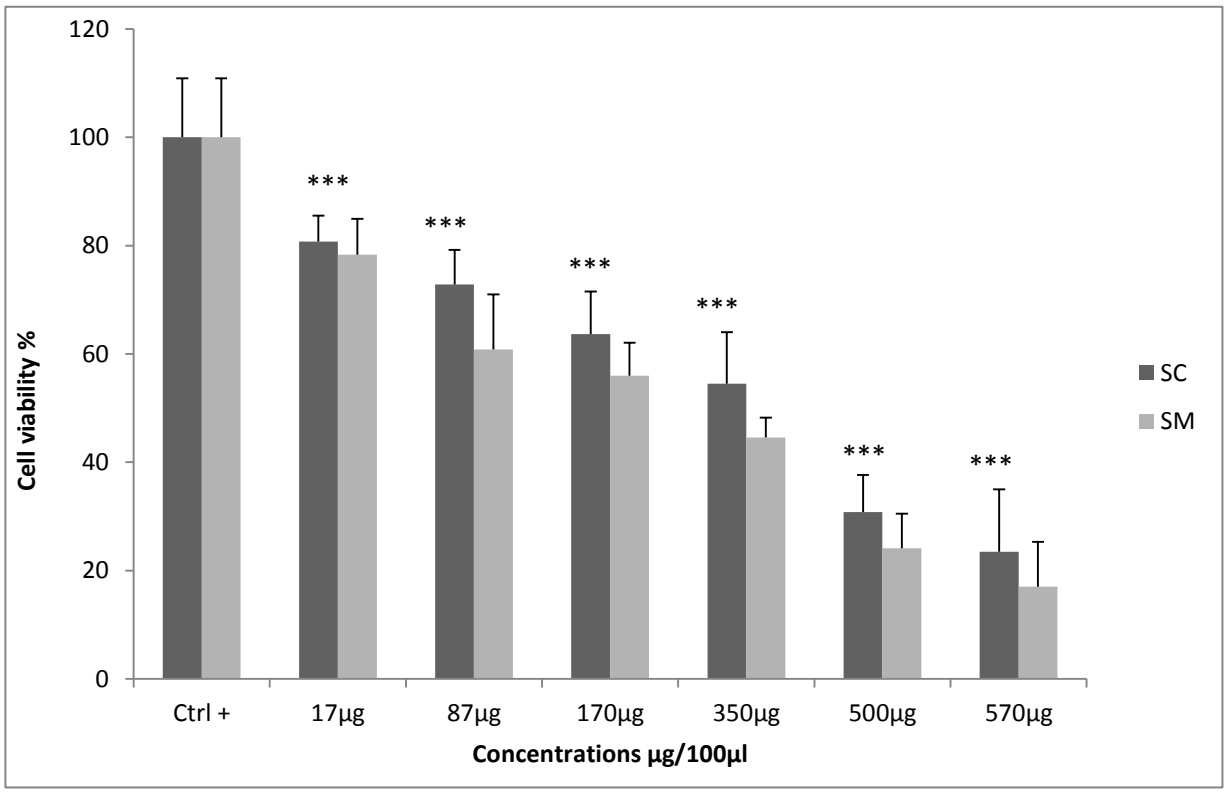

Figure 31: Comparison between the cytotoxic activity of S. *mialhesi* and *S. circinata* extracts on Hepatocarcinoma cell line (HepG2).

Cells were treated with increasing doses of the extract."Ctrl" [cell +medium]. Cells were incubated with the extracts for 24 h at 37 °C, and subjected to MTT assays to measure % cell viability. The data were obtained from three independent assays using three wells for each assay.

In figure 31, *Stachys mialhesi* extract showed a significantly higher viability-reducing effect than *Stachys circinata* extract in Hepatocarcinoma cell line (P=0.000) (17µg: SM=77.89%, SC=80.72%, 170µg: SM=55.96%, SC=63.66%, 570µg: SM=17.06%, SC=23.48%).

III.6.3. Effect of *Stachys mialhesi* on Breast cancer cells (MCF7)

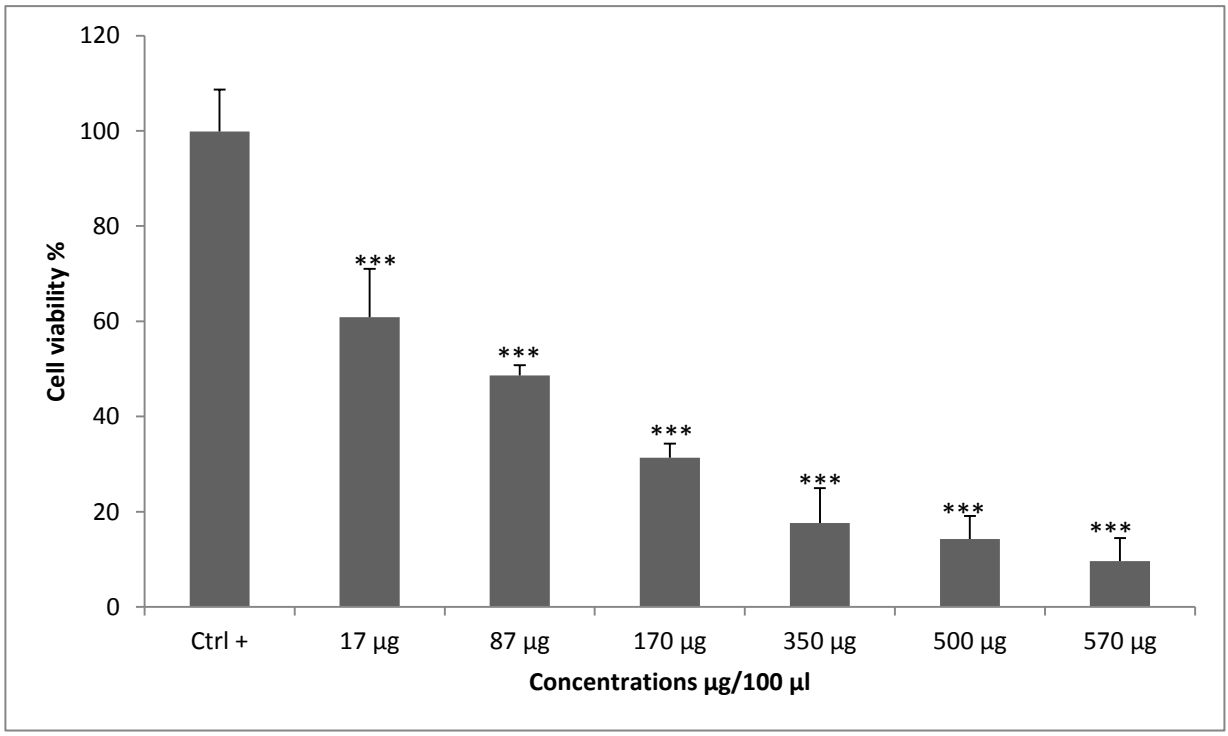

Figure 32: Dose-dependent cytotoxic activity of S. *mialhesi* extract on Human breast cancer cell line (MCF7).

Cells were treated with increasing doses of the extract."Ctrl" [cell +medium]. Cells were incubated with the extracts for 24 h at 37 °C, and subjected to MTT assays to measure % cell viability. The data were obtained from three independent assays using three wells for each assay

The figure 32 showed that the extract *Stachys mialhesi* added to the cell culture could decrease the cell viability to 9.62% at the concentration of 570 µg/100µl compared to the control (P=0.000). The MCF7 cell viability significantly decreased in a concentration-dependent manner.

III.6.4. Effect of *Stachys circinata* on Breast cancer cells (MCF7)

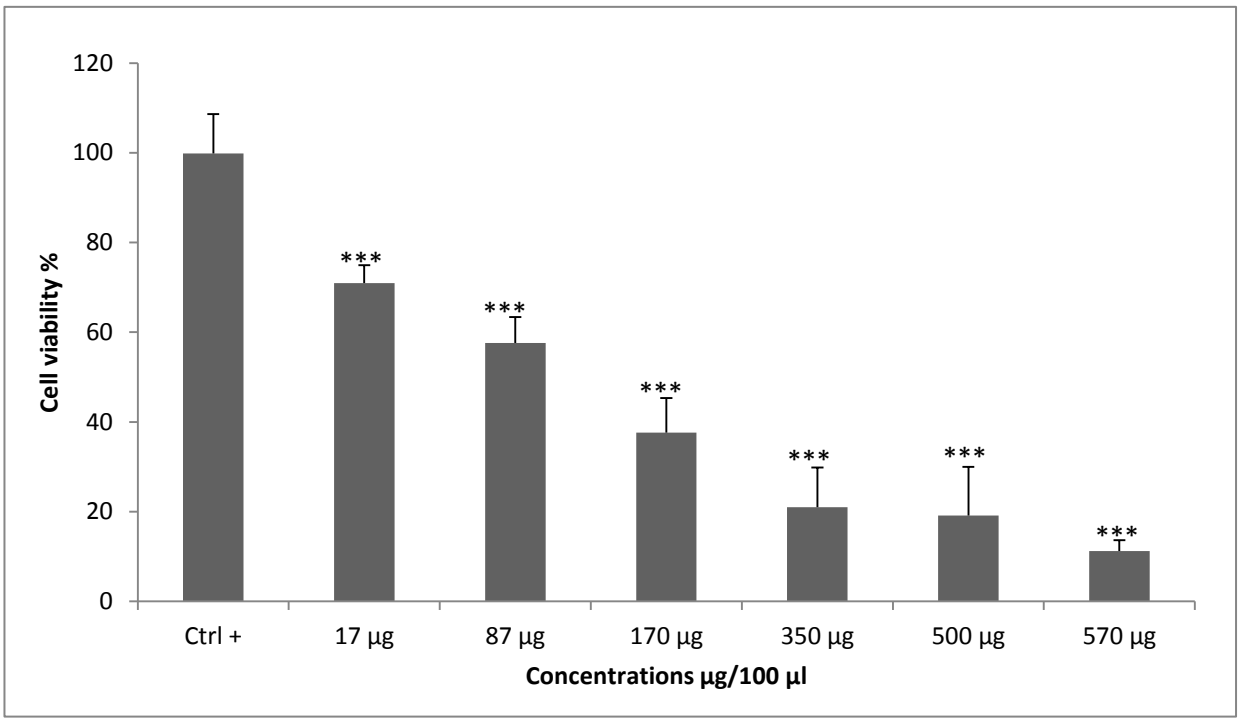

Figure 33: Dose-dependent cytotoxic activity of S. *circinata* extract on Human breast cancer cell line (MCF7).

Cells were treated with increasing doses of the extract."Ctrl" [cell +medium]. Each cell type was incubated with the extracts for 24 h at 37 °C, and subjected to MTT assays to measure % cell viability. The data were obtained from three independent assays using three wells for each assay.

The results in figure 33 concerning the effect of *Stachys circinata* extract on the proliferation of MCF7, showed a high decrease in the cancer cells viability (P=0.000). As can be seen from the proliferation data, the proliferation rate decreased with the increase of treatment dose, at 17, 87, 350 and 570 µg/100µl of *S. circinata*, the percentage of cell viability was 70.94, 57.61, 21.01 and 11.23% respectively.

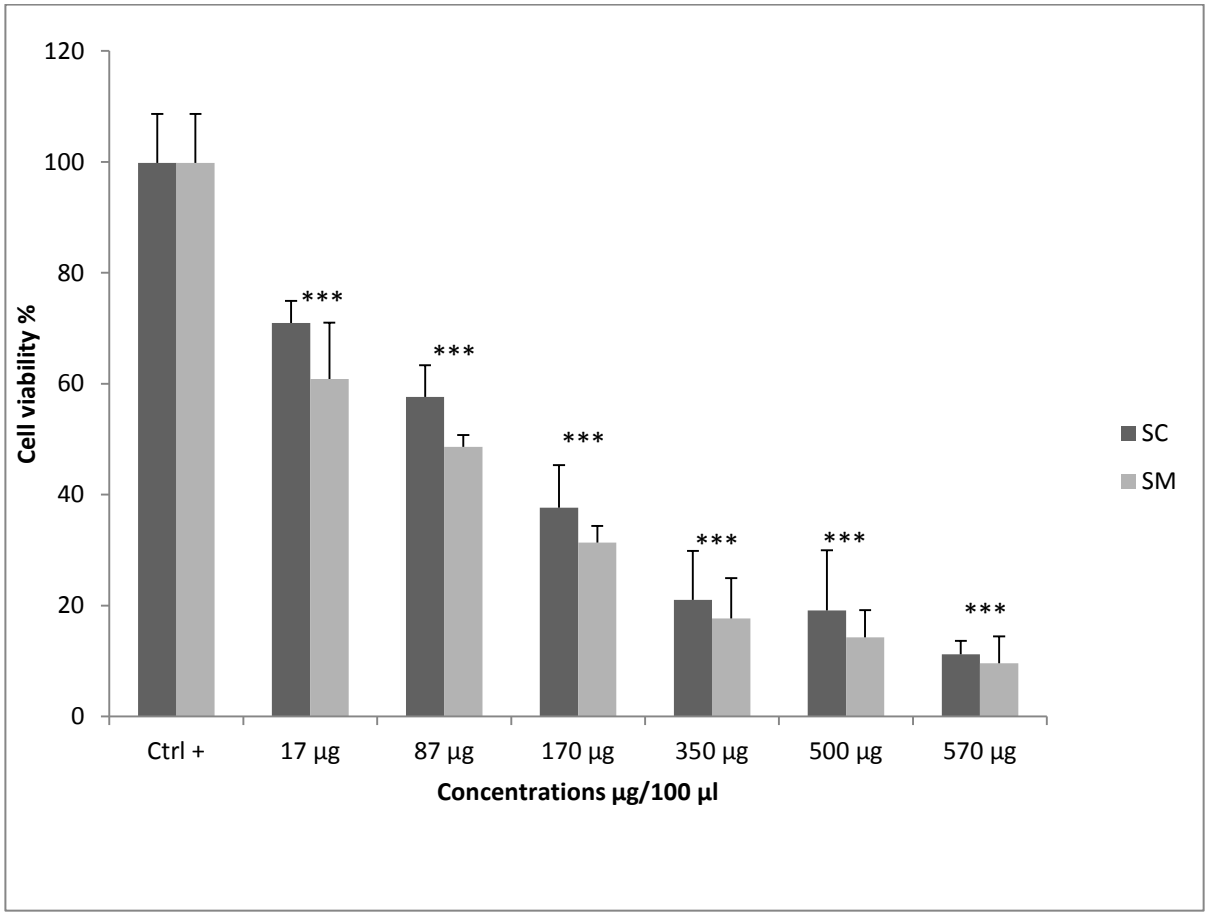

Figure 34: Comparison between the cytotoxic activity of S. *mialhesi* and *S. circinata* extracts on Human breast cancer cell line (MCF7).

Cells were treated with increasing doses of the extract."Ctrl" [cell +medium]. Cells were incubated with the extracts for 24 h at 37 °C, and subjected to MTT assays to measure % cell viability. The data were obtained from three independent assays using three wells for each assay.

In figure 34, *Stachys mialhesi* extract showed a significantly higher viability-reducing effect than *Stachys circinata* extract in MCF7 (P=0.000) (17µg: SM=60.87%, SC=70.94%, 87µg: SM=48.60%, SC=57.61%, 170µg=31.38%, 350µg: SM=17.65%, SC=20.01%, 570µg: SM=9.62%, SC=11.23%).

III.6.5. Effect of *Stachys mialhesi* on Phaeochromocytoma cell line (PC12)

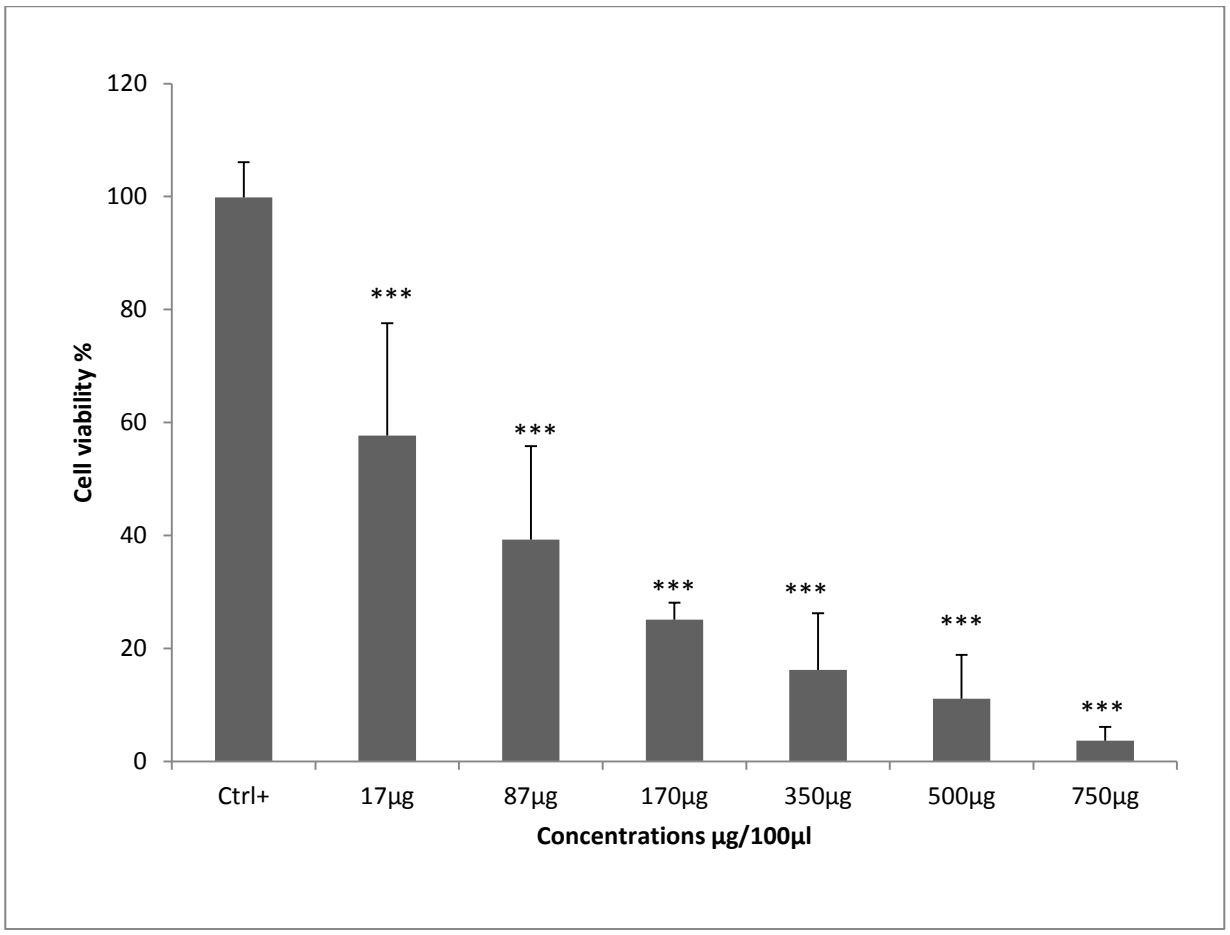

Figure 35: Dose-dependent cytotoxic activity of *S. mialhesi* extract on phaeochromocytoma cell line (PC12).

Cells were treated with increasing doses of the extract."Ctrl" [cell +medium]. Each cell type was incubated with the extracts for 24 h at 37 °C, and subjected to MTT assays to measure % cell viability. The data were obtained from three independent assays using three wells for each assay.

The figure 35 showed that a strong reduction of cell viability compared to the control (P=0.000). The extract of *Stachys mialhesi* (570 µg/100µl) added to the cell culture could decrease the cell viability to 3.63%.We can observed that with the increase of treatment concentration, the PC 12 cell viability was decreased

III.6.6. Effect of *Stachys circinata* on Phaeochromocytoma cell line (PC12)

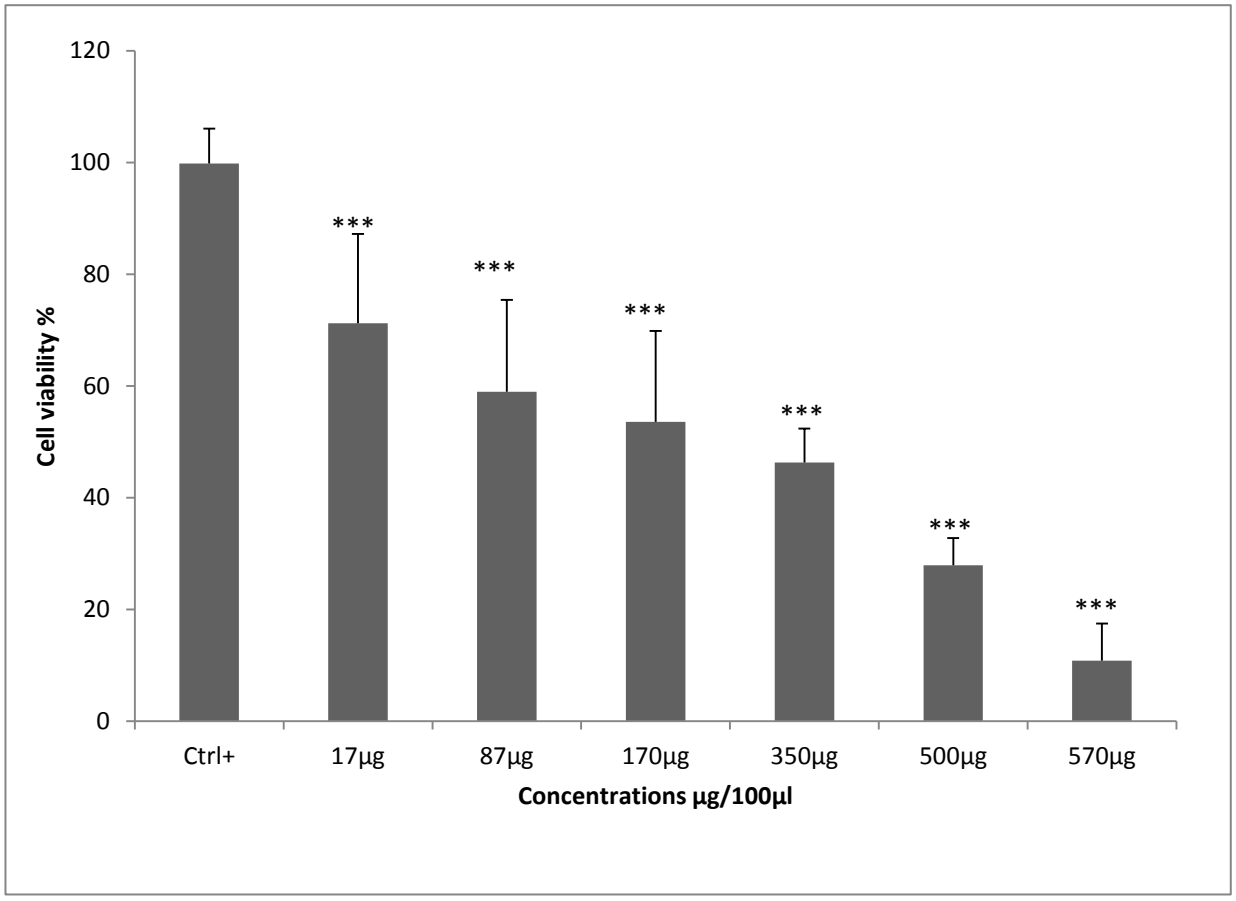

Figure 36: Dose-dependent cytotoxic activity of *S. circinata* extract on Phaeochromocytoma cell line (PC12).

Cells were treated with increasing doses of the extract."Ctrl" [cell +medium]. Cells were incubated with the extracts for 24 h at 37 °C, and subjected to MTT assays to measure % cell viability. The data were obtained from three independent assays using three wells for each assay.

The figure 36 showed strong decrease in the proliferation and the viability of the PC12 (P=0.000), the extract at dose dependent reduction in cell viability was found.

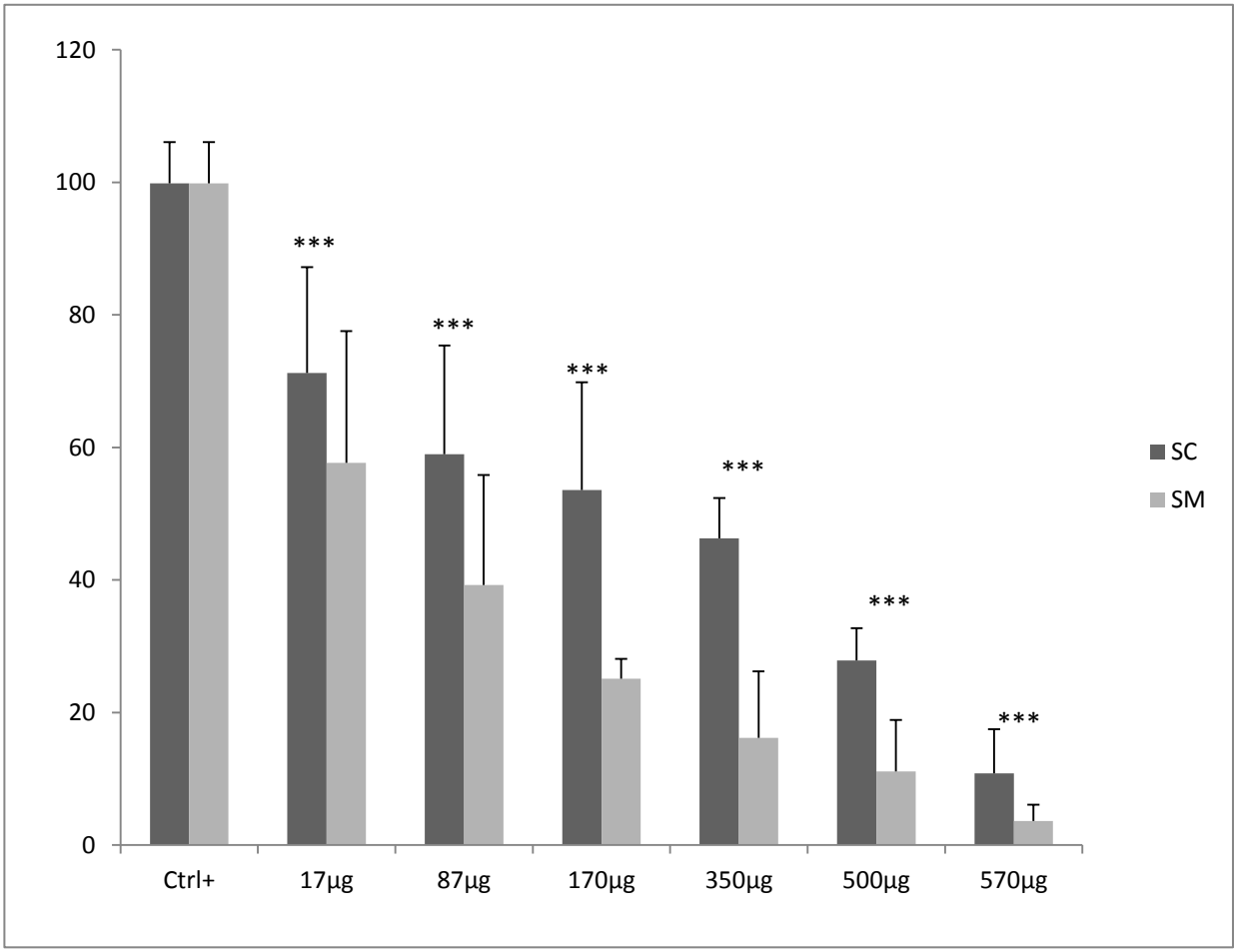

Figure 37: Comparison between the cytotoxic activity of S. *mialhesi* and *S. circinata* extracts on Phaeochromocytoma cell line (PC12).
Cells were treated with increasing doses of the extract."Ctrl" [cell +medium]. Cells were incubated with the extracts for 24 h at 37 °C, and subjected to MTT assays to measure % cell viability. The data were obtained from three independent assays using three wells for each assay.

In figure 37, *S. mialhesi* extract showed a significantly higher viability-reducing effect than *S. circinata* extract in PC12 (P=0.000) (17µg: SM=57.65%, SC=71.23%, 87µg: SM=39.26%, SC=58.97%, 350µg: SM=16.2%, SC=46.27%, 570µg: SM=3.63%, SC=10.80%).

III.6.7. Effect of *Stachys mialhesi* on normal Human Umbilical Vein Endothelial Cell (HUVEC)

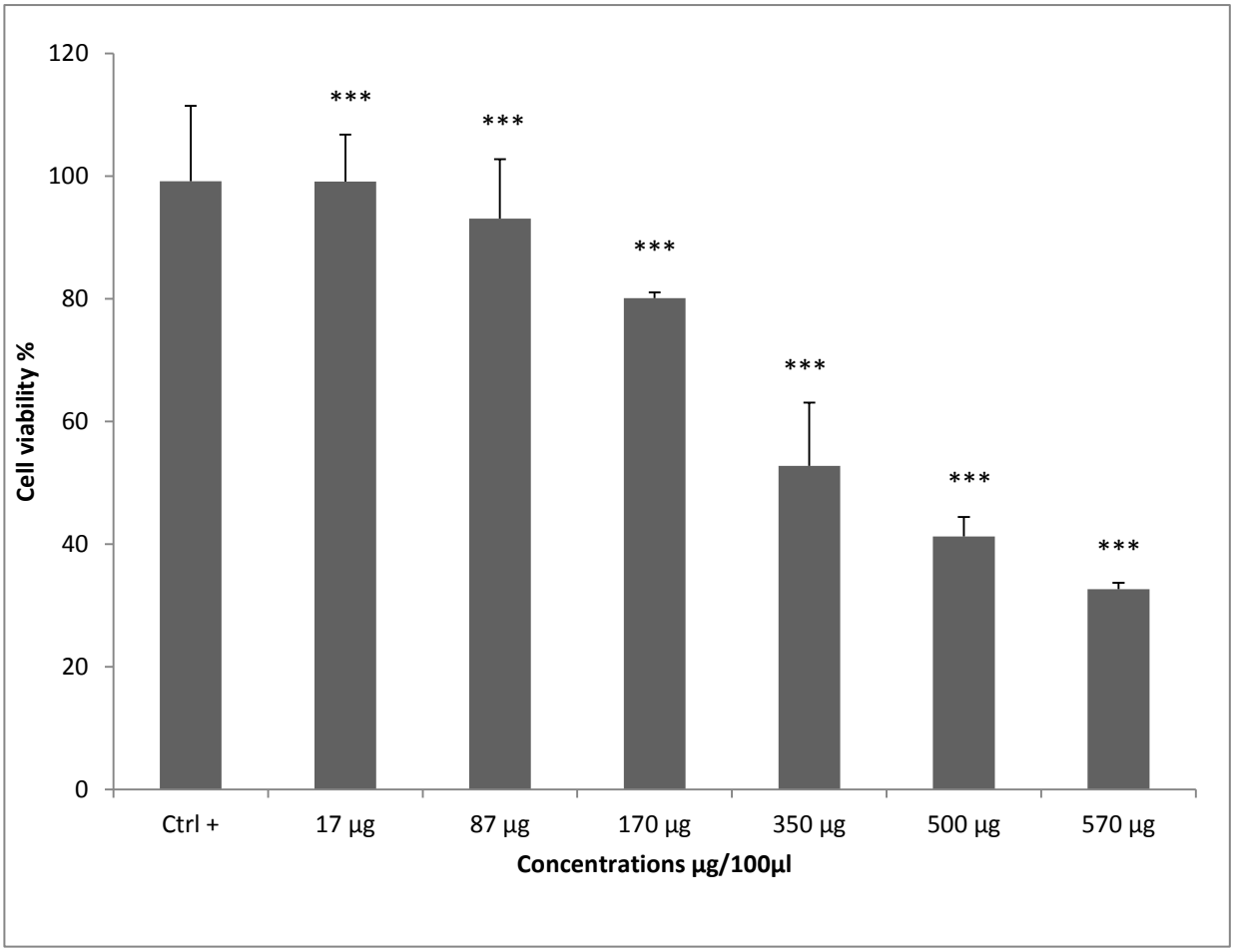

Figure 38: Dose-dependent cytotoxic activity of *S. mialhesi* extract on normal Human Umbilical Vein Endothelial Cell (HUVEC).

Cells were treated with increasing doses of the extract."Ctrl" [cell +medium]. Cells were incubated with the extracts for 24 h at 37 °C, and subjected to MTT assays to measure % cell viability. The data were obtained from three independent assays using three wells for each assay

The figure 38 showed a highly significant difference in cells viability (P=0.000). There was dose or concentration dependent decrease in cells viability more the concentration increased more the viability decreased.

III.6.7. Effect of *Stachys circinata* on normal Human Umbilical Vein Endothelial Cell (HUVEC)

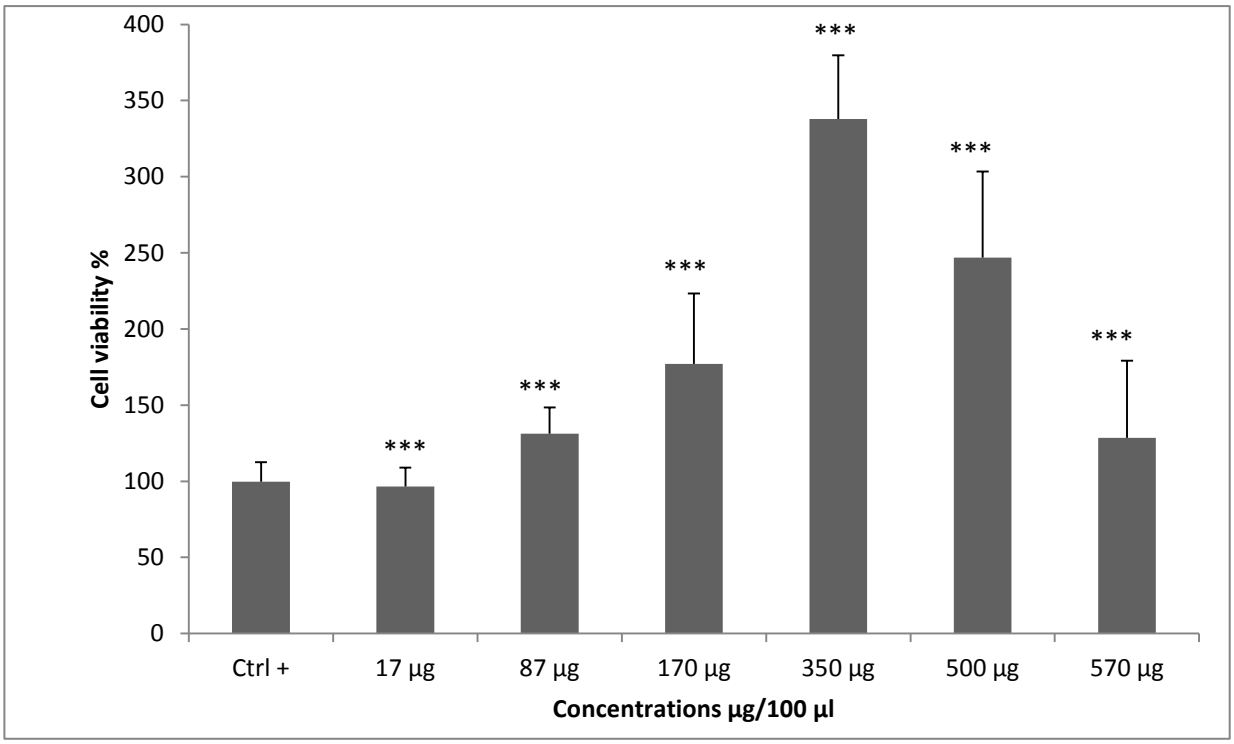

Figure 39: Dose-dependent cytotoxic activity of *S. circinata* extract on normal Human Umbilical Vein Endothelial Cell (HUVEC).
Cells were treated with increasing doses of the extract."Ctrl" [cell +medium]. Cells were incubated with the extracts for 24 h at 37 °C, and subjected to MTT assays to measure % cell viability. The data were obtained from three independent assays using three wells for each assay

As shown in Figure 39, *Stachys circinata* extract promoted HUVEC cell proliferation in a dose-dependent manner. The maximum increase of cell viability induced by *S. circinata* was increased significantly at 350 µg/ 100µl (P=0.000), compared to control.

III.7. Evaluation of *in vitro* antioxidant activities of plant extracts

III.7.1. *In vitro* antioxidant activities of *Stachys mialhesi*

III.7.1.1. Cellular catalase activity

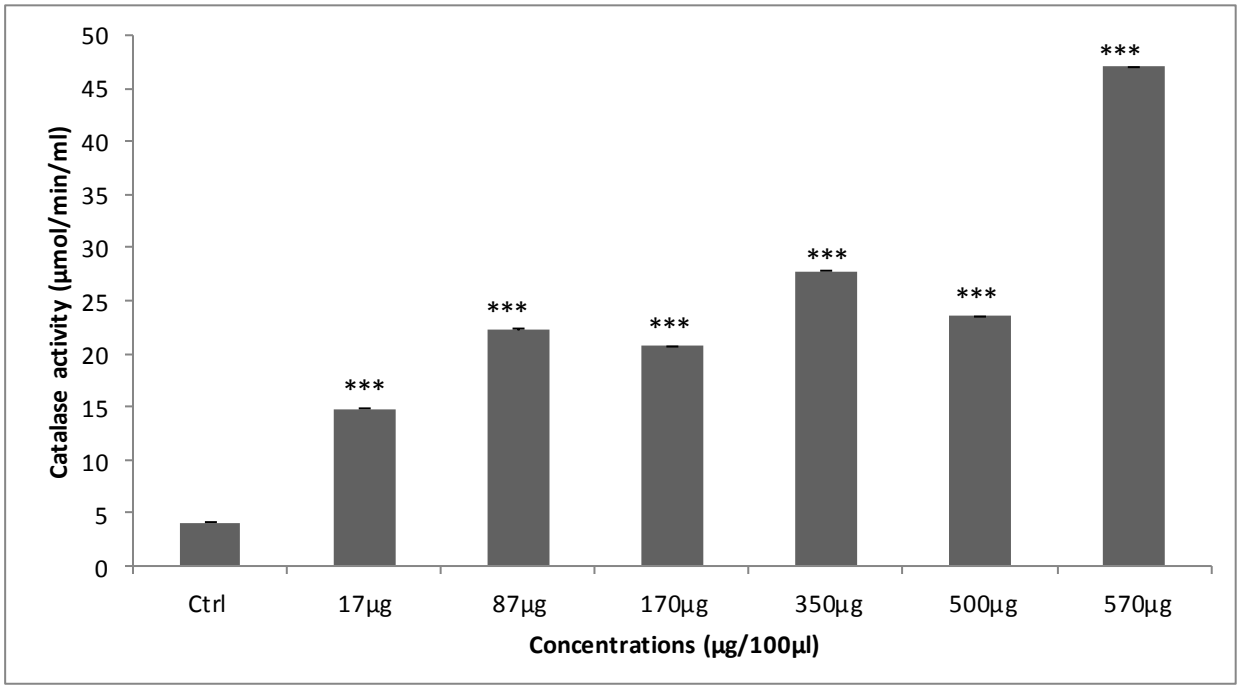

Figure 40: Effect of *S. mialhesi* on cellular catalase activity (HepG2). Data are expressed as mean ± SD (n = 3).

The figure 40 showed that treating cells with *S. mialhesi* extracts enhanced catalase activity compared to control (P=0.000) however, did not differ significantly ($p > 0.05$) in cells treated with different concentrations of *S. mialhesi*.

III.7.1.2. Cellular GSH values

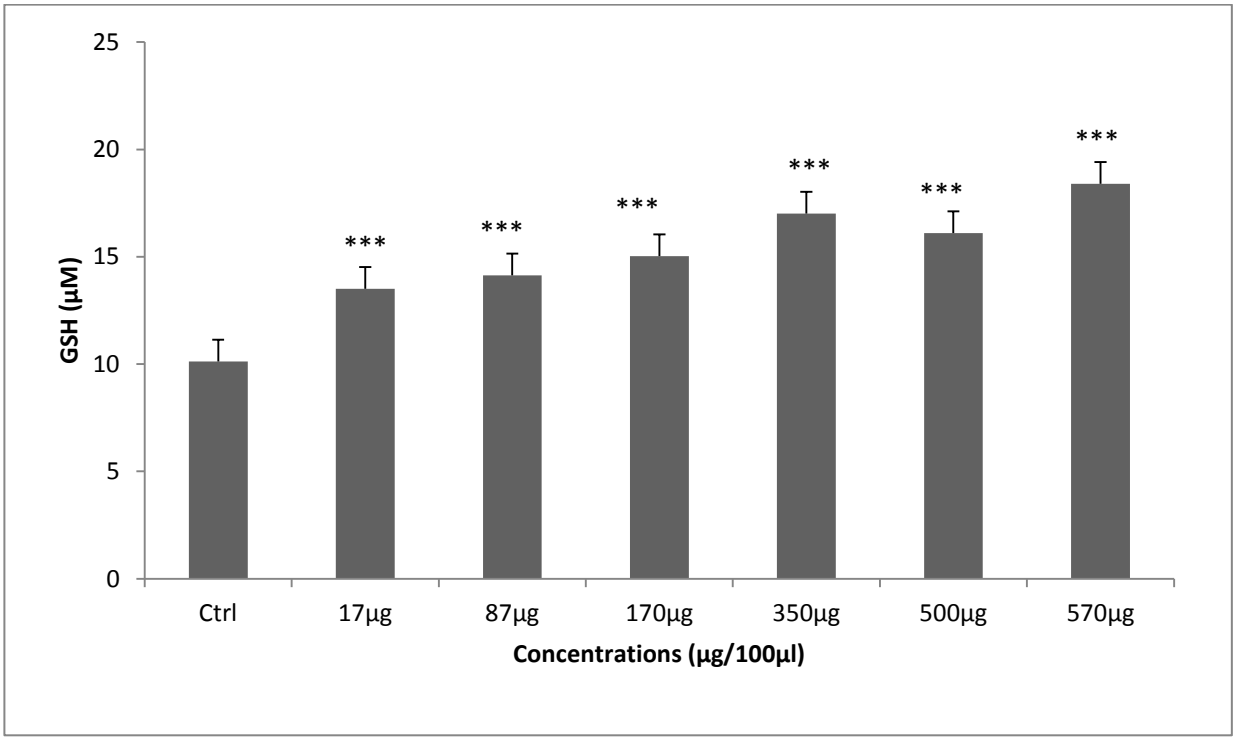

Figure 41: Effect of *S. mialhesi* on cellular GSH levels (HepG2). Data are expressed as mean ± SD (n = 3).

The figure 41 showed a high significant increase (p = 0.000) of GSH levels in the cells treated with *S. mialhesi* extract compared to control. GSH levels were significantly higher in cells treated with 570 µg *S. mialhesi* extract than the other groups.

III.7.2. *In vitro* antioxidant activities of *Stachys circinata*

III.7.2.1. Cellular catalase activity

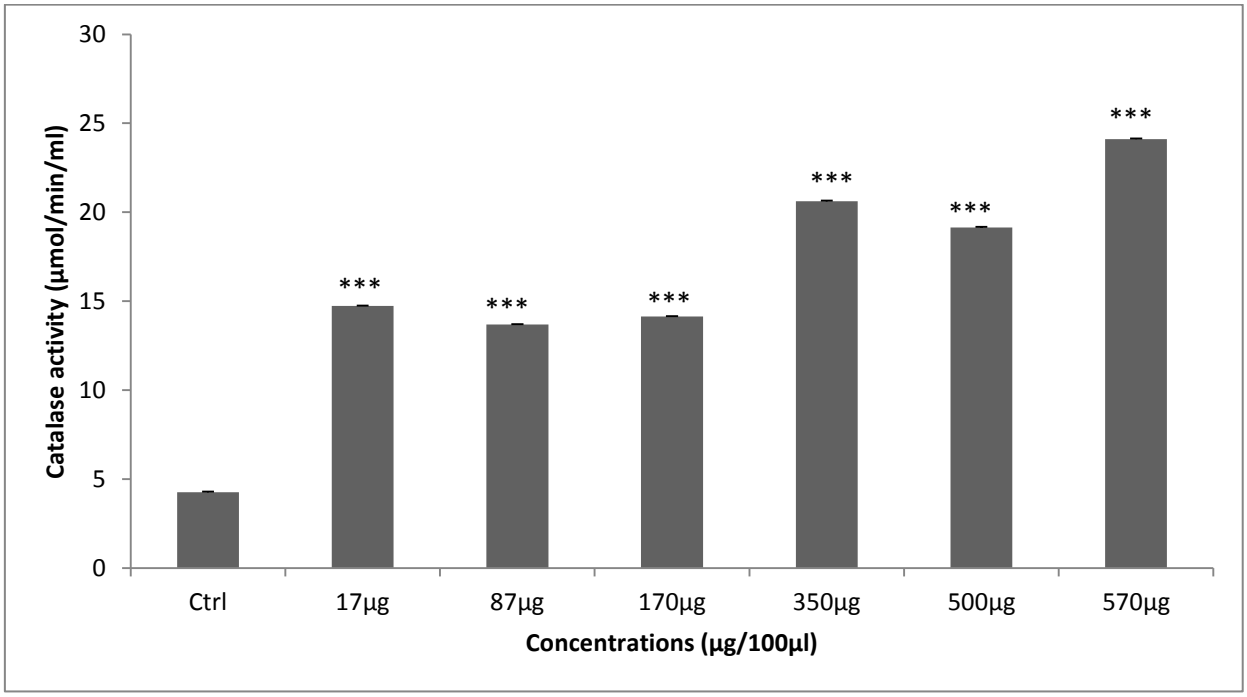

Figure 42: Effect of *S. circinata* on cellular catalase activity (HepG2). Data are expressed as mean ± SD (n = 3).

The catalase activity was significantly higher in cells treated with *S .circinata* compared to control (p= 0.000), CAT activity was significantly higher in cells treated with 570µg *S. cicinata* extract than the other groups (figure 42).

III.7.2.2. Cellular GSH values

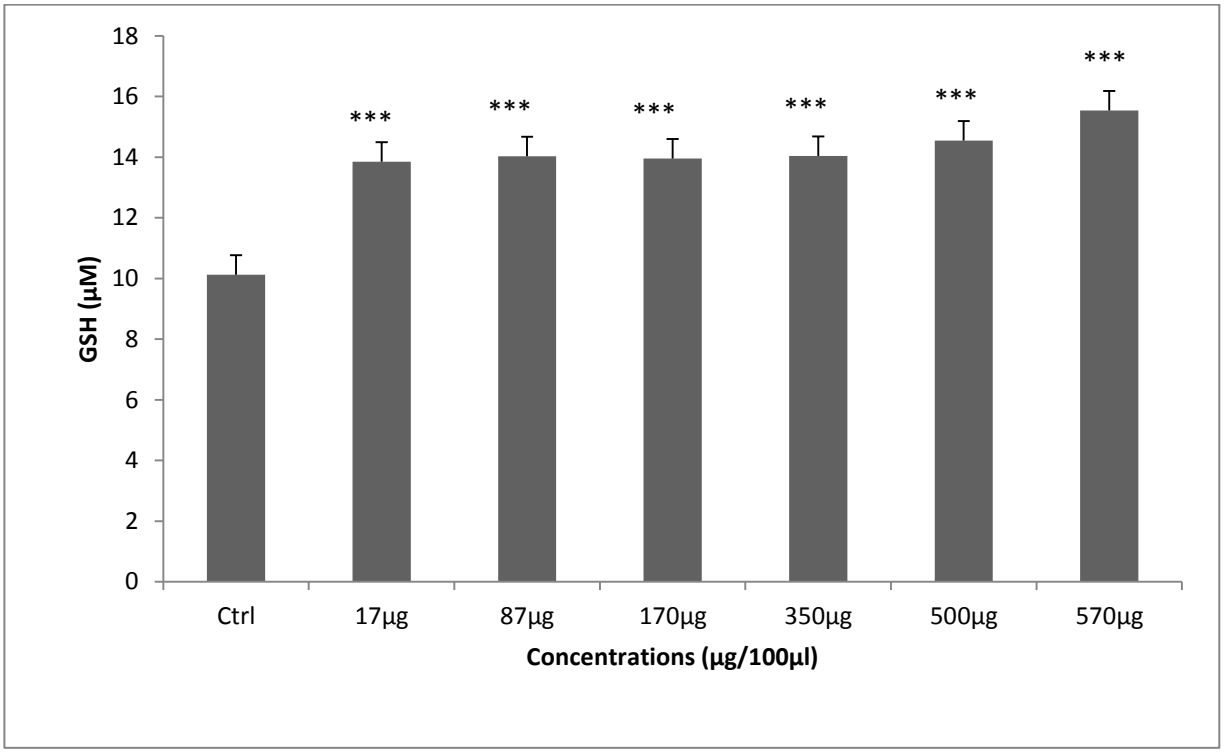

Figure 43: Effect of *S. circinata* on cellular GSH levels (HepG2). Data are expressed as mean ± SD (n = 3).

Treating cells with *S. circinata* extract showed a very high significant enhance of GSH levels compared to control in figure 43 (P=0.000).

Chapter IV

Discussion

IV. Discussion

In the acute oral toxicity study represented by up and down method, both extracts are toxically safe by oral administration in mice. All five mice survived until the end of the observation period. The use of herbal medicines as alternative treatments has been increasing worldwide and gaining popularity in developing countries. Although medicinal plants may have biological activities that are beneficial to humans, the potential toxicity of these bioactive substances has not been well established. Thus, the safety and efficacy of these plants must be studied thoroughly to maximise their benefits for mankind. To achieve this objective, a toxicological evaluation is performed using an experimental animal to provide guidelines for selecting a "safe" dose for human uses. To evaluate the toxicity of plant extracts, experimental animals, are treated at specific doses for a defined period of time. At this period, the mice are controlled (Elsnoussi *et al*., 2011).

In our study, we have demonstrated that the dichloromethane extract of *S. circinata* and the *n* butanolic extract of *S. mialhesi* were not toxic by oral administration in mice up to 2000 mg/kg, similar toxicological concentrations were achieved for plant extracts of *Argania spinosa*, *Citrullus colocynthis* and *Boswellia serrate* by Aribi, 2015.

The second experimental approach in this study was planned to evaluate the immunomodulatory effect of *S. circinata* and *S. mialhesi* extracts which took account that the immune cells and mediators are directly involved in the processing of antigens, removal of microorganisms by, phagocytosis, lysis of bacteria, viruses or tumour cells. Many malignant diseases are caused by, a decreased number or function of immune competent cells. Hence, modification of immune response either through suppression or stimulation may be helpful in avoiding diseases related to the immune system (Sharma *et al*., 2012).

Therapeutics for immunomodulation can be referred to as a therapeutic approach to intervene or adjust the auto-regulating immune responses to a desired level via immune-stimulation, immune suppression or induction of immunologic tolerance. An immunomodulator can be defined as a substance or agent than can elicit immunomodulatory activities by altering or affecting immune cell systems to produce the desired immune response through dynamic regulation of the target immune systems (Wen *et al.*, 2012).

Currently several of the available therapeutic drugs have potential side effects. Thus, medicinal plants and their active components as a source of immunomodulatory agents are gaining importance (Rasheed *et al*., 2016).

The carbon clearance assay was done to evaluate the effect of extracts on the reticuloendothelial system (RES). It is a diffuse system containing phagocytic cells (Singh *et al*., 2012).

In this study we found that *S. circinata* and *S. mialhesi* may stimulate cell mediated immunity as shown by an increase in macrophage induced phagocytosis in carbon clearance test. When ink containing colloidal carbon is injected into the systemic circulation, the macrophages engulf the carbon particles of the ink and the rate of clearance of ink from blood is known as phagocytic index (George *et al.*, 2014). *Stachys circinata* and *S. mialhesi* extracts stimulated the RES by, a high significant increase in the phagocytic index.

Our results are in agreement with those of Benmebarek *et al*. (2014) who indicated that *Stachys ocymastrum* extract appears immune stimulatory at low concentrations and immunosuppressive at high concentrations as it exhibited a biphasic effect on the phagocytic activity of the RES and with those of Nassar *et al*. (2015) who reported that the *n* butnolic extract of *Stachys circinata* increased the phagocytic index at 150 mg/kg. Mazumder *et al*. (2012) reported that *Glycyrhiza glabra* L roots, was found to stimulate the phagocytic activity of the macrophages, as evidenced by an increase in the rate of carbon clearance.

These results are also agree with those of Kehili *et al*. (2014) who reported that *Phoenix Dactylifera* revealed an immune-stimulatory effect on the reticuloendothelial system activity with higher effect by the administration of 50 mg/kg and with those of Patel *et al.* (2010) who reported that both low dose (100 mg/kg, *p.o*) as well as high dose (500 mg/kg, *p.o*) of *Aegle marmelos* stimulates immune system and Hajra *et al*. (2012) who demonstrated that *Swietenia mahagoni* seeds stimulate the reticuloendothelial system by significant increase in the phagocytic index.

The improvement in phagocytic function by *S. circinata* and *S. mialhesi* extracts maybe due to a number of actions of different effector components of the phagocytes. However, the most important mechanism is the up-regulation of receptors that are required to interact with the pathogens which include mannose and toll like receptors. It may also be due to the increased opsonization of carbon particles by, complement protein and immunoglobulins. So, the

immunostimulate activity of the *S. circinata* and *S. mialhesi* extracts which acted by, activating the function of the RES is due to the fact that it contains natural physiologically active substances such as terpenoids, phenolic compounds and flavonoids (Laggoune *et al.*, 2016), which increase the humoral response, by stimulating the macrophages and B-lymphocytes subsets involved in antibody synthesis (Dash *et al.*, 2006).

Reactive oxygen species such as hydrogen peroxide (H_2O_2) and superoxide anion (O^{2-}), reactive nitrogen species, and peroxynitrite are generated in the first minutes of macrophages stimulation through the respiratory burst (Castaneda *et al*., 2017, Gollo *et al.*, 2020). Respiratory burst plays an important role in the immune system. It is a crucial reaction that occurs in phagocytes to degrade internalized particles and bacteria. NADPH oxidase, an enzyme family widely expressed in many types of cells, produces superoxide, which spontaneously recombines with other molecules to produce reactive free radicals. To combat infection, immune cells use NADPH oxidase to reduce O^{2-} to an oxygen free radical and then H_2O_2 (Yang *et al.*, 2016).

In a healthy human being generation of ROS is keeps in check by cellular antioxidants such as SOD, CAT, GPx, GSH and glutathione reductase (GR) (Salla *et al*., 2016). However, over production of free radicals can cause an imbalance in cellular redox status producing oxidative damage to biomolecules, (lipids, proteins, DNA) (Rouabhi *et al*., 2015), which has major implications in the etiology of chronic diseases such as cancer, diabetes and cardiovascular conditions (Choi and Kim, 2013).

Bioactive compounds from plant origin have the potential to subside the biochemical imbalances induced by various toxins associated with free radicals. They provide protection without causing any side effects and therefore, development of drugs from plant products is desired (Anbuselvam *et al*., 2007). The findings of this study demonstrated the antioxidant ability of extracts of *S. mialhesi* and *S. circinata* by several *in vivo* and *in vitro* methods.

While screening the immunomodulatory activity, most of the studies employ agents like carbon in order to induce cytotoxicity, this agent is known to generate free radicals in the biological system and thereby cause oxidative stress (Lee *et al*., 2011). Therefore, *in-vivo* antioxidant evaluation from liver homogenate on oxidative stress markers as catalase (CAT) and reduced glutathione (GSH) from mice was investigated. Moreover, the *in-vitro* antioxidant activity of the extracts was evaluated on the hepatocarcinoma cell line HepG2.

Reduced glutathione (GSH) is a natural antioxidant produced inside the cell, playing both a role of co-factor for glutathione peroxidase and an active scavenger to eliminate reactive species, as hydroxyl radical, carbon centered radicals, peroxynitrite, and singlet oxygen molecule. Reduced glutathione has the role to preserve cellular redox status. It is possible that an increase in GSH levels will minimize ROS levels thus antagonizing oxidative stress (Housseini-Zijoud *et al.,* 2016).

Glutathione is involved in many cellular functions. These include protein and DNA synthesis; enzyme activation; amino acid transport (Knight, 2000) and plays a key role in both innate and adaptive immunity; it boosts innate immunity by aiding in the production of T-lymphocytes and dendritic cell function and affecting the ability of neutrophils to destroy foreign pathogens by phagocytosis. Glutathione is essential in the process of breaking disulfide bonds in order to digest the pathogen by APCs into fragments that can then bind with the major histocompatibility complex (MHC) and be displayed to T-cells that are specific to that antigen recognize this and are then activated (Ghezzi, 2011).

Glutathione, in its reduced form (GSH), is able to act as a very good antioxidant which can donate electrons to free radicals. Its ability to do this comes from the free sulfhydryl group (-SH) on its molecular structure (Biwas and Rahman, 2009) and it's antioxidant function is accomplished largely by GPx- catalyzed reactions, which reduce H_2O_2 and lipid peroxide as GSH is oxidized to GSSG. Glutathione disulfide in turn is reduced back to GSH by GSSG reductase at the expense of NADPH, forming a redox cycle (Shelly and Lu, 2013).

Catalase shows one of the fastest turnover rates for all enzymes: under optimal conditions, each subunit can convert about 6 million hydrogen peroxide to water and oxygen per minute. Catalase is present in all aerobic cells, but the highest concentration is found in liver and erythrocytes (Kurutas, 2016).

The results showed, a potential antioxidant effect of the *S. mialhesi* and *S. circinata* extracts, presented by a high significant decrease in the CAT and GSH values from liver in the groups treated by different doses of the extracts, with a higher effect at the dose of 150 mg/kg comparing to the control group for the two extracts, the result suggest that *S. mialhesi* and *S. circinata* extracts stimulate the release of GSH and CAT from liver, this result agrees with the result of (Kehili *et al*., 2014), who reported that the *Phoenix dactylifera* extracts, have a potential antioxidant effect by stimulating the excretion of the GSH from the liver, which will lead to eliminate the reactive oxygen species and protect the organism, our results are in agreement with

the results of (Theo *et al*., 1989) and (Chahar *et al*., 2012) who reported that the liver releases GSH and CAT mainly into the systemic blood, as part of an interorgan turnover, thereby maintaining a thiol redox balance in the plasma. It was suggested that the hepatic glutathione transport may function in the protection against increased generation of reactive oxygen species in the blood caused by inflammatory processes or during extreme physical exercise. Indeed, under such conditions GSH and CAT levels in liver and other organs were significantly decreased (Theo *et al*., 1989; Chahar *et al*., 2012).

Stimulating the release of liver glutathione and catalase by *S. circinata* and *S. mialhesi* extracts maybe due to the fact that the plant extracts accelerate the activation of the GSH and CAT transporter molecules.

In the liver, a major site of GSH synthesis and export, GSH is released at high rates into both blood plasma and bile. GSH transport into bile functions as a driving force for bile secretion and plays an important role in the transport and hepatic detoxification of reactive compounds of both endogenous and exogenous origin. GSH is also released at high rates across the sinusoidal membrane into blood plasma, for delivery to other tissues. Although the molecular identity of GSH transporters has remained elusive, recent studies have implicated a major role for some multidrug resistance-associated proteins (Mrp/Abcc) in this process (Ballatori *et al.*, 2009).

Controversial issues concerning the interference between chemotherapy, ROS and antioxidants needs to be clarified in order to improve combined therapies (Peiris-Pagès *et al*., 2015; Sznarkowska *et al.*, 2017; Hegedűs *et al*,. 2018; Cockfield *et al*., 2019). Within this context, more and more molecules and/or phytocomplexes have been reported to influence intracellular levels of free radicals associated with carcinogenesis, some acting as antioxidants and others as antioxidant enzyme inducers (Tariq *et al.*, 2015; Stagos *et al.*, 2018).

The interest in phytotherapic drugs is growing considering their potential use in combination with other approaches and/or be supplied as diet in the recovery stage to prevent tumor recurrence (Prasad, 2004; Roleira *et al.* 2015; Xie and Zhou, 2017).

Therefore, the potent antioxidant properties of flavonoids are well established disclosing their ability to regulate the enzymes involved in oxidative stress processes, such as SOD, CAT and GR (Zhao *et al*., 2014), this antioxidant capability is due to the presence of hydroxyl groups (·OH) in the skeleton of these classes of molecules. Moreover, other authors have proven that phenolic and flavonoid contents are associated to anti-oxidant properties, rendering them

excellent stabilizers for the lipid peroxidation (Bekhouche *et al.*, 2018). Namvar *et al*. (2018) revealed that there was a strong correlation between total phenolic content and the antioxidant capacity of the *Stachys turcomanica* extracts. Furthermore, Sadeghi *et al*. (2020) revealed that the nephroprotecitve and antioxidant effects of *Stachys pilifera Benth* against cisplatin induced nephrotoxicity due to the presence of compounds such as diterpenes, phenylethanoid glycosides, saponins, terpenoids and flavonoids in *Stachys* species. In another study, Alizadeh *et al*. (2020) revealed that the presence of components such as polyphenols, phenolic acids, and flavonoids in *Stachys sylvatica* hydroalcoholic extract is the reason of its antioxidant potential. Alpay *et al.* (2017) reporting that *Stachys annua (l.) L. Subsp. Annua var. Annua* had an antioxidant effect on cervical cancer cells (HeLa) and prostatic cancer cells (PC3).

The results of the *in-vivo* and *in-vitro* antioxidant activity proved that the extracts of *S. circinata* and *S. mialhesi* have a significant antioxidant capacity, these results are explained by the richness of the extracts of phenolic compounds and flavonoids (Laggoune *et al.*, 2016).

In our work we are searching for a new therapeutic drugs for arthritis because synthetic drugs, in recent years, are accompanied by numerous unwanted side effects, such as the non steroidal anti-inflammatory drugs (NSAIDs) that produce gastric ulcer, and as major side effects glucocorticoids are associated with adrenal suppression (Boddawar *et al*., 2016). In addition, their use cannot halt the development of rheumatoid arthritis and disease modifying antireheumatic drugs (DMARDs) have been impeded by their potential of long-term side effects, toxicity and immunosuppression (Tag *et al.*, 2014). So, it is very important to search for new therapeutic drugs from a natural source with greater efficiency and lower toxicity.

Findings of the present study have revealed that *S. circinata* and *S. mialhesi* treatment exerts anti-arthritic effect. It decreased the inflammation compared to the control group as observed by the decrease in the edema size, the concentration of ACCP and CRP values. These results agree with those of Mazumder *et al.* (2012) who reported that in the formaldehyde induced arthritis inflammation test (FIA), the methanol extract of *Barleria lupulina* owned a significant inhibition of the edema formation during the experimental period of 10 days. Our results are in agreement with those of Kehili *et al.* (2016) who reported that ACCP and CRP are decreased in mice injected with formalin and treated with Algerian *Phoenix dactylifera* fruit. Benmebarek *et al*. (2013) reported a decrease of hs-CRP when mice were treated with extracts of *S. mialhesi* following an inflammation induction by hyperhomocysteinemia.

Formalin-induced arthritis is one of most commonly used acute models for assessing anti-arthritic potential of plant extract (Kore *et al*., 2011). Injection of formalin into hind paw produced a biphasic pain response: an early neurogenic component followed by a later tissue-mediated response (Shastry *et al*., 2011).

The paw edema induced by formalin involves several chemical mediators such as histamine, serotonin, bradykinin, and prostaglandins (Sowemimo *et al*., 2015). The initial phase of the edema is due to the release of histamine and serotonin and the edema is maintained during the plateau phase by kinin like substance and the second accelerating phase of swelling due to the release of prostaglandin like substances (Mangesh *et al*., 2010), this phase is an inflammatory response with inflammatory pain that can be inhibited by anti-inflammatory drugs (Chao *et al.*, 2009).

The anti-inflammatory activities of many plants have been attributed to their flavonoids saponin, terpenoids and steroids contents (Shastry *et al*., 2011). Flavonoids were considered to be the active components responsible for the biological actions of the genus *Stachys*. In addition, this genus has been shown to possess various biological properties related to antioxidant, anti-nociceptive and anti-inflammatory mechanisms by targeting reactive oxygen species and prostaglandins which are involved in the late phase of acute inflammation and pain perception (Kehili *et al.*, 2016; Laggoune *et al*., 2016). These results are compatible with those of Peng *et al.* (2016) who reported that the flavonoids and alkaloids in the ethanol extract of the roots of *Caragana pruinosa* might be responsible for its anti-arthritic activity. In our research, we evidence that *S. mialhesi* and *S. circinata* extracts had accelerated the anti-inflammatory activity in a similar pathway as diclofenac and this is remarkable by lower volume of edema, CRP and Anti-CCP values.

One of the potential markers for increased risk of rheumatoid arthritis may be CRP. C-reactive protein has been shown to be of great value as an inflammatory marker in RA and has been suggested to mediate part of the complement activation in RA (Singh *et al*., 2013). In addition CRP may also contribute directly to the pro-inflammatory state. C-reactive protein is a plasma protein that increases during the systemic response to inflammatory states and its synthesis increase rapidly within hours after tissue injury or infection, it also contributes to host defense as a player of the innate immune response (Naderi *et al*., 2016). C-reactive protein an acute phase protein is synthesized by hepatocytes in response to pro-inflammatory cytokines in particular IL-6 and it stimulates monocyte release of inflammatory cytokines such as IL-1β, IL-6

and TNF-α and may also directly act as a pro-inflammatory stimulus to phagocytic cells (Shrivastava *et al*., 2013; Yu *et al*., 2017).

The presence of ACPAs usually measured as anti-CCP is highly specific for RA (Boissier *et al*., 2012). ACPAs recognize citrullinated peptides found in many matrix proteins such as filaggrin, keratin, fibrinogen, and vimentin and found also in alpha-enolase (Svärd *et al*., 2013). Citrulline derives from arginine by post-translational modification by peptidyl arginine deiminases (PADs) (Sakkas *et al*., 2014). Citrullination of synovial antigens, especially fibrin, is an active process during synovial inflammation that probably allows the induction of anti-CCP antibody in RA patients (Del Val Del Amo *et al*., 2006).

The initiation of RA involves the activation of auto-reactive T cells and the recruitment of these T cells along with other leukocytes into the joints. These leukocytes produce a variety of mediators of inflammation. Prominent among these mediators are arachidonic acid metabolites, pro-inflammatory cytokines, free radicals and matrix-degrading enzymes. These mediators modulate the processes relating to cell migration into the joints as well as angiogenesis and degradation of the extracellular matrix within the joints, leading to the arthritic inflammation (Venkatesha *et al*., 2011).

Flavonoids have an anti-inflammatory potential since they inhibit the production of inflammatory mediators by modulating the arachidonic acid pathway, inhibiting various enzymes like, cyclooxygenase, prostaglandin, ATPase, lipoxygenase, NADH oxidase, protein kinase, hydrolases, peroxidases, metallopeptidases, tyrosinases, and phospholipases (Nunes *et al.*, 2020).

The anti-inflammatory capacity of many flavonoid aglycones such as kaempferol, quercetin, apigenin, luteolin, genistein, and hesperetin was evaluated. Among them, luteolin was the most active in the inhibition of NO and TNF-α (Nunes *et al.*, 2020). Besides, luteolin and apigenin were found in *S. circinata* and *S. mialhesi* extracts (Laggoune *et al.*, 2016) which exert potent anti-inflammatory effects. In addition Naderi *et al*. (2016) reported that phenolic compounds in the Ginger decrease the pro-inflammatory factors of TNF-α and IL-1β involved in the inflammation and degradation of joints, these crucial compounds suppress the synthesis of prostaglandin and leukotriene by inhibiting the COX-2 and lipoxygenase pathways and other inflammation-involved pathways.

Serum CRP, a surrogate marker of disease severity that correlates with final outcome of arthritis is also a potent endogenous ligand for TLR-2 present on the surfaces of synovial

fibroblasts, polymorphonuclear leukocytes and macrophages and its transcription is regulated by pro-inflammatory cytokines including IL-6 (Adhikary *et al*., 2016). Serum concentration of CRP, the most common type of acute phase proteins was tested in our experiments and was found to be significantly attenuated in case of formalin-induced arthritis in mice treated with *S. mialhesi* and *S. circinata* extracts. This explains a protective role of the extracts against liver damage and inflammatory reactions in hepatic tissues during pathogenesis of FIA.

Immune complexes (IC) deposited into the synovial joints elevate pro-inflammatory cytokines in serum, through induction of mononuclear cells, these immune complexes can stimulate PMNs and macrophages to secrete pro-inflammatory cytokines, like TNF-α. In both ways, there is activation of synovial macrophages which ultimately results in increased production of TNF-α, IL-1β and other pro- inflammatory cytokines like IL-6, IL-12 and IL-15 which are involved in RA pathogenesis (Adhikary *et al*., 2016). However, IC containing citrullinated fibrinogen have been detected in the peripheral blood of ACPA-positive RA patients and also in synovial pannus (Fisher, 2014). These IC stimulate macrophage TNFα production and the accumulation of multiple ACPA specificities is correlated with preclinical inflammation (elevation of TNF-α, IL-6, and IFN-γ) preceding clinical arthritis (Sakkas *et al.*, 2014).

A considerable improvement in the management of RA has been obtained since the advent of biological agents such as (TNF)-α inhibitors (adalimumab, certolizumab, etanercept, golimumab or infliximab) anti-B cell agent (rituximab), anti-IL-6 receptor inhibitor (tocilizumab), T cell modulator (abatacept) (Atzeni *et al.,* 2013). A problem facing the practicing physician is to prescribe the most appropriate biological agent to individual patient, in other words, to match a biological agent with a patient profile, given the high cost of biological (Sakkas *et al*., 2014). The presence of ACPAs was associated with reduced response to anti-TNFα agents (Potter *et al*., 2009).

In our research, we have confirmed the protective effects of the extracts of *S. circinata* and *S. mialhesi* by histological analysis which was carried out on the mouse hind paws.

Formalin-induced arthritis model is known to present histologic similarities to human RA, with comparable synovitis, infiltration of inflammatory cells, erosion of joint cartilage, pannus formation and bone destruction which results in the destruction of joint stability and function disability (Nishat and Sayeda Jabeen, 2016).

However, oral administration of mice with *S. circinata, S. mialhesi* extracts and diclofenac as reference drug greatly reduced the histological changes caused by formalin-induced arthritis. The significant anti-arthritic effect of our extracts may be due to the suppression of the inflammatory mediators. Therefore it can propose that the presence of phenolic and flavonoids which are the major constituents of *Stachys* species may be explain the anti-arthritic properties of the extracts.

Sadeghi *et al*. (2014) revealed that the pathological analysis of the paws in carrageenan test showed that hydroalcoholic extract of *Stachys pilifera* reduced tissue destruction, cellular infiltration, and subcutaneous edema and reported that the presence of phenolic compounds such as polyphenols, phenolic acids, flavonoids, iridoids, and phenylethanoid glycosides in the extract of *Stachys* genus may the major reason of it's anti-inflammatory effect.

Inaddition, our study reports investigations on the antiproliferative effect of *S. circinata* and *S. mialhesi* extracts by screening for cytotoxic activity against normal human cell line (HUVEC) and several cancer cell lines, breast cancer (MCF7) hepatocarcinoma (HepG2) and rat phaeochromocytoma cell line (PC12).

Cancer is a common most fatal disease that affects the majority of the population, annually (Balabhaskar *et al.,* 2019). The malignant, metastatic, self-governing cell proliferation, termed 'cancer or tumor' arise in mainly all organs, but lungs are the most affected totaling 11,6% of diagnosed cases and 18,4% of total recorded deaths in combined sexes (Bray *et al.*, 2018).

In men, mortality is also high by liver and stomach cancer while, in woman breast cancer is the main cause of death (Siegel *et al.,* 2020). It is worth mentioning, that cancer epidemiology substantially vary among and within countries, depends on age and sex; economic status; social and life style (Weir *et al.,* 2015). Indeed, early diagnoses, access to health care and right/personalized therapies result in a significant improvement of cancer survival. Among therapies, surgery is the best option, but is limited to confined metastases (~10-15% of cases). Other cures include, radiotherapy, chemotherapy, targeted therapy, virotherapy, immune checkpoint inhibitor therapy, vaccine and combinations of them (Schirrmacher, 2019).

Nevertheless, tumor recurrence, drug resistance, treatment toxicity, and high heterogeneity of cancer cells indicate the need for new personalized therapies and molecules able to control a

broader range of cancer cell aberrations (Dagogo and Show 2018; Wang *et al.*, 2019; Falzone *et al.*, 2018).

Carcinogenesis generates a burst in intracellular reactive oxygen species (ROS) affecting survival of neighboring somatic cells and regulating tumor development (Snezhkina *et al.*, 2019). Indeed, free radical over production cause an imbalance in the cellular redox homeostasis with oxidative damage to biomolecules (lipids, proteins, DNA), (Rouabhi *et al.*, 2015), which has major implications in the etiology of chronic diseases such as cancer, diabetes and cardiovascular conditions (Choi and Kim. 2013).

Thus, it is advisable that new therapeutic drugs for cancer control take into account the effect on the redox status of cancer cells (Yang *et al.*, 2018).

Our results demonstrated that the extracts of *S .circinata* and *S. mialhesi* led to a selective cytotoxic activity against MCF7, HepG2, PC12 cell lines while, no cytotoxicity against normal human umbilical vein endothelial cell (HUVEC) could be observed. Ferhi *et al*. (2019) revealed that the extracts obtained from grape leaves grown in the Medea region (Algeria) exhibited an antiproliferative effect on MCF-7 and HepG2 cells.

With reference to our previous results, *S. circinata* and *S. mialhesi* extracts have been shown to increase antioxidant levels. Besides, Alpay *et al.* (2017) reported that increased antioxidant levels are reducing cancer progression.

S .circinata and *S. mialhesi* have been a subject of intensive phytochemical research and are characterized mainly by the presence of flavonoids including apigenine, isocsutellarein, luteolin, isorhamnetin, triterpenoids such as betulinic acid, ursolic acid, olealonic acid, sterols as stigmasterol and β-sitosterol (Laggoune *et al.*, 2016). Furthermore, Jassbi *et al*. (2014) reported that the presence of cytotoxic compounds with different polarities in several *Stachys* species like *S. pilifera*, dichloromethane extract (that may hold more nonpolar agents such as terpenoides) showed a stronger effect. Nevertheless, some plants as *S. persica*, 80% methanol extract (containing more polar molecules as phenolic compounds) was also able to exhibit even a higher cytotoxic effect.

Previous study has also demonstrated that chloroform fraction of *S. setifera* greatly inhibited the proliferation of breast ductal carcinoma cell line (T-47D) (IC50 2.44 µg/mL), as compared to normal cells (IC50 394.88 µg/mL), whose major components were found to be terpenoids and flavonoids (Ostad *et al.*, 2014). Several works have shown that luteolin inhibits

cell proliferation. Seelinger *et al.* (2008) reported that luteolin, isolated from two Asian plants traditionally used as anticancer medicines, *Epimedium koreaonum* and *Terminalia arjuna*, was found to inhibit MCF-7 and HepG2 proliferation in a dose-dependent manner. The cytotoxic effect of luteolin and apigenin was also previously demonstrated on human chronic myelogenous leukemia (K562) and bladder cancer (RT112) cells, in a dose- and time-dependent manner (Kilani-Jaziri *et al.*, 2012). Furthermore, apigenin also exhibited broad anticancer effects in various human cancers; this flavone inhibits cancer cell proliferation by triggering cell apoptosis, inducing autophagy and modulating cell cycle (Yan *et al.*, 2017).

In other studies, isorhamnetin was able to inhibit lung cancer cells proliferation *in vitro* and *in vivo*, being able to counteract also other carcinoma cell line proliferation as MCF-7. Its mechanisms of action may involve apoptosis of cells by down-regulating oncogenes and inducing apoptotic genes (Li *et al.*, 2015).

In another study, oleanolic acid exerted cytotoxic activity against HepG2, by arresting the cell cycle, and inducing apoptosis and DNA fragmentation (Zhu *et al.*, 2015). Interestingly, Zarei and Yaghoobi. (2017) reported that the extracts of *Fritillaria imperialis L.* were toxic for human liver cancer cells (LCL-PI 11) and breast adenocarcinoma cells (MCF-7) probably inducing cell cycle arrest or inducing intrinsic apoptosis.

The results showed that the extracts of *S. circinata* and *S. mialhesi* were strongly cytotoxic against HepG2, MCF-7 and PC12 cells, these extracts induced a significant inhibition of cell viability. In addition, the results showed that the normal HUVEC cell lines were not sensitive to extracts and that no cytotoxicity was seen, our results corroborate with those of much work done on plant varieties. Ferhi *et al.* (2019) reported that extracts obtained from grape leaves cause any damage to HUVEC as non-cancerous cells. These results are further deduced by the work of Aghbali *et al.* (2013), reporting that *Vitis vinifera* had a pro-apoptotic effect, inhibiting cell growth, while no cytotoxic activity was observed on HUVEC cells.

Conclusion and Perspectives

Our work which is a contribution to the study of biological activities of *Stachys circinata* dichloromethane and *Stachys mialhesi n*-butanolic extracts, allowed us to understand that the field of plants still remains a valid field of scientific research. Thus the study shows the immunostimulant potential of the extracts as evident from the increased phagocytic index in the reticuloendothelial system and supports their use, for the control and management of diseases, in which the immune system needs to be stimulated. The protective effect of these medicinal plants on formalin-induced paw edema, might be due to the significant alleviation of inflammatory response. Thus, the plants seem to be ones of promising candidates for inflammatory conditions like rheumatoid arthritis through their anti-inflammatory property.

In vitro and *in vivo* methods have been developed to evaluate the antioxidant effect. The activity of CAT and GSH level were increased after being treated with these plants, considering the results obtained from this study we can conclude that the plant extracts my participate in cellular protection not only directly as an antioxidant molecule but also indirectly as a stimulator of antioxidant enzymes. Through the results obtained from the *in vitro* study we can conclude that the extracts used from *Stachys circinata* and *Stachys mialhesi* have an anti-proliferative potential against three cancer cell lines (breast cancer cells (MCF7), human hepatocarcinoma (HepG2) and rat phaeochromocytoma cell line (PC12)) and no toxic effect on normal human cell lines (normal Human Umbilical Vein Endothelial Cells HUVEC).

Based on the present results, our future work and perspectives can evaluate many topics:

- ✓ Purification of the bioactive molecules presented in the plants extracts.

- ✓ Evaluate the effect of *Stachys mialhesi* and *Stachys circinata* extracts on gene expression of proinflamatory cytokines such as IL-1, IL-6, TNFα.

- ✓ Evaluate the effect of the tested extracts and fractions on some regulators of cell death and proliferation such as caspase-8, caspase-9, nuclear factor-κB (NF-κB).

- ✓ Prepare liposomal formulations encapsulating flavonoids in order to overcome their insolubility in water and prepare suitable formulations for *in-vivo* and *in- vitro* study.

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Appendices

Phosphate buffer 100 Mm, pH 7.5

- **molcular weight (NaH2PO4) ⟶ 1000 mMole = 1L**
  X (NaH2PO4) ⟶ 100 mMole

$$x = \frac{\text{molecular weight (NaH2PO4)} \times 100}{1000\ \text{mMole}}$$

- **molecular weight (Na2HPO4) ⟶ 1000 mMole = 1L**
  X (Na2HPO4) ⟶ 100 mMole

$$y = \frac{\text{weight molecular (Na2HPO4)} \times 100}{1000\ \text{mMole}}$$

H_2O_2 30%

- **30 g (H_2O_2) ⟶ 882 Mole**
  X (H_2O_2) ⟶ 500 Mole

$$z = \frac{30\text{g} \times 500\ \text{Mole}}{882\ \text{mMole}}$$

Sulfo-salicylic acid solution 0.25%

Dissolve 250 mg of salicylic acid in 100 ml of distilled water.

Tris (0.4 M), EDTA (0.02 M), pH 9.6

Dissolve 12.114 g of Tris and 1.871 g EDTA in 250 ml of distilled water and adjust the pH to 9.6 by adding Hcl or NaOH.

DTNB solution (0.01 M)

Dissolve 200 mg of DTNB in 50 ml of absolute methanol.

Bradford method

We need 1ml of Bradford solution to measure the protein concentration for each sample.

1ml of Bradford solution = 200µl Bradford + 800µl H_2O.

- ✓ Put 1ml Bradford solution + 1µl from each sample in the cuvette and mix the solution,

one cuvette serve as blanc and consists of mixing 1ml Bradford solution + 1µl Laemmli Buffer;

✓ Measure the absorbance of samples using spectrophotometer at 595 nm

Treatment dose calculation

✓ Plant extracts given dose (100mg/kg)

0,1g ⟶ 1000g

Xg ⟶ Mouse weight (g)

$$\textbf{Plant extract given dose} = \frac{\textbf{0.05g x Mouse weight (g)}}{\textbf{1000}}$$

Histological section procedure

- **Tissue fixation**

- The joint tissues are immersed into a fixative (10 % formol) for a period of time (minimum 24 hours) to allow the fixative to diffuse into the tissue.

- The joints are decalcified in 5% formic acid for three days; this step is to facilitate the sectioning.

- The tissues are then dehydrated by bathing through a graded series of mixtures of ethanol ethanol solution (50%, 70%, 96%), each step is placed for 30 min.

- The tissues are finally kept in small containers filled with butanol for one week, after that they are immersed in xylene solution for 10 min

- **Embedding in paraffin**

The tissues are immersed in paraffin and the sectioning is performed with a microtome.

- **Staining**

Paraffin slices, 5µm thick were stained with hematotoxylin eosin staining.

Hematoxylin eosin staining

- ✓ Dip slides in alcohol for 5minutes;
- ✓ Rinse with water;
- ✓ Stain slides in hematoxylin for 4 minutes;
- ✓ After rinsing stain with eosin for 10 minutes;
- ✓ Rinse with water;
- ✓ Dip slides in alcohol for 1 minute
- ✓ After rinsing and drying, the editing is done using xylene

Protocol for cryopreservering cultured cell

The cryopreservation medium was prepared by using the appropriate complete growth medium for each cell type and 10% of DMSO (cryoprotective agent).

- ✓ The spent cell culture media was removed and discarded from the flask.
- ✓ The cells were washed by adding 3 ml of PBS to the side of the flask to avoid disturbing cell layer and the flask was gently rocked back and forth several times.
- ✓ The PBS was removed and discarded from the flask.
- ✓ 1.5 ml of the Trypsin-EDTA was added to the cells and incubated in CO2 incubator for 5 min.
- ✓ The cells were observed under the microscope for detachment.
- ✓ 6.5 ml of the medium was added to the flask and by pipetting over the cell layer surface several times, the cells were dissociated and the mix was then transferred to a sterile conical tube and centrifuged for 5min/700 RPM.
- ✓ The supernatant was decanted and the cell pellet was resuspended in the cryopreservation medium.
- ✓ Aliquots of the cell suspension were dispensed into cryovials of 1ml and the cells were mixed frequently and gently to maintain a homogeneous cell suspension.

The cryovials containing the cells were stored at – 80°C.

Paper

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Immunomodulatory and Anti-Arthritic Activities of *Stachys circinata*.

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Abstract

The present study reports on the immunomodulatory and anti-arthritic activity of the dichloromethane extract of *Stachys circinata* L'her dried aerial parts (DMESC). Male *Mus Musculus* Albinos were used in all *in vivo* experiment. The toxicity was determined by the acute oral toxicity test administering DMESC orally, while the effect on phagocytosis was monitored by the blood carbon clearance assay. The formalin-induced arthritis (FIA) approach was used to measure the edema size during a 10 d period and quantify the C-reactive protein (CRP) and anti-cyclic citrullinated peptide (ACCP) at the end of the experiment. DMESC, did not produce visible signs of toxicity nor mortality and the LD resulted > than 2000 mg/kg. Phagocytic activity increased at all tested DMESC concentrations (50, 150 and 200 mg/kg) as evidenced by the half-life of colloidal carbon in the blood, the clearance rate was faster at 150 mg DMESC/kg. Also in the FIA test, DMESC supply at 150 mg/kg, revealed a significant decrease of the edema size, anti-CCP values ($P=0.000$) and CRP ($P<0.05$). As conclusion, the results clearly evidence that DMESC owns immune-stimulatory and anti-arthritic activity.

Keywords: *Stachys circinata*, Phagocytic activity, anti-inflammatory, CRP, Anti- CCP

1. Introduction

A large number of plants and their isolated constituents have been shown to potentiate health by exerting anti-inflammatory, anti-stress and anti-cancer effects by modulating the immune function (Bin-Hafeez *et al*., 2003). Macrophage is the first cell to recognize infectious agents and is central to cell-mediated and humoral immunities. It is a specialized phagocytic cell that attacks, destroys, and ingests cancer cells, foreign substances, and infectious microbes, by secreting pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukine-1 (IL-1) (Kim *et al*., 2013).

Rheumatoid arthritis (RA) is a frequent chronic inflammatory disease (Boissier *et al.*, 2012). RA is an unremitting multisystem disease accompanied by immune hyperactivity, persistent synovitis, and synovial hyperplasia along with deposition of autoantibodies to immunoglobulins leading to articular cartilage damage and resorption of osseous matter (Hasan and Alamgeer, 2018).

C-reactive protein (CRP) is an acute phase protein synthesized by hepatocytes in response to proinflammatory cytokines, in particular interleukin (IL)-6. It has been shown to be of great value as an inflammatory marker in RA and has been suggested to mediate part of the complement activation in RA (Singh *et al.*, 2013).

The presence of anti-citrullinated protein/peptide antibodies (ACPAs usually measured as anti-CCP) is highly specific for RA. ACPAs recognize citrullinated peptides found in many matrix proteins such as filaggrin, keratin, fibrinogen, and vimentin and found also in alpha-enolase (Svard *et al*., 2013). Citrulline derives from arginine by post-translational modification by peptidyl arginine deiminases (PADs) (Sakkas *et al.*, 2014). Citrullination of synovial antigens, especially fibrin, is an active process during synovial inflammation that probably allows the induction of anti-CCP antibody in RA patients (Del Val Del Amo *et al*., 2006).

The genus Stachys (Lamiaceae) is widely known in folk medicine and is worldwide distributed accounting for 300 species. In Algeria, this genus is represented by 14 species. Nassar *et al.* (2015) has revealed that three plant extracts belonging to Lamiaceae family exert antioxidant and immunostimulant effects. The study of Laggoune *et al*. (2016) has shown that the n-butanolic extract of the aerial parts of *Stachys mialhesi* exhibited significant antioxidant, antinociceptive and anti-inflammatory effects in laboratory animals. *In vivo* studies have revealed that *Stachys pilifera* possess significant anti-inflammatory effect (Sadeghi *et al.*, 2014), and considerable cytotoxic and anti-proliferative properties on HT-29 colorectal cell line (Panahi Kokhdan *et al*., 2018).

The current study was designed to evaluate the immunomodulatory and the anti-arthritic effects of the endemic species *S .circinata.*

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2. Materials And Methods

2.1. Plant Collection and authentication

Aerial parts of *S. circinata* L'Her were collected from Djebel El-Ouahch-Constantine (North Eastern Algeria) in April 2013 during the flowering stage. A voucher specimen (LOST SC04/13) has been deposited in the Laboratory of therapeutic substances, University frères Mentouri-Constantine and authenticated by Prof. G. De Belair (University of Annaba, Algeria).

2.2. Preparation of the dichloromethane extract

Air-dried and powdered aerial parts (1kg) of *S. circinata* were macerated three times at room temperature with MeOH-H2O (7:3, v/v) for 24h. After filtration, the filtrate was concentrated and dissolved in water (600 mL). The resulting solution was extracted successively with petroleum ether, CH_2CL_2, EtOAc and *n*-butanol. Concentration in vacuo at room temperature led to the following extracts: petroleum ether (2.3 g), dichlomethane (9 g) EtOAc (5 g) and *n*-butanol (25 g). The resulting dichlomethane extract of *S. circinata* (DMESC) was then used in all experiments.

2.3. Animals

Adult male *Mus Musculus* Albinos mice (2-2.5 mths old) were obtained from central pharmacy Institute, Constantine, Algeria. The animals used in all experiments had a weight range between 26 and 35 g. All the mice were kept under standard laboratory conditions at 24 ± 1°C relative humidity 55% with a 12 h light/dark cycle. They were fed with a stock rodent diet and tap water. The animal studies were conducted after obtaining clearance from Institutional Animal Ethics Committee and the experiments were conducted in strict compliance according to ethical principles and guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

2.4. Acute oral toxicity

The present study was conducted according to the guideline proposed by the Organization for Economic Cooperation and Development (OECD) revised up-and-down procedure for acute toxicity testing. This guideline is based on the procedure of Bruce *et al*. 1985.

A 2000 mg/kg dose was used in five adult male mice, the dose was given to a sole mouse, with the aim to monitor mortality and clinical signs (behaviors recorded: unusual aggressiveness, unusual vocalization, restlessness, sedation and somnolence movements, paralysis, convulsion, fasciculation, prostration and unusual locomotion). Observations lasted 48 h and were performed during the first hour and then each 3 h until the end. Upon survival of this mouse, four additional mice were given the same dose sequentially at 48 h intervals and again, clinical signs were monitored. All of the experimental animals were maintained under close observation for 14 d following DMESC administration, and the number of mice that died within the experimental period was noted. The lethal dose 50 (LD50) was established to be above 2000 mg/kg if no health disorders nor death was registered in three or more mice.

2.5. Phagocytosis (carbon clearance method)

Phagocytic activity of reticuloendothelial system (RES) was assayed by carbon clearance test. Phagocytic index was calculated as a rate of carbon elimination of RES by carbon clearance test determined by a reported method (Halpern *et al*., 1953).

Mice were divided into four groups each of 7 animals: group I (Control) received 0.5 mL of a 0.9% NaCl saline solution via intraperitoneal (i.p.) injection; groups II, III and IV were administred by i.p injection with 50, 150 and 200 mg/Kg of DMESC, respectively.

Forty eight hours after the i.p. injection of the treatment, a colloidal carbon ink suspension was injected via tail vein to all groups at a dose of 0.1 mL/10g. The ink suspension consisted of black carbon ink 3 ml, saline 4ml and 3% gelatin solution 4ml. Then, blood samples (≈14 drops or 25µL) were withdrawn from the retro-orbital plexus via heparin glass capillaries at interval of 5 and 15 min after carbon ink injection. Collected blood samples were lysed in a 0.1% sodium carbonate solution (4 mL) and optical density measured spectrophotometrically at 675nm. At the end of the experiment, liver and spleen were removed from each mice, weighted and values used to calculate the phagocytic index K.

Clearance kinetic was expressed by: 1) the phagocytic index K, which follows an exponential function of concentration to time and measures all the RES activity in contact with the circulating blood, and 2) the corrected phagocytic index α, which expresses this activity by, unit of active weight organs (liver and spleen). Finally, the clearance rate was expressed as the half-life period (t½, min) of the carbon ink in the blood. Parameters have been calculated using the following formulas according to Biozzi *et al*., 1970.

$$K = \frac{\text{LogOD1} - \text{LogOD2}}{t2 - t1} \qquad t\tfrac{1}{2} = \frac{0{,}693}{K}$$

$$\alpha = \sqrt[3]{K}\frac{\text{Body weight of animal}}{\text{Lever} + \text{spleen wt}}$$

OD1 and OD2 are the optical densities (at 675nm) recorded at time t1 (5 min) and t2 (15 min), respectively.

2.6. Formalin-induced arthritis

To perform this test, mice (20-30 g) were divided into four groups (F, FF, SC, D) of five animals each. Group 'F' remained untreated (negative control); group 'FF' (positive control) was subjected to the sole formalin treatment; group 'SC' to formalin + DMESC at 150 mg/kg and group 'D' to formalin + the anti-inflammatory standard drug (diclofenac of sodium) at 10 mg/kg. The administration was done orally by mixing the plant extract or diclofenac of the treated groups into a flour balls. The delivery of DMESC or diclofenac of sodium was carried out while maintaining the standard diet. In this experiment, the concentration with the highest efficacy of DMESC (150 mg/kg) was employed according to the results attained in the phagocytosis experiment.

According to the protocol of Mazumder *et al.* (2012), Formalin treatments were performed by injecting into the sub-plantar of the right hind paw 100 µL of formalin (2%) on the 1st and 3rd day of the experiment. Then, diclofenac and the DMESC were daily administered until the end of the experiment. During the 10 d experiment, a daily

measurement of the edema size was realized with a digital caliper.

2.7. Blood investigation

At the end of the experiments, animals from the entire groups were water-fasted overnight before collecting the blood samples. Blood samples were withdrawn as reported in the phagocytosis paragraph. The separated plasma was assayed for hs-C-reactive protein by an immunoturbidimetric method on a Cobas integra 400 plus analyser (Roche) and anti-CCP was measured by Stratec Biomedical Systems Gemini 6280 Automated Compact Microplate Processor.

2.8. Statistical analysis

The data are reported as mean ± SEM (standard error of the mean). Statistical analyses of the results were performed using one–way ANOVA test and Tukey's multiple comparison tests (SPSS version 20). The values of, $^{***}P<0.001$, $^{**}P< 0.01$and $^{*}P< 0.05$ were considered to indicate the significant levels.

3. Results

3.1. Acute toxicity study

According to the preliminary toxicity test, the DMESC was found to be safe up to 2000 mg/kg. Indeed, during the 14 day-assessment time, mice were not affected by the amendment of floor balls with 2000 mg/kg of DMESC and they remained healthy and with no visible signs of toxicity nor mortality. This result stand up for an LD50 higher than 2000 mg/kg.

3.2. Phagocytic activity

The results show a significant increase of K index mean values in mice belonging to DMESC supplied groups if compared to the control (NaCl group) with *P*= 0.001 (Fig. 1A). Index values for the DMESC administered groups were: 0.031 ± 0.004; 0.038 ± 0.005 and 0.035 ± 0.007 with 50, 150 and 200 mg/kg, respectively. The NaCl group attained a mean index value of 0.017 ± 0.005. The highest activity was monitored in the group of mice fed with 150 mg DMESC /kg (65.3% increase), but difference among DMESC doses was not significant. This indicates that DMESC enhanced the phagocytic activity by stimulating the RES. and according to the results, it seems that the tested concentrations lower or higher than 150 mg/kg do not improve the phagocytic index value.

DMESC supply to mice influenced significantly the calculated half time (t½) of colloidal carbon clearance which decreased by nearly 50% compared to the control (NaCl). Among DMESC supplied groups the probability was *P*= 0.01 with a t½ of 23.01 ± 3.14 min; 18.65 ± 2.52 min and 20.56 ± 2.83 min with 50, 150 and 200 mg/kg, respectively (Fig. 1B). The NaCl group owned a t½ of 40.62 ± 20.35 min. Compared to control, the clearance rate with150 mg DMESC/kg was lowered more than twice.

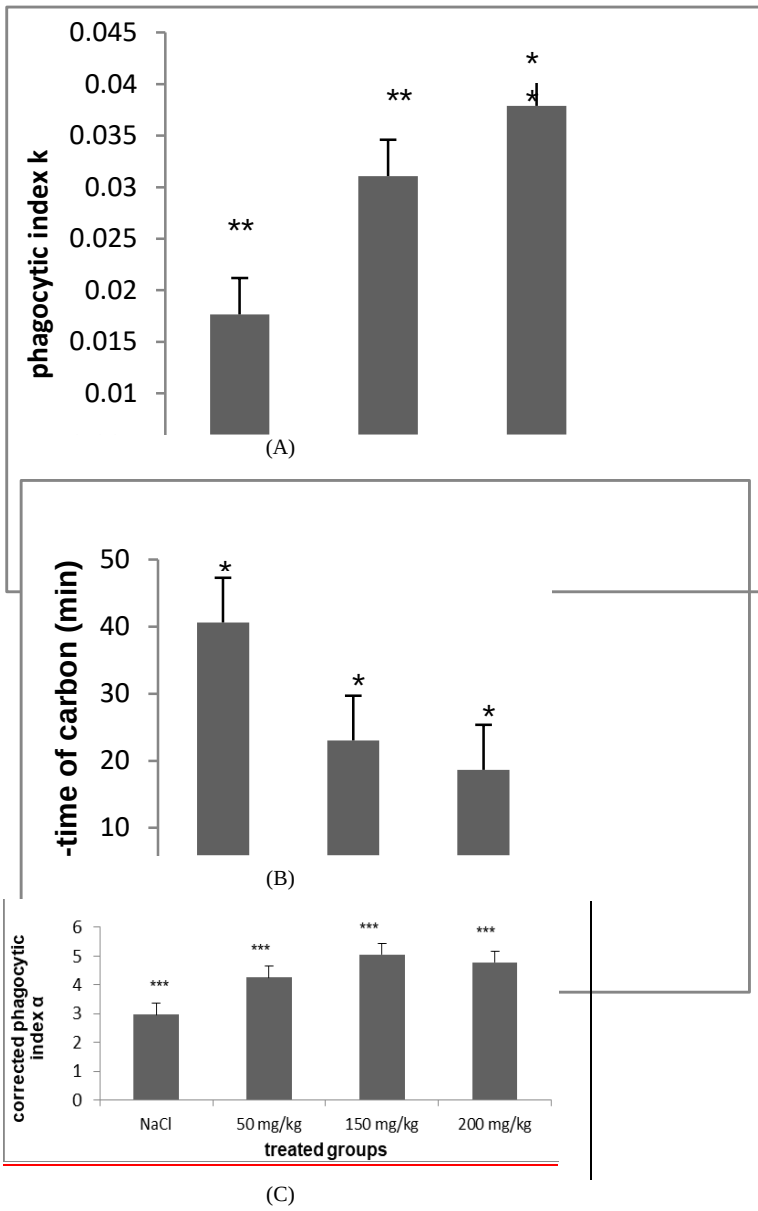

Fig. 1. Effect of dichloromethane extract *S. circinata* of aerial parts on phagocytic activity expressed as: (A) index phagcytic K; (B) half-time t1/2 of carbon in the blood; (C) corrected phagocytic index α. Values are mean ± SEM (n=7) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001

GI: Control group received NaCl; **GII:** group received dichloromethane extract of *S. circinata* at dose 50mg/kg; **GIII:** group received dichloromethane extract of *S. circinata* at dose 150mg/kg; **GIV:** group received dichloromethane extract of *S. circinata* at dose 200mg/kg.

In addition to the results reported in Fig.1A, B a significant increase in the corrected phagocytic index α occurred between DMESC groups *P*=0.000 (α= 4.26 ± 0.74; 5.05 ± 0.57; 4.78 ± 0.60 with 50, 150 and 200 mg/kg, respectively) and the control group (α= 2.97 ± 0.50) (Fig. 1C).

3.3. Formalin induced arthritis

The results evidenced a significant inhibition in the edema size in group 'SC' and 'D' (*P*=0.000) while, on the other hand a significant increase of the size occurred in

group 'FF' (*P*=0.000) in comparison to the negative control, group 'F' (*P*=0.000) (Fig. 2).

One day following the first injection of formalin, the edema size enlarged significantly in groups 'FF', 'SC' and 'D' (3.24 ± 0.37; 3.26 ± 0.08 and 3.10 ± 0.24 mm, respectively) in comparison to the negative control group 'F' (1.71 ± 0.016 mm), while, after the second injection of formalin, size increase on the 3rd day was negligible.

On the 4th d, size decreased significantly in group 'SC' and 'D' (2.98 ± 0.13 and 3.04 ± 0.11 mm, respectively) compared to the group 'FF', positive control, where the edema size increased until 5 days after the 2nd formalin injection (3.83 ± 0.28 mm).

From the 5th d until the end of the experiment, the sizes in group 'SC' and 'D' decreased slightly and mean values were almost identical, whereas a significant decrease occurred in the positive control group 'FF' (Fig. 2).By comparing the edema size of each group during the experimental period, the size of the negative control group 'F' remained nearly stable throughout the experiment with an average of 1.7 mm, while compared to the edema size in group 'FF' a decrease of about 77,6 and 79,2% was attained in treated groups 'SC' and 'D', respectively.

It is noteworthy to evidence that the edema development in group 'FF' progressed differently from group 'SC' and 'D' following the 2nd injection of formalin at the 3rd day. Indeed, in group 'FF' size continue to enlarge until day 8 and then decreased till 10th d (3.27 ± 0,44 mm). On the other hand, starting from day 4, edema in group 'SC' and 'D' slowly underwent a similar decease evidencing clearly a comparable anti-inflammatory effect of DMESC and diclofenac of sodium.

The C-reactive protein (CRP) concentration in mice blood following formalin injection decreased by supplying mice with 150 mg/kg DMESC (group 'SC') or with 10 mg/kg diclofenac of sodium (group 'D') and values were about 1.03 ± 0.74 and 0.68 ± 0.26 mg/L, respectively. However, the mean value of CRP (1.62 ± 0.82 mg/L) in group FF was increased but not significantly when it's compared to group 'SC' and 'D' (Fig. 3A).

The ACCP values were also influenced by treatments and had a similar trend to those of CRP but differences resulted a significantly lower (*P* = 0.000) in blood of mice supplied with DMESC or diclofenac of sodium. The concentration of ACCP is decreased in the groups 'SC' and 'D' (2.14 ± 0.38 and 1.57 ± 0.56 UI/mL, respectively) when it's compared to the group 'FF' (positive control) which had the highest ACCP values (3.13 ± 0.96 UI/mL) (*P* = 0.000), while the group 'F' (negative control) had the lowest (0.62 ± 0.32 UI/mL) values as shown in Fig. 3B.

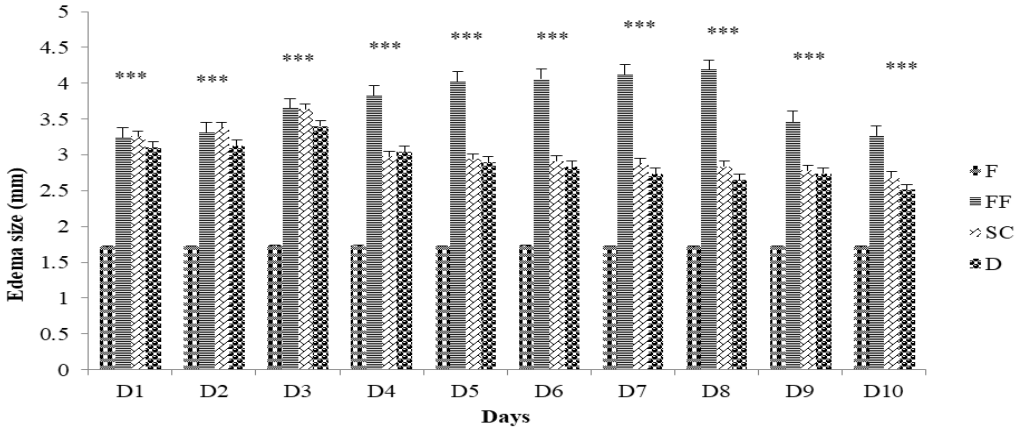

Figure 2: Anti-inflammatory effect dichloromethane extract of *S. circinata* aerial parts on the formalin induced mice hind paw edema during a 10 day experimental period. Values are mean ± SEM (n=8) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001. Treatments: F= untreated (negative control); FF= (positive control) sole formalin treatment; SC= formalin + DMESC (150 mg/kg); D= formalin + diclofenac of sodium (10 mg/kg).

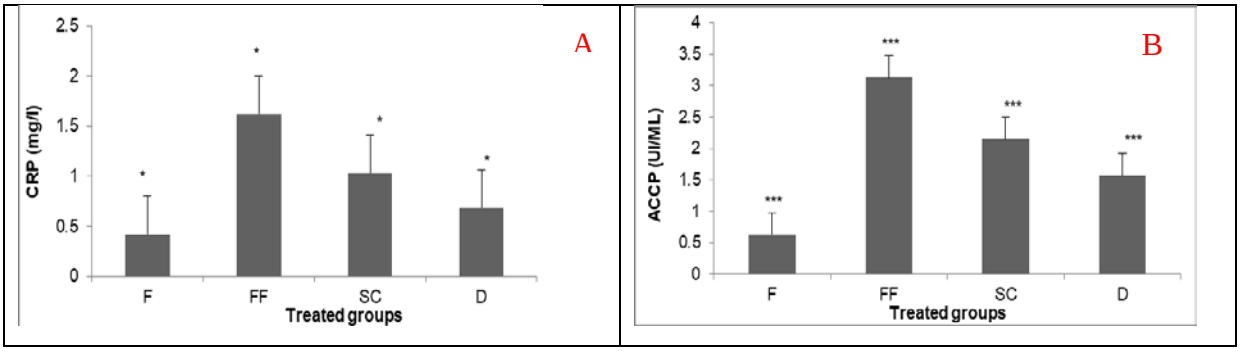

Figure 3: Effect of dichloromethane extract of *Stachys circinata* on serum parameters in Formalin-induced arthritis in mice. (A) Blood levels of C-Reactive Protein (CRP) in mice following formalin-induced arthritis as influenced by the dichloromethane extract of *S. circinata* aerial parts (DMESC) and diclofenac of sodium; (B) Anti-cyclic citrullinate peptide (ACCP) levels in mice blood following formalin-induced arthritis as influenced by the dichloromethane extract of *S. circinata* aerial parts (DMESC) and diclofenac of sodium. Values are mean ± SEM (n=7) and significant difference from the control group is shown as *p<0.05, **p<0.01, ***p<0.001. Group F: untreated (negative control); Group FF: (positive control) formalin inflammation; Group SC: formalin inflammation+ DMESC (150 mg/kg); Group D: formalin inflammation+ diclofenac of sodium (10 mg/kg).

4. Discussion

The use of herbal medicines as alternative or adjuvant treatment has been increasing worldwide and gaining popularity in developing countries where ethnobotanical practices are still very popular. Although medicinal plants may have biological activities that are beneficial to humans, the potential toxicity of these bioactive substances has not been well established. Thus, the safety and efficacy of these plants must be studied thoroughly to maximize their benefits for mankind (Elsnoussi *et al*., 2011).

In our study, we have demonstrated that the dichloromethane extract of *S. circinata* is not toxic by oral administration in mice up to 2000 mg/kg, similar toxicological concentrations were achieved for plant extracts of *Argania spinosa, Citrullus colocynthis* and *Boswellia serrata* by Aribi, 2015.

The evaluation of the immunomodulatory effect of DMESC took into account that the immune cells and mediators are directly involved in the processing of antigens, removal of microorganisms by, phagocytosis, lysis of bacteria, viruses or tumor cells. Many malignant diseases are caused by, a decreased number or function of immune competent cells. Hence, modification of immune response either through suppression or stimulation may be helpful in avoiding diseases related to the immune system (Sharma *et al*., 2012).

Currently several of the available therapeutic drugs have potential side effects. Thus, medicinal plants and their active components as a source of immunomodulatory agents are gaining importance (Sharma *et al*., 2012).

The results of the present study showed that DMESC may stimulate cell mediated immunity as shown by an increase in macrophage induced phagocytosis in carbon clearance test. When ink containing colloidal carbon is injected into the systemic circulation, the macrophages engulf the carbon particles of the ink and the rate of clearance of ink from blood is known as phagocytic index (George *et al*., 2014). DMESC stimulated the RES by, a high significant increase in the phagocytic index. Our results are in agreement with those of Benmebarek *et al*. (2014) who indicated that *Stachys ocymastrum* extract appears immune stimulatory at low concentrations and immunosuppressive at high concentrations as it exhibited a biphasic effect on the phagocytic activity of the RES and with those of Nassar *et al.* (2015) who reported that the *n* butnolic extract of *Stachys circinata* increased the phagocytic index at 150 mg/kg.

The improvement in phagocytic function by DMESC may be due to a number of actions of different effector components of the phagocytes. However, the most important mechanism is the up-regulation of receptors that are required to interact with the pathogens which include mannose and toll like receptors. It may also be due to the increased opsonization of carbon particles by complement proteins and immunoglobulins. So, the immunostimulate activity of the DMESC which acted by activating the function of the RES is due to the fact that it contains natural physiologically active substances such as terpenoids, phenolic compounds and flavonoids (Laggoune et al., 2016), which increase the humoral response, by stimulating the macrophages and B-lymphocytes subsets involved in antibody synthesis (Dash *et al*., 2006).

In our work we are searching for a new therapeutic drugs for arthritis because synthetic drugs, in recent years, are accompanied by numerous unwanted side effects, such as the Non Steroidal Anti-Inflammatory Drugs (NSAIDs) that produce gastric ulcer, and as major side effects glucocorticoids are associated with adrenal suppression (Boddawar *et al*., 2016). In addition, their use cannot halt the development of rheumatoid arthritis and Disease Modifying Antireheumatic Drugs (DMARDs) have been impeded by their potential of long-term side effects, toxicity and immunosuppression (Tag *et al.,* 2014). So, it is very important to search for new therapeutic drugs from a natural source with greater efficiency and lower toxicity.

Findings of the present study have revealed that DMESC treatment exerts anti-arthritic effect. It decreased the inflammation compared to the control group as observed by the decrease in the edema size, the concentration of ACCP and CRP values. These results agree with those of Mazumder *et al.* (2012) who reported that in the formaldehyde induced arthritis inflammation test (FIA), the methanol extract of *Barleria lupulina* owned a significant inhibition of the edema formation during the experimental period of 10 days. Our results are in agreement with those of Kehili *et al.* (2016) who reported that ACCP and CRP are decreased in mice injected with formalin and treated with Algerian *Phoenix dactylifera* fruit. Benmebarek *et al*. (2013) reported a decrease of hs-CRP when mice was treated with extracts of *S. mialhesi* following an inflammation induction by hyperhomocysteinemia.

FAI is one of most commonly used acute models for assessing anti-arthritic potential of plant extract (Kore *et al*., 2011). Injection of formalin into hind paw produces localized pain and inflammation, which is biphasic response, an early neurogenic component followed by a later tissue-mediated response (Shastry *et al*., 2011).

The initial phase of the edema is due to the release of histamine and serotonin and the edema is maintained during the plateau phase by kinin like substance and the second accelerating phase of swelling due to the release of prostaglandin like substances (Manguesh *et al*., 2010); this phase seems to be an inflammatory response with inflammatory pain that can be inhibited by anti-inflammatory drugs (Vasudevan *et al.*, 2006).

The anti-inflammatory activities of many plants have been attributed to their saponin, terpenoids, flavonoids and steroids contents (Shastry *et al*., 2011). Flavonoids were considered to be the active components responsible for the biological actions of the genus *Stachys.* In addition, this genus has been shown to possess various biological properties related to antioxidant, anti-nociceptive and anti-inflammatory mechanisms by targeting reactive oxygen species and prostaglandins which are involved in the late phase of acute inflammation and pain perception (Kehili *et al*., 2016; Laggoune *et al*., 2016). These results are compatible with those of Peng *et al.* (2016) who reported that the flavonoids and alkaloids in the ethanol extract of the roots of *Caragana pruinosa* might be responsible for its anti-arthritic activity. In our research, we evidence that DMESC had accelerated the anti-inflammatory activity in a similar pathway as diclofenac and this is remarkable by lower volume of edema, CRP and Anti-CCP values.

The initiation of RA involves the activation of auto-reactive T cells and the recruitment of these T cells along

with other leukocytes into the joints. These leukocytes produce a variety of mediators of inflammation. Prominent among these mediators are arachidonic acid metabolites, pro-inflammatory cytokines, free radicals and matrix-degrading enzymes. These mediators modulate the processes relating to cell migration into the joints as well as angiogenesis and degradation of the extracellular matrix within the joints, leading to the arthritic inflammation (Venkatesha *et al*., 2011).

Naderi *et al*.(2016) reported that phenolic compounds in the Ginger decrease the pro-inflammatory factors of TNF-α and IL-1β. Both mediators induce NF-kB, which is a ubiquitous eukaryotic transcription factor with a pivotal role in inflammatory pathways. In addition, these crucial compounds suppress the synthesis of prostaglandin and leukotriene by inhibiting the COX-2 and lipoxygenase pathways and also inflammation-involved pathways diminishing the inflammation. Therefore the active compounds obtained by Lggoune *et al*.(2016), could play same role for inhibition of TNF-α and IL-1β.

Serum CRP, a surrogate marker of disease severity that correlates with final outcome of arthritis is also a potent endogenous ligand for TLR-2 present on the surfaces of synovial fibroblasts, PMNs and macrophages and its transcription is regulated by pro-inflammatory cytokines including IL-6 (Adhikary *et al*., 2016).Serum concentration of CRP, the most common type of acute phase proteins was tested in our experiments and was found to be significantly attenuated in case of FAI in mice treated with DMESC. This explains a protective role of DMESC against liver damage and inflammatory reactions in hepatic tissues during pathogenesis of FIA.

Immune complexes (IC) deposited into the synovial joints elevate pro-inflammatory cytokines in serum, through induction of mononuclear cells, these immune complexes can stimulate PMNs and macrophages to secrete pro-inflammatory cytokines, like TNF-α. In both ways, there is activation of synovial macrophages which ultimately results in increased production of TNF-α, IL-1β and other pro- inflammatory cytokines like IL-6, IL-12 and IL-15 which are involved in RA pathogenesis (Adhikary *et al*., 2016). However, IC containing citrullinated fibrinogen have been detected in the peripheral blood of anti-citrullinated protein/peptide antibody (ACPA) -positive RA patients and also in synovial pannus (Fisher, 2014). These IC stimulate macrophage TNFα production and the accumulation of multiple ACPA specificities is correlated with preclinical inflammation (elevation of TNF-α, IL-6, and IFN-γ) preceding clinical arthritis (Sakkas *et al*., 2014).

A considerable improvement in the management of RA has been obtained since the advent of biological agents such as (TNF)-α inhibitors (adalimumab, certolizumab, etanercept, golimumab or infliximab) anti-B cell agent (rituximab), anti-IL-6 receptor inhibitor (tocilizumab), T cell modulator (abatacept) (Atzeni *et al*., 2013). A problem facing the practicing physician is to prescribe the most appropriate biological agent to individual patient, in other words, to match a biological agent with a patient profile, given the high cost of biological (Boissier *et al*., 2012). The presence of ACPAs was associated with reduced response to anti-TNFα agents (Potter *et al*., 2009).

From the DMESC, a variety of secondary metabolites were isolated and fifteen known compounds have been identified, among them flavonoids such as luteolin, apigenin, isorhamnetin, triterpenoids such as betulinic acid, ursolic acid and olealonic acid, sterols such as stigmasterol and β-sitosterol (Lggoune *et al*., 2016). However the effect shown by DMESC that ameliorates FIA induced inflammation and progressive bone damage is an outcome of a cumulative effect of all these bioactive compounds; therefore, further studies are warranted on these chemical constituents from DMESC after purifying them and administering them separately in animal models with respect to formalin-induced arthritis.

5. Conclusion

These findings provide a basis for the therapeutic potential of *S. circinata* for the control and management of diseases in which the immune system needs to be stimulated and for inflammatory conditions like rheumatoid arthritis.

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Conflicts Of Interest

All authors report no conflicts of interest regarding this manuscript.

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Résumé en Français

Introduction

Les produits naturels d'origine végétale et biologique demeurent une source illimitée et non condensée de nouveaux composés phytochimiques et nutraceutiques (Ahmad *et al.,* 2017). Les plantes médicinales sont prescrites et largement utilisées depuis des milliers d'années pour traiter plusieurs troubles et affections dans les systèmes de phytothérapie populaire partout dans le monde et ont été considérées comme une approche efficace (Hiwa, 2016) pour contrôler divers troubles tels que les maladies auto-immunes (Singh *et al.,* 2020), l'inflammation et les cancers (Panahi Kokhodan *et al.,* 2018).

Le système immunitaire est un mécanisme de défense hautement sophistiqué dans les organismes vivants qui protège l'hôte des agents pathogènes étrangers (Xu et Larbi, 2017). On pense que le dysfonctionnement du système immunitaire est l'une des principales étiologies responsables du développement de nombreuses conditions anormales telles que les maladies auto-immunes, le rejet de greffe d'organe, les maladies infectieuses et le cancer. Cependant, la réponse modulatrice du système immunitaire est restée d'un grand intérêt depuis longtemps pour atténuer ces maladies (Rasheed *et al.,* 2016).

L'inflammation est caractérisée par une séquence d'événements comprenant une phase d'induction, qui conduit au pic de l'inflammation et suivie progressivement d'une phase de résolution. La phase d'induction de l'inflammation est conçue pour permettre une activation immunitaire rapide et robuste nécessaire à une défense efficace de l'hôte. Elle est initiée par la détection de signaux de danger exogènes et endogènes résultant de lésions tissulaires induites mécaniquement, chimiquement ou biologiquement, suivies du recrutement de cellules effectrices, qui orchestrent une réponse inflammatoire caractérisée par la libération de médiateurs lipidiques et protéiques de l'inflammation (Schett et Neurath, 2018).

L'inflammation aiguë fait partie de l'immunité innée initiée par les cellules immunitaires qui ne persiste que pendant une courte période. Cependant, si l'inflammation persiste, la deuxième étape de l'inflammation appelée inflammation chronique commence, ce qui entraîne de nombreuses maladies chroniques, notamment l'arthrite, le cancer, les maladies cardiovasculaires respiratoires, le diabète et les maladies neurodégénératives (Kannumakkara *et al.,* 2018).

La polyarthrite rhumatoïde (PR) est une maladie inflammatoire systémique chronique qui se caractérise par une synovite étendue entraînant des érosions du cartilage articulaire et de l'os marginal qui entraînent la destruction des articulations (Chimenti *et al.,* 2015). La libération des cytokines, en particulier le facteur de nécrose tumorale α (TNF-α), l'interleukine-6 (IL-6) et

l'IL-1, provoque une inflammation synoviale. En plus de leurs effets articulaires, les cytokines pro-inflammatoires favorisent le développement d'effets systémiques, y compris la production de protéines en phase aiguë (Zampeli et al., 2015). La PR est considérée comme une maladie auto-immune puisque la production du facteur rhumatoïde (RF), un auto-anticorps dirigé contre les déterminants sur le fragment Fc des molécules d'immunoglobuline (Ig) G, a été observé pour la première fois. Les auto-anticorps les plus pertinents semblent être les anticorps anti-protéine citrullinée (ACPA). La citrullination est l'étape critique pour la reconnaissance de plusieurs protéines (fibrine, vimentine, fibronectine, collagène de type II) fortement exprimées par l'ACPA dans la membrane synoviale lors de l'inflammation. La pathogenèse de la PR est un processus en plusieurs étapes qui commence par le développement de l'auto-immunité, se poursuit avec une inflammation locale et finalement induit une destruction osseuse (Chimenti *et al.,* 2015).

Le processus inflammatoire induit un stress oxydatif (Khansari *et al.,* 2009) qui constitue une perturbation causée par un déséquilibre entre la génération et l'accumulation d'espèces réactives à l'oxygène (ROS) dans les cellules et la capacité d'un système biologique à détoxifier ces produits réactifs (pizzino *et al.,* 2017). Ces ROS sont générés sous le stimulus de cytokines pro-inflammatoires dans les cellules phagocytaires et non phagocytaires par l'activation de la signalisation des protéines-kinases. Ainsi, le TNF-α augmente la formation des ROS par les neutrophiles et d'autres cellules, tandis que l'interleukine- 1 β (IL-1β), le TNF - α et l'interféron (IFN) -γ stimulent l'expression de l'oxyde nitrique synthase inductible dans l'inflammation et les cellules épithéliales (Federico *et al.,* 2007).

Chez un être humain en bonne santé, la génération des ROS est contrôlée par des antioxydants cellulaires tels que la superoxyde dismutase (SOD), la catalase (CAT), la glutathion peroxydase (GPx), le glutathion (GSH) et le glutathion réductase (GR) (Salla *et al.,* 2016). La SOD catalyse la dismutation du radical superoxyde en oxygène (O_2) et en peroxyde d'hydrogène (H_2O_2), et la CAT catalyse la décomposition du H_2O_2 nocif en eau et O_2. Le disulfure de glutathion (GSSG) est réduit en GSH catalysé par la GR et fournit des ions hydrogène (H +) par le nicotinamide adénine dinucléotide phosphate (NADPH) avec une diminution des taux de NADPH (Zhao *et al.,* 2014). Cependant, la surproduction de radicaux libres peut provoquer un déséquilibre du statut redox cellulaire produisant des dommages oxydatifs aux biomolécules, (lipides, protéines, acide désoxyribonucléique (ADN) (Rouabhi *et al.,* 2015), ce qui a des implications majeures dans l'étiologie des maladies chroniques telles que le diabète, les maladies cardiovasculaires et le cancer (Choi et Kim. 2013).

Le cancer est l'une des principales causes de mortalité à l'origine de 8,2 millions de décès dans le monde en 2012 (Meneses-Sagrero *et al.*, 2017). Il peut être décrit comme un ensemble de processus complexes impliquant une mort cellulaire altérée, une prolifération cellulaire illimitée et des changements temporo-spatiaux de la physiologie cellulaire, qui conduisent souvent à la formation de tumeurs malignes entraînant l'invasion de tissus distants, pour former des métastases (George et al., 2017) . La carcinogenèse est un processus multi étapes qui implique une série d'événements comprenant des changements génétiques et épigénétiques conduisant à l'initiation, à la promotion et à la progression du cancer. La carcinogenèse peut résulter de dommages importants à l'ADN, souvent causés par l'exposition à une variété d'agents exogènes et endogènes, notamment les rayons ultraviolets (UVR), les rayonnements ionisants (IR), les produits chimiques mutagènes, les agents environnementaux, les agents thérapeutiques ou l'imagerie diagnostique (George *et al.*, 2017).

Une grande attention est portée aux produits naturels, en raison des effets cancérigènes des antioxydants synthétiques utilisés dans l'industrie alimentaire (Demiroz *et al.*, 2020) et des effets secondaires des anti-inflammatoires, y compris les lésions gastro-intestinales et l'hépatotoxicité (Ou et al., 2019)). L'antioxydant naturel joue un rôle essentiel dans la prévention des dommages oxydatifs, qui peuvent entraîner de nombreuses maladies (Xia *et al.*, 2017). En outre, les connaissances traditionnelles et les rapports scientifiques démontrent que les plantes médicinales sont de riches sources de composés biologiquement actifs qui peuvent être utilisées pour la prévention ou le traitement de diverses maladies, y compris certains types de cancers (George *et al.*, 2017).

Cette étude a révélé le mode d'action de deux espèces endémiques, appartenant au genre *Stachys* (Lamiaceae) et nous avons tenté de cibler ces objectifs:

Tester la sécurité d'utilisation des extraits de *Stachys mialhesi* et *Stachys circinata* et déterminer les doses létales à l'aide de tests de toxicité chez l'animal.

- ✓ Évaluation de l'effet immunomodulateur des extraits de *Stachys mialhesi* et *Stachys circinata* à l'aide du test de clairance du carbone.

- ✓ Évaluation de l'effet antioxydant des extraits de *Stachys mialhesi* et *Stachys circinata* en effectuant le dosage du GSH et de la CAT sur le foie des souris.

- ✓ Examen de l'effet des extraits de *S. mialhesi* et *S. circinata* sur l'inflammation et l'arthrite par le dosage de la hs-CRP plasmatique et de l'Anti-CCP.

- ✓ Confirmation de l'action des extraits de plantes sur l'arthrite induite par le formol et de sur l'inflammation articulaire par étude histologique.

- ✓ Évaluation de l'activité antiproliférative des extraits de plantes sur le cancer du foie (lignée cellulaire d'hépatocarcinome HepG2), cancer du sein (les cellules MCF7) et sur les cellules du phéochromocytome (Tumeur neuro-endocrinienne) de médullosurrénale de rat (PC12) en utilisant des techniques de culture cellulaire.

- ✓ Etudier l'effet des extraits *S. mialhesi* et *S. circinata* sur la viabilité cellulaire des cellules humaines saines: cellules endothéliales des vaisseaux sanguins HUVEC.

- ✓ Évaluation des activités antioxydantes *in-vitro* (CAT et GSH) des extraits de S. *mialhesi* et *S. circinata* sur le cancer du foie (lignée cellulaire hépatocarcinome HepG2).

Matériel et méthodes

Extrait de plante

Des parties aériennes de *S. circinata* et de *S. mialhesi* ont été récoltées de la région de Constantine Djebel El-Ouahch en avril 2013 pendant la phase de floraison. Elles ont été identifiées par le Professeur Gérard De Bélair (faculté des sciences, Université Badji-Mokhtar, Annaba). Un échantillon de *S. mialhesi* a été déposé au Musée Botanique de la ville d'Angers (France) sous la référence MBAng2005.10 et un spécimen de référence (LOST SC04 / 13) de *S. circinata* a été déposé au Laboratoire des substances thérapeutiques, Université frères Mentouri-Constantine.

Préparation des extraits

1 kg des parties aériennes séchées à l'air de *S. circinata* et *S. mialhesi*, ont été mises à macérer séparément pendant trois jours à température ambiante dans un mélange hydroalcoolique (MeOH-H20 ; 70:30, v / v). Après filtration, les extraits de chaque plante ont été réunis, concentrés puis dissous dans 300 ml d'eau. La solution résultante a été extraite successivement avec l'éther de pétrole, puis le dichloromethane (CH_2CL_2), puis l'acétate d'éthyle (EtOAc) et en dernier le n-butanol. Les phases organiques récupérées sont concentrées sous pression réduite. L'extrait au dichlorométhane résultant de *S. circinata* et l'extrait butanolique de *S. mialhesi* ont ensuite été utilisés dans toutes les expériences.

Animaux

Les animaux utilisés dans notre étude *in-vivo* étaient des souris mâles, adultes et saines appartenaient à l'espèce *Mus musculus* provenant de l'institut de pharmacie, Constantine, Algérie. Les animaux ont été acclimatés dans les conditions du laboratoire pendant une semaine avant le début de l'expérience.

Les cellules utilisées dans la culture cellulaire

Dans notre étude, quatre lignées cellulaires ont été utilisées : trois d'entre elles cancéreuses : les cellules du cancer du sein (MCF7), cellules de cancer du foie (HepG2), les cellules du phéochromocytome (PC12) et une lignée cellulaire saine endothéliale de la veine ombilicale humaine (HUVEC). Ces cellules ont été obtenues du laboratoire civil de Cagliari en Italie et du laboratoire de pharmacologie de l'université de Sassari, Italie.

Méthode

Toxicité orale aiguë

La présente étude a été menée conformément à la directive proposée par l'organisation de coopération et de développement économique (OCDE), qui est basée sur la procédure de (Bruce, 1985). Une dose de 2000 mg / kg d'extrait de *S. circinata* et *S. mialhesi* a été utilisée sur cinq souris saines. Cette dose a été d'abords administrée à une seule souris, dans le but de surveiller et la mortalité et les signes cliniques (comportements enregistrés: agressivité inhabituelle, vocalisation inhabituelle, agitation, mouvements de sédation et de somnolence, paralysie, convulsions, fasciculation, prostration et locomotion inhabituelle), la souris était observée à la première heure puis chaque trois heures pour une durée de 48h.

Lors de la survie de cette souris, quatre souris supplémentaires avaient reçu la même dose à 48 h d'intervalle, les signes cliniques ont été à nouveau surveillés. Tous les animaux expérimentaux ont été maintenus sous étroite observation pendant 14 jours après l'administration des extraits, et le nombre de souris qui sont mortes pendant la période expérimentale a été noté. La dose létale 50 (DL50) a été établie comme étant supérieure à 2000 mg / kg si aucun trouble de santé ni décès n'a été enregistré chez trois souris ou plus.

Evaluation de l'activité immunomodulatrice des extraits de plantes

De nombreux extraits de plantes ont longtemps été décrits comme possédant des actions anti-inflammatoires et immunomodulatrices. La première ligne de défense du corps humain contre les agents pathogènes envahissants est le système immunitaire inné par les macrophages. Dans la présente étude, l'activité phagocytaire du système réticuloendothélial (RES) a été évaluée par un test de clairance du carbone. L'indice phagocytaire a été calculé comme un taux d'élimination du carbone du RES par un test de clairance du carbone déterminé par la méthode de Halpern *et al*. (1953). Les souris ont été divisées en quatre groupes de 7 animaux chacun comme indiqué sur le tableau 1 et 2:

Tableau 1: Traitement des souris dans le test du taux de clairance du carbone *S. circinata* n=7

Groupes expérimentaux	**Traitement**	**Dose**
GI	NaCl 0.9 %	0.5ml/souris
GII	*S. circinata*	50mg/kg
GIII	*S. circinata*	150mg/kg
GIV	*S. circinata*	200mg/kg

Tableau 2: Traitement des souris dans le test du taux de clairance du carbone *S. mialhesi* n=7

Groups expérimentaux	Taitement	Dose
GI	NaCl 0.9 %	0.5ml/souris
GII	*S. mialhesi*	50mg/kg
GIII	*S. mialhesi*	150mg/kg
GIV	*S. mialhesi*	200mg/kg

Après quarante-huit heures de traitement par voie intra péritonéale, une suspension d'encre de carbone colloïdal à une dose de 0,1 ml / 10 g de poids a été injectée via la veine de la queue à tous les groupes d'animaux. Ensuite, des échantillons de sang (≈14 gouttes ou 25 µL) ont été prélevés du plexus rétro-orbitaire via des capillaires en verre d'héparine à des intervalles de 5 et 15 minutes. Les échantillons de sang collectés ont été lysés dans une solution de carbonate de sodium à 0,1% (4 ml) et la densité optique (DO) a été mesurée par spectrophotométrie à 675 nm. À la fin de l'expérience, le foie et la rate de chaque souris ont été prélevés, pesés et les valeurs obtenues ont été utilisées pour calculer l'indice phagocytaire K comme suit :

$$\mathbf{K} = \frac{\mathbf{Log\ DO1 - Log\ DO2}}{\mathbf{t2 - t1}} \qquad \mathbf{t½} = \frac{\mathbf{0.693}}{\mathbf{K}}$$

$$\boldsymbol{\alpha} = \sqrt[3]{\mathbf{K}}\ \frac{\mathbf{le\ poides\ de\ l'animal}}{\mathbf{Le\ poides\ de\ la\ rate + le\ poids\ du\ foie}}$$

DO1 et DO2 sont les densités optiques enregistrées à 675 nm au temps t1 (5 min) et t2 (15 min), respectivement.

Evaluation de l'activité anti-oxydante des extraits de plantes

Le glutathion réduit a été estimé en utilisant une technique colorimétrique selon la méthode de Weckbeker et Corey. (1988), tandis que l'activité de la catalase a été mesurée selon la méthode de Aebi (1983). Les concentrations totales de protéines du foie ont été mesurées par la méthode de Bradford (1976), les souris ont été sacrifiées et les foies ont été rapidement retirés .L'homogénat hépatique a été utilisé pour effectuer le dosage de la catalase et le glutathion réduit.

GSH

La mesure de la concentration intracellulaire de GSH est basée sur l'absorbance optique de l'acide 2-nitro benzoique(DTNB) selon l'équation suivante :

$$\boldsymbol{SH}\left(\frac{\boldsymbol{nmol}}{\boldsymbol{mg\ protein}}\right) = \frac{\boldsymbol{DO\ X\ 1\ X\ 1.525}}{\mathbf{13100\ X\ 0.8\ X\ 0.5\ X\ }\boldsymbol{mgprotein}}$$

Catalase

L'activité de la catalase (CAT) est mesurée selon Aebi, (1983) à l'aide d'un spectrophotomètre par la variation de la densité optique consécutive à la dismutation du peroxyde d' hydrogène (H_2O_2) en faisant réagir dans 100 mM de tampon phosphate pendant 1 mn à pH 7.5, 100 µl de H_2O_2, 500 mM sur 20 µl du d'homogenat, à une température d'incubation de 25°C.

Les résultats ont été exprimés en µmole d' H_2O_2 par minute et par mg de protéine **(Tableau 4 page 27).** La diminution de la densité optique est due à la décomposition du peroxyde d'hydrogène qui a été mesurée par rapport à un blanc à 240 nm. **(voir annexes)**

L'activité spécifique a été exprimée en termes d'unités par gramme de tissu selon cette formule:

$$\boldsymbol{AT\ (\mu mol/\ mn/\ mg\ Hb)} = \frac{\boldsymbol{(\Delta\ Do\ \times 10)}}{\boldsymbol{\varepsilon \times L \times Y\ mg\ de\ prot\acute{e}ines}}$$

Evaluation de l'activité antiarthritique des extraits de plantes

Cette étude a été réalisée selon le protocole de Mazumder *et al.* (2012). Les souris ont été divisées en quatre groupes (F, FF, SC / SM, D) de cinq animaux chacun. Le groupe (F) (témoin

négatif); le groupe FF (témoin positif) a été soumis au formol uniquement; groupe (SC), (SM) au formol + *S. circinata* et *S. mialhesi* respectivement à 150 mg / kg et groupe (D) au formol + le médicament standard anti-inflammatoire (diclofénac de sodium) à 10 mg / kg (**Tableau 3**).

Une injection de 100 µL de formol (2%) a été administrée dans l'espace sous-plantaire de la patte arrière droite des souris le premier et le 3ème jour de l'expérience. Par conséquent, le diclofénac et les extraits (SC, SM) ont été administrés quotidiennement jusqu'à la fin de l'expérience de 10 jours, pendant cela, une mesure quotidienne de la taille de l'œdème a été réalisée avec un pied à coulisse numérique.

A la fin des expériences, les animaux de tous les groupes ont été soumis à une diète hydrique à la veille du prélèvement de sang. Le dosage de la protéine C réactive (CRP) a été évalué sur un analyseur Cobas integra 400 plus (Roche) quant aux anti-CCP ont été mesurés par le processeur de microplaques compact automatisé Gemini 6280 de Stratec Biomedical Systems au laboratoire Iben Sina. Le dosage de ces paramètres a été réalisé à partir du plasma sanguin.

Tableau 3: Traitement des souris dans le test d'arthrite induite par le formol

Groups expérimentaux	Farine (g)	Formol (µl)	Dose
F	**0,1**	**0**	**/**
FF	**0,1**	**100**	**/**
SC	**0,1**	**100**	**150mg/kg**
SM	**0,1**	**100**	**150mg/kg**
D	**0,1**	**100**	**10mg/kg**

Etude histologique des articulations

A la fin de l'expérience, les animaux ont été sacrifiés avec du chloroforme et les pattes postérieures ont été coupées et fixées dans le formol à 10% pendant 24 heures ensuite

décalcifiées dans 0,3% d'acide formique. Après avoir subi une déshydratation à l'alcool, les tissus ont été inclus dans la paraffine, coupés en sections de 5 µm d'épaisseur et enfin colorées (voir annexe).

Evaluation de l'activité antiproliférative des extraits de plantes

L'activité antiproliférative des extraits de chacune des deux espèces a été évalué *in-vitro* sur des lignées cellulaires cancéreuses du foie (HepG2), du sein (MCF7), du phéochromocytome (PC12) et sur des cellules endothéliales humaines saines (HUVEC) en utilisant le test colorimétrique MTT.

Brièvement, une fois que les cellules HepG2, MCF-7, PC-12 et HUVEC ont été ensemencées dans des plaques à une densité de 5000 cellules / puit dans 100 µL de milieu et maintenues à 37 ° C dans un incubateur avec atmosphère humide et 5% de CO_2 pendant 24heures, le milieu a été aspiré et les cellules ont été traitées par six différentes concentrations de chaque extrait puis incubées pendant 24 heures supplémentaires, après cela, les extraits ont été retirés et remplacés par 100 µL de MTT (0,65 mg / mL), les cellules en culture ont été incubées encore 2h.

Finalement, le MTT a été éliminé et 200 µl / puit de DMSO a été ajouté afin de solubiliser les cristaux de formazan.

La densité optique de chaque échantillon a été mesurée afin de déterminer le pourcentage de prolifération cellulaire suivant la formule de Patel *et al.* (2009).

$$\textbf{\% Cell proliferation} = (\boldsymbol{At - Ab / Ac - Ab}) \times \mathbf{100}$$

At = Valeur d'absorbance du composé testé (*S.mialhesi* / *S.circinata*).

Ab = valeur d'absorbance du blanc (milieu de culture).

Ac = valeur d'absorbance du contrôle (milieu de culture + cellules).

Evaluation de l'activité antioxydante *in-vitro* des extraits de plantes

Dans le but de valider les résultats obtenus du pouvoir antioxydant de nos plantes dans l'étude *in-vivo*, une étude *in-vitro* a été réalisée sur les cellules cancéreuses du foie (HepG2) qui ont été

exposées à six différentes concentrations 17, 87, 170, 350, 500 et 570 µg / 100 µL des extraits étudiés pendant 24h afin d'évaluer l'activité de la catalase et du glutathion réduit.

La catalase

L'activité de la catalase est déterminée par une méthode de dosage basée sur la mesure du peroxyde d'hydrogène restant après l'action de la catalase. Tout d'abord, la catalase convertit le peroxyde d'hydrogène en eau et en oxygène, puis cette réaction enzymatique est arrêtée avec de l'acide de sodium. Une aliquote du mélange réactionnel est ensuite dosée pour quantifier le peroxyde d'hydrogène restant par une méthode colorimétrique. Par conséquent, l'activité catalase présente dans l'échantillon est inversement proportionnelle au signal obtenu.

Des cellules HepG2 ont été ensemencées dans une plaque (250 000/ puit dans 500 µl), maintenues à 37 ° C dans un incubateur sous 5% de CO_2, 95 % d'air et à saturation en vapeur d'eau pendant 24 heures. Le milieu a été enlevé et les cellules ont été traitées par nos extraits avec six différentes concentrations et ont été encore mises en incubation pendant 24 heures.

Les extraits ont été aspirés et un processus de digestion enzymatique (trypsine), de dilution et de centrifugation a été établi sur les cellules adhérentes pour obtenir un culot cellulaire auquel on avait rajouté 300µl de tampon de dilution enzymatique et a été précédé par une étape de centrifugation à 1200 g pendant 10 minutes. La concentration en protéines (mg/ml) dans le surnageant a été mesurée par spectrophotomètre

La réaction enzymatique de la catalase a été démarrée en ajoutant 25 µl de la solution de substrat de dosage colorimétrique (200 mm H_20_2), l'absorbance a été mesurée à 490 nm.

GSH

Les cellules HepG2 ont été ensemencées dans des boîtes de Pétri (5 000 000 cellules / boite dans 6 ml) maintenues à 37 ° C dans un incubateur avec atmosphère humide et 5% de CO2 pendant 24heures. Ensuite, le milieu de croissance a été retiré et remplacé par différentes concentrations d'extrait de plantes incubés à 37 ° C pendant 24 h.

Les extraits ont été aspirés et un processus de digestion enzymatique (Trypsine-EDTA), de dilution et de centrifugation a été établi sur les cellules adhérentes pour obtenir un culot cellulaire auquel on avait rajouté 180µl de solution d'acide sulfosalicylique à 4%, la suspension obtenue a été congelée dans de l'azote liquide puis décongelée au bain marie et maintenue à 2-8°C pendant 5 minutes. Le surnageant a été récupéré après une centrifugation (10000g) de 10 minutes. Une solution de NADPH diluée a été ajoutée.

L'absorbance dans chaque puit a été mesurée en utilisant un lecteur de plaque à 405 nm et lue à des intervalles de 1 mn pendant 5 mn.

Résultats

Selon le test de toxicité préliminaire, les extraits de *S. circinata* et *S. mialhesi* se sont avérés non toxiques jusqu'à 2000 mg / kg. Au total, cinq souris mâles adultes ont été traitées par voie orale avec le même extrait à la même dose et ont été observées pendant 14 jours, ces souris sont restées en bonne santé et sans signes visibles de toxicité ni de mortalité. Les résultats obtenus de l'activité immunomodulatrice des extraits de *S. circinata* et *S. mialhesi* ont montré une stimulation importante de l'activité phagocytaire dans tous les groupes de souris traités, cependant la dose de 150mg/kg a fortement stimulé l'activité phagocytaire par apport au témoin et aux autres groupes $p<0.05$ et cela pour les deux extraits (figures 8,10, 11,13), les résultats montrent aussi que l'apport des extraits aux souris a influencé de manière significative la demi-durée calculée (t½) de la clairance du carbone colloïdal qui a diminué de près de 50% par rapport au témoin (NaCl) $p<0.05$ (Figure 9 et 12).

Notre étude sur la capacité antioxydante des extraits de *S. mialhesi* et *S. circinata* s'est étalée à l'étude de l'évaluation des taux des enzymes antioxydants notamment la catalase et le glutathion (GSH) hépatique. Le taux de GSH et de la catalase déterminé chez les groupes de souris traités s'est révélé significativement élevé à comparer aux groupe témoin ($P<0.001$) comme a été observé dans les figures 14, 15, 16, 17.

Les résultats de l'étude menée pour évaluer l'activité antiarthritique des extraits en utilisant le test de l'arthrite induite par le formol ont mis en évidence une inhibition significative de la taille de l'œdème des pattes de souris dans les groupes traités par les extraits ainsi que le groupe traité par l'anti-inflammatoire (diclofenac) ($P = 0,000$) tandis que, d'autre part, une augmentation significative de la taille s'est produite dans le groupe contrôle positif (formol) ($P = 0,000$) par rapport au contrôle négatif ($P = 0,000$) (figure 18 et 21), ces résultats ont été confirmés par la diminution significative des taux d'anti-CCP ($P = 0,000$) et de la CRP ($P <0.05$) (figure 19, 20,22,23).

Comme l'illustre la figure 25, l'évaluation histologique de l'articulation de la cheville a montré que l'injection sous-plantaire de formol a induit des lésions cartilagineuses et une érosion osseuse, cependant, le groupe des souris non immunisées (groupe F) a montré un cartilage hyalin intact, une absence d'infiltrat dans la synoviale et un espace articulaire normal (figure 24).

L'examen histologique de l'articulation de la cheville de souris traitées avec *S. mialhesi* et *S.circinata* (150 mg / kg), a montré un cartilage hyalin intact et une synoviale normale, cependant nous avons observé dans le groupe traité par *S. circinata* une dégénérescence superficielle sur le cartilage de l'articulation en un seul endroit (figures 27 et 28), tandis que le traitement par le diclofénac (10 mg / kg) dans le groupe (D) a résulté un tissu conjonctif normal de l'articulation de la cheville avec moins d'œdème (figure 26).

De plus, dans la présente thèse, nous avons réalisé une étude *in-vitro* pour évaluer l'effet antiprolifératif des extraits étudiés, sur des lignées cellulaires du cancer du foie (HepG2), du sein (MCF7), du phéochromocytome (PC12) et sur des cellules endothéliales humaines saines (HUVEC), nous avons étudier ensuite l'effet antioxydant des extraits de plantes sur les cellules HepG2. Les résultats ont révélé que les extraits de plantes conduisaient à une activité cytotoxique sélective contre les lignées cellulaires cancéreuses alors qu'aucune cytotoxicité contre les cellules normales n'était observée. Nos résultats ont également montré que le traitement des cellules cancéreuses du foie (HepG2) avec les différentes concentrations des extraits de plantes augmentait la libération de l'enzyme anti-oxydante CAT et le GSH.

Discussion

Bien que les plantes médicinales puissent avoir des activités biologiques bénéfiques pour l'homme, la toxicité potentielle de ces substances bioactives n'a pas été bien établie. Ainsi, l'innocuité et l'efficacité de ces plantes doivent être étudiées de manière approfondie pour maximiser leurs avantages pour l'humanité. Pour atteindre cet objectif, une évaluation toxicologique est effectuée à l'aide d'un animal de laboratoire afin de fournir des lignes directrices pour la sélection d'une dose «sûre» à usage humain. Pour évaluer la toxicité des extraits végétaux, les animaux de laboratoire sont traités à des doses spécifiques pendant une durée définie. A cette période, les souris sont contrôlées (Elsnoussi *et al.*, 2011).

Dans notre étude, nous avons démontré que les souris subissant une administration orale des extraits de plantes pendant 14 jours sont restées en bonne santé et sans signes visibles de toxicité ni de mortalité. Ce résultat correspond à une DL50 supérieure à 2000 mg / kg.

La deuxième approche expérimentale de cette étude a été prévue pour évaluer l'effet immunomodulateur des extraits de *S. circinata* et *S. mialhesi* en tenant compte du fait que les cellules immunitaires et les médiateurs de l'inflammation sont directement impliqués dans l'élimination des antigènes. De nombreuses maladies malignes sont causées par une diminution du nombre ou de la fonction des cellules immunitaires compétentes. Par conséquent, la

modification de la réponse immunitaire par suppression ou stimulation peut être utile pour éviter les maladies liées au système immunitaire (Sharma *et al.,* 2012).

Actuellement, beaucoup de médicaments thérapeutiques disponibles ont des effets secondaires potentiels, ainsi, les plantes médicinales et leurs composants actifs en tant que source d'agents immunomodulateurs gagnent une importance (Rasheed *et al.*, 2016).
Le test de clairance du carbone a été effectué pour évaluer l'effet des extraits sur le système réticuloendothélial (RES). C'est un système diffus contenant des cellules phagocytaires (Singh et *al.,* 2012).

Dans cette étude, nous avons constaté que *S. circinata* et *S. mialhesi* peuvent stimuler l'immunité à médiation cellulaire par l'augmentation de la phagocytose induite par les macrophages dans le test de clairance du carbone. Lors de l'injection d'encre contenant le carbone colloïdal, dans la circulation systémique, les macrophages engloutissent les particules de carbone d'encre et le taux de clairance d'encre dans le sang est appelé indice phagocytaire (George *et al.,* 2014). Les extraits de *S. circinata* et de *S. mialhesi* ont stimulé le RES par une forte augmentation significative d'indice phagocytaire. Nos résultats sont en accord avec ceux de Benmebarek *et al*. (2014) qui ont indiqué que l'extrait de *Stachys ocymastrum* apparaît immunostimulant à faibles concentrations et immunosuppresseur à fortes concentrations car il présente un effet biphasique sur l'activité phagocytaire du RES et sont en accord avec ceux obtenus par Nassar *et al.* (2015) qui ont rapporté que l'extrait n butnolique de *Stachys circinata* augmentait l'indice phagocytaire à 150 mg / kg. Mazumder *et al.* (2012) ont rapporté que les racines de *Glycyrhiza glabra* L stimulaient l'activité phagocytaire des macrophages, comme en témoigne une augmentation du taux de clairance du carbone.

L'amélioration de la fonction phagocytaire par les extraits de *S. circinata* et de *S. mialhesi* peut être due à un certain nombre d'actions de différents composants effecteurs des phagocytes. Cependant, le mécanisme le plus important est la régulation à la hausse des récepteurs qui sont nécessaires pour interagir avec les agents pathogènes qui comprennent le mannose et les récepteurs de type péage. Cela peut également être dû à une opsonisation accrue des particules de carbone par les protéines du complément et les immunoglobulines. Ainsi, l'activité immunostimulante des extraits de *S. circinata* et *S. mialhesi* qui ont agit en activant la fonction du RES est due au fait qu'il contient des substances naturelles physiologiquement actives telles que des terpénoïdes, des composés phénoliques et des flavonoïdes (Laggoune *et al.,* 2016), qui augmentent la réponse humorale, en stimulant les macrophages et les sous-ensembles de lymphocytes B impliqués dans la synthèse des anticorps (Dash *et al.,* 2006).

Les espèces réactives de l'oxygène telles que le peroxyde d'hydrogène (H_2O_2) et l'anion superoxyde (O^{2-}), les espèces réactives de l'azote et le peroxynitrite sont générées dans les premières minutes de stimulation des macrophages par le sursaut respiratoire (Castaneda *et al.*, 2017, Gollo *et al.,* 2020). La poussée respiratoire joue un rôle important dans le système immunitaire en étant une réaction cruciale qui se produit dans les phagocytes pour dégrader les particules internalisées et les bactéries. La NADPH oxydase, une famille d'enzymes largement exprimée dans de nombreux types de cellules, produit du superoxyde, qui se recombine spontanément avec d'autres molécules pour produire des radicaux libres réactifs. Pour lutter contre l'infection, les cellules immunitaires utilisent la NADPH oxydase pour réduire l'O^{2-} en un radical libre d'oxygène, puis en H_2O_2 (Yang *et al.*, 2016).

Chez un être humain en bonne santé, la génération de ROS est contrôlée par des antioxydants cellulaires tels que la SOD, la CAT, le GPx, le GSH et la glutathion réductase (GR) (Salla *et al.*, 2016). Cependant, la surproduction de radicaux libres peut provoquer un déséquilibre du statut redox cellulaire produisant des dommages oxydatifs aux biomolécules, (lipides, protéines, ADN) (Rouabhi *et al.,* 2015), ce qui a des implications majeures dans l'étiologie des maladies chroniques comme le cancer , diabète et maladies cardiovasculaires (Choi et Kim, 2013).

Les composés bioactifs d'origine végétale ont le potentiel de pallier les déséquilibres biochimiques induits par diverses toxines associées aux radicaux libres. Ils fournissent une protection sans provoquer d'effets secondaires, par conséquent, le développement de médicaments à partir de produits végétaux est souhaité (Anbuselvam *et al.*, 2007). Les résultats de cette étude ont démontré la capacité antioxydante d'extraits de *S. mialhesi* et *S. circinata* par plusieurs méthodes *in -vivo* et *in -vitro.*

Lors du criblage de l'activité immunomodulatrice, la plupart des études utilisent des agents comme le carbone afin d'induire la cytotoxicité, cet agent est connu pour générer des radicaux libres dans le système biologique et provoquer ainsi un stress oxydatif (Lee *et al.,* 2011). Par conséquent, l'évaluation des antioxydants *in-vivo* de l'homogénat de foie sur des marqueurs de stress oxydatif comme la catalase (CAT) et le glutathion réduit (GSH) de souris a été étudiée. De plus, l'activité antioxydante *in-vitro* des extraits a été évaluée sur la lignée cellulaire cancéreuse d'hépatocarcinome (HepG2).

Le glutathion réduit (GSH) est un antioxydant naturel produit à l'intérieur de la cellule, jouant à la fois un rôle de cofacteur pour le glutathion peroxydase et un piégeur actif pour éliminer les espèces réactives comme le radical hydroxyle, les radicaux centrés sur le carbone, le

peroxynitrite et la molécule d'oxygène singulet. Le GSH a le rôle de préserver le statut redox cellulaire. Il est possible qu'une augmentation des niveaux de GSH minimise les niveaux de ROS, antagonisant ainsi le stress oxydatif (Housseini-Zijoud *et al.*, 2016).

Le glutathion est impliqué dans de nombreuses fonctions cellulaires. Ceux-ci comprennent la synthèse des protéines et de l'ADN; l'activation enzymatique; le transport des acides aminés (Knight, 2000) et joue un rôle clé dans l'immunité innée et adaptative; il renforce l'immunité innée en aidant à la production de lymphocytes T et au fonctionnement des cellules dendritiques et en affectant la capacité des neutrophiles à détruire les agents pathogènes étrangers par phagocytose. Le glutathion est essentiel dans le processus de rupture des liaisons disulfure afin de digérer le pathogène par les cellules présentatrices d'antigène en fragments qui peuvent ensuite se lier au CMH et être présentés aux lymphocytes T qui sont spécifiques à cet antigène, le reconnaissent et sont ensuite activés (Ghezzi, 2011).

Le glutathion, sous sa forme réduite (GSH), est capable d'agir comme un très bon antioxydant qui peut donner des électrons aux radicaux libres. Sa capacité à le faire, provient du groupe sulfhydryle libre (-SH) sur sa structure moléculaire (Biwas et Rahman, 2009) et sa fonction antioxydante est accomplie en grande partie par des réactions catalysées par le GPx, qui réduisent le H_2O_2 et le peroxyde lipidique lorsque le GSH est oxydé en GSSG. Le GSSG est à son tour réduit en GSH par la GSSG réductase aux dépens du NADPH, formant un cycle redox (Shelly et Lu, 2013).

La catalase présente l'un des taux de renouvellement les plus rapides pour toutes les enzymes: dans des conditions optimales, chaque sous-unité peut convertir environ 6 millions de peroxyde d'hydrogène en eau et en oxygène par minute. La catalase est présente dans toutes les cellules aérobies, mais la concentration la plus élevée se trouve dans le foie et les érythrocytes (Kurutas, 2016).

Les résultats ont montré un effet antioxydant potentiel des extraits de *S. mialhesi* et *S. circinata*, présenté par une forte diminution significative des valeurs de CAT et de GSH du foie dans les groupes traités par différentes doses des extraits, avec un effet plus élevé à la dose de 150 mg / kg par rapport au groupe témoin pour les deux extraits, le résultat suggère que les extraits de *S. mialhesi* et *S. circinata* stimulent la libération de GSH et de CAT par le foie, ce résultat est en accord avec le résultat de (Kehili *et al.* , 2014) qui a rapporté que les extraits de *Phoenix dactylifera* ont un effet antioxydant potentiel, en stimulant l'excrétion du GSH par le foie, ce qui conduira à éliminer les espèces réactives de l'oxygène et à protéger l'organisme, dans le même

contexte, (Theo *et al.,* 1989) et (Chahar *et al.,* 2012) ont prouvé que le foie libère le GSH et la CAT principalement dans le sang systémique, dans le cadre d'un renouvellement interorganique, en maintenant ainsi un équilibre redox thiol dans le plasma, ils ont rapporté aussi que le transport hépatique du glutathion pourrait jouer un rôle dans la protection contre la génération accrue d'espèces réactives de l'oxygène dans le sang causée par des processus inflammatoires ou lors d'un exercice physique extrême. En effet, dans de telles conditions, les niveaux de GSH et de CAT dans le foie et d'autres organes étaient significativement diminués.

La stimulation de la libération de glutathion et de catalase hépatiques par des extraits de *S. circinata* et *S. mialhesi* peut être due au fait que les extraits végétaux accélèrent l'activation des molécules de transport GSH et CAT.

Dans le foie, site majeur de synthèse et d'exportation du GSH, le GSH est libéré à des taux élevés dans le plasma sanguin et la bile. Le transport du GSH dans la bile fonctionne comme une force motrice de la sécrétion biliaire et joue un rôle important dans le transport et la détoxification hépatique des composés réactifs d'origine endogène et exogène. Le GSH est également libéré à des taux élevés à travers la membrane sinusoïdale dans le plasma sanguin, pour être administré à d'autres tissus. Bien que l'identité moléculaire des transporteurs de GSH soit restée insaisissable, des études récentes ont impliqué un rôle majeur pour certaines protéines associées à la résistance multidrogue (Mrp / Abcc) dans ce processus (Ballatori *et al.,* 2009).

Au cours des processus métaboliques, des ROS sont normalement générés chez les humains en bonne santé, la concentration de ces molécules dangereuses est contrôlée par des enzymes antioxydantes cellulaires telles que la superoxyde dismutase (SOD), la catalase (CAT), la glutathion peroxydase (GPx), le glutathion (GSH), le glutathion réductase (GR) et des molécules antioxydantes (He *et al.,* 2017; Salla *et al.,* 2016). Néanmoins, les questions controversées concernant l'interférence entre la chimiothérapie, les ROS et les antioxydants doivent être clarifiées afin d'améliorer les thérapies combinées (Cockfield *et al.,* 2019; Hegedűs *et al.,* 2018; Sznarkowska *et al.,* 2017; Peiris-Pagès *et al.,* 2015). Dans ce contexte, de plus en plus de molécules et / ou de phytocomplexes influent sur les niveaux intracellulaires de radicaux libres associés à la cancérogenèse, certains agissant comme antioxydants et d'autres comme inducteurs d'enzymes antioxydantes (Tariq *et al.,* 2015; Stagos *et al.,* 2018).

L'intérêt pour les médicaments phytothérapiques est croissant, compte tenu de leur utilisation potentielle en combinaison avec d'autres approches et / ou être fournis sous forme de régime en

phase de récupération pour prévenir la récidive tumorale (Xie et Zhou, 2017; Roleira *et al.*, 2015; Prasad, 2004).

Par conséquent, les puissantes propriétés antioxydantes des flavonoïdes sont bien établies, révélant leur capacité à réguler les enzymes impliquées dans les processus de stress oxydatif, tels que la SOD, le CAT et le GR (Zhao *et al.*, 2014), cette capacité antioxydante est due à la présence d'hydroxyle groupes (· OH) dans le squelette de ces classes de molécules. De plus, d'autres auteurs ont prouvé que les teneurs phénoliques et flavonoïdes sont associées à des propriétés anti-oxydantes, ce qui en fait d'excellents stabilisants pour la peroxydation lipidique (Bekhouche *et al.*, 2018). Dans ce contexte, Namvar *et al*. (2018) ont révélé qu'il existait une forte corrélation entre le contenu phénolique total et la capacité antioxydante des extraits de *Stachys turcomanica*. De plus, Sadeghi *et al.* (2020) ont révélé que les effets néphroprotectrices et antioxydants de *Stachys pilifera Benth* contre la néphrotoxicité induite par le cisplatine en raison de la présence de composés tels que les diterpènes, les glycosides phényléthanoïdes, les saponines, les terpénoïdes et les flavonoïdes chez les espèces *Stachys*. Dans une autre étude, Alizadeh *et al.* (2020) ont révélé que la présence de composants tels que les polyphénols, les acides phénoliques et les flavonoïdes dans l'extrait hydroalcoolique de *Stachys sylvatica* est la raison de son potentiel antioxydant. De plus, Alpay *et al.* (2017) rapportant que *Stachys annua (l.) L. Subsp. Annua var. Annua* a eu un effet antioxydant sur les cellules cancéreuses du col utérin (HeLa) et les cellules cancéreuses prostatiques (PC3).

Les résultats de l'activité antioxydante *in-vivo* et *in -vitro*, ont prouvé que les extraits *S. circinata* et de *S. mialhesi* possèdent une capacité antioxydante importante, ces résultats sont expliqués par la richesse des extraits de composés phénoliques et de flavonoides.

Dans notre travail, nous recherchons de nouveaux médicaments thérapeutiques pour l'arthrite, car les médicaments synthétiques ces dernières années, sont accompagnés par de nombreux effets secondaires indésirables, tels que les anti-inflammatoires non stéroïdiens (AINS) qui produisent un ulcère gastrique, quant aux glucocorticoïdes sont associés à la suppression surrénalienne (Boddawar *et al.*, 2016). De plus, leur utilisation ne peut pas arrêter le développement de la polyarthrite rhumatoïde et les médicaments antirhumatismaux modificateurs de la maladie (DMARD) ont été entravés par leur potentiel d'effets secondaires à long terme, de toxicité et d'immunosuppression (Tag *et al.*, 2014). Il est donc très important de rechercher de nouveaux médicaments thérapeutiques à partir d'une source naturelle avec une plus grande efficacité et une moindre toxicité.

Les résultats de la présente étude ont révélé que le traitement par *S. circinata* et *S. mialhesi* exerce un effet antiarthritique confirmé par une diminution significative de la taille de l'œdème des pattes de souris, des taux d'anti-CCP et de la CRP. Ces résultats concordent avec ceux de Mazumder *et al.* (2012) qui ont rapporté que dans le test d'inflammation de l'arthrite induite par le formaldéhyde (FIA), l'extrait de méthanol de *Barleria lupulina* possédait une inhibition significative de la formation d'œdème pendant la période expérimentale de 10 jours. Nos résultats sont en accord aussi avec ceux de Kehili *et al.* (2016) qui ont rapporté que l'ACCP et la CRP sont diminuées chez les souris injectées de formol et traitées avec le fruit algérien de *Phoenix dactylifera*. Benmebarek *et al.* (2013) ont rapporté une diminution de la hs-CRP lorsque les souris étaient traitées avec des extraits de *S. mialhesi* suite à une induction d'inflammation par hyperhomocystéinémie.

L'arthrite induite par le formol est l'un des modèles aigus les plus couramment utilisés pour évaluer le potentiel antiarthritique de l'extrait de plante (Kore *et al.,* 2011). L'injection de formol dans la patte arrière induit une réponse douloureuse biphasique; on pense que la première phase résulte de l'activation directe des neurones sensoriels afférents primaires, alors que la deuxième phase a été proposée pour refléter les effets combinés de l'entrée afférente et de la sensibilisation centrale dans la corne dorsale (Shastry *et al.,* 2011).

L'œdème de la patte induit par le formol implique plusieurs médiateurs chimiques tels que l'histamine, la sérotonine, la bradykinine et les prostaglandines (Sowemimo *et al.,* 2015). La phase initiale de l'œdème est due à la libération d'histamine et de sérotonine où l'œdème est maintenu pendant la phase de plateau par une substance de type kinine et la deuxième phase d'accélération de l'enflure due à la libération de substances de type prostaglandine (Mangesh *et al.,* 2010), cette phase est une réponse inflammatoire avec une douleur inflammatoire qui peut être inhibée par des anti-inflammatoires (Chao *et al.,* 2009).

Les activités anti-inflammatoires de nombreuses plantes ont été attribuées à leur teneur en flavonoïdes en saponine, terpénoïdes et stéroïdes (Shastry *et al.,* 2011). Les flavonoïdes étaient considérés comme les composants actifs responsables des actions biologiques du genre *Stachys.* De plus, il a été démontré que ce genre possède diverses propriétés biologiques liées aux mécanismes antioxydants, anti-nociceptifs et anti-inflammatoires en ciblant les espèces réactives de l'oxygène et les prostaglandines qui sont impliquées dans la phase tardive de l'inflammation aiguë et de la perception de la douleur (Kehili *et al.,* 2016; Laggoune *et al.,* 2016). Ces résultats sont compatibles avec ceux de Peng *et al.* (2016) qui ont rapporté que les flavonoïdes et alcaloïdes contenus dans l'extrait éthanolique des racines de *Caragana pruinosa* pourraient être

responsables de son activité anti-arthritique. Dans notre recherche, nous prouvons que les extraits de *S. mialhesi et S. circinata* avaient accéléré l'activité anti-inflammatoire dans une voie similaire à celle du diclofénac et cela est remarquable par une diminution du volume d'œdème, des valeurs de CRP et d'Anti-CCP.

L'un des marqueurs potentiels d'un risque accru de la polyarthrite rhumatoïde peut être la CRP. La protéine C-réactive s'est avérée être d'une grande valeur en tant que marqueur inflammatoire dans la PR et a été suggérée comme médiatrice d'une partie de l'activation du complément dans la PR (Singh *et al.,* 2013). De plus, la CRP peut également contribuer directement à l'état pro-inflammatoire. La protéine C-réactive est une protéine plasmatique qui augmente pendant la réponse systémique aux états inflammatoires et sa synthèse augmente rapidement dans les heures suivant une lésion tissulaire ou une infection, elle contribue également à la défense de l'hôte en tant qu'acteur de la réponse immunitaire innée (Naderi *et al.,* 2016). Protéine C-réactive une protéine de phase aiguë est synthétisée par les hépatocytes en réponse à des cytokines pro-inflammatoires en particulier IL-6 et elle stimule la libération de cytokines inflammatoires telles que IL-1β, IL-6 et TNF-α et peut également agir directement comme stimulus pro-inflammatoire des cellules phagocytaires (Shrivastava *et al.,* 2013; Yu *et al.,* 2016).

La présence d'ACPA habituellement mesurée comme anti-CCP est hautement spécifique de la PR (Boissier *et al.,* 2012). Les ACPA reconnaissent les peptides citrullinés présents dans de nombreuses protéines matricielles telles que la filaggrine, la kératine, le fibrinogène et la vimentine et que l'on trouve également dans l'alpha-énolase (Svärd *et al.,* 2013). La citrulline dérive de l'arginine suite à une modification post-traductionnelle par les peptidyl arginine déiminases (PAD) (Sakkas *et al.,* 2014). La citrullination des antigènes synoviaux, en particulier la fibrine, est un processus actif au cours de l'inflammation synoviale qui permet probablement l'induction d'anticorps anti-CCP chez les patients atteints de PR (Del Val Del Amo *et al.,* 2006).

L'initiation de la PR implique l'activation de cellules T auto-réactives et le recrutement de ces cellules T avec d'autres leucocytes dans les articulations. Ces leucocytes produisent une variété de médiateurs de l'inflammation. Parmi ces médiateurs figurent les métabolites de l'acide arachidonique, les cytokines pro-inflammatoires, les radicaux libres et les enzymes dégradant la matrice. Ces médiateurs modulent les processus liés à la migration cellulaire dans les articulations ainsi que l'angiogenèse et la dégradation de la matrice extracellulaire au sein des articulations, conduisant à l'inflammation arthritique (Venkatesha *et al.,* 2011).

La capacité anti-inflammatoire de nombreux aglycones flavonoïdes tels que le kaempférol, la quercétine, l'apigénine, la lutéoline, la génistéine et l'hespérétine a été évaluée. Parmi eux, la lutéoline était la plus active dans l'inhibition du NO et du TNF-α (Nunes *et al.*, 2020). En outre, la lutéoline et l'apigénine ont été trouvées dans des extraits de *S. circinata* et *S. mialhesi* (Laggoune *et al.,* 2016) qui exercent de puissants effets anti-inflammatoires. De plus, Naderi *et al.* (2016) ont rapporté que les composés phénoliques du gingembre diminuent les facteurs pro-inflammatoires tels que le TNF-α et l'IL-1β impliqués dans l'inflammation et la dégradation des articulations, ces composés cruciaux suppriment la synthèse de prostaglandine et de leucotriène en inhibant la COX-2 et voies de la lipoxygénase et autres voies impliquées dans l'inflammation.

La CRP sérique, un marqueur de substitution de la gravité de la maladie qui est en corrélation avec le résultat final de l'arthrite, est également un puissant ligand endogène du TLR-2 présent sur les surfaces des fibroblastes synoviaux, des PMN et des macrophages. Sa transcription est régulée par des cytokines pro-inflammatoires dont l'IL- 6 (Adhikary *et al.*, 2016). La concentration sérique de CRP a été testée dans nos expériences et s'est avérée être significativement atténuée chez les souris traitées avec des extraits de *S. mialhesi* et *S. circinata*. Ceci explique un rôle protecteur des extraits contre les lésions hépatiques et les réactions inflammatoires des tissus hépatiques lors de la pathogenèse de l'arthrite induite par le formol.

Les complexes immuns (IC) déposés dans les articulations synoviales augmentent les cytokines pro-inflammatoires dans le sérum, grâce à l'induction de cellules mononuclées, ces complexes immuns peuvent stimuler les PMN et les macrophages à sécréter des cytokines pro-inflammatoires, comme le TNF-α. Dans les deux cas, ce qui en résulte activation des macrophages synoviaux qui aboutit finalement à une production accrue de TNF-α, IL-1β et d'autres cytokines pro-inflammatoires comme l'IL-6, IL-12 et IL-15 qui sont impliquées dans la pathogenèse de la PR (Adhikary *et al.*, 2016). Cependant, des complexes immuns contenant du fibrinogène citrulliné ont été détectés dans le sang périphérique de patients atteints de la PR ACPA-positifs et également dans le pannus synovial (Fisher, 2014). Ces complexes immuns stimulent la production de TNFα des macrophages et l'accumulation de multiples spécificités ACPA est corrélée à l'inflammation préclinique (élévation du TNF-α, de l'IL-6 et de l'IFN-γ) précédant l'arthrite clinique (Sakkas *et al.*, 2014).

Une amélioration considérable de la prise en charge de la PR a été obtenue depuis l'avènement des agents biologiques tels que les inhibiteurs du (TNF) -α (adalimumab, certolizumab, étanercept, golimumab ou infliximab) agent anti-lymphocyte B (rituximab), anti-

IL-6 inhibiteur des récepteurs (tocilizumab), modulateur des lymphocytes T (abatacept) (Atzeni *et al.,* 2013). Un problème auquel est confronté le médecin en exercice est de prescrire l'agent biologique le plus approprié à chaque patient, c'est-à-dire de faire correspondre un agent biologique avec un profil de patient, étant donné que le coût est élevé de ces produits biologiques (Sakkas *et al.,* 2014). La présence d'ACPA était associée à une réponse réduite aux agents anti-TNFα (Potter *et al.,* 2009).

Les effets anti-inflammatoires et antiarthritiques observés des extraits de *S. circinata* et *S. mialhesi* peuvent être attribués à la présence de leurs composants bioactifs tels que les flavonoïdes, les terpénoïdes, les stéroïdes qui peuvent être responsables de l'inhibition des médiateurs inflammatoires.

Pour confirmer les effets protecteurs des extraits de *S. circinata* et *S. mialhesi,* une analyse histologique a été réalisée sur les pattes postérieures de souris.

L'arthrite induite par le formol est connue pour présenter des similitudes histologiques avec la PR humaine, avec une synovite comparable, une infiltration de cellules inflammatoires, une érosion du cartilage articulaire, une formation de pannus et une destruction osseuse qui entraîne la destruction de la stabilité articulaire et une incapacité fonctionnelle (Nishat et Sayeda Jabeen, 2016). L'inflammation chronique implique la libération d'un certain nombre de médiateurs qui sont responsables des changements indiqués (Lin *et al.,* 2014). Cependant, l'administration orale des extraits de *S. circinata* et *de S. mialhesi* et du diclofénac aux souris a considérablement réduit les changements histologiques causés par l'arthrite induite par le formol. L'effet anti-arthritique significatif de nos extraits peut être dû à la suppression des médiateurs inflammatoires. Il est donc conclu que la présence de composés phénoliques et de flavonoïdes qui sont les principaux constituants des espèces *Stachys* puisse expliquer les propriétés antiarthritiques des extraits.

Sadeghi *et al.* (2016) ont révélé que l'analyse pathologique des pattes dans le test de carraghénane a montré que l'extrait hydroalcoolique de *Stachys Pilifera* réduisait la destruction des tissus, l'infiltration cellulaire et l'œdème sous-cutané et a rapporté que la présence de composés phénoliques tels que les polyphénols, les acides phénoliques, les flavonoïdes, les iridoïdes, et les glycosides phényléthanoïdes dans l'extrait du genre *Stachys* peuvent être la principale raison de son effet anti-inflammatoire.

Dans cette partie, une approche *in-vitro* a été réalisée pour évaluer l'effet antiprolifératif des extraits étudiés sur des lignées cellulaires du cancer du foie (HepG2), du sein (MCF7), du phéochromocytome (PC12) et sur des cellules endothéliales humaines saines (HUVEC),

Les maladies cardiovasculaires et le cancer sont les principales causes de mortalité humaine dans le monde (The Lancest, 2019). La prolifération cellulaire maligne, métastatique et autonome, appelée `` cancer ou tumeur ", survient principalement dans tous les organes, mais les poumons sont les plus touchés, totalisant 11,6% des cas diagnostiqués et 18,4% du total des décès enregistrés chez les deux sexes (Bray *et al.,* 2018).

Chez les hommes, la mortalité est également élevée par cancer du foie et de l'estomac, tandis que chez la femme, le cancer du sein est la principale cause de décès (Siegel *et al.,* 2020). Il convient de mentionner que l'épidémiologie du cancer varie considérablement d'un pays à l'autre et à l'intérieur des pays, dépend de l'âge et du sexe; statut économique; social et style de vie (Weir *et al.,* 2015). En effet, les diagnostics précoces, l'accès aux soins de santé et les thérapies adaptées / personnalisées se traduisent par une amélioration significative de la survie au cancer. Parmi les thérapies, la chirurgie est la meilleure option, mais se limite aux métastases confinées (~ 10 à 15% des cas). Les autres remèdes comprennent la radiothérapie, la chimiothérapie, la thérapie ciblée, la virothérapie, la thérapie par inhibiteur de point de contrôle immunitaire, le vaccin et leurs combinaisons (Schirrmacher, 2019).

Néanmoins, la récidive tumorale, la résistance aux médicaments, la toxicité du traitement et la grande hétérogénéité des cellules cancéreuses indiquent le besoin de nouvelles thérapies personnalisées et de molécules capables de contrôler un plus large éventail d'aberrations cellulaires cancéreuses (Wang *et al.,* 2019; Dagogo et Show 2018; Falzone *et al.*, 2018).

La carcinogenèse génère une explosion d'espèces intracellulaires réactives de l'oxygène (ROS) affectant la survie des cellules somatiques voisines et régulant le développement de la tumeur (Liou et Storz, 2010). En effet, la surproduction de radicaux libres provoque un déséquilibre dans l'homéostasie redox cellulaire avec des dommages oxydatifs sur les biomolécules (lipides, protéines, ADN), (Rouabhi et al., 2015), ce qui a des implications majeures dans l'étiologie des maladies chroniques comme le cancer, diabète et maladies cardiovasculaires (Choi et Kim. 2013).

Ainsi, il est conseillé que les nouveaux médicaments thérapeutiques pour la lutte contre le cancer prennent en compte l'effet sur le statut redox des cellules cancéreuses (Yang et al., 2018).

Nos résultats ont démontré que les extraits de *S. circinata et S. mialhesi* conduisaient à une activité cytotoxique sélective contre les lignées cellulaires MCF7, HepG2, PC12 alors qu'aucune cytotoxicité contre les cellules endothéliales de la veine ombilicale humaine normale (HUVEC) n'a pu être observée. Ferhi *et al.* (2019) ont révélé que les extraits obtenus à partir des feuilles de vigne cultivées dans la région de Médée (Algérie) présentaient un effet antiprolifératif sur les cellules MCF-7 et HepG2.

En référence à nos résultats précédents, il a été démontré que les extraits de *S. circinata* et de *S. mialhesi* augmentent les niveaux d'antioxydants. En outre, Aplay *et al.* (2017) ont rapporté que l'augmentation des niveaux d'antioxydants réduit la progression du cancer.

S. circinata et *S .mialhesi* ont fait l'objet de recherches phytochimiques intensives et se sont caractérisées principalement par la présence de flavonoïdes dont l'apigénine, l'isocsutellaréine, la lutéoline, l'isorhamnétine, les triterpénoïdes tels que l'acide bétulinique, l'acide ursolique, l'acide oléalonique, les stérols comme le stigmastérol et le β -sitostérol (Laggoune *et al.,* 2016). De plus, Jassbi *et al.* (2014) ont rapporté que la présence de composés cytotoxiques avec des polarités différentes dans plusieurs espèces de *Stachys* comme l'extrait dichlorométhane de *S. pilifera,* (qui peut contenir plus d'agents non polaires tels que les terpénoïdes) a montré un effet plus fort. Néanmoins, certaines plantes comme l'extrait méthanolique à 80% de *S. persica* (contenant plus de molécules polaires comme composés phénoliques) ont également pu présenter un effet cytotoxique encore plus élevé.

Une étude antérieure a également démontré que l'extrait chloroformique de *S. setifera* inhibait grandement la prolifération de la lignée cellulaire de carcinome canalaire du sein (T-47D) (IC50 2,44 µg / mL), par rapport aux cellules normales (IC50 394,88 µg / mL), dont les composants se sont révélés être des terpénoïdes et des flavonoïdes (Ostad *et al.,* 2014). Plusieurs travaux ont montré que la lutéoline inhibe la prolifération cellulaire. Seelinger *et al.* (2008) ont rapporté que la lutéoline, isolée de deux plantes asiatiques traditionnellement utilisées comme médicaments anticancéreux, *Epimedium koreaonum* et *Terminalia arjuna*, s'est avérée inhiber la prolifération du MCF-7 et du HepG2 de manière dose-dépendante. L'effet cytotoxique de la lutéoline et de l'apigénine a également été démontré précédemment sur les cellules de leucémie myéloïde chronique humaine (K562) et de cancer de la vessie (RT112), de manière dose-dépendante du temps (Kilani-Jaziri *et al.,* 2012). En outre, l'apigénine a également présenté de larges effets anticancéreux dans divers cancers humains; ce flavone inhibe la prolifération des cellules cancéreuses en déclenchant l'apoptose cellulaire, en induisant l'autophagie et en modulant le cycle cellulaire (Yan *et al.,* 2017).

Dans d'autres études, l'isorhamnetine a été révélée capable d'inhiber la prolifération des cellules cancéreuses du poumon *in-vitro* et *in-vivo* et également capable de contrer la prolifération d'autres lignées cellulaires de carcinome comme le MCF-7. Ses mécanismes d'action peuvent impliquer l'apoptose des cellules en régulant à la baisse les oncogènes et en induisant des gènes apoptotiques (Li *et al.*, 2015).

Dans une autre étude, l'acide oléanolique a exercé une activité cytotoxique contre HepG2, en arrêtant le cycle cellulaire et en induisant l'apoptose et la fragmentation de l'ADN (Zhu *et al.*, 2015). Des résultats similaires ont été rapportés par Zarei et Yaghoobi. (2017) concernant les extraits de *Fritillaria imperialis L.* qui se sont révélées toxiques pour les cellules cancéreuses du foie humain (LCL-PI 11) et les cellules d'adénocarcinome du sein (MCF-7) induisant probablement un arrêt du cycle cellulaire ou une apoptose intrinsèque.

Les résultats ont montré que les extraits de *S. circinata* et *S. mialhesi* étaient fortement cytotoxiques contre les cellules HepG2, MCF-7 et PC12, ces extraits ont induit une inhibition significative de la viabilité cellulaire. En outre, les résultats ont montré que les lignées de cellules normales HUVEC n'étaient pas sensibles aux extraits et qu'aucune cytotoxicité n'a été aperçue, nos résultats corroborent avec ceux de beaucoup de travaux réalisés sur des variétés de plantes. Ferhi *et al.* (2019) ont rapporté que les extraits obtenus à partir de feuilles de vigne ont un effet anti-prolifératif sur les cellules HepG2 et MCF-7 sans induire de dommages à HUVEC en tant que cellules non cancéreuses. Ces résultats sont en outre déduits par les travaux d'Aghbali *et al.* (2013), rapportant que *Vitis vinifera* avait un effet pro-apoptotique, inhibant la croissance cellulaire, alors qu'aucune activité cytotoxique n'a été observée sur les cellules HUVEC.

Abstract

The natural products from plants and biological sources still remain an unlimited and uncondensed source of new phytochemicals and nutraceuticals. Medicinal plants have been prescribed and used widely for thousands of years to treat several disorders and ailments in folk herbal medicine systems all around the world and have been considered as an effective approach to control various disorders such as autoimmune diseases, inflammation and cancers.

In the present study, we evaluated the *in vivo* effect of two endemic species belonging to the genus *Stachys* (*Stachys circinata* and *Stachys mialhesi*) harvested in Djebel El-Ouahch Constantine on the toxicity, immunomodulatory, anti-oxidant and anti-arthritis activities.

The toxicity assessment was evaluated using the "up and down" method, the immunomodulatory activity was performed in the reticulo-endothelial system using carbon clearance assay, the *in vivo* antioxidant evaluation from liver homogenate on oxidative stress markers as catalase (CAT) and reduced glutathione (GSH) was investigated and the anti-arthritic effect was studied by the formalin-induced arthritis test. All the *in-vivo* experiments were performed in *Mus musculus* mice.

The results showed that the extracts of medicinal plants did not produce any signs of toxicity and the LD resulted > than 2000mg/kg, also increased significantly the phagocytic activity at all tested concentrations but the clearance rate was faster at 150mg/kg for both extracts, and have antioxidant effect by stimulating the release of CAT and GSH from liver. Furthermore, the extracts of our plants have accelerate the anti-inflammatory activity by a significant decrease of the edema size, anti-CCP values and CRP.

Moreover, in the present thesis, we performed a study *in-vitro* to evaluate the anti-proliferative effect of the plant extracts on liver cancer cell line (HepG2), breast cancer cell line (MCF7), phaeochromocytoma cell line (PC12) and on healthy cells: human endothelial cells (HUVEC). Another study has been carried to assess the anti-oxidant effect of plant extracts on HepG2 cells. The results revealed that the extracts of *Stachys circinata* and *Stachys mialhesi* led to a selective cytotoxic activity against cancer cell lines in dose dependent manner while, no cytotoxicity against normal cells. Our results showed also a high significant increase of CAT activity and GSH levels in liver cancer cells (HepG2) treated with the different concentrations of plant extracts, compared to control.

Keywords:

Stachys mialhesi, *Stachys circinata*, immunomodulatory, cancer, anti-arthritis activity, anti-oxidant activity, anti-proliferative activity, reduced glutathione, catalase.

إن المنتجات الطبيعية ذات الأصل النباتي و البيولوجي ستضل دوما مصدرا أساسيا غير محدود و غير مكثف لمواد و مركبات جديدة ذات مصدر كيميائي نباتي أو غذائي. تم وصف النباتات الطبية و استعمالها على نطاق واسع منذ آلاف السنين لعلاج العديد من الاضطرابات و الأمراض وفقا لنظام التداوي بالأعشاب الشعبية المعروفة في جميع أنحاء العالم ، و اعتبرت بمثابة نهج فعال للسيطرة على الاضطرابات المختلفة مثل أمراض المناعة الذاتية و الالتهابات و السرطانات.

في هذه الدراسة، قمنا بتقييم التأثير الحيوي لنبتتين مستوطنتين تنتميان إلى جنس *Stachys* وهما *Stachys mialhesi , Stachys circinata* والتي تم قطفهما من منطقة جبل الوحش المتواجدة بقسنطينة ، على السمية و على نشاط تعديل الجهاز المناعي كذلك تم تقييم تأثيرهما على كل من مضادات الأكسدة و التهاب المفاصل.

تم تقييم السمية باستخدام طريقة Up and down test ، أما نشاط تعديل الجهاز المناعي فقد تم إجراءه باستخدام تقنية التخلص من جزيئات الكربون من الدم، أما بالنسبة لتقييم النشاط المضادات للأكسدة فقد تم عن طريق التحديد الطيفي لكمية الجلوتاثيون المختزل في الكبد و ذلك باستخدام مسحوق الكبد المتجانس ، كذاك استعمل هذا الأخير لمعايرة نشاط إنزيم الكتالاز. تم إجراء دراسة أخرى لتقييم تأثير مستخلص النبتتين المستعملتين و ذلك ضد التهاب المفاصل المحفزة بواسطة الفورمول، و ذلك باستعمال اختبار التهاب المفاصل. الفئران المستعملة خلال الدراسات داخل العضوية *In-vivo* كانت من نوع *Mus musculus*

أظهرت النتائج أن مستخلصات النبتتين المستعملتين في هذه الدراسة ليس لها أي تأثير سام بجرعة 2000 مغ/كغ و بذلك تكون الجرعة المميتة اكبر من 2000 مغ/كغ، كما لاحظنا زيادة كبيرة في نشاط البلعمة للمجموعات المعالجة بمستخلصات النبتتين وقد تم اختبار ذلك بتراكيز مختلفة . من ناحية أخرى لوحظ إن نصف العمر الحيوي لجزيئات الكربون في الدم قد انخفض بقيمة أسرع في المجموعات المعالجة بالمستخلصين و قد لوحظت هذه النتيجة عند استعمالنا لجرعة قدرها 150 مغ/كغ. تشير دراستنا أيضا إلى أن مستخلصات النبتين المستعملتين لديها تأثير مضاد للأكسدة و ذلك عن طريق تحفيز إفراز كل من الجلوتاثيون و الكتالاز من قبل الكبد (GSH, CAT) ، كما أن لها تأثير على التهاب المفاصل و قد تأكدنا من ذلك من خلال الانخفاض الكبير في حجم انتفاخ قدم الفئران و في كمية الأجسام المضادة (ACCP). لوحظ كذلك هذا الانخفاض الكبير في البروتين سي التفاعلي (CRP)

بالإضافة إلى ذلك، قمنا في هذه الرسالة بدراسة خارج العضوية *(In-vitro)* لتقييم تأثير مستخلص النبتتين على الخلايا السرطانية للكبد(HepG2) و الثدي(MCF7) و على سرطان لب الغدة الكظرية (PC12) كذلك تم دراسة تأثير هذين المستخلصين على الخلايا البطانية البشرية السليمة (HUVEC) كما تم إجراء دراسة أخرى لتقييم تأثير مضادات الأكسدة على الخلايا (HEPG2) أظهرت النتائج إن مستخلص النبتتين أدى إلى تثبيط في تكاثر الخلايا السرطانية مع عدم تثبيط الخلايا الطبيعية.

كما أظهرت نتائج دراستنا أن علاج خلايا سرطان الكبد (HEPG2) بتراكيز مختلفة من مستخلص النبتتين زاد من إفراز الإنزيمات المضادة للأكسدة المتمثلة في كل من الجلوتاثيون و الكتالاز

كما أظهرت النتائج أن العلاج بستة تراكيز مختلفة من مستخلص النبتتين أعطى تناسبا طرديا بين التراكيز المستخدمة و نسبة انخفاض انقسام و تكاثر الخلايا السرطانية ، دون أن يكون لهما أي سمية على الخلايا السليمة .

الكلمات المفتاحية

Stachys mialhesi , Stachys circinata ، نشاط تعديل الجهاز المناعي، الأمراض السرطانية، النشاط المضاد للالتهاب، النشاط المضاد للأكسدة، الجلوتاثيون المختزل، الكتالاز.

Résumé

Les produits naturels d'origine végétale et biologique restent une source illimitée et non condensée de nouveaux composés phytochimiques et nutraceutiques. Les plantes médicinales sont prescrites et largement utilisées depuis des milliers d'années pour traiter plusieurs troubles et affections dans les systèmes de phytothérapie populaire du monde entier et ont été considérées comme une approche efficace pour contrôler divers troubles tels que les maladies auto-immunes, l'inflammation et les cancers.

La présente étude a été initiée dans le but d'étudier *in-vivo* les activités biologiques de deux espèces endémiques appartenant au genre *Stachys (Stachys circinata* et *Stachys mialhesi*) récoltées de Djebel El-Ouahch, Constantine. La toxicité de ces plantes a été évaluée en utilisant la méthode «up and down», l'activité immunomodulatrice a été réalisée par le test de l'épuration sanguine d'une dose de carbone colloïdal, quant au pouvoir anti-oxydant a été étudié sur l'homogénat hépatique des souris en effectuant le dosage de la catalase (CAT) et du glutathion réduit (GSH). Une autre étude a été menée pour évaluer l'effet antiarthritique des extraits de plantes en adaptant le test d'arthrite induite par le formol. Toutes les expériences *in-vivo* ont été réalisées sur des souris *Mus musculus.*

Les résultats ont montré que nos extraits ne produisaient aucun signe de toxicité à la dose 2000 mg / kg, nous avons également constaté une augmentation hautement significative de l'activité phagocytaire des groupes traités par des différentes concentrations d'extraits étudiés, en revanche un taux de clairance plus rapide a été nettement observé dans les groupes traités par les deux extraits à la dose 150 mg / kg. Nos résultats indiquent aussi que les extraits de *S. mialhesi* et *S. circinata* ont révélé un effet antioxydant en stimulant ainsi la libération de la CAT et du GSH par le foie et un effet antiarthritique confirmé d'une part par une diminution significative de la taille de l'œdème des pattes de souris, des taux d'anti-CCP et de la CRP et d'autre part par une étude histologique.

Une approche *in-vitro* a été réalisée pour évaluer l'effet antiprolifératif des extraits étudiés, sur des lignées cellulaires du cancer du foie (HepG2), du sein (MCF7), du phéochromocytome (PC12) et sur des cellules endothéliales humaines saines (HUVEC), nous avons étudié ensuite l'effet antioxydant des extraits de plantes sur les cellules HepG2. Les résultats ont révélé que les extraits de plantes conduisaient à une activité cytotoxique sélective contre les lignées cellulaires cancéreuses d'une manière dose dépendante cependant, aucune cytotoxicité contre les cellules normales n'était observée. Nos résultats ont également montré

que le traitement des cellules cancéreuses du foie (HepG2) avec les différentes concentrations des extraits de plantes augmentait la libération de l'enzyme anti-oxydante CAT et le GSH.

Mots clés

Stachys mialhesi, *Stachys circinata*, activité immunomodulatrice, cancer, activité-antiarthritique, activité anti-oxydante, glutathion réduit, catalase.

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Biological activities of medicinal plant extracts on arthritis induced by formalin and on tumoral process

Thèse en vue de l'obtention du diplôme de doctorat 3ème cycle

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Une approche *in-vitro* a été réalisée pour évaluer l'effet antiprolifératif des extraits étudiés, sur des lignées cellulaires du cancer du foie (HepG2), du sein (MCF7), du phéochromocytome (PC12) et sur des cellules endothéliales de la veine ombilicale humaines saines (HUVEC), nous avons étudié ensuite l'effet antioxydant des extraits de plantes sur les cellules HepG2. Les résultats ont révélé que les extraits de plantes conduisaient à une activité cytotoxique sélective contre les lignées cellulaires cancéreuses d'une manière dose dépendante cependant, aucune cytotoxicité contre les cellules normales n'était observée. Nos résultats ont également montré que le traitement des cellules cancéreuses du foie (HepG2) avec les différentes concentrations des extraits de plantes augmentait la libération de l'enzyme anti-oxydante CAT et le GSH.

Mots clés:
Stachys mialhesi, *Stachys circinata*, activité immunomodulatory, cancer, activité antiarthritique, activité anti-oxydant, activité anti-proliferative, glutathione, catalase.

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