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The Evaluation of Anti-Inflammatory and Anti-Oxidant Activities of Ethanolic Extract of *Aristolochia Longa* Roots in Mice

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Order Number …….

Dedication

Every challenging work needs efforts as well as guidance of elders especially those who were very close to our heart.

My humble effort I dedicate to my sweet and loving

Father Ahcene & Mother Noura,

Whose affection, love, encouragement and prays of day and night make me able to get such success and honor,

Along with all hard working and respected

Teachers.

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First of all, i thank ALLAH, the Almighty, my creator, for giving me the strength, the will, the patience and the courage to accomplish this modest work;

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Thank you all .

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The acute inflammatory response is the body's first system of alarm signals that are directed toward containment and elimination of microbial invaders (Charles et al., 2010). However, when inflammation is uncontrolled, it leads to chronic inflammatory and autoimmune diseases. Extensive investigations within the last two decades revealed that most chronic illnesses including cancer, neurological, autoimmune, diabetes, and cardiovascular diseases are mediated through inflammation. Thus, suppressing inflammation has the potential to delay, prevent, and treat these diseases. Therefore, inhibition of inflammatory biomarkers could be a target to treat inflammatory disorders. (Begum et al., 2014).

When inflammation is an immune response oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, reactive oxygen species (ROS) that are formed during metabolism, may contribute to pathogenesis of many diseases such as diabetes, cancer and cardiovascular diseases (Zovko et al., 2010).

Medicinal plants have been known among different nations since thousands of years ago and have been used throughout many centuries according to the traditional medicine of most countries. In recent decades and despite the emergence of synthetic drugs, medicinal plants continue to be welcomed and are being used in many countries due to safe use, efficiency, cultural acceptance, and fewer side effects than synthetic drugs(Madiseh.2016). There is accumulating evidence suggesting medicinal plants are unlimited reservoirs of drugs. The amazing structural diversity among their active components makes them a useful source of novel therapeutic compounds (Saeed Arayne and Sultana, 2007).

For a long time plants were used as treatment against inflammation and infections in Algerian population like the plant *Aristolochia longa* or as the local population calls it (baraztam) which has been used to treat many health's disorders including skin infections and gangrene and It is an antidote against snake bites (Cherif et al., 2014). Also it is used in cancer treatment (Benarba, 2015).

Aristolochia longa was a subject of many researches including its cytotoxicity its anti-cancer activity and its anti-microbial activity but none of these researches have investigated the anti-inflammatory and antioxidant activities of the ethanolic extract of *Aristolochia longa* roots. For this, the present study was carried out to investigate the anti-inflammatory by the red blood cells membrane stabilization method and the assessment of induced oedema in paw of mice by carageenan, MDA and GSH levels will also estimated, a phytochemical study will be performed by the estimation of secondary metabolites content.

I.1. Generals about inflammation

I.1.1. Inflammation

Inflammation is an essential tissue response to extrinsic/intrinsic damage, is a very dynamic process in terms of complexity and extension of cellular and metabolic involvement. The aim of the inflammatory response is to eliminate the pathogenic initiator with limited collateral damage of the inflamed tissue, followed by a complex tissue repair to the preinflammation phenotype (Gallo *et al*, 2017). This response requires innate immunity and, in some cases, an adaptive immune response, which are the two main integral components of the host's defense system (Lawrence *et al.,* 2002). Inflammation occurs in two forms: as an acute (short-term) or chronic (long-term).

I.1.2. Acute Inflammatory Response

The acute inflammatory response is defined as a series of tissue responses that can occur within the first few hours following injury. It is thought to be beneficial to the injured organism with the aim of restoring the disturbed physiological homeostasis (Charles *et al.,* 2010).

The acute phase of inflammation is characterized by the rapid influx of blood granulocytes, typically neutrophils, followed swiftly by monocytes which mature into inflammatory macrophages that subsequently proliferate and thereby affect the functions of resident tissue macrophages. This process causes the cardinal signs of acute inflammation: rubor (redness), calor (heat), tumor (swelling) and dolor (pain) (Ricciotti and Fitzgerald, 2011), and by the increased movement of plasma and innate immune system cells, such as neutrophils and macrophages, from the blood into the injured tissues. The standard signs of inflammation are expressed by increased bloodflow, elevated cellular metabolism, vasodilatation, release of soluble mediators, extravasation of fluids and cellular influx (Sarkhel, 2015).

The acute phase response is general and nonspecific. The acute inflammation can occur in different organs but wherever it occurs its mechanism is the same. That is, an acute inflammation in the skin has the same mechanisms. The process of this inflammation is divided into three steps:

I.1.2.1 Vascular events

The local increase of vascular permeability resulting in cell infiltration and exudation of protein-rich fluid is the most important event in the initial phase of the inflammation. The initial vascular events in inflammation are accompanied by an increased leukocyte adhesion to the endothelium of small venules (dahlen *et al.*, 1981). a process called vasoconstriction. Following this transient event, which is believed to be of little importance to the inflammatory response, the blood vessels dilate (vasodilation) increasing blood flow into the area. Vasodilation may last from 15 minutes to several hours (Benly.2005) (Fig 1).

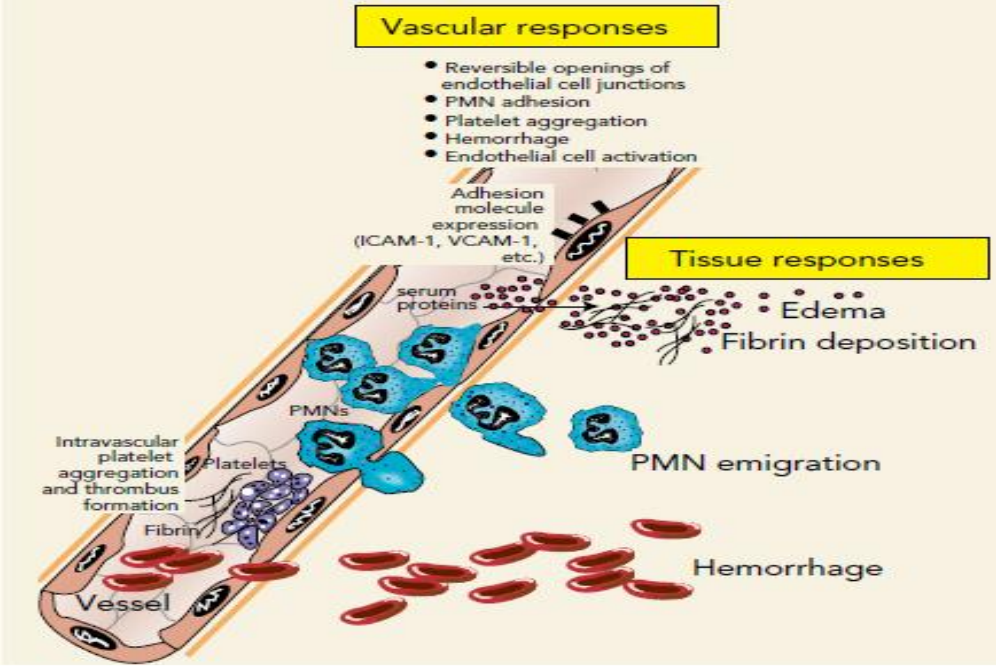


Fig 1. Acute inflammatory tissue injury (Charles *et al.*, 2010).

I.1.2.2 Cellular events

Cellular events are. Upon initial challenge, protein exudation increases and polymorphonuclear leukocytes (neutrophils) accumulate in inflamed tissue. Neutrophil infiltration follows a rapid response from sentinel cells prestationed in the tissues at the time of injury, including macrophages and mast cells (Charles *et al.*, 2010).

I.1.2.3 Resolution of inflammation

Recent data indicate that the resolution of inflammation is an active process controlled by endogenous mediators that suppress proinflammatory gene expression and cell trafficking, as well as induce inflammatory-cell apoptosis and phagocytosis, which are crucial determinants of successful resolution (Lawrence *et al*., 2002).

For tissues to return to normal, all of the events involved in the evolution of inflammation must be reversed. In the very simplest example of an acute inflammatory reaction this must include: removal of the inciting stimulus, dissipation of the mediators generated by the inciting influence, cessation of granulocyte emigration from blood vessels, return of normal microvascular permeability, limitation of granulocyte secretion of potentially histotoxic and pro-inflammatory agents, cessation of the emigration of monocytes from blood vessels and their mutation into inflammatory macrophages, and finally, the removal of extravasated fluid, proteins, bacterial and cellular debris, neutrophils and macrophages. With the completion of these events, the stage should be set for the recovery of normal tissue architecture and function. The mechanism of which the inflammatory reaction started and ended is explained in (Fig 2) (Haslett, 1992)

Apoptosis and the clearance of apoptotic cells have been recognized as important mechanisms for the resolution of inflammation. The signals that promote leukocyte apoptosis are important for the resolution of inflammation; however, apoptotic cells themselves can also promote the resolution of inflammation. Studies have shown that the phagocytic clearance of apoptotic cells by macrophages promotes the release of TGF-α1 and suppresses the proinflammatory activity of the macrophages (Lawrence *et al.,* 2002). An acute inflammation that fails to heal may become chronic.

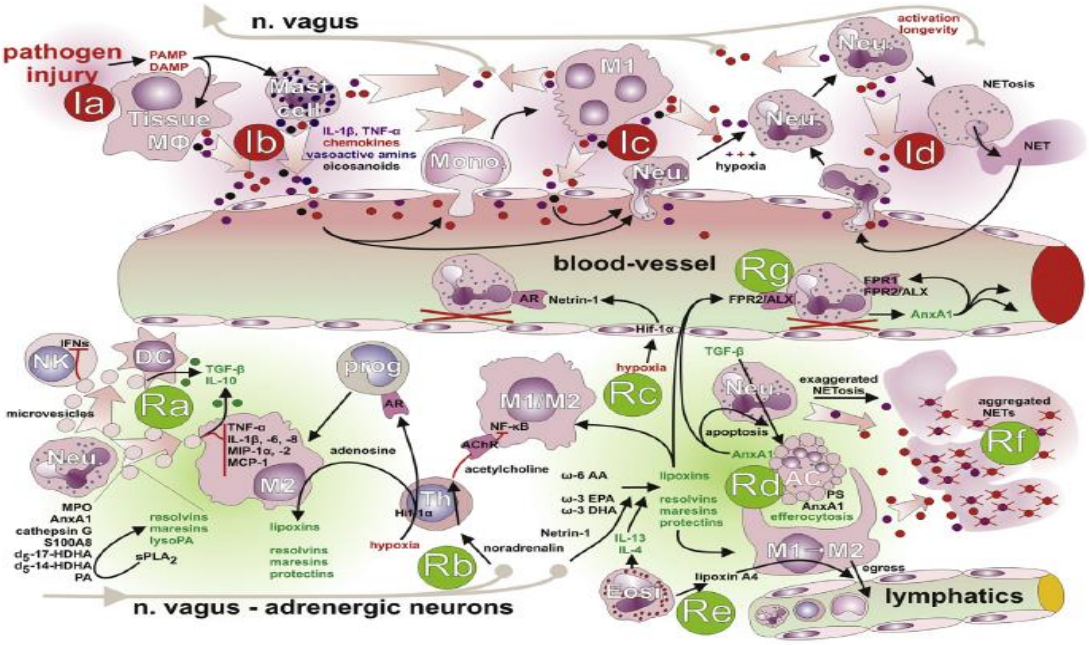


Fig 2. Start and resolution of inflammation (Gallo *et al,* 2005).

I.1.3. Chronic inflammation

Chronic inflammation is characterized by the prolonged stimulation of the production of immune blood cells from the lymphoid and myeloid lineages and the release of various mediators, notably inflammatory cytokines, in blood vessels and in tissues. The production of polyclonal megakaryocytes and platelets is frequently increased, to ensure thrombus formation and homeostasis in case of damaged blood vessels in inflamed tissues. The accumulation and persistence of leukocytes is a hallmark of chronic inflammation (Lawrence *et al.,* 2002; Hermouet *et al.,* 2016).

Chronic inflammation is originated from increased production of pro-inflammatory cytokines and chemokines in adipose tissues. The accumulating chemokines recruit additional immune cells, most notably macrophages, which in turn produce more chemokines in a vicious cycle of chronic inflammation (Benson *et al.,* 2018).

Chronic inflammation leads to cancer development and in recent times experimental and clinical studies have supported this hypothesis, which is now globally accepted. The epidemiological studies have identified chronic infections and inflammation as major risk factors for various types of

cancer. It has been estimated that the underlying infections and inflammatory reactions are linked to 15–20% of all cancer deaths (Gautam and Jachak, 2009).

I.1.4. Triggers of inflammation

Inflammation is a pathological process typically triggered by an external aggression, which may be a physical or chemical injury, irradiation, or infection (Hermouet *et al.,* 2015). And it can also be triggered by an oxidative stress (reactive oxygen species, especially oxidized lipids), psychological stress, viral infections and nutrient deficient/poor diet. Massive trauma, post-ischemic or toxic necrosis, and hemorrhage and resuscitation can each trigger an inflammatory response, and also accumulated products of metabolism, noxious chemical agents, and immunologic tissue injury (Nathan, 2002).

I.1.5. Indicators of inflammation

As well as the clinical evaluation of symptoms and signs indicating inflammation (pain, fever, and localized redness, swelling and tenderness), laboratory investigations are frequently used to support the diagnosis of inflammatory processes.

I.1.5.1 C-reactive protein (CRP)

CRP is a precise, sensitive and specific marker of the inflammatory reaction. It appears within six hours of acute inflammation. Its rate increases and reaches the maximal after two days. And it can drop in less than 6 hours when the source of inflammation has been eradicated. In the presence of calcium, CRP specifically binds to phosphocholine residues. Phosphocholine is found in microbial polysaccharides. CRP activates in this way the classical pathway of the complement in the absence of antibodies, and opsonizes the ligands, for their phagocytosis. Phosphocholine is also present in PAF (Platelet-Activating factor) and polynuclear cells, CRP attenuate the coagulation pathway and activation of inflammatory cells (Diallo, 2010).

I.1.5.2 Erythrocyte sedimentation rate

The erythrocyte sedimentation rate is a marker of the acute phase reaction. During an inflammatory reaction, the sedimentation rate is affected by increasing concentrations of fibrinogen, the main clotting protein, and alpha globulins. The test mainly measures the plasma viscosity by assessing

the tendency for red blood cells to aggregate and 'fall' through the variably viscous plasma (Harrison, 2015).

I.1.6. Inflammatory mediators

Inflammation involves a complex interaction of many different inflammatory cells that release a spectrum of chemical mediators ultimately affecting various target tissues. It is now understood that the inflammatory reaction consists of an early-phase response primarily involving mast cell degranulation accompanied by the release of histamine and other mediators including cytokines and a late-phase response that is characterized by the migration of inflammatory cells from the circulation (White, 1999). Inflammatory mediators such as cytokines, serotonin, histamine, prostaglandin and leukotrienes that increase vascular permeability, facilitate the migration of leukocytes to the site of inflammation (Sarkhel, 2015).

I.1.6.1 Cell derived mediators

I.1.6.1.1 Histamine

Histamine (b-Imidazolylethylamine) is a vasodilator, a constrictor of smooth muscle, and a potent stimulant of vascular permeability, respiratory mucus, and gastric acid secretion. It exerts its effects on a variety of cell types including smooth muscle cells, neurons, glandular cells (endocrine and exocrine), blood cells, and cells of the immune system. Histamine can exert its H2-receptor- mediated anti-inflammatory activity, including inhibition of human neutrophil lysosomal enzyme release, inhibition of IgE-mediated histamine release from peripheral leukocytes, and activation of suppressor T lymphocytes (White, 1999).

I.1.6.1.2 Prostaglandins

Prostaglandins are lipid autacoids derived from arachidonic acid. They sustain homeostatic functions and mediate pathogenic mechanisms, including the inflammatory response. They are generated from arachidonate by the action of cyclooxygenase (COX) isoenzymes and their biosynthesis is blocked by nonsteroidal anti-inflammatory drugs (NSAIDs), including those selective for inhibition of COX-2. Despite the clinical efficacy of NSAIDs, prostaglandins may function in both the promotion and resolution of inflammation (Ricciotti and Fitzgerald, 2011).

I.1.6.1.3 Neutrophilic proteases

Neutrophilic proteases destroy proteins and cell membranes and are responsible for proteolytic activation of the complement, coagulation, and kinin cascade (Riede and Werner, 2004).

I.1.6.1.4 Platelet activating factor

It is a phospholipid formed by phospholipase A2 that is produced by thrombocytes, granulocytes, macrophages, and endothelial cells. Its effects include, increasing vascular permeability, platelet aggregation and bronchoconstriction (Riede and Werner, 2004).

I.1.6.1.5 Cytokines

They are secreted from immune cells and some other cell types, such as fibroblasts and endothelial cells, which are responsible for immune regulation (Wang *et al.*, 2017). They accepted as one of the key regulatory of inflammation. Those cytokines can be secreted from different cell types, have different targets and activate different pathways (Sonmez and Sonmez, 2017).

Cytokines such as IL-1, IL-2, IL-4, IL-5, IL-6, TNF-α influence the course of inflammation by modulating the activity of inflammatory cells including macrophages, T cells, B cells, and eosinophils. IL-1 enhances the growth of T-helper cells and the growth and proliferation of B cells (White, 1999). They regulate a wide range of events, for example, the migration, proliferation, and function of inflammatory and immune cells (phagocytic, secretory, etc.) (Table.1) (Gallo *et al.*, 2017).

Chemokines influence the recruitment of circulatory monocytes and neutrophils and other cells to inflamed tissues. These cells govern, among others, the processes in which the initiator of inflammation can be eradicated with or without the help of the adaptive immune system. There is a growing body of evidence that chemokines also contribute to tissue regeneration and repair. Importantly, the inflammatory mediators are both antagonistic and dominant over homoeostatic molecules mainly because of the conflict with the goals of homoeostatic and inflammatory processes (Gallo *et al.*, 2017).

I.1.6.1.6 Tumor necrosis factor alfa (TNF-α)

TNF-α is a pleiotropic cytokine produced by a variety of cells including macrophages, endothelial cells, and smooth muscle cells. TNF-α, along with interferon-γ and IL-1, stimulates IL-6 production by smooth muscle cells. IL-6 gene transcripts are expressed in human atheromatous lesions (Blake and Ridker, 2001).

Table 1. Major biological functions of the cytokines, IL-1, IL-6 and TNF- α (Petersen *et al.*, 2004).

Cytokine	**Common functions**
IL-1, IL-6 and TNF-α	-Induction of hepatic acute phase response. -Induction of fever. -Activation of T, B and NK cells. -Induction of IL-2 in T-cells.
Cytokine	**Specific functions**
IL-1	-Activation of stroma, chondrocytes and epithelium in response to localized tissue damage. - Regulation of B-lymphopoiesis in bone marrow. -Mediation of tissue infiltration of leucocytes (via IL-8). -Osteoblast activation, bone and cartilage degradation.
IL-6	-Participates in induction and differentiation of cytotoxic T lymphocytes. -Stimulates the differentiation of hematopoietic stem cells. -Modulates the production of IL-1 and TNF.
TNF-α	-Induction of IL-1 production. -Participates in cell destruction by suppressing protein synthesis with resulting cachexia. -Elicits local endothelial damage.

I.1.6.2 Plasma derived mediators

I.1.6.2.1 Kinins

Kinins are potent peptide hormones formed in body fluids and tissues during inflammation. They are derived from α2 globulins (high and low molecular weight kininogens) through proteolytic cleavage by a variety of enzymes, the most important of which are plasma and tissue kallikreins. The pharmacologic properties of kinins include their abilities to increase vascular permeability, to cause vasodilatation and pain, to contract most smooth muscle preparations, and to stimulate arachidonic acid metabolism (Proud, 1988).

I.1.6.2.2 The leukotrienes (LT)

Leukotrienes are potent chemotactic and inflammatory factors. Leukotriene A4 (LTA4) is the first to be synthesized and is then processed into LTB4 or LTC4, then LTD4 and LTE4, (Buckwalter and Lotz, 2007). The leukotrienes (LT) are potent mediators generated by almost all cell types known to participate in inflammatory reactions (White, 1999).

The cysteinyl leukotrienes, comprising leukotriene (LT) C4 and its major metabolites LTD4 and LTE4, are inflammatory lipid mediators derived from metabolism of arachidonic acid by 5-lipoxygenase. LTC4 and its metabolites are present in other fluids removed from the blood, such as tears, skin blister fluids, gastric fluids, joint fluid, middle ear fluid, cerebral spinal fluid, bile, and nasal fluids (Rogers and Donnelly, 2001).

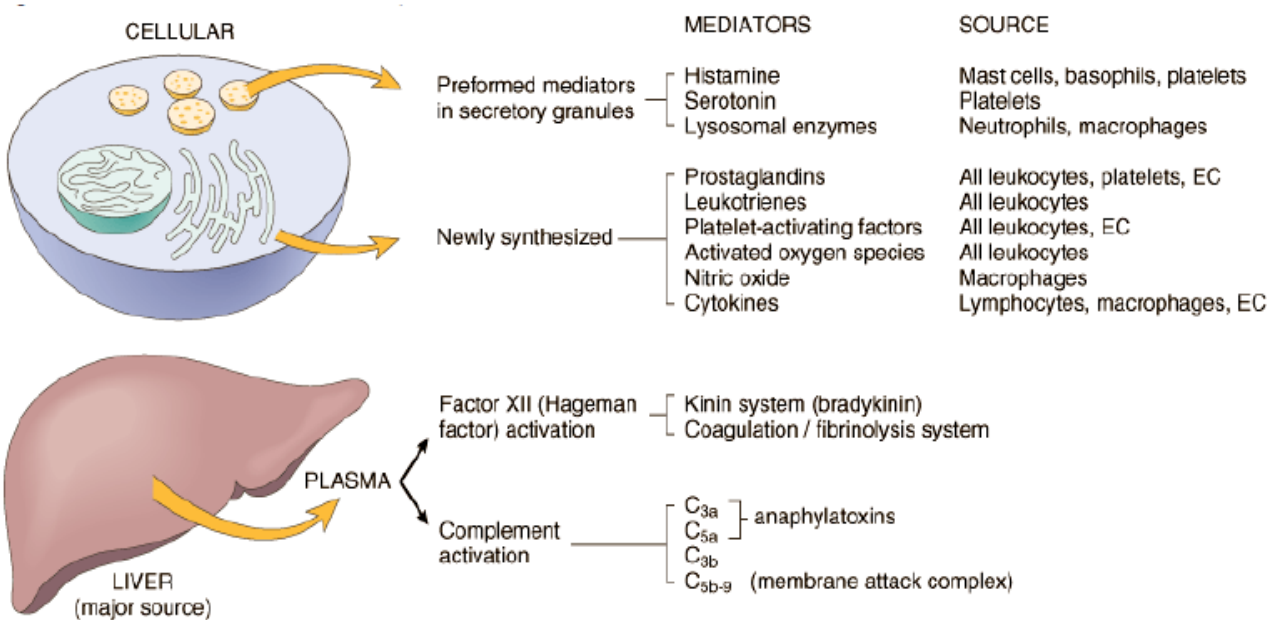


Fig 3. Chemical mediators of inflammation (Kumar *et al.*, 2005).

I.1.7. Anti inflammatory drugs

I.1.7.1 Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs have been defined as those substances, other than steroids which suppress one or more compounds of the inflammatory process. The group is generally restricted only to those substances that act by inhibiting components of the enzyme system in the metabolism of arachidonic acid and formation of eicosanoids (Swan, 1991).

Almost all NSAIDs are weak organic acids, or salts and esters of weak organic acids (Kurowski, 1992). These medications are used in rheumatic disorders such as rheumatoid arthritis (RA), osteoarthritis (OA) and spondylarthropathies, as well as in peri-articular disorders and soft-tissue rheumatism (Russell and Gabriel, 2001). However, NSAIDs have important adverse effects, including gastrointestinal (GI) bleeding, peptic

ulcer disease, hypertension, edema, and renal disease. More recently, some NSAIDs have also been associated with an increased risk of myocardial infarction (Saraf, 2008).

I.1.7.1.1 Mechanism of action

NSAIDs reduce pain and inflammation by blocking cyclo-oxygenase (COX), enzymes that are needed to produce prostaglandins. Most NSAIDs block two different cyclo-oxygenases called COX-l and COX2. COX-2, found in joint and muscle, contributes to pain and intlammation. NSAIDs cause bleeding because they also block the COX-l enzyme which protects the lining of the stomach from acid. NSAlDs differ in their selectivity for COX-2 how much they affect COX-2 relative to COX-I. An NSAlD that blocks COX-2 but not COX-I might reduce pain and inflammation in joints but leave the stomach lining alone. (Saraf, 2008).

NSAIDs have been found to interfere with the production of certain types of prostaglandins (PGs), a form of eicosanoid which have a multitude of effects on blood vessels, nerve endings and cells involved in the inflammatory cascade. Eicosanoid synthesis begins with the release of arachidonate from membrane phospholipids via the activity of phospholipase A2 (PLA2). Subsequently, two different COX isozymes convert arachidonic acid into various PGs. It is here that NSAIDs, by interfering with the activity of the COX enzymes inhibit the production of PGs (Saraf, 2008).

I.1.7.2 Steroidal anti-inflammatory drugs (SAIDS)

Steroidal anti-inflammatory or Glucocorticoids (GC) are steroid hormones, mostly synthesized by the adrenal glandcortex under the control of pituitary Adreno-Cortico-Trophic-Hormone (ACTH) and hypothalamic corticotrophin releasing hormone (CRH) (Vitellius *et al*., 2018). GC are pleiotropic hormones with a wide range of physiological effects. They are well known as anti-proliferative, pro-apoptotic and anti-inflammatory hormones in the lung. They are currently among the most effective and widely used medications to treat chronic and acute inflammation (Rebeyrol *et al.*, 2012). They are also used in the treatment of asthma, rheumatoid arthritis, inflammatory bowel disease and autoimmune diseases (Lawrence *et al*., 2002).

Glucocorticoids rely on several molecular mechanisms, and because some of these mechanisms are also involved in physiologic signaling rather than inflammatory signaling, the therapeutic effects of glucocorticoids in inflammation are often accompanied by clinically significant side effects (Rhen *et al.*, 2005).

I.1.7.2.1 Mechanism of action

Most of the actions of glucocorticoids require binding to cytoplasmic steroid hormone receptors that migrate to the nucleus and antagonize proinflammatory gene transcription. However, glucocorticoids also induce the expression of regulatory proteins that have anti-inflammatory actions, of which the peptide Annexin 1 it has been shown to inhibit the production of prostaglandins, as well as neutrophil and monocyte migration, *in vivo* (Fig.I-4) (Lawrence *et al.*, 2002).

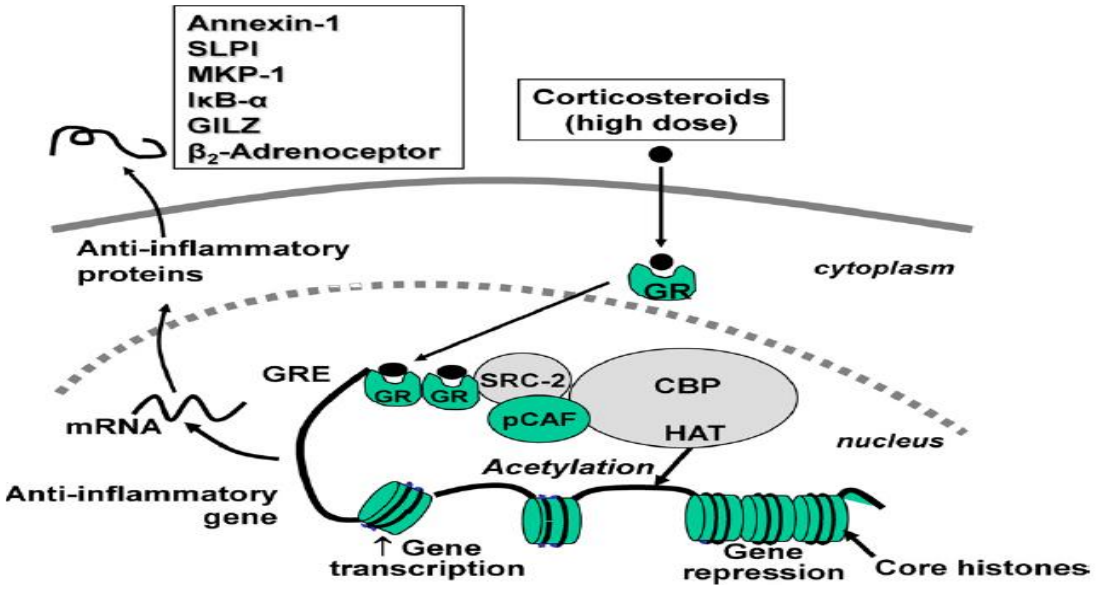


Fig 4. Glucocorticoid activation of anti-inflammatory gene expression (Barans, 2010).

I.1.8. Plants as anti-inflammatory agents

Plants have been used traditionally to treat inflammation. They have a potential to serve as sources of future drugs for treatment of various inflammatory diseases. Plants has been extensively investigated and a number of chemical constituents from the stem bark, leaves, and seeds of the plant have previously been reported which includes triterpenoides, flavonoides, sterols, coumarin, saponins, and tannins (Table.2) (Begum *et al.*, 2014).

Table 2. The anti-inflammatory effect of some medicinal plants (Wiart, 2006, Rick-Leonid et *al*., 2018, Dong Su *et al*., 2018).

Plant	Mechanism of action
Lophira procera A.	A strong anti-inflammatory activity manifest by the inhibition of protein denaturation and a strong antiangiogenic activity marked by the inhibition of vascularization in CAM.
Sanguisorba officinalis L.	Significantly suppressed the activation of NF-jB P65 and NF-jB translocation into the nucleus.
Salix alba L.	Salicin is antipyretic and analgesic and has been used in the treatment of rheumatic fever; Acetylsalicylic acid, the acetylated form of salicin. Aspirin is one of the most commonly used pharmaceuticals containing salicin.

I.2. Oxidative stress

Oxidative stress appears in a cell when the balance between pro-oxidant species and antioxidant is broken in favor of pro-oxidants. In living systems, a physiological production of reactive oxygen species (ROS) is done continuously. In pathological conditions or caused by exogenous factors, an overproduction of these reagents is possible. Antioxidant defenses, part of which is dependent on food, may be insufficient for prevent cell damage that can cause oxygen free radicals (Valko et *al.*, 2007). Excess ROS that are not neutralized by the body's defense system can damage vital cellular components causing abnormal gene expression, cell proliferation, or cell death. These imbalances can be a result of normal metabolic processes, aging, a large number of disease states (Chirico, 2012).

Oxidative stress has been implicated in numerous pathologies including cancer, atherosclerosis and neurodegenerative diseases (Shirley *et al.,* 2014).

I.2.1. Free radicals

A free radical is any chemical species that contains one or more unpaired electrons. Unpaired electrons alter the chemical reactivity of an atom or molecule, usually making it more reactive than the corresponding non-radical, because they act as electron acceptors and essentially "steal" electrons from other molecules. This loss of electrons is called oxidation, and free radicals are referred to as oxidizing agents because they tend to cause other molecules to donate their electrons (Table.3) (Gilgun-Sherki et *al*., 2001).

ROS and RNS (reactive nitrogen species) are known to play a dual role in biological systems, since they can be either harmful or beneficial to living systems. Beneficial effects of ROS involve physiological roles in cellular responses to noxia, as for example in defence against infectious agents and in the function of a number of cellular signaling systems (Valko *et al*., 2006).

Table 3. Some reactive oxygen species (Birben *et al*., 2012).

Oxidant	Formula
Superoxide anion	$O_2^{\bullet -}$
Hydrogen peroxide	H_2O_2
Hydroxyl radical	$OH^{\bullet}$
Hypochlorous acid	HOCl
Peroxyl radicals	ROO^-
Hydroperoxyl radical	HOO^-

I.2.2. Principal sources of reactive oxygen species

ROS are produced continuously inside and outside the eukaryotic cell by various mechanisms. Therefore, the source of ROS can be divided into endogenous ROS and exogenous ROS.

I.2.2.1 Endogenous sources of ROS

ROS are produced from molecular oxygen as a result of normal cellular metabolism. ROS can be divided into two groups: free radicals and nonradicals. Molecules containing one or more unpaired electrons and thus giving reactivity to the molecule are called free radicals. When two free radicals share their unpaired electrons, nonradical forms are created. The three major ROS that are of physiological significance are superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (OH), and hydrogen peroxide (H2O2) (Birben *et al* .,2012).

Superoxide anion ($O_2^{\bullet -}$) can be formed by nicotine adenine dinucleotide phosphate [NAD(P)H] oxidase or xanthine oxidase or by mitochondrial electron transport system by the addition of 1 electron to the molecular oxygen (Birben et *al.,* 2012). Hydrogen peroxide (H_2O_2) can be produced by Xanthine oxidase during ischemia / reperfusion (Valko *et al.,* 2007). There are other endogenous sources of ROS like uncoupled nitric oxide synthases, cyclooxygenase, and cytochrome P-450, which can potentially generate and/or release ROS (Belcastro, 2016).

I.2.2.2 Exogenous sources of ROS

There are many factors outside of the body that can produce ROS by different mechanisms, like cigarette smoke that contains many oxidants and free radicals and organic compounds, such as superoxide and nitric oxide. In addition, inhalation of cigarette smoke into the lung also activates some endogenous mechanisms, such as accumulation of neutrophils and macrophages. The metal (iron, copper, chromium, cobalt, vanadium, cadmium, arsenic, nickel) -mediated formation of free radicals (Valko et *al.,* 2006). Also ozone exposure, hyperoxia and the ionizing radiation can lead to the production of ROS (Birben et *al.,* 2012).

I.2.3. Biological defense against oxidative stress

In order to reduce ROS-induced oxidative damage, the cells normally possess intracellular molecules and enzymes to keep the homeostasis of ROS at a low signaling level. The antioxidant defense system includes enzymatic (i.e superoxide dismutase (SOD), catalase (CAT), Glutathione (GPx), Glutathione reductase (GR), Glutathione-S-transferase (GST), Thioredoxin reductase (TrxR), metal chelating and free radical scavenging activities to neutralize these radicals after they have been formed (Table.4) (Belcastro, 2016).

Table 4. Enzymatic defense against ROS (Zielinski and Portner, 1999).

Enzyme	**Mechanism of action**	**Equation**
Superoxide dismutase(SOD)	Convert the Superoxide ($O2^{-}$) in to hydrogen peroxide	$O2^{-}2H2O \rightarrow 2H2O2$
Catalase	removes H2O2	$2H2O2 \rightarrow 2H2O+O2$
Glutathione peroxidase (GPX)	Catalyzes the transformation reaction of hydrogen peroxides H2O2 and lipid ROOH	$H2O2+2GSH \rightarrow 2H2O+GSSH$
Glutathione Reductase (GR)	Regenerate GSH from GSSG using NADPH as an electron donor.	$GSSH+2H2O \rightarrow H2O2+2GSH$

There is also a non enzymatic defense line against oxidative stress which is represented by the ubiquinol, carotenoids, uric acid, reduced glutathione (GSH) ascorbic acid (vitamin C), and α–tocopherol (vitamin E) (Siamilis, 2009).

I.2.4. The principal oxydative damages

Reactive oxygen species and reactive nitrogen species, which are deleterious by products of metabolic processes cause damage to cellular biomolecules such as proteins, DNA and lipids, leading to oxidative stress that is a major causative factor of number of chronic diseases such as cancer, aging, heart diseases, diabetes mellitus gastric problems, immune suppressions and neurodegenerative diseases (Perera et *al.,* 2016). Reactive oxygen species modulate the function of all classes of biomolecules, targeting almost all substrates in the cell (Pisoschi and Pop, 2015).

I.2.4.1 Nucleic acids damage

One of the most major consequences of oxidative stress is the DNA and the RNA damage. Exposure of nucleic acids to reactive species may result in a standard breakage, nucleic acid-protein cross-linking and nucleic base modification. Base modification, cross-linking of DNA-DNA and DNA-proteins, sister chromatid exchange and single or double strand breaking may lead to disruption of transcription, translation and DNA replication (Saadallah, 2013).

I.2.4.2 Proteins damage

The backbone of a protein polypeptide chain is highly susceptible to reaction with ROS. Certain amino acids (i.e. histidine, arginine and lysine) contain redox active sulphur-hydryl (-SH) groups, also called thiol groups that similarly to some ROS contain unpaired outer shell electrons. As a result, during heightened periods of oxidative stress, amino acid side chains can be targeted by ROS such as $OH^{\cdot}$, causing oxidative modifications that stabilize outer shell stability of both molecules. Mildly oxidized proteins can be reduced by antioxidant systems or degraded and removed by the proteasome system; however excess protein oxidation can lead to protein aggregation and inhibition of cellular and non-cellular processes. Common protein adducts include dityrosine, protein-protein cross linking and protein carbonyl groups are all results of oxidative stress (Wadley, 2013).

I.2.4.3 Lipid damage

Lipids are the most susceptible to undergo oxidation: polyunsaturated fatty acids, especially arachidonic acid and docosahexaenoic acid, which lead to malondialdehyde and 4-hydroxynonenal, recognized markers of lipid oxidative (Pisoschi and pop, 2015).

Unsaturated phospholipids of cell membrane are sensitive to oxidation. The most common fatty acid in cells is linoleic acid. Fatty acid oxidation products are short lived hydroperoxides and their reaction with metals creates, for example, aldehydes and epoxides. Malondialdehydes (MDA) are mutagenic products of lipid oxidation and can further react with DNA. 4-hydroxy-2-nonenal (HNE) 4-hydroxy-2-nonenal is weakly mutagenic but seems to be the major toxic product of lipid peroxidation and it has effects on signal transduction pathways. Nucleotide excision repair pathways and mismatch repair can fix mutations (Fig 5) (Eerola, 2015).

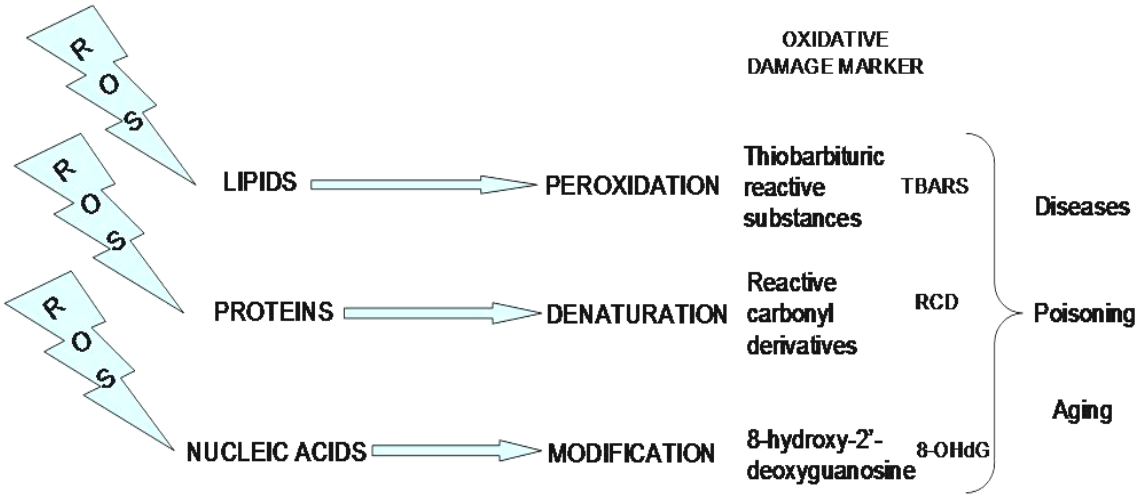


Fig 5. The major impact of ROS on macromolecules (Siamilis, 2009).

I.2.5. Link between oxidative stress and inflammation

There is a very strong link between the inflammation and oxidative stress because ROS such as OH, H_2O_2, and ($O_2^{\bullet -}$), are produced in response to activation by cytokines, including TNFα and IL-1. The production of ROS activates redox-sensitive signaling pathways that induce inflammatory gene expression. In adequate quantities, ROS are considered to be second messengers. ROS have been

considered to be general messengers for the induction of NF-B activation, although possibly not through direct mechanisms. Recent evidence supports the notion that ROS may oxidize KF-B subunits, by impairing DNA binding and transcriptional activities of NF-B. In addition, ROS can strongly activate JNK though the oxidative inactivation of the endogenous JNK inhibitors (Chirico 2012).

Moreover, inflammatory processes also involve in the generation of free radicals by leukocyte activation. Hence, screening for antioxidant potential may reveal important information about anti-inflammatory properties of drug candidates (Perera et al., 2016).

I.2.6. Plants as anti oxidants

Many herbal plants contain antioxidant compounds which protects cells against degenerative effects of reactive oxygen species. Plants are important source for the discovery of new products of medicinal value for drug development and plants secondary metabolites are unique sources for pharmaceuticals food additives (Maroua et al., 2018). This antioxidant activity of the plant is possible because they have the ability of scavenging free radicals. Recent researches reports have indicated that plant based antioxidants are of great importance as therapeutic agents to combat oxidative stress associated chronic diseases including inflammatory diseases. Medicinal plants remain as potent sources of new anti-inflammatory agents and antioxidants (Perera et al., 2016).

I.3. Aristolochia longa

I.3.1. Introduction

Medicinal plants are a group of species that accumulate different active principles, useful in treating various human or animal diseases. The long term use of herbs in medicine is a sure indication of their value and usefulness in the future (Oancea et al., 2013). Over the past decade there has been an increasing interest in the use of natural products. Since 1978, the World Health Organization (WHO) had extended the use of plants in health ecosystem. WHO has estimated that 80% of the world population relies chiefly on traditional medical system (Bouzabata, 2017).

In general, plants can be classified into three categories: food plants or edible plants, which represent a very important part of the food intake of humans and herbivorous animals; medicinal plants

which are the botanical species used in herbal medicine and folk medicine to cure certain diseases in humans and animals, and poisonous plants that can cause toxicological accidents in the individuals who ingest them (Lamnaour, 2009).

I.3.2. *Aristolochia longa*

I.3.2.1 Habitat and geographic repartition

Aristolochia longa belongs to the genus Aristolochia (Aristolochiaceae), the largest genus of the Aristolochiaceae family. That genus consists of about 500 species of herbaceous perennials, under shrubs or shrubs mostly distributed along tropical, subtropical, and mediterranean regions of the world (Benarba et al., 2012). It is widely distributed in Asia, Africa, North and South America (Dhouioui et al., 2016). It is a climber that grows in thickets, mountain slopes, and moist valleys (Wiart 2006). In Algeria it grows in the medal and in west up the mountains of Tlemcen, Cherea Medea, and in Reghaia (Blida). It also grows in Tizi ouzou and Laghout (Benarb, 2013). In Jijel, it is found in the mountains of Djimla.

I.3.2.2 Scientific classification

Kingdom: Plantae
Clade: Angiosperms
Order: Piperales
Family: Aristolochiaceae
Genus: Aristolochia
Species: A. longa

Common names: *Aristolochia longa* has different names; in French it is called sarrasine, aristoloche of the vigne. In Algeria is called belrosstom or zerwand el taouil (Benarba, 2013).

I.3.2.3 Botanical description

Aristolochia longa is a perennial almost glabrous, green, slightly glaucous green, can grows to 20 to 50 cm long, oval triangular leaves in heart and peduncle, sinus widely open at the base, entire, smooth, weak veins (Fig.7). The yellowish flowers with brown streaks have a short longuette. The flours striped with tubular brown horn-shaped at the top curved at the head of cobra baffled at the base.

The fruit is large ovoid and elongated (Fig .6). It is hermaphrodite (has both male and female organs) (M'hammed, 2017).

Fig 6. *Aristolochia longa* fruits (Lahiaoun i, 2016).

Fig 7. *Aristolochia longa* leaves (M'hammed, 2017).

I.3.2.4 Traditional use

Aristolochia longa have been used as medicinal herb since ancient time in different cultures. It has multiple applications and virtues, and used in traditional medicine. It has been reported that the most widely uses of A. longa in Algeria are in cancer treatment. In Morocco, the rhizomes of this plant are used to treat acute toxicities (Benarba, 2015).

In Iran it has been used as an antidote for snakebites. In Ireland it was given to the women for abortive purposes for its ability to remove the dead child. Other uses are to remove all fluids in the womb after childbirth and can also be used to promote menstruation and urination. Also, it was given to children for rickets. The people in Germany use it as a stimulant and an emmenagogue and to prevent arthiritis, (Heinrich et al., 2009).

Aristolochia L. has also used against the constipation and intestinal disorders. It was recommended for ovarian failure, healing diuretic analgesic, and as an anti-inflammatory and anti-mitotic (Cherif et al., 2014). Its roots, sprayed, associated with honey were used to treat skin diseases, constipation and intestinal infections. They had also used, only in decoction, against asthma, for the treatment of the palpitations of the aorta, as emetic-cathartic and diuretic (Bammi and Douira, 2002).

The powder of roots of *Aristolochia longa* L. with salted butter is used to treat skin infections and gangrene; it was used as anti-cancer in case of sclerosis, uterine and nasal cancer (Cherif et al., 2014, Benarba, 2015).

I.3.2.5 Biological and pharmacological properties

The genus of Aristolochia is rich in aromatic and medicinal plants which some of them showed pharmacological properties and biological activities. The plants of that genus have several biological activities including, anti-microbial, anti-tumor, anti-allergic, anti-oxidant, anti-fungal, anti inflammatory, and anti-venomous (Benarba, 2013). These biological activities were attributed to a wide array of bioactive components including flavonoids alkaloids, fixed oil, tannic acid, resin nitrophenanthrene carboxylic acids, sugar and aristolochic acids (Dhouioui et al., 2016).

The anti-inflammatory property of Aristolochia species is probably the result of a direct interaction between aristolochic acid and derivatives of phospholipase A2. Aristolochia indica L., Aristolochia kaempferi, and Aristolochia recurvilabra Hance are used for the treatment of inflammatory conditions (Wiart, 2006).

In the last two decades there has been a considerable interest in *Aristolochia longa*, which have been a subject of many chemical and pharmacological studies. Many researches indicate that Aristolochia L. have several biological activities that are presented in the table below (Table 5).

Table.5. Biological activities of *Aristolochia longa.*

Effect	Part of plant	Preparation	Reference
anti-septic	Root	aqueous extract	(Daoudi and Benboubker, 2015)
Anti-cancer	Root	aqueous extract	(Benarba and Elmallah, 2016)
Anti-fungal	Root	aqueous extract	(Benarba and Meddah, 2014)
Anti-diabetic	The rhizome	Decoctions	(Ghourri *et al.*, 2013)

I.3.2.6 Chemical composition

It is well known that there is a close relation between the chemical composition of the plant and its biological activities. There are only a small number of studies carried out on the chemical composition of *Aristolochia longa*. They have shown the presence of polyphenols, flavonoids, cheterosides, carbohydrates, and saponins in the aqueous extract of the plant. Also, it has been reported that the flavonoid glycosides are the major compounds in the extract of the plant (Benarba and Elmallah, 2016). Aristolochia L. like the other members of the genus is rich with terpènes. It contains:

I.3.2.6.1 Monoterpenoids

There are a lot of monoterpenoids that has been identified in Aristolochia L. like the linalool, bornyl acetate, and the bornanol.

I.3.2.6.2 Sesquiterpenoids

Only one sesquiterpenoids has been isolated from the rhizomes of Aristolochia L. which is the maaliol, there are another sesquiterpenoids like βcaryophyllene and caryophllene (Shung Wu et al., 2003).

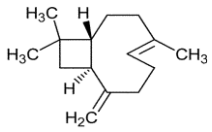
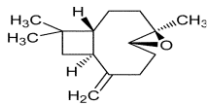
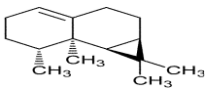
I.3.2.6.3 Aristolochic acids and aristolactams

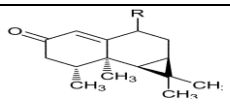
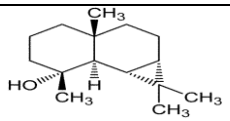
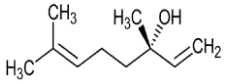
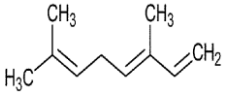
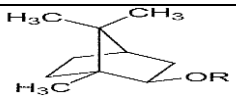
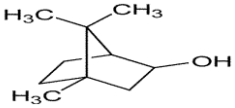
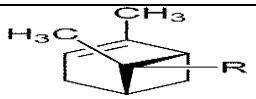
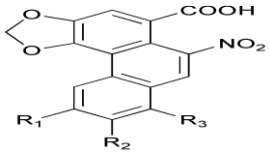
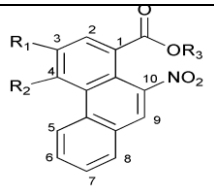
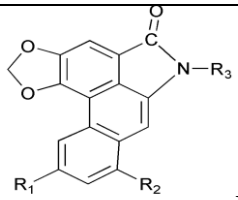
The phenantherenic compounds are one of genus Aritolochia characteristics, they contains the aristolochic acids which are unique to this genus. All aristolochic acid derivatives in Aristolochia species have a methylenedioxy group on ring A. However, 3-hydroxy-4-methoxy-10-nitrophenanthrene-1-carboxylic acid methyl ester has the possibility to be a species marker, which is useful to characterize the species (Kumar et al., 2003).

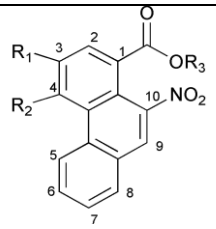
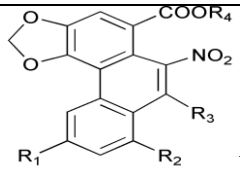
I.3.2.6.4 Chemical composition of essential oils

Cherif et al., 2009 in their study they have proved that the lycopene, 1,2-dihydro-1-hydroxy is the majer component in essential oils of the rhizomes. The other major compounds included are : s-Indacen-1- (2H) -one, 3,5,6,7-tetrahydro-3, 3,5,5- tetramethyl-8- (3-methylbutyl) , 2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde , and Carda-4,20 (22) -dienolide, 3 - [(6-deoxy-3-O-methyl-alpha-D-allopyranosyl) oxy] -1,4-dihydroxy-, (1.beta., 3 beta) (Cherif et al., 2009). The table below (table 6) represents some of the major components of *Aristolochia longa*.

Table 6. Major components of *Aristolochia longa* (Shung Wu et al., 2003 ; Kumar et al., 2003 ; Aneb et al., 2016).

Compound	**Structure**
β-Caryophyllene	
Caryophyllene oxide	
Δ 1(10)-Aristolene (calarene)	

*Δ*1(10)-Aristolone (2-oxocalarene)	 R=H
Maaliol	
(*S*)-Linalool	
β-Ocimene	
Bornyl acetate	 R=COCH3
Bornanol	
α-Pinene	 R=CH3
Aristolochic acid IV (6-methoxyaristolochic acid)	 R1=R3=OCH3; R2=H
Aristolochic acid II	 R1=R2=OCH2O ; R3=H
Aristolactam Ia	 R1=R3=H; R2=OH

2-Phenanthro-[3,4-d]-1,3-dioxole-6-nitro-5-carboxamidopropanoic acid methyl ester	 R3=CH(CH3)COOH3; R1=R2=OCH3O
Aristolochic acid A (aristolochic acid I)	 R1=R3=R4=H; R2=OCH3

I.3.2.7 Toxicity

Aristolochia longa owes its toxicity to aristolochic acid which is one of the most active components in the plant. The World Health Organization considers it as carcinogenic (capable of causing cancer) (El idrissi et al., 2018). There is a an accumulating evidence from researches in China and Europe that the ingestion of herbal remedies of this plant is associated with the rare form of nephropathy that can result in the subsequent development of transitional cell cancer (Heinrich et al., 2009). This nephropathy is characterized by chronic renal failure, tubule-interstitial fibrosis and urothelial cancer (Benzakour et al., 2012). Aristolochic acids include mutagenic and carcinogenic molecules for the stomach, bladder, kidneys and testicles (El idrissi et al., 2018). Following these dangerous health consequences, a decision was taken to prohibit the use of any natural remedy containing this acid (Cherif et al., 2014).

This active ingredient is terrifying because it is considered a highly toxic substance. Plant toxicity, not only related with aristolochic acid but also some of its volatile oils, includes nausea, vomiting, headache, dyspnae, fever, hypertension, spasm, convulsions, coma and respiratory disorders that may lead to death (Ait Haj Said et al., 2015).

II. Materials and methods

II.1. Materials

II.1.1. Plant materials

Fresh plant material samples of *Aristolochia longa* . were collected in may 2018, from the region of Djimla (Jijel). The species of the plant has been identified by the botanist KHANOUF Hanane at University of Mohammed Esseddik Ben Yahia, Jijel.

II.1.2. Animal materials

The experiments were carried out by male adult mice supplied by the animal factory of the Pasteur Institute, weighing between 30 g and 38 g. The rats were divided into 5 homogeneous lots of 2 in plastic cages with free access to standard food (croquettes) and water. These rats were acclimated for a period at room temperature with a natural cycle of light and darkness prior to experimentation.

II.2. Methods

II.2.1. Preparation of plant extract

The plant was harvested from its original environment. The roots (Fig.10) were separated and rinsed severally with clean tap water to make it dust and debris free. Then they were dried in the shady condition for 3 to 4 days until they become moisture free. Dried roots were ground in electric chopper to get fine powder form for further use.

Fig 8. Roots of *Aristolochia L.*

According to Salehabadi *et al.,* (2014), 30 grams from dried powdered material (roots) were mixed with 300 ml of 70 % aqueous ethanol initial warmed at 30°C. The mixture was kept at room temperature (23 to 25 °C) for 48h under maceration. Then it was filtered through Whatman filter paper. The filtrate was concentrated using the rotary evaporator at 50°C and stored until used for further analysis.

II.2.2. Determination of extraction yield

The yield of the extract was calculated using equation below (Mabiki *et al.,* 2013). The percentage yield obtained was used to evaluate the efficiency of ethanol extraction method.

$$\%\text{Yield} = \frac{\text{Amount (g)of the dry extract obtained}}{\text{Amount (g) of the dry sample used}} \times 100$$

II.2.3. Phytochemical analysis of the extract

Natural phytophenols are considered antioxidant compounds which have ability to damage free radical in the organisms related to their bioactivity to,scavenge free radicals and to chelate metals (Jaradat *et al.*, 2015). Total phenolic content was measured using the Folin–Ciocalteu reagent according to the protocol explained by Djeridane *et al*., (2006).

The Folin-Ciocalteu test relies on the transfer of electrons in an alkaline environment from phenolic compounds to acid complexes phosphomolybdic/ phosphotungstic which are determined spectrophotometer at 765 nm (Ainsworth and Gillespie, 2007).

To realize the estimation, 0.2 mL of sample (250µg/ml) was added to 1.5 ml (1/10) of the Folin-Ciocalteu reagent. The solution was mixed and incubated at room temperature for 5 min. Then 1.5 ml of 7.5% sodium carbonate (Na2CO3) reagent was added and shaken. The final mixture was incubated for 90 min in the dark at room temperature. The absorbance was measured at 765 nm using spectrophotometer (Specord 50 plus). The polyphenol content in the extract is calculated by reference to the calibration curve made under the same conditions using gallic acid. The average polyphenol

content is expressed in mg of gallic acid per gram of crude extract (mg GAE / g). All assays wered performed three times.

II.2.3.1 Estimation of total flavonoids content

The total flavonoid content of the ethanolic extract was calculated according to the aluminum chloride (AlCl3) method explained by Djeridane *et al*., (2006). It is based on the formation of a complex between the aluminum ion Al3 and the carbonyl and hydroxyl groups of flavonols that produce a yellow color (Pontis *et al.*, 2013).

To realize the reaction, 1.5 ml of the ethanolic extract (2 mg/ml) was added to 1.5 ml of AlCl3 (2%). After incubation for 30 minutes at room temperature, the absorbance was measured at 430 nm against a blank.

The different flavonoids concentrations of the extract were determined by the use of quercetin as standard. The average flavonoids content is expressed in mg of quercetin per gram of crude extract (mg Q / g). All reactions were performed three times.

II.2.3.2 Estimation of total flavonols content

Total flavonols content was measured according to the method of Abdel-Hameed, (2009). A solution of 1 ml of extract (2 mg/ml), was mixed with1 ml of $AlCl_3$ (2 %) and 3 ml of CH3CO2Na (5%) was added. After incubation for 30 min at room temperature and in the dark, the absorbance was measured at 440 nm. Flavonol content was concluded from calibration curve of quercetin and it is expressed in mg of quercetin per gram of crude extract (mg Q/g). All reactions were repeated three times.

II.2.3.3 DPPH radical-scavenging activity

The DPPH method is the most frequently used assay for the evaluation of the free radical-scavenging capacity of plant extracts. The reaction mechanism involves the H transfer from a phenolic compound to the DPPH radical (Fig.11). Interaction of the DPPH radical (purple-coloured) with a phenolic compound, which is able to neutralize its free radical character ,leads to the formation of yellow colorless hydrazine and the resulting effect can be quantified spectrophotometrically at 515 nm (Bujor, 2018).

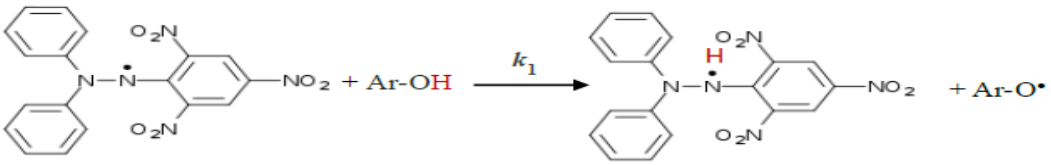


Fig 9. The H-transfer reaction from a phenolic compound (AR-OH) to DPPH.

To evaluate the scavenging effect for DPPH radical, the method of Bougandoura and Bendimerad, (2012) was used. For this, 50 ul of extract with different concentrations (78.5, 150, 250, 500, 1000, 2500, 5000 ug/ml) were added to 1.95 ml DPPH (0.025g/l). The mixtures were left to stand at room temperature in the dark for 30 min. than the absorbance was read at 515 nm. The mixture that contains 50 ul of ethanol and 1.9 of DPPH were considered as a control. The same procedure was carried out with ascorbic acid. Radical-scavenging activity for DPPH free radical was expressed in percentage and calculated using the following formula:

$$\%\ \text{radical scavenging activity} = \frac{\text{absorbace of contol} - \text{absorbace of test}}{\text{absorbace of control}} \times 100$$

II.2.4. Study of the anti-inflammatory activity

To evaluate the anti-inflammatory effect of ethanolic extract of *Aristolochia L.* roots, two methods have been used:

II.2.4.1 *In vitro* study

The anti-inflammatory in vitro has been realized by the following method:

II.2.4.1.1 Stabilization of red blood cells membrane

The principle of this method is based on the stabilization of the red blood cells membrane by the extract with reference to a standard solution. This method is carried out according to kar *et al.*, (2012) with some modifications.

a. Preparation of red blood cells suspension

The blood was collected (2 ml) from a male rat and mixed with an equal volume of the sterilized Alsevers solution (2% glucose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl in distilled water) and centrifuged at 3000 rpm. The packed cells (pellet) were washed with isosaline water and prepared with normal saline to prepare at 10% (V / V) suspension and held at 4 ° C before use.

b. Hemolysis induced by heat

Different concentrations of the ethanol extract of *Aristolochia longa* and the aspegic were prepared (50, 100, 200, 500 and 1000 µg / 0.5 ml in normal saline). Aspegic was used as a standard and physiological saline was used as a negative control. These concentrations were mixed separately with 1 ml of phosphate buffer, 2 ml of hypo-saline and 0.5 ml of the 10% red blood cell suspension. All the mixtures were heated at 56°C for 30 min and centrifuged at 2500 rpm for 10 min (SIGMA 3-16PK),. The supernatants were recovered and the absorbance was read by the spectrophotometer at 560 nm.The percentage of membrane stabilization of red blood cells or inhibition of hemolysis of red blood cells was calculated using the following formula:

$$\text{stabilisation percentage (\%)} = \frac{\text{absorbace of control} - \text{absorbace of test}}{\text{absorbace of control}} \times 100$$

II.2.4.2 *In vivo* anti-inflammatory activity

To evaluate the anti-inflammatory action of *Aristolochia longa* roots, was carried according to the protocol of (Ndiaye *et al*.,2006) with same modifications .The ethanol extract was used as a treatment against carrageenan induced paw oedema in mice. The anti-inflammatory model that has been used was an acute inflammation type. The paw oedema was induced by the injection of 0.1ml of 1% carrageenan into the sub plantar tissue of right hind paw of each mouse except negative control. The extract at concentration of 300mg/kg was administered orally one hour prior to carrageenan administration.

To realize this activity, eight mice were fasted 16 hours before assay and were divided into four (each group contains two mice) as the following:

Group I: (negative control) receive 1 ml of physiologic water (0.9%).

Group II: (positive control) receive 1 ml of physiologic water (0.9)

Group III: receive 1ml of acetylsalicylic acid (Aspegic drug) at 150 mg/kg one hour prior to carrageenan administration.

Group IV: receive 1 ml of root extract of *Aristolochia longa* (300mg/kg).

Paw volumes were measured at 30min, 1h, 1.5h, 2h and 4h using digital caliper. The importance of the oedema measuring appears in the determination of the percentage of increase in mouse paw volume. The equation below was used to calculate the percentage of increase (PI)

$$PI = \frac{V(t) - V0}{V0} \times 100$$

PI: percentage of increase.

V0: the paw size at T (0) before the injection.

V (t): the paw size after different periods of the injection (30 min, 60min,…).

Fig.10. The paw measuring before the injection

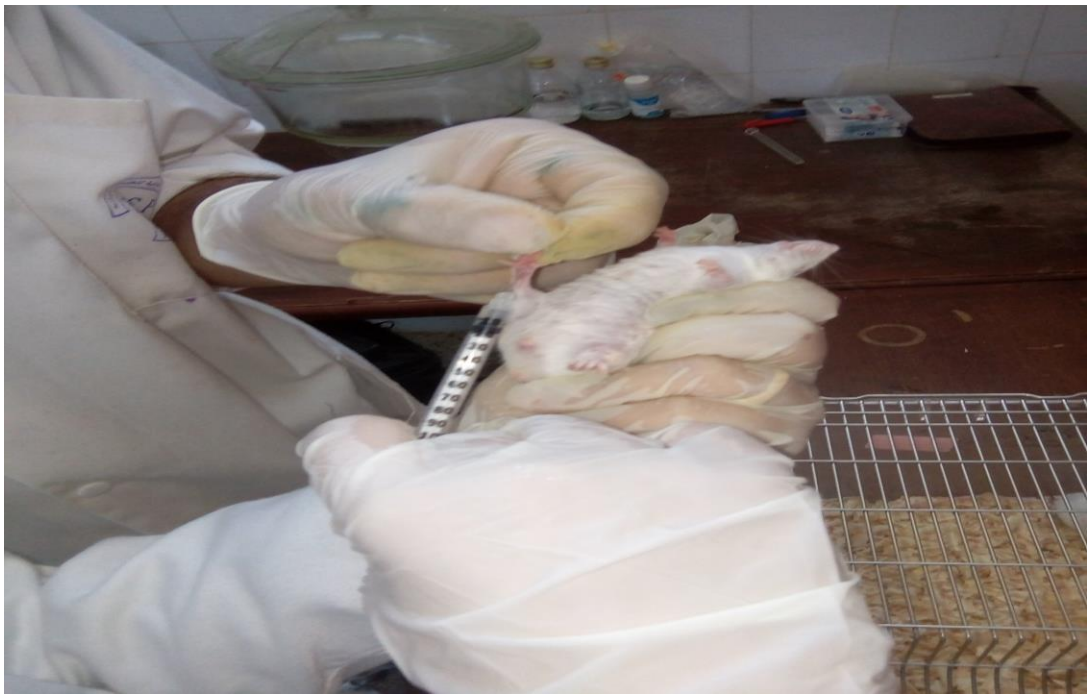


Fig.11 Carrageenan injection

The anti-inflammatory activity was calculated according to the percentage of inhibition of the oedema (PIH).

$$\%PIH = \frac{\%(PI)\ reference - \%(pI)\ treated}{\%(PI)\ reference} \times 100$$

PIH: percentage of inhibition of the edema.

After the sacrifice of the animals, organs were collected and preserved in standard conditions to be used later in different tests.

II.2.5. Evaluation of oxidative stress in liver

II.2.5.1 Preparation of cytosolic fraction

The preparation of the cytosolic fraction was carried out according to the protocol of Iqbal *et al* (2003), 1 g of the liver was removed and directly immersed with three volumes of phosphate buffer KH_2PO_4 (0.1 M; pH 7.4) containing 1.17% of KCl. After cutting the liver into thin pieces and homogenizing, the homogenate was centrifuged at 2000 rpm for 15 minutes at 4°C to remove nuclear debris and the supernatant was centrifuged again at 9600 rpm for 30 minutes at 4°C. The final supernatant was used to carry out the different tests including the ones measuring the oxidative stress.

a. Estimation of tissue total proteins

The determination of total proteins was performed according to the method of Bradford (1976) that is based on ametachromatic shift of the dye when its bind to a protein. The amount of dye remaining in solution after reaction with an immobilized protein is measured by a spectrophotometer (Sapan and Lundblad, 2015).

The experiment was realized by using 4 ml of Coomassie blue was added to 100 µl of the enzymatic source diluted ten times. The mixture was incubated at room temperature for 15 minutes. The absorbance was measured at 595 nm. A calibration curve was performed using bovine serum albumin (BSA) (0.062-1 mg/ml) as standard.

b. Estimation of cytosolic malondialdehyde (MDA)

MDA represents the most used biomarker in lipid peroxidation, it's a metabolic product resulting from lipid peroxidation reactions that attack polyunsaturated membrane lipids by ROS generation. MDA assay is based on a condensation reaction of two molecules of TBA with one molecule of MDA (Ohkawa et al.,1976). After treatment with the hot acid, the aldehydes react with Thiobarbituric acid (TBA) to form a chromogenic condensation product with a pink color. The intense

absorption of this chromogen is measured at 530 nm. In this study, the method of Ohkawa *et al*., (1979) has been used to evaluate the concentration of MDA at the cytosolic fraction.

0.5 ml of the cytosolic fraction of the liver was added to 1ml of TBA (0.67 %) and 0.5 ml of TCA (20%), and then the mixture was incubated at 100°C for a period of 15 min. After the incubation the mixture was cooled and 4 ml of n-butanol were added. In the next step the mixture was centrifuged at 3000 t/min for 15min. The absorbance was read at 530 nm.

The MDA was calculated using a standard curve that was prepared in the same conditions, using the tétraetoxypropane (TEP) which is given after the MDA is hydrolyzed.

c. Estimation of cytosolic glutathione (GSH)

The method of sedlak and raymond (1967) was used to evaluate the GSH in the mice. The assay is based on the reaction of DTNB (Ellman's reagent) with GSH that produced the TNB (5-thio-2-nitrobenzoic acid) and GS-TNB (Fig 12). The rate of formation of TNB, measured at 412 nm, is proportional to the concentration of GSH in the sample (Rahman *et al*., 2007).

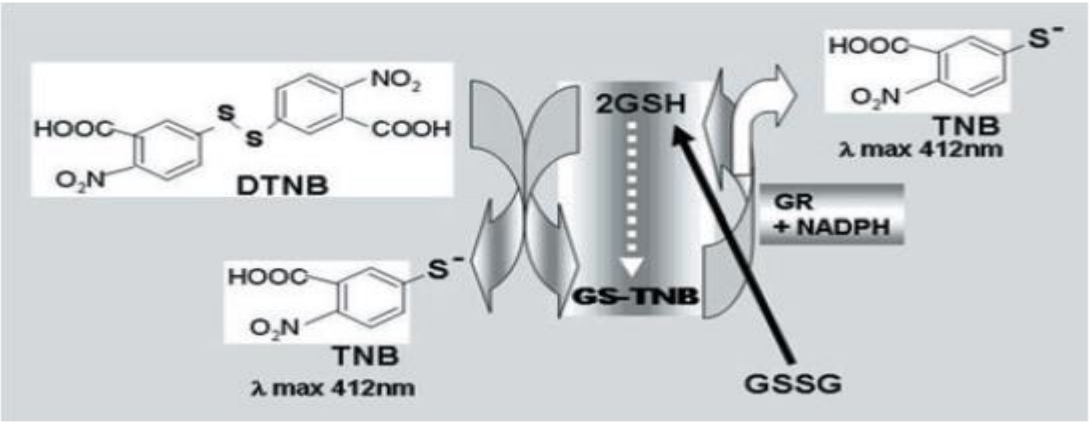


Fig 12. The reaction of which TNB is produced (Rahman *et al*.,2007).

For this assay 50 µl of the enzymatic source was diluted in 10 ml of phosphate buffer (0.1 M, pH = 8). Next 20µl of Ellman's reagent (0.01M DTNB) was added to 3 ml of the dilution mixture. The incubation was carried out at room temperature for 15 minutes. The absorbance was read at 412 nm against a blank prepared under the same conditions.

GSH levels were concluded from a standard curve of glutathione that was prepared in the same conditions as the assay. The concentrations were expressed in mMol of glutathione on gramm of total protein.

II.2.6. Statistical analysis

The results were expressed as the mean ± standard deviation. The statistical comparison between groups was done using Student's t-test, to determine whether there were any significant differences P-Values < 0.05 (*), < 0.01(**), < 0.001(***) were considered significant. Data were subjected to analysis using the Microsoft Excel 2007.

III. Rsults and discussions

III.1. Extract yield

Ethanolic extraction by maceration of *Aristolochia L.* roots is shown a weak yield (10.66 %). This result is similar to the finding of (Merouani *et al.,* 2016) who has found a yield of 10.05%. This result was affected by several parameters, which are plants related. These parameters include the chemical composition of the plant and its physical characteristics, and it's also can be effected by extraction method (Merouani *et al.,* 2016).

III.1.1.Phenolic, flavonoids and flavonols content in the roots of *Aristolochia L.* extract

The total phenolic content was expressed as gallic acid equivalent (GAE) using the following equation based on calibration curve: y = 0,021x - 0,013 (Annex). The phenolic content of the root extracts is 662,89mg GAE/g. This result is higher than obtained from Benarba and Meddah, 2014 who have conducted a study on the same plant (Fig.13).

Chlorur aliminium assay was used to calculate the flavonoid content in the ethanol extract of *Aristolochia longa* roots. The Results were expressed with mg of quercetin equivalents (QE)/mg of dried extract. The flavonoids content in root extract is 1337.69 mg QE/G. The concentration was concluded from the calibration curve (annex.2)

The flavnols content in the extract is 1585.77 mg QE/g. which is considered the highest between the quantified secondary metabolites. Flavonols concentration was obtained using the equation in the calibration curve (annex.3)

The flavonoids and the flavonols contents were both lower than found by Merouani *et al.,* 2016 in their study on methanolic extract. The difference in the concentration of bioactive compounds may be caused by several factors like the extraction method and the change in the environmental conditions also the soil composition can be a factor (Treutter, 2010).

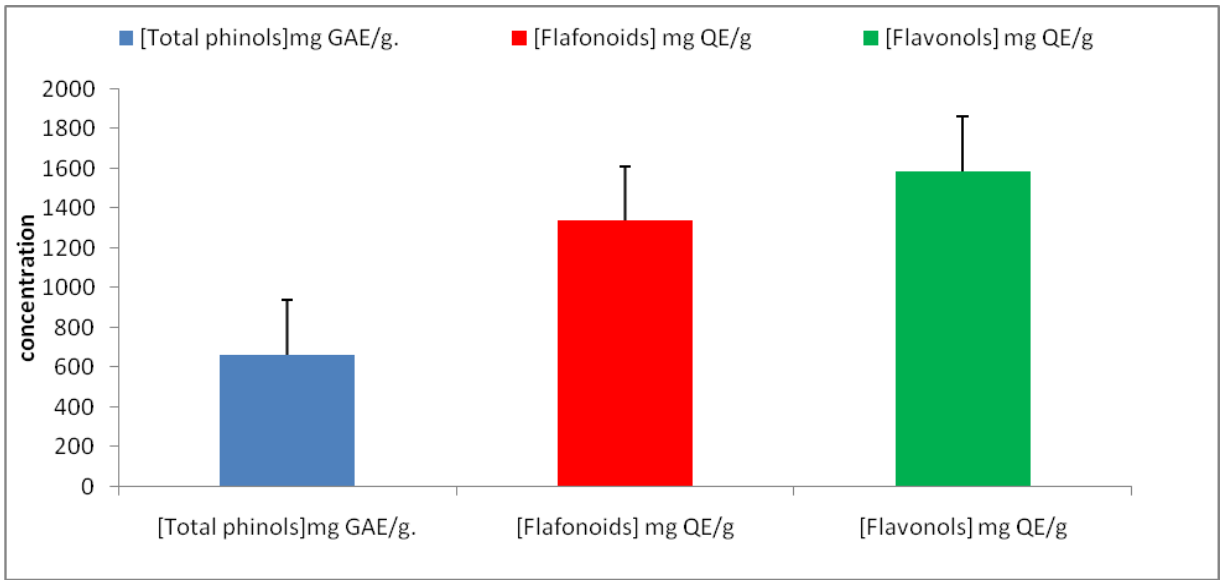


Fig 13. Contents of total phenolics, flavonoids and flavonols in the ethanol root extract of *Aristolochia L.*

III.1.2. Radical scavenging activity

The free radical scavenging activity of the plant has been evaluated by the DPPH assay. The results are expressed with percentage of inhibition of DPPH (Fig.14).

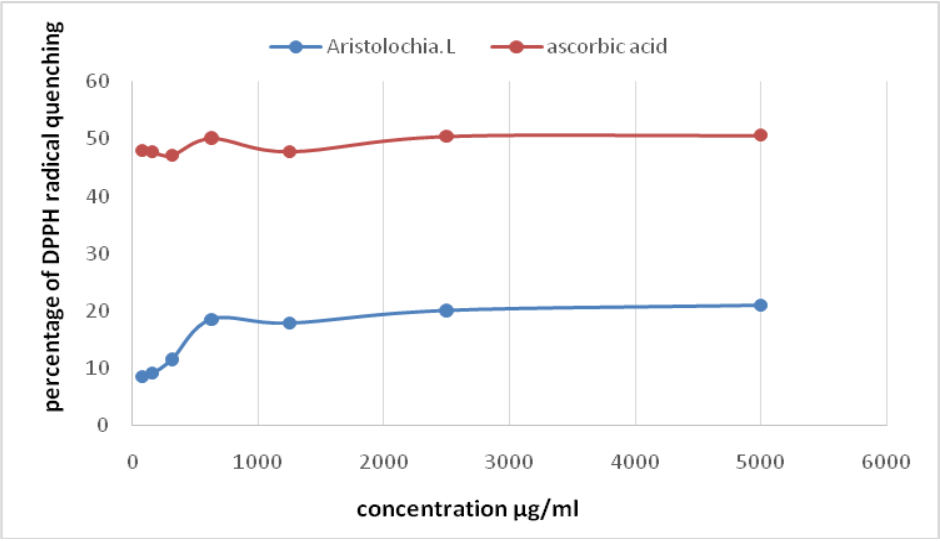


Fig 14. DPPH radical quenching activity of various concentrations of ethanolic extract and ascorbic acid.

The results of DPPH assay showed that ethanolic extract of *Aristolochia L.* exhibited a less free radical scavenging activity than the ascorbic acid at concentrations from 78.25 to 5000 µg/ml. At the concentration of 5000µg/ml (21.04% ±0.005) the extract showed highly significant ($p< 0,001$) free radical scavenging activity than ascorbic acid and also significant ($p< 0,05$) less activity in a concentration (78,25µg/ml)(8.49±%0.005), than ascorbic acid (48.02%±0.02) and (50.68%±0.0004) respectively in the same concentrations.

In this study the ethanol extract showed weak antioxidants capacity in a comparison with the ascorbic acid. These results are correlated with the results of Merouani *et al,* 2016 who were studied the aerials parts of the plant in the manner of comparison between the plant and the standard anti-oxidant.

The DPPH inhibition activity is related to the plant composition of flavonoids and other phenolic compounds (maroi *et al*, 2008), in the case of *Aristolochia L.* it has been showed a high concentration in flavonols .

III.2. Study of anti- inflammatory activity

III.2.1. Results of the in vitro study

III.2.1.1 Red blood cells membrane stabilization

The ethanol extract was tested to evaluate its ability to protect the red blood cells against an induced hypo tonicity. The percentage of hemolysis inhibition (stabilization of membrane) by the extract and the drug was calculated (Fig 15).

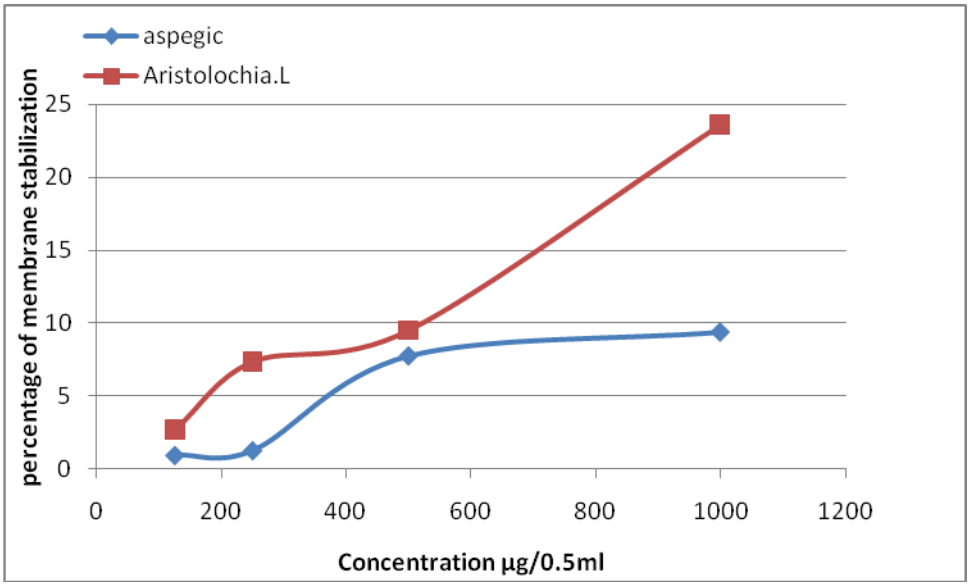


Fig 15. The percentage of red blood cells membrane stabilization treated with ethanolic extract of *A. longa* roots and Aspegic at different concentrations.

These results showed that the ethanolic extract of *A. longa* roots present a high inhibition of red blood cells hemolysis at different concentrations compared with the standard (aspegic drug). The percentage of stabilization was found to be 23.65% at concentration of 1000 µg/ml of *Aristolochia L.* extract (9.34 % at the same concentration in aspegic). The evaluation of percentage inhibition indicates that the ethanolic extract of *A. long* roots have an anti-inflammatory activity by inhibition of hemolysis of red blood cells by heat at different concentrations.

These results are so much lower than reported by Abou-elella and ahmed, (2015) who were studied the ability of *Enteromorpha compress* extract to protect the red blood cells membrane against an hypo tonicity. This can be explained by the lack of ethanolic extract of biological membrane stabilization properties that may due to the chemical composition of bioactive compounds. Gaamoune, (2016) suggested that the secondary metabolites plays a major role in stabilization of the red blood cells membrane and in the case of *Aristolochia.L* it may not contain high enough concentration of these compounds.

III.2.2. Results of in vivo study

In this study the anti-inflammatory activity of *Aristolochia L.* roots was evaluated using an acute inflammatory type based on induce of a paw edema bay the carragennan. The change in size of the edema was evaluated and the percentage of inhibition and increase was calculated (Table 7) (Fig.16).

Table 7. The means of increase and percentages of inhibition of the paw size in the different groups of the experiment.

Treatment(mg/kg)	Mean increase in paw volume						% Decrease in paw volume 4h
	00	30 min	1h	1.5 h	2 h	4 h	
Aspegic (150)	2,51±0,09	3,52±0,33	2,9±0,38	2,955±0,13	2,84±0,11	2,69 ±0,2	85,63*
Positive Control	2,68±0,12	3,6±0,4	3,18±0,09	3,48±0,4	3,09±0,07	4,14±0,50	
Aristolochia.L. (300)	2,55±0,22	3,9±0,39	3,39±0,18	3,415±0,33	3,3±0,69	2,84± 0,62	80,31*

These results showed that there is no significant different between the effect of the ethanol extract and aspegic in reducing the paw size in the fourth hour after treatment with the carragennan 80,31 % and 85,63 % respectively). The plant extract effect was almost good as the effect of the anti inflammatory standard aspegic in the fourth hour and showed a significant increase comparing with the positive control.

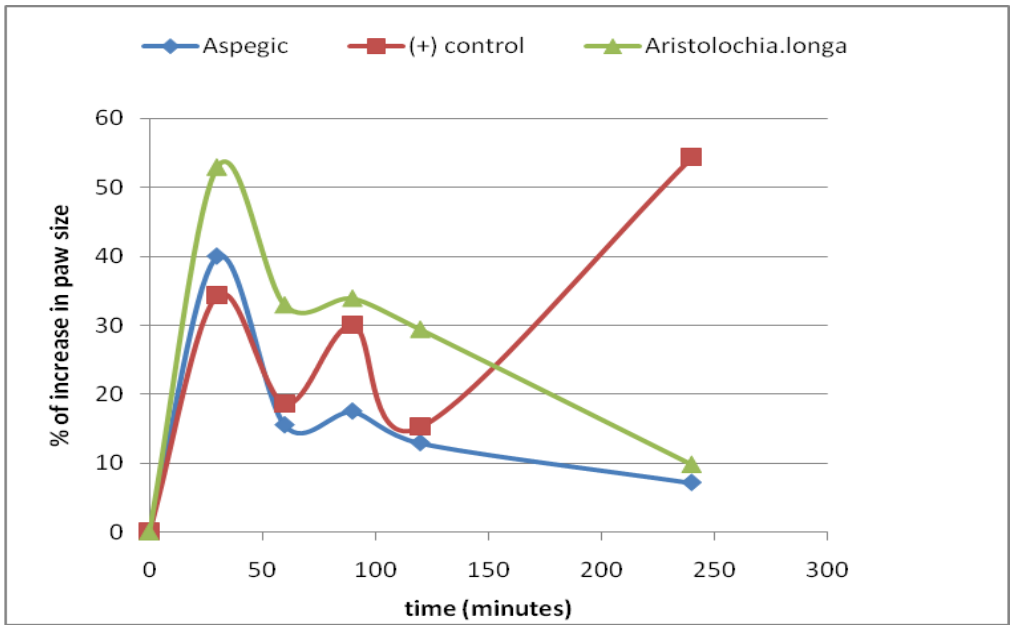


Fig 16. Percentages of increase in the paw size.

The results showed a high increase in all the groups of mice in the first 30 min after the carragennan injection ,after the 30 the levels of increase started to fall to 17% in the the group that has treated whit aspegic and 30,03% and 33,92% in positive control and ethanol extract respectively in min 90 .After a small increase in all groups there was a diminution until the fourth hour in the groups tha was treated with aspegic and the ethanol extract but in the case of the positive control that last diminution was followed by an increase until the fourth hour to reach 7,15%, 9,80%, 49,81% in aspegic ,ethanol extract and positive control respectively .These results have showed that the effect of the ethanol extract is time dependent .

These results are consistent with the study of Ruth *et al*., 2003 about the anti-inflammatory activity of *Aristolochia ringens* roots that shows a decrease in the paw size. The injection of carrageenan causes the release of several chemical mediators that are responsible for the inflammatory process (hafeez *et al*.,2013)

The decrease in the mice paw may be because of the presence of aristolochic acid in the roots of *Aristolochia.L* which have an anti-inflammatory action. Aristolochic acid acts by its interaction with the derivatives of phospholipase A2 (Rosenthal et al 1998) which is an enzyme responsible for the

synthesis of prostaglandin (Wiart, 2006). The inhibition of prostaglandin production is one of the most important paths in the elimination of inflammatory reaction.

III.3. Oxydative stress results

III.3.1. Lipid peroxidation levels

The levels of cytosolic MDA in the livers of all mice in the forth groups were measured. The results were expressed in nmol MDA/mg of protein (Fig 17).

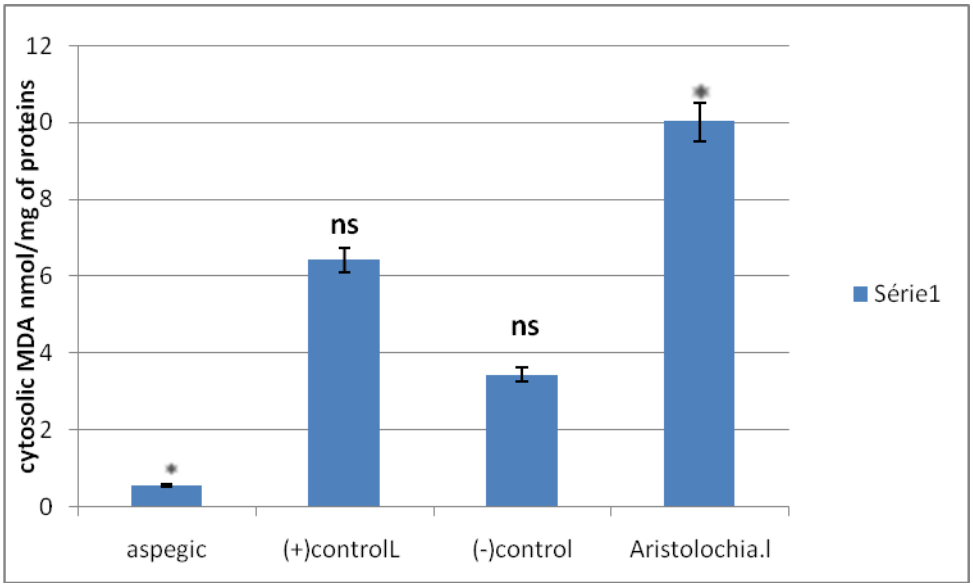


Fig 17. Levels of cytosolic MDA in all groups of the experiment.

These results shows that the levels of MDA in liver of the mice that was treated with the ethanol extract (10.025 ±0,013) are significantly higher ($P < 0.05$) than the ones treated with Aspegic (0,56±0,008). Also the results showed no significant difference between the group treated with the ethanol extract and the of the (+) contol .Also no significant difrrence between the (-) control and the (+) control. MDA concentration was obtained using the equation from the calibration curve (Annex.6)

These results do not correspond to what has been obtained by derouich *et al.*2018. .A significant production of oxygen free radicals can lead to an increased of lipid peroxidation by attacking the double bonds of the fatty acids composing the lipidic membranes (Gaamoune, 2016). That

increase in lipid peroxidarion leads to a increase in the MDA concentration. The ethanolic extract may have no protective effect against the lipid peroxidation induced by ROS. In fact, it may even aggravate it considering the high levels of MDA.

III.3.2. Reduced glutathione (GSH) concentrations

The GSH levels are considered as health state marker of the person .The levels of cytosolic GSH in the liver of all mice that were measured (Fig 18).

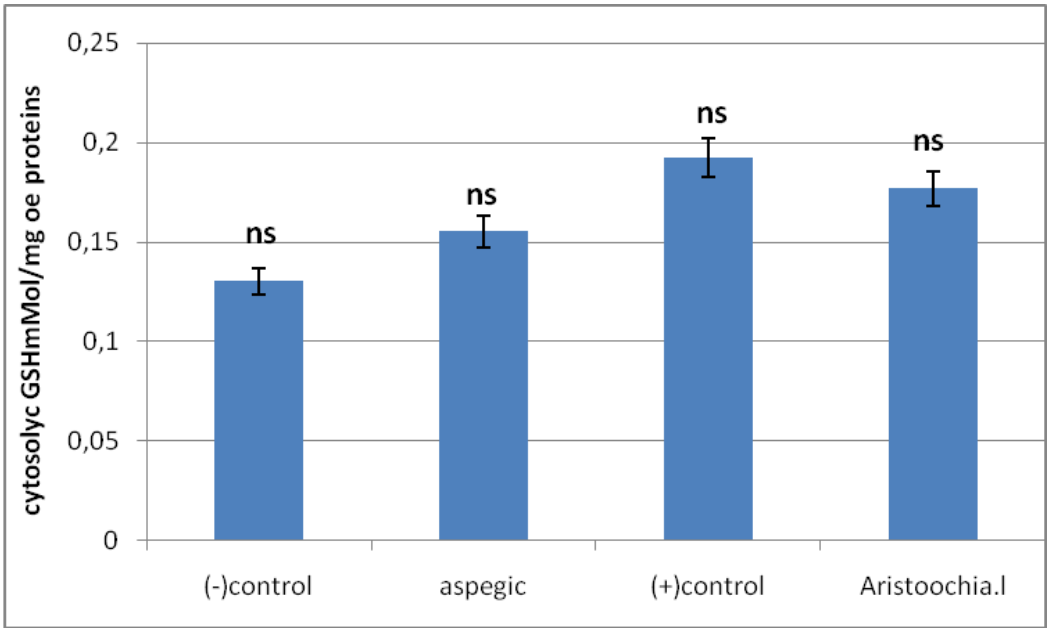


Fig 18. The results were expressed as the mean ± standard deviation (n=2) followed by t test of Student t to determine whether there were any significant differences (P < 0.001 or P < 0.05)

These results show that the GSH concentration is not significantly increased in the group that was treated with the ethanol extract (0,1767 ±0,9) comparing with aspegic (0,15641±0,06). Our result showed no significant elevated level of the GSH in the group of positive control (0,192±0,052) and also these result have showed that the consentration of GSH in positive control is not significantly higher than the negative control. These concentration were obtained using the equation from the calibration curve (annex.6)

The results are in consistent with the results of Derouiche *et al*., 2018 in a study about the effect of *Aristolochia.L* on an induced acute liver injury, a correlation was found in the increase of the cytosolic GSH comparing with the (-) control group. The increase in cytosolic GSH can be explained by the increase of free radicals because the generation of the ROS in response to their activation by cytokines, including TNFα and IL-1.ROS also acts as second messengers (Chirico, 2012).

The objective of the current study was to evaluate anti-inflammatory and anti-oxidant effects of *Aristolochia longa* roots. The results obtained in this study shows that the roots of *Aristolochia L.* contain considerable concentrations of phenols, flavonoids and flavonols but have a weak radical scavenging activity (antioxidant activity).

Also they exhibit that the ethanolic extract of the roots a high ability to reduce the edema size in the acute inflammation induced by carrageenan. The result was almost good as the standard anti-inflammatory drug (aspegic).

Since *Aristolochia longa* is widely used in the Algerian medicine, more researches are needed about this plant.

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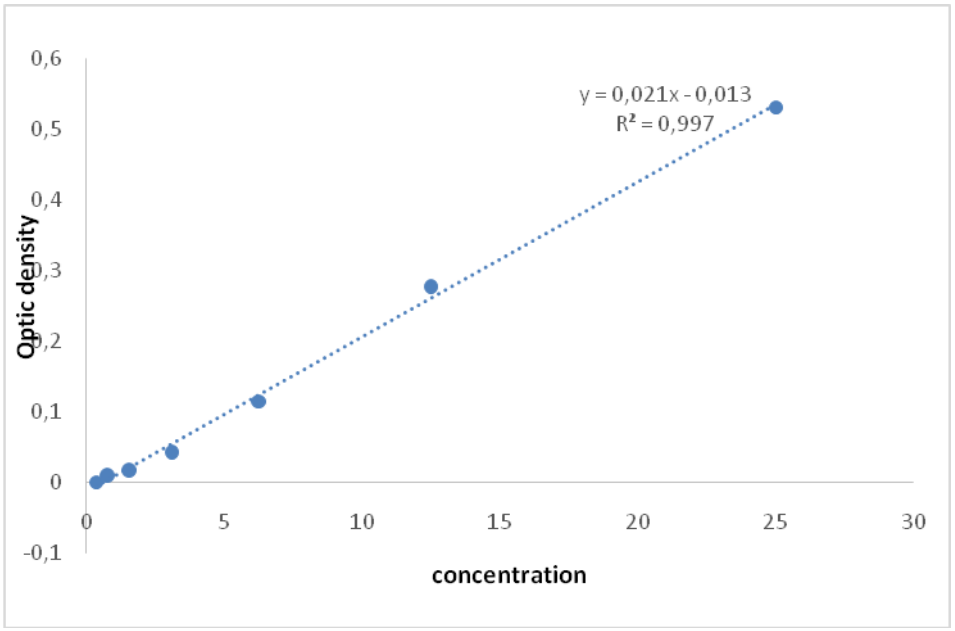
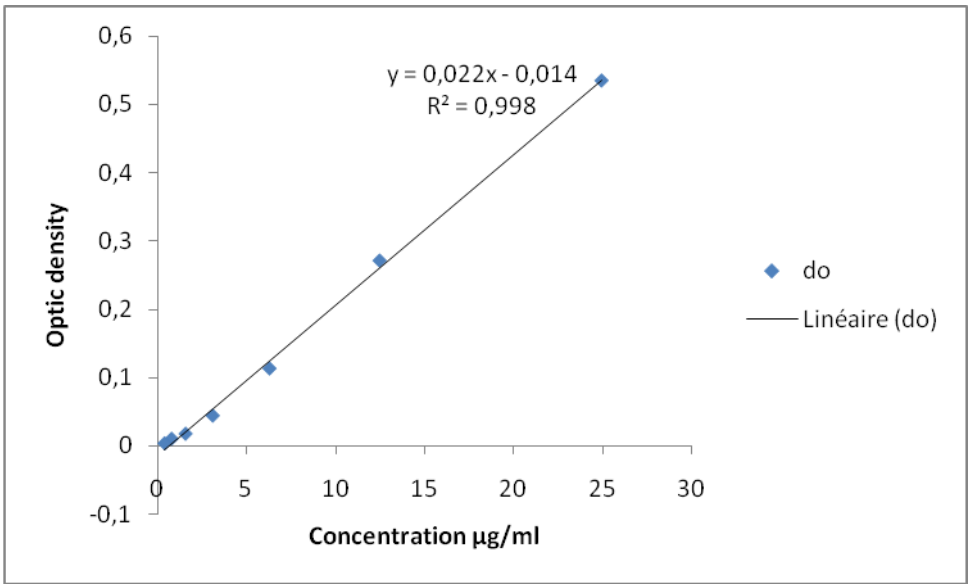
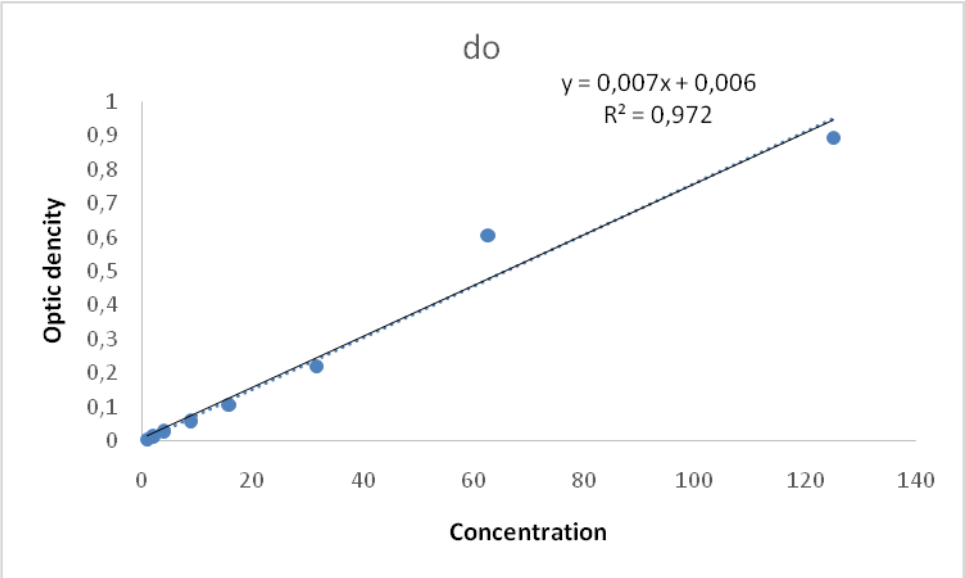


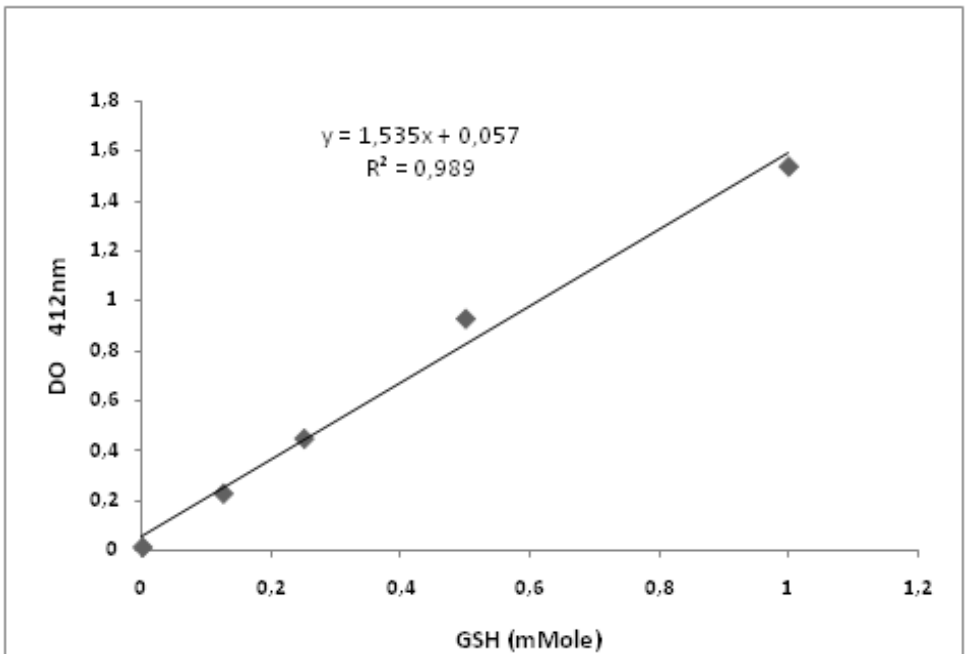
Annex 01: Calibration curve of gallic acid (estimation of polyphenols content).



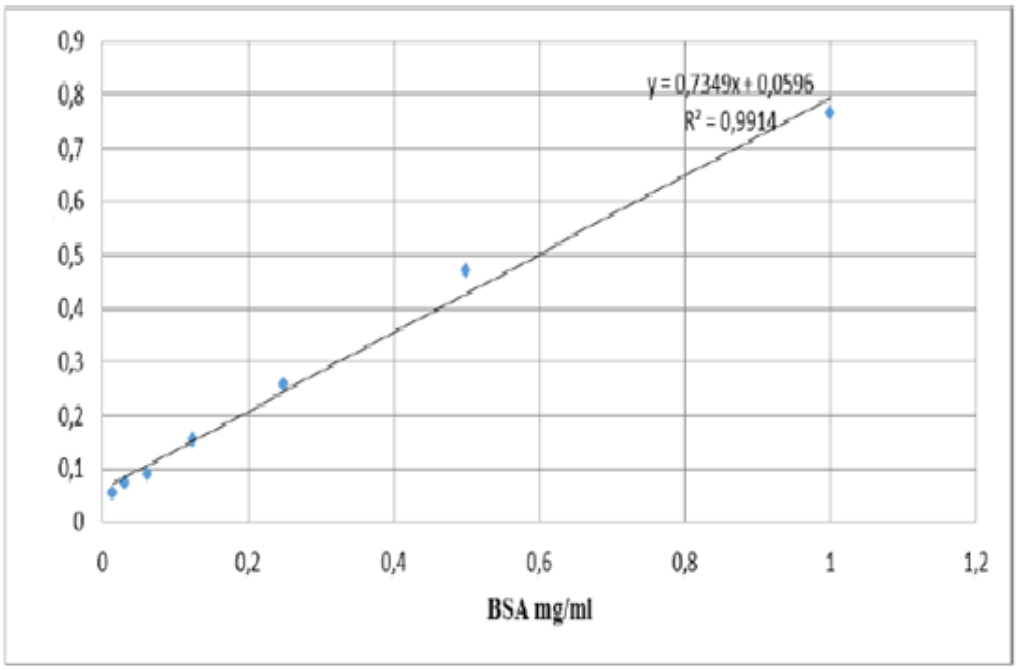
Annex 02: Calibration curve of quercetin (estimation of flavonoids content).



Annex 03: Calibration curve of quercetin (estimation of flavonols content).

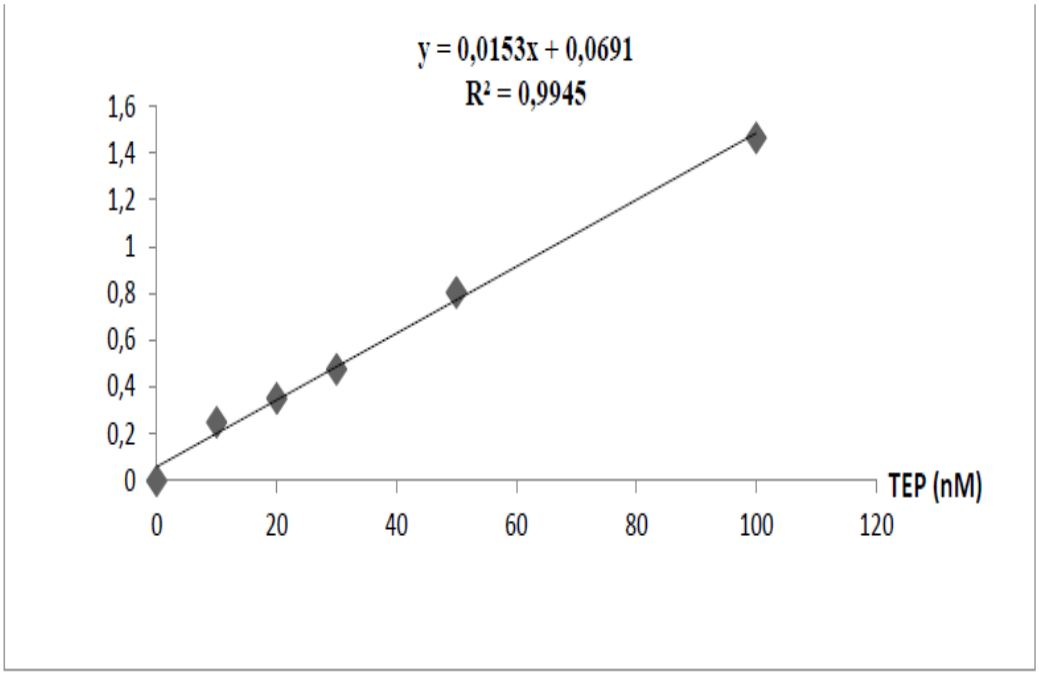


Annex 04: GSH calibration curve.



Annex 05: BSA calibration curve.

A



Annex 06: TEP calibration curve (estimation of MDA).

Presented by:	**Theme:**
Khenchil Amir	The Evaluation of Anti-Inflammatory and Anti-Oxydant Activities of Ethanolic Extract of *Aristolochia Longa* Roots in Mice.

Summary

Aristolochia longa is one of the plants that have been used in Algerian traditional medicine and worldwide. An ethanolic extraction was performed from the roots. Determination of total polyphenols and flavonoids as well as flavonols showed considerable levels of these components in the extract but the DPPH test revealed a weak anti-radical activity. The anti-inflammatory activity was investigated in vitro using red blood cell membrane stabilization method and in vivo using Carrageenan-induced oedemain mice. The plant exhibited a significant anti-inflammatory effect in vivo by reducing the size of the inflamed paw with 80.31 % but showed a low ability in stabilizing the red blood cells membranes.Some parameters of oxidative stress have been measured in the liver of miceand the results showed no effect against lipid peroxidatin caused by ROS but the GSH level was increased,that can be considered as a considerable anti-oxidant activity.These results are encouraging but another wide study is highly recommended for more perfect results.

Key words: *Aristolochia longa;* anti-oxidant; anti-inflammatory; secondary metabolites.

Résumé

Aristolochialonga est l'une des plantes qui ont été utilisées dans la médecine traditionnelle algérienne et dans le monde entier. Une extraction éthanolique a été réalisée à partir des racines. La détermination des polyphénols totaux et des flavonoïdes ainsi que des flavonols ont montré des niveaux considérables de ces composants dans l'extrait mais le test DPPH a révélé une faible activité anti-radicalaire. L'activité anti-inflammatoire a été étudiée in vitro en utilisant la méthode de stabilisation de la membrane des globules rouges et in vivo en utilisant l'œdème induit par la carragénine chez la souris. La plante a présenté un effet anti-inflammatoire significatif in vivo en réduisant la taille de la patte enflammée avec 80.31% mais a montré une faible capacité à stabiliser les membranes des globules rouges. Certains paramètres du stress oxydatif ont été mesurés dans le foie des souris et les résultats n'ont montré aucun effet contre la peroxydatine lipidique causée par les ROS, mais le taux de GSH a augmenté, ce qui peut être considéré comme une activité antioxydante considérable. Ces résultats sont encourageants, mais une autre étude approfondie est fortement recommandée pour obtenir des résultats plus parfaits.

Mots clés :*Aristolochialonga;*anti-oxydant; anti-inflammatoire; métabolites secondaires.

ملخص :

أريسطواكيا لونغا تعتبر من احدى النباتات التي تستعمل في الطب التقليدي في الجزائر وفي العالم اجمع . تم القيام بتحصيل المستخلص الايثانولي للجدور, وتم تقدير كميات متعددات ا لفيونولوالفلافونويدات , وقد أظهر المستخلص الايثانولي قدرة ضعيفة على انقاص المركبات الجدرية في اختبار DPPH .وتم ايضا اختبار قدرة المستخلص على القضاء على الالتهابات واختبار قدرته ايضا على حماية أغشية الخلايا الدموية الحمراء, حيث أظهر قدرة ضعيفة على حمايتها لكن في المقابل أظهر قدرة عالية على اضمار الالتهاب الناتج عن الحقن بالكراجينين بنسبة اضمار تقدر بـ80.31% في قدم الفأر. تم أيضا اختبار قدرة المستخلص الايثانولي ضد الجهد الناتج عن المركبات الجدرية, وقد اظهر قدرة ضعيفة على الحماية ضد تحطيم اللبيدات في الكبد ,وهدا لارتفاع مستويات MD A ، تم أيضا قياس GSH إد أظهر ارتفاعا هدا الارتفاع قد يأخد كقدرة على انقاص الجهد ضد المركبات الجدرية .هده النتائج مشجعة لكن يجب إدراء اختبارات اخرى من اجل نتائج افضل .

الكلمات المفتاحية :اريسطواكيا لونغا ، الإجهاد التأكسدي ، مضاد الالتهاب ، المستقلبات الثانوية .