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The effect of *Vitis vinifera* on inflammatory bowel disease caused by hyperhomocysteinemia

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Dedication

♥ *Thanks to ALLAH who have lightened my path and who granted me the ability to accomplish this work.*

♥ *Special thanks to my parents* ***Fatima*** *and* ***Nacereddine*** *for their help and care the whole my life,*

♥ *Big thanks for all my teachers from primary school to the university*

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♥ *To my univercity* ***MENTOURI*** *my home for five years, to my mice,.., thank you all.*

Your
Meriemma

Dedication

♥ *Al hamd li ALLAH, for giving us a chance to be what we are today..*
♥ *This thesis is dedicated to all the people who never stop believing on me..*
♥ *A special feeling of gratitude to my loving mom « **Abla** » your prayers are ringing in my ears..*
♥ *Daddy « **Layeche** », GOD bless you, i hope that you are proud of your lovely daughter..*
♥ *I also dedicate this thesis to auntie « **Souheila** » and his husband « **Anis** », auntie « **Fadia** » auntie « **Nadjia** » and my cousin « **Maissous** »..*
♥ *For my teachers from primary to the university and my classmates..*
♥ *My sister who shared with me the good & the bad moments, & every thing along 11 years « **Meryoumti** », I love you so much & i can't imagine my life without you..*
♥ *Thank you my little lovely cute « **mice** », i'm sorry for dissecting you, in fact, your sacrifice is benefic for the humanity..*
♥ *Thank you my lovely friend « **Maroua** », for your advices along my work ..*
♥ *Without forgetting « **Rahma** », « **Fouzia** » and « **Ramla** ».*
♥ *I dedicate this work and give special thanks for « **Mr. Dehrib Samir** » who teached me how i make my own happiness, and his wife « **Zeineb** », my friends « **Seifeddine** » & « **Oussama** », i hope for you all the happiness of the world.*

And all who helped me from near or far..

♥ *Who loves you too much: **Sarrouha** ♥*

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Liste of abreviations

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ATP : Adenosine Triphosphate

BSA : Bovine Serum Albumin

CD : Crohn's Disease

CRP : C-Reactive Protein

DTNB : 5,5'-Dithiobis-(2-Nitrobenzoic Acid)

GSE : Grape Seed Extract

GSH : Glutathione

GSH-Px : Glutathione Peroxidase

GSPE : Grape Seed Proanthocyanidin Extract

GT : Gastrointestinal Tract

Hcy : Homocysteine

hHcy : Hyperhomocysteinemia

IBD : Inflammatory Bowel Disease

IgG : Immunoglobuline G

IL-1 : Interleukine-1

IL-6 : Interleukine-6

MCP-1 : Monocyte Chemotactic Protein-1

MS : Methionine Synthase

mtGSH : Mitochendrial Glutathione

MTHFR : Methylene Tetrahydrofolate Reductase

NO : Nitric Oxide

NSAIDs : Non-Steroid Anti-Inflammatory Drugs

ROS : Reactive Oxygen Species

SAH : S-Adenosyl-L-Homocysteine

SAM : S-Adenosyl-Methionine

TBS : Tris-Buffered Saline

tHcy : Total Homocysteine

TNF-α : Tumor Necrosis Factor- α

UC : Ulcerative Colitis

Vv : *Vitis vinifera*

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Introduction

Inflammatory bowel disease (IBD) is a group of inflammatory situations of the colon and small intestine. crohn's disease (CD) and ulcerative colitis (UC) are 2 types of idiopathic IBD that present chronic relapsing and progressive inflammation that may affect the entire gastrointestinal tract and the colonic mucosa, respectively [1]. The clinical presentation of IBD includes bloody diarrhea, and abdominal cramping pain [2].

Hyperhomocysteinemia seems to be a common phenomenon in both patients with ulcerative colitis and crohn's disease. Many factors including deficiencies of cobalamin, folate and pyridoxine, smoking habits, alcohol and coffee intake, some medications and age may predispose subjects to hyperhomocysteinemia.

Homocysteine level in patients with inflammatory bowel disease is mostly normal or slightly elevated. Disease activity does not have an impact on homocysteine level. Folic acid is the most important factor having an influence on homocysteine level in patients with inflammatory bowel disease [3].

Grape (*Vitis vinifera L.*) has a traditional relevance as wellness food and health promoting fruits based on their high content of bioactive polyphenols including flavan-3-ols, hydroxycinnamic acids, flavonols, stilbenes, anthocyanins [3]. It's a species of *Vitis*, native from Mediterranean region [4].

Since ancient times, *Vitis vinifera* leaves have been used in medicine due to various biological activities including hepatoprotective, spasmolytic, hypoglycemic and vasorelaxant effects, as well as, antibacterial, antifungal, anti-inflammatory, antinociceptive, antiviral and particularly antioxidant [5-12].

Grape consumption has been stressed as beneficial for inflammation and cardiovascular pathologies as well as pathophysiological processes of aging [13].

Leaves of the plant have been used to stop bleeding and to treat inflammatory disorders and pain [14].

The aim of this research is to focus on this objectives :

1-Evaluate the effect of L-methionine and *Vitis vinifera* on the weight of mice.

2-examinate the effect of homocysteine on the inflammatory bowel disease, through the measurement of the plasma hs-CRP marker, glutathione reduced and histological sections of segmoid part of the colon.

Bibliographic part

I. Inflammation

I.1. Definition

The word inflammation comes from the Latin *"inflammo"*, meaning *"I set alight, I ignite"* [15]. Inflammation is a process to protect the host against infection and danger signals [16], it's defined as a series of protective and regenerative responses of the body [17], which participates importantly in host defenses against infectious agents and injury [18].

The inflammatory response constitutes an important part of both innate and acquired immunity [19]. It's an integral part of the innate immune mechanism that is triggered in response to a real or perceived threat to tissue homeostasis, with a primary aim of neutralizing infectious agents and initiating repair to damaged tissue [20].

The inflammatory process is characterized by the local activation of inflammatory cells as well as vascular and parenchymal cells [16]. In the 1st century; four cardinal signs of inflammation were identified by the Roman Cornelius Celsus : rubor, tumor cum calore and dolore (redness, swelling, heat and pain), and loss of function [21].

Inflammation comes in two types: chronic inflammation, which can be defined as a dysregulated form of inflammation, and acute inflammation, which can defined as a regulated form [22].

I.2. Types of inflammation

I.2.1. The acute inflammation

The acute inflammation starts rapidly, within minutes after injury, the inflammatory process begins with activation and increased concentration of pharmacologically powerful substances such as a group of proteins known as acute-phase proteins. An important member of the acute-phase proteins is C-reactive protein [19]. Signs and symptoms are only present for a few days, but in some cases may persist for a few weeks. Examples of diseases which can result in acute inflammation include acute bronchitis, acute appendicitis [15].

The Inflammation goes chronic when there is a persistent stimulus [23].

I.2.2. The chronic inflammation

The chronic inflammation is a long-term inflammation, which can last for several months and even years. It can result from failure to eliminate whatever was causing an acute inflammation, an autoimmune response to a self-antigen. The immune system attacks healthy tissue, considering it as harmful pathogens [15].

Examples of diseases which can result in chronic inflammation include: Asthma, Rheumatoid arthritis, Ulcerative colitis and crohn's disease, chronic active hepatitis [15].

I.3 C-reactive protein (CRP)

I.3.1. Definition

C-reactive protein (CRP) is known to most clinicians as a marker of inflammation [24]. It was first discovered in 1930 by Tillet and Francis in the serum of patients with pneumonia, but it was not actually isolated until 1941. The name is derived from the ability of the C-reactive protein to react with C-polysaccharide isolated from pneumococcal cell walls [25].
The serum CRP has (12-18 h) half-life [26], that is independent of any physiological or pathophysiological circumstances or of the concentration of CRP in the serum.
Under normal conditions, the baseline concentration of CRP in the plasma is around 0.8 mg/L and is in part genetically regulated [27].

C-reactive protein (CRP) is one of the most important proteins that is rapidly produced by hepatocytes during an acute-phase response upon stimulation by interleukin-6 (IL-6), Tumor Necrosis Factor- α (TNF-α), and Interleukin-1 (IL-1), originating at the site of inflammation or pathology [24].

I.3.2. Structure

CRP consists of five identical, noncovalently associated 23-kDa protomers arranged symmetrically around a central pore.The term “pentraxins” has been used to describe the family of related proteins with this structure. Each protomer has a recognition face with a phosphocholine binding site consisting of two coordinated calcium ions adjacent to a hydrophobic pocket [28] (Figure 1), it’s molecular formula is $C_{62}H_{93}N_{13}O_{16}$ (Figure 2) [29]

I.3.3. Role

The C-reactive proteine interacts with IgG receptors in man and mouse, eliciting a response from phagocytic cells [30]. The CRP is considered as a marker in inflammatory bowel disease [24].

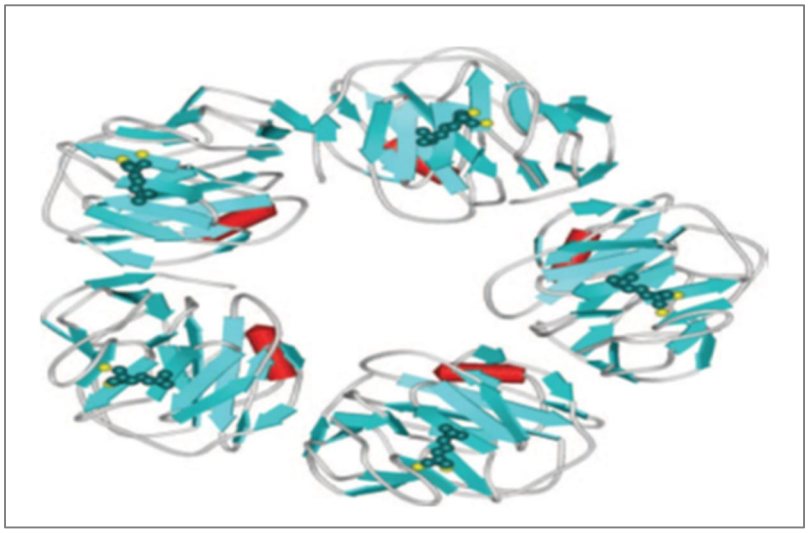

Figure 1: Crystal structure of C-reactive protein complexed with phosphocholine. The calcium ions are yellow, and phosphocholine is green [31].

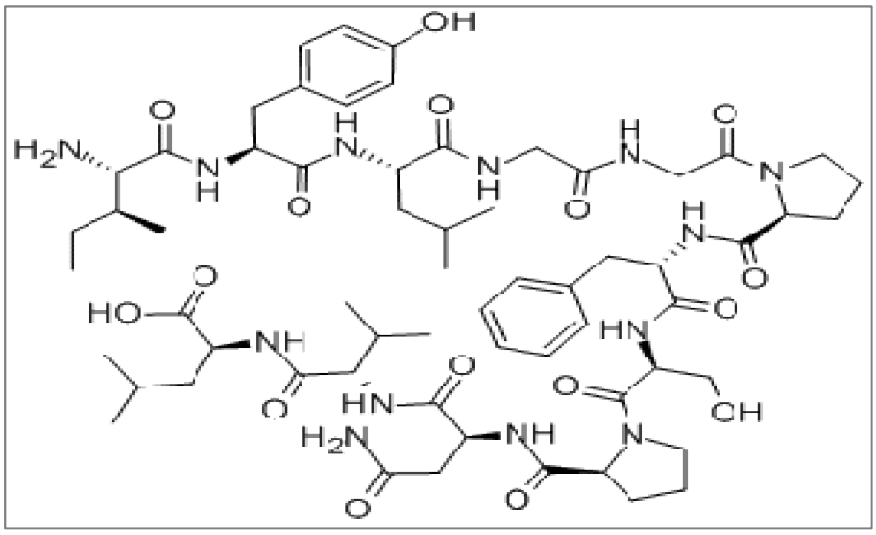

Figure 2: Molecular formula of C-reactive protein [29]

C-reactive protein in patients with quiescent ulcerative colitis (UC) was not different from that of non-inflammatory bowel disease patients (non-IBD patients), suggesting that CRP is a marker especially to differentiate active IBD from functional bowel disorders [24].

I. Inflammatory bowel disease (IBD)

II.1. Definition

Inflammatory bowel disease (IBD) comprises a variety of chronic disorders affecting the gastrointestinal tract [32]. It's a group of inflammatory situations of the colon and small intestine [33]. Inflammatory bowel disease may affect male and female adults and children equally, and it has a peak age at onset between 15 and 25 years of age, a lesser peak age at onset exists between 55 and 65 years of age [34]. The symptoms include -but are not limited to- abdominal pain, tiredness, diarrhea, vomiting, and cramping [35]. Inflammatory bowel disease are usually classified under two major relapsing conditions, crohn's disease (CD) and ulcerative colitis (UC) [36]. Usually distal ileum and colon can be affected by crohn's disease, whereas ulcerative colitis affects only the colon [37] (Figure 3).

II.2. Colon's anatomy

The colon is a part of the gastrointestinal tract, it absorbs water and salt from the food and serves as a storage place for waste matter. It has 4 sections: the first section is called **the ascending colon**. It starts with a small pouch (the cecum) where the small bowel attaches to the colon and extends upward on the right side of the abdomen. The second section is called **the transverse colon**, since it goes across the body from the right to the left side in the upper abdomen. The third section, called **the descending colon**, continues downward on the left side. The fourth and last section is known **as the sigmoid colon**, because of its "S" or "sigmoid" shape. The waste matter that is left after going through the colon is called feces or stool. It goes into the rectum, the final 6 inches of the digestive system, where it is stored until it passes out of the body through the anus [38] (Figure 4).

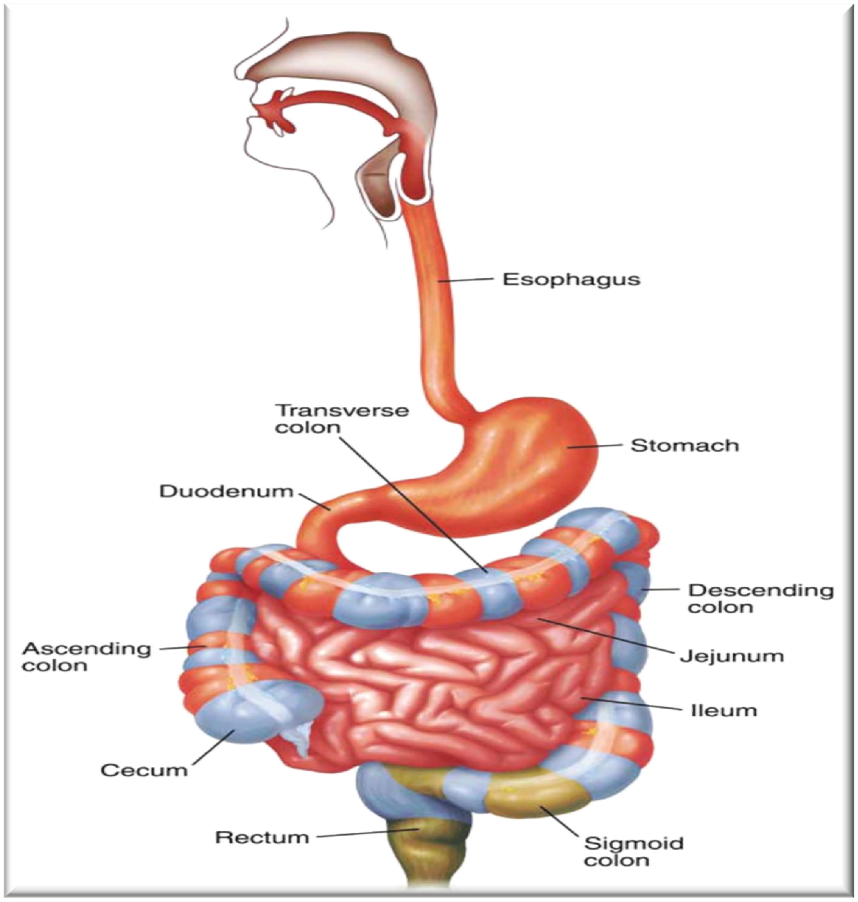

Figure 3: Anatomical regions affected by IBD [34]

UC (green) is characterized by mucosal inflammation of the rectum and colon. CD (red) has transmural inflammation of any gastrointestinal tract segment except the rectum. The areas shaded in blue and red indicate locations where both UC and CD may be encountered. The areas shaded in blue and green indicate locations where UC predominates and CD is less frequent.

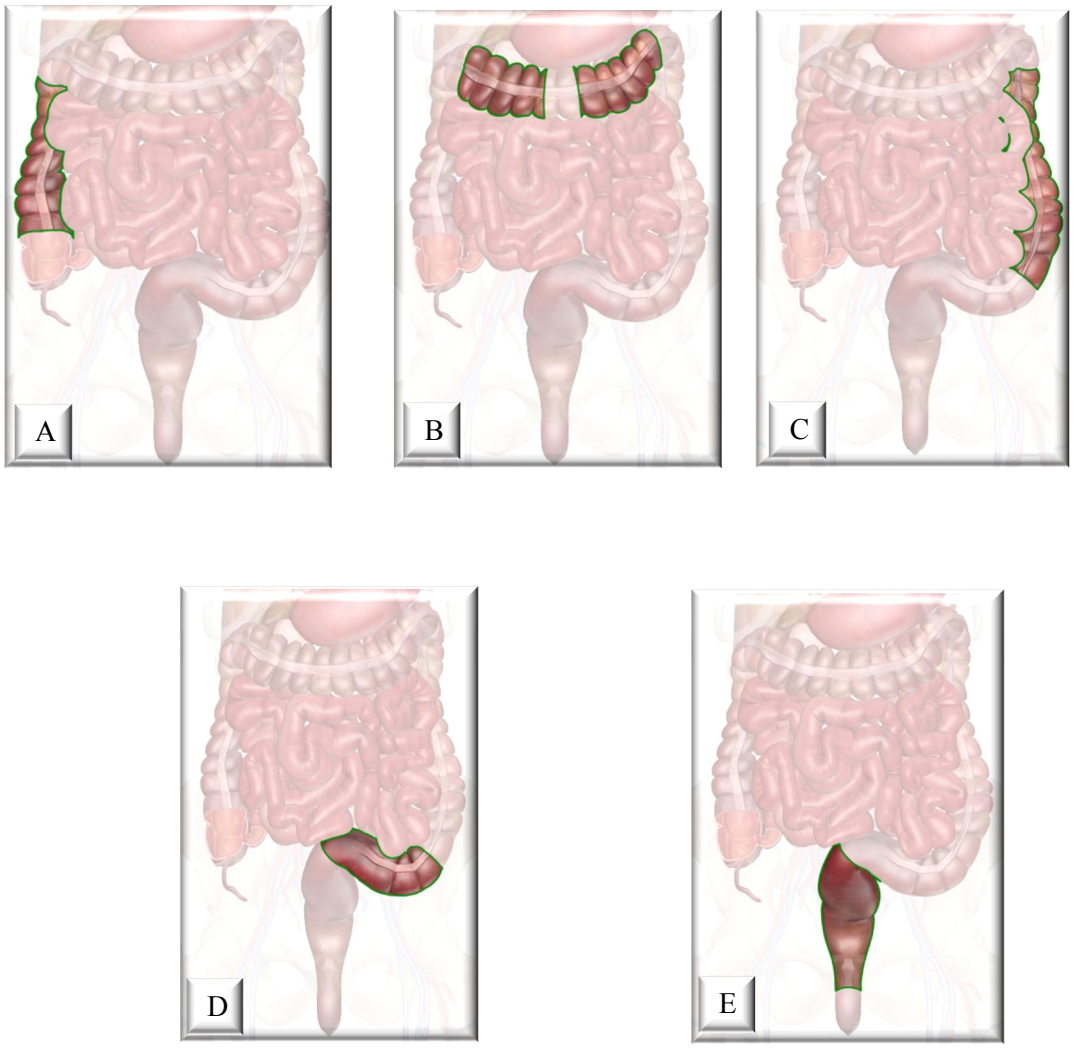

Figure 4 : colon's anatomy [46]

A: ascending colon, **B**: Transverse colon, **C**: descending colon, **D**: Sigmoid colon, **E**: Rectum

II.3. The colonic mucosa

The colonic mucosa of the lower gastrointestinal tract carries out several important functions in the human organism: digestion and absorption of nutrients, uptake of water and electrolytes, elimination of digestive waste products, and the protection of the body against toxic or carcinogenic compounds ingested with food. For the latter purpose, a complex defense system is present. The epithelial cells are protected by a barrier of mucus [39], it have a relatively short life-time [40], and a complex system of enzymes that are able to metabolize harmful compounds in a way ultimately leading to the excretion of innocuous metabolites via bile, feces, or urine [41].

II.4. Types of inflammatory bowel disease

II.4.1. Crohn's disease (CD)

Crohn's disease (CD) is a major chronic inflammatory bowel disease in humans [42]. It's characterised by granulomatous inflammation, which may affect any site along the gastrointestinal tract from the mouth to the anus. The physician Giovanni Battista Morgagni, made the first description of crohn's disease in 1769 when he diagnosed a man with chronic diarrhea. In 1930, Dr. Burrill Crohn and his colleagues described the features of this disease to the American Medical Association. The CD was named after Burrill Crohn and it became an official medical entity in 1932 [43]. Patients with crohn's disease are at risk for early small bowel and colorectal cancer. The risk is even higher with an uncontrolled inflammation [44].

II.4.2. Ulcerative Colitis (UC):

Ulcerative colitis (UC) is a relapsing and remitting disease characterized by acute non infectious inflammation of the colorectal mucosa, extends proximally from the anal margin. The rectal mucosa is invariably affected. Virtually all patients with UC have rectal bleeding or bloody diarrhea and other colonic symptoms. These symptoms may persist for days, weeks, or months [45].

II.5. Causes of inflammatory bowel disease

It does not have a single cause, however, some IBD has been linked to heredity. It can also be caused by problems with the immune system.

- **Heredity:** Scientists think IBD may have a genetic component.
- **The Immune System:** The immune system also plays a role in IBD. Normally, the immune

system defends the body from pathogens. A bacterial or viral infection of the digestive tract can trigger an immune response. The digestive tract becomes inflamed as the body tries to fight off the invaders [47].

III. Homocysteine

III.1. Definition

Homocysteine is not a classic amino acid (Figure 5), it's found in dietary protein or used for the endogenous synthesis of proteins, but rather a sulfur-containing amino acid derived from the metabolism of methionine via methyl group metabolism [48].

III.2. Homocysteine metabolism

The homocysteine is produced from S-adenosyl-L-homocysteine (SAH) by S-adenosyl-homocysteine hydrolase. SAH is a transmethylation product from S-adenosyl-methionine (SAM) that is made from methionine and ATP. In the metabolic cycle, homocysteine is either remethylated to methionine through methionine synthase or degraded to cysteine through cystathionine beta-synthase. Intracellular homocysteine is also released into blood and urine. Vitamin B12, folate, and B6 are needed in both homocysteine remethylation and transsulfuration pathway [49] (Figure 6).

III.3. Sources of homocysteine

Homocysteine is derived primarily from the methionine in dietary proteins, foods contain only trace amount of homocysteine, which reflects the notion that homocysteine albeit an essential intracellular metabolite, is maintained at low concentration in both animal and plant cells (Table 1) [50].

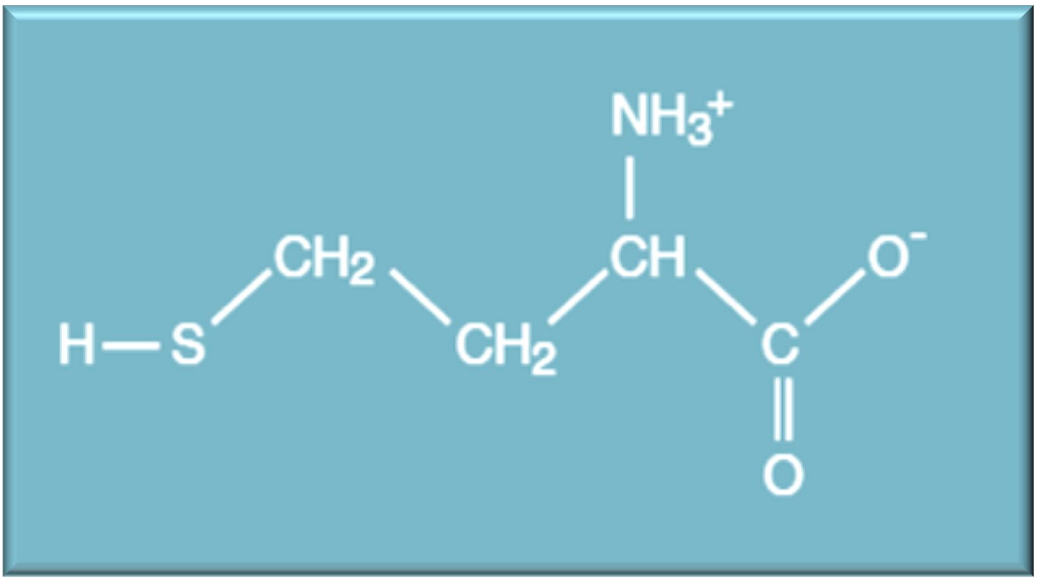

Figure 5: Molecular formula of homocysteine [29]

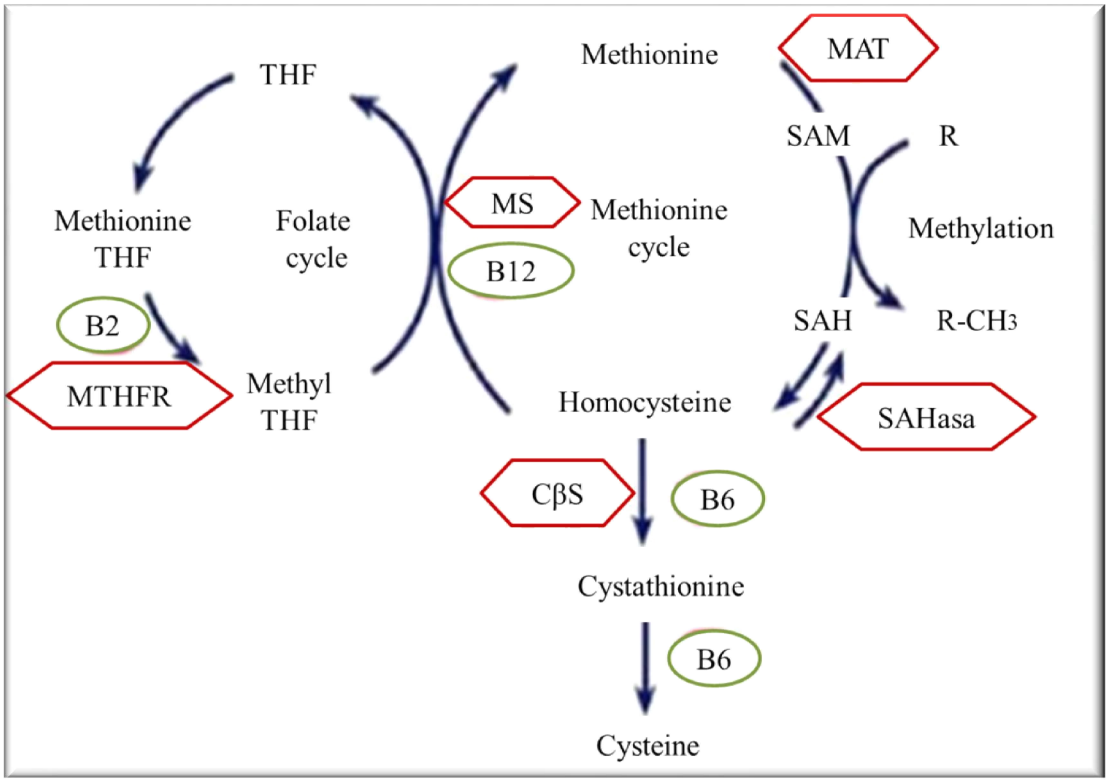

Figure 6 : Methionine cycle [49]

Table 1 : The methionine content of food protein [50]

Source		Methionine (g per 100 g of protein)	Exceptions
Plant	Fruits	0.9	Peaches/Grapes 3,6 g
	Vegetables	1.2	
	Nuts	1.4	Brazil nuts : 5,6 g
	Cereals	1.8	
Animal	Meat & fish	2.7	
	Cow's milk	2.9	Human milk 1,4g
	Eggs	3.2	

IV. Hyperhomocysteinemia

IV.1. Definition

Elevated plasma homocysteine (hcy) levels, also known as hyperhomocysteinemia (hhcy) [51], it occurs when Homocysteine metabolism is disturbed [52]. The causes of hyperhomocysteinemia range from aging and vitamin deficiency to genetic defects [53]. In addition, there are numerous external factors that affect Hcy levels [54] such as diet, genetic factors, certain drugs, and renal function [55]. Hcy levels increase with age and are higher in post-menopausal women [56].

IV.2. Types of hyperhomocysteinemia

IV.2.1. Severe hyperhomocysteinemia (plasma tHcy > 100 μmol/L) is classically caused by rare genetic defects in the metabolism of methionine, folate, or vitamin B12, but it also can occur in individuals with severe vitamin B12 deficiency due to pernicious anemia [55].

IV.2.2. Moderate hyperhomocysteinemia (plasma tHcy of 10 to 100 μmol/L) can be caused by renal disease, nutritional deficiencies of folate or vitamin B12, or a common genetic variant in the methylene tetrahydrofolate reductase (MTHFR) gene. Moderate hyperhomocysteinemia is highly prevalent in most populations [55].

IV.3. Causes of Hyperhomocysteinemia

B vitamin deficiency : B6, B12, and folic acid are needed for the enzymes involved in homocysteine metabolism [58].

Enzyme deficiency Genetic defects in genes encoding for enzymes such as methylene tetrahydrofolate reductase (MTHFR) and methionine synthase (MS) which are involved in the homocysteine metabolic pathways [58].

Renal dysfunction: Renal failure patients have extremely high Hcy levels due to less efficient renal clearance of Hcy [58].

Drug interaction: Certain drugs such as Methotrexate and Cyclosporine A cause elevation of Hcy [58].

Age: Hcy levels increase with age [58].

Sex: The average Hcy levels in men are higher than those of women [58].

Life style: Stress, physical inactivity, smoking, and coffee drinking cause elevation of Hcy [58].

Health Status: Hcy levels are low in healthy subjects but are elevated in subjects with suboptimal health conditions [30]. It seems to be a common phenomen on in both patients with ulcerative colitis and crohn's disease [58].

IV.4. The risk of hyperhomocysteinemia

It has been reported that, in patients with inflammatory bowel disease (IBD), the Hcy levels in plasma and colonic mucosa are increased [59].

The risk of hyperhomocysteinemia is significantly higher in IBD patients when compared with healthy controls [60], (Table 2).

Hyperhomocysteinemia is a well-known risk factor for venous thromboembolism in the general population, and it is thought that it may increase hypercoagulability in CD patients due to the frequent malabsorption of vitamin B12 and folic acid observed in patients with ileal involvement. However, there is still disagreement regarding the magnitude of the association between hyperhomocysteinemia and CD [61].

In human, vitamins therapy is traditionally considered to normalize increased plasma homocysteine concentrations originating from metabolic defects in the enzymatic control of hcy metabolism as well as low levels of involved cofactors (folate, B12, B6) [62].

Elevated Hcy levels may lead to endothelial damage through intensification of intravascular oxidative stress and a decrease in bioavailability of nitric oxide (NO) and in consequence to impairment of endothelium-dependent vascular relaxation [63,64]**.**

That's why is considered as one of factors contributing to a state of hypercoagulability in IBD resulting from endothelial dysfunction [65]

Hcy can increase oxidative stress possibly through its auto-oxidation and thiolactone formation, and inhibition of glutathione peroxidase (GSH-Px) and other antioxidant enzymes [66]. Although the relationship between Hcy and oxidative stress is affected by the glutathione level, whether the detected antioxidant capacity of HCY remains to be elucidated [67].

Table 2 : Serum homocysteine in patients with IBD and healthy controls [71]

	Ulcerative colitis (N=53)	Crohn's disease (N=56)	Healthy controls (N=74)
tHcy (mmol/l)	15.87	13.62	9.59

V. Oxydative stress :

V.1. Definition

Oxidative stress is the presence of reactive oxygen species in excess of the buffering capacity of available antioxidants [68]. It has been implicated in atherosclerosis, cancers, pre-eclampsia, and many other human diseases [69]. It could be a major contributing factor to the tissue injury that characterize inflammatory bowel disease (IBD). Homocysteine (Hcy) could cause oxidative damage to the colon tissue in ulcerative colitis (UC) patients [70].

V.2. Antioxydant marker

V.2.1. Glutathione (GSH)

Glutathione (GSH) is a tripeptide containing glutamic acid, cysteine, and glycine (Figure 7). It has many important functions, such as maintaining the reduced state of proteins and protecting the cells against reactive oxygen species (ROS), drugs or heavy metal ions. Glutathione and its related enzymes are essential for these enzymatic defense systems in the colonic mucosa [39].

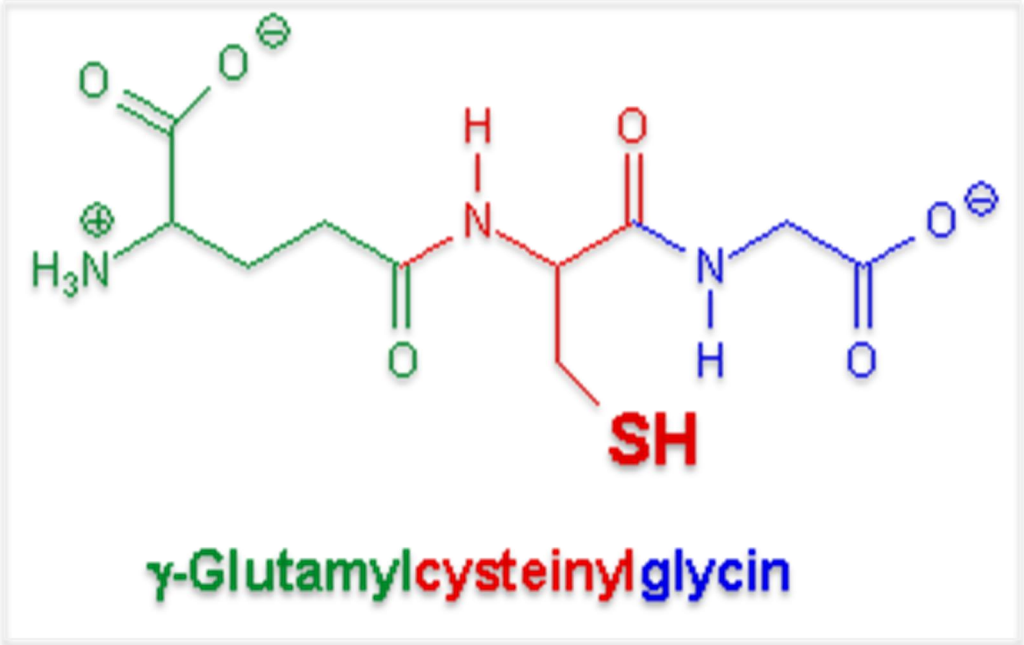

Figure 7 : Structure of Glutathione [72]

VI. Inflammatory bowel disease treatment:

The inflammation is the most important feature of diseases and need to be treated with a therapeutic agent such as glucocorticoids and immunosuppressive drugs which have a new anti-inflammatory agent with low levels of adverse effects [73].

VIII. ***Vitis vinifera*** **L**

VII.1. Definition

Vitis vinifera L. (Vitaceae), Turkish name, "Asma", is a perennial woody vine, usually climbing by tendrils, native to Asia Minor and then introduced in Europe and other continents (Photo 01) [74]. The medicinal and nutritional value of grapes (*Vitis vinifera*) has been indicated for thousands of years. Egyptians consumed this fruit at least 6,000 years ago and several ancient greek philosophers praised the healing power of grapes [75].

VI..2. Classification of *Vitis Vinifera* [76]

Domain- Eukaryotic

Kingdom- Plantae

Phylum- Angiospermae

Class- Magnoliopsida

Order- Rhamnales

Family- Vitaceae

Genus- *Vitis*

Species- *Vitis vinifera*

Photo 01 : *Vitis vinifera*

VII.3. The composition of *Vitis vinifera*

Vitis vinifera contains many chemical constituents such as phenolic acids, Vitamin E, procyanidins (linoleic acid) [77], flavonoids; including anthocyanins and proanthocyanidins, sugars, sterols, amino acids and minerals [78].

VII.4. The pharmaceutical effect of *Vitis vinifera*

Grape is known as the "queen of fruits" because of cleansing properties. A "grape cure" or grape fast involves eating 3–6 pounds of grapes to detoxify and improve liver function [79]. Additionally, it has been reported that grape has important role in controlling of some liver diseases, high blood pressure and anemia. Also fibers and fruit acids in grape have vital role in cleaning blood functions of digestive system and kidney [80].

In Iran, grape leaves are used for treatment of diarrhea and bleeding, vomiting and varicose treatment [11], hepatitis and stomachaches [81], also it has been used in Ayurvedic (Indian) and traditional medicine as a diuretic, to soothe the digestive tract, improve circulation, control swelling and bleeding, and detoxify the body [79].

European folk healers developed a balm from the sap of grape vines to cure skin and eye diseases. Among other beneficial effects, the active compounds in grape seed are believed to have antioxidant properties [75].

Long before the ancient, *Vitis vinifera* constituted the most important element of botanical landscape of Algeria, since 1925 [82].

Grape (*Vitis vinifera L.*) has a traditional relevance as wellness food and health-promoting fruits based on their high content of bioactive phenols. In this regard, grape consumption has been stressed as beneficial for inflammation and cardiovascular pathologies as well as pathophysiological processes of aging [83].

VII.5. The anti-inflammatory effect of *Vitis vinifera*

Several natural compounds have proven to have anti-inflammatory activities; among the most important are polyphenolic compounds [78], which may help to inhibit enzyme systems that are responsible for the production of free radicals and that are associated with inflammatory reactions. Procyanidins intervene in the synthesis and release of many substances that promote inflammation for example, histamine, serine protease, prostaglandins and leukotrienes [84], and the metabolic extract of *Vitis vinifera* has an analgesic activity [85].

In other studies, it was indicated that grape seed proanthocyanidin extract (GSPE) reduces the expression of IL-6 and MCP-1(monocyte chemotactic protein-1) and enhances the production of the anti-inflammatory adipokine adiponectin suggest that GSPE may have a beneficial effect on low-grade inflammatory diseases [86].

GSPE has various biological functions such as antibacterial, antiviral, anti-inflamatory, antiallergic and vasodilatory actions and it's the most effective anti-inflamatory compound in grape seed extract [87]. We suggest the use of GSE as suplemental drug in patients with acute or chronic inflamatory diseases [88].

Material and methods

Material and methods

1. Materials

1.1 .Animals

The investigations were performed on male young mice (*Mus Musculus*), (1-2 months) weighing between (22-28g). All animals were birth in animal house in the pharmacy institute of Constantine, and housed in cages with free access to water and diet every day at room temperature (25°c). Composition of diet is shown at table 3.

1.2 blood samples

After each experiment, animals were fasted overnight and the blood was obtained from the sinus venipuncture and collected into heparin tubes.

1.3 light microscope samples :

The animals were sacrified and samples for light microscopy were obtained from segmoid colon, and liver for the dosage of glutathione.

1.4 Chemicals

L-Methionine purity 98% was obtained from acros organics (belgium), Chloroforme, Formol 10%, Ethanol (different concentrations 60%-70%-96%), Butanol, Paraffin, Xylene, Distilled water, NaCl 0,9%, Picric acid, Acetic acid, hematoxylin eosin, bovin serum albumin, Tris, EDTA, dithio-bis-nitrobenzoic acid (DTNB), sulfosalicylic acid, Coomassie Brilliant Blue (G250), orthophosphoric acid (85%), NaOH.

1.5 Equipements

Balance (0,001), precision balance (0,0001) (OHAUS®), agitator, light microscope, incubator (labtech), hote (chemcap™ filter), microtome, dissection kit, centrifuge, spectrophotometer, heparinized tubes, microscope system with camera, pH meter, magnetic stirrer with hotplate, homogenizer and spectrophotometer,

Table 3 : Composition of diet taken by mice during 40 days of treatement (ONAB)

Food material	Amount in g/kg diet	Pourcentage %
Corn	620	62
Soy	260	26
Phosphate	16	1,6
Limestone	9	0,9
Cellulose	10	1
Minerals	10	1
Vitamins	10	1

2. Methods

2.1 . Biochemical analysis :

- **Animal's treatment**

This study was performed on 28 males mice in four experimental groups.

First group (F) was administered with white bread (0.08 mg/mouse/day), the second group (M) was administered with L-Methionine (500mg/kg/day). The third group (PM) was treated with the extract of *Vitis vinifera* (200 mg/kg/day) and administered with L-Methionine (500 mg/kg/day). The fourth group (P) was treated with the extract of *Vitis vinifera* (200 mg/kg/day) (Table 4).

The dose of Methionine and *Vitis vinifera* were calculated relatively to body weight, and given with white bread, incorporated into the flour and administered in the form of balls. The weight of mice was taken every day at the same time. After 40 days, the animals were fasted overnight, and the blood was removed for biochemical analysis.

2.1a. hs-CRP determination

- **Objective of hs-CRP determination**

The hs-CRP measurement is a useful tool for evaluating possible infective or inflammatory disease [51].

The plasma hs-CRP values were measured by immunoturbidimetric method on auto analyzer Biochimie mindray 300, the analysis was performed in the medical laboratory KENZI Lekhroub (Constantine).

Table 4 : treatement of mice

Experimental group	Substance administered	Number of animals	Duration of the experiment	Daily dose
F	Flour	7	40	0,6 g/7 mice
M	Flour + methionine	7	40	500 mg/Kg
M-P	Flour+methionine+plant	7	40	500 mg/Kg + 200 mg/Kg
P	Flour + plant	7	40	200 mg/Kg

2.1b. Dosage of glutathione reduced

o Preparation of the homogenate

First, the weight of 0,5g of the liver was homogenized in 2 ml of TBS buffer solution, then the homoginates were centrifuged at 9000 tours/min for 15 min at 4°C, after that the supernatent was used for determination of glutathione reduced (GSH).

o Glutathione assay principle

The glutathione reduced content in the liver was measured spectrophotometrically by using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) as a coloring agent, following the method of [105].

The concentration of the GSH are measured by the method of Weckbecker and Cory as shwon below, the spectrophotometric reader assay method for GSH involves oxidation of GSH by the sulfhydryl reagent 5,5'-dithio-bis2-nitrobenzoic acid (DTNB) to form the yellow derivative 5'-thio-2-nitrobenzoic acid (TNB), measurable at 412 nm.

Liver homogenate sample 0,8 ml was deproteinized with 0,2 ml 5-sulfosalicylic acid solution (0,25%) and was allowed stane on ice for 10 min. Following centrifugation at 1000 tours/mn, during 5 min to remove the pricipitated protein. 0,5 ml supernatant was mixed with 1ml Tris/EDTA buffer (pH= 9,6), and 0,025 ml DTNB-reagent (0,01 M 5,5'-dithio-bis2-nitrobenzoic acid) and left at room temperature for 5 min. Then the absorbtion at 412 nm was measured on spectrophotometer by comparing to the blank reaction.

Glutathione concentration was obtained by direct calculation the following formulae :

$$\text{GSH (nmol/mg of protein)} = \frac{OD \times 1 \times 1{,}525}{13100 \times 0{,}8 \times 0{,}5 \times mg\ P}$$

OD : optical density

1 : total volume of sulutions in the deproteinisation (0,8 ml homogenate + 0,2 ml 5-sulfosalicylic acid)

1,525 : total volume of the solutions used in the assay of GSH (0,5 ml supernatant + 1 ml Tris/EDTA + 0,025 ml DTNB)

13100 : absorbance coefficient at groupement –SH to 412 nm

0,8 : volume of homogenate sample.

0,5 : volume of supernatant.

2.2. Histological analysis

- **Dissection**

After the blood samples collection, the animals were sacrified and samples for light microscopic investigations were obtained. For histological investigations, only the segmoid colon was used.

- **Preparation of histological sections**

First, the sigmoid colon was fixed in the formol 10% for 1 month, and alcohol bouin for 20 minutes, then it was carried in different concentrations of ethanol (60%, 70%, 96%) for 4h30 minutes, and 3 days in butanol for deshydratation, and followed by inclusion into paraffin, and finally, the paraffin cubes were cuted using the microtome at 5µm, and colored with hematoxylin eosin. The slices were observed by a photomicroscope connected to computer.

2.3. Statistical analysis :

Results were analysed for differences between the groups across dietary treatments by one-way ANOVA test and tukey's multiple comparison tests (SPSS version 20).

Results and discussion

1. Body weight

- **Control group (group F)**

The weight of mice group (F) during the first 10 days was (24,91 g ± 0,95), (25,75 g ± 1,65) in the second 10 days, (23,47 g ± 2,37) in the third 10 days, and (27,00 g ± 1,18) in the fourth 10 days.

- **Methionine group (group M)**

The weight of mice group (M) during the first 10 days was (27,05g ± 0,59), (26,06g ± 0,23) in the second 10 days, (25,91g ± 0,68) in the third 10 days and (29,93g ± 0,77) in the fourth 10 days.

The results showed that the weight of mice increased in groups F and M (Figure 8). Our result is in agreement with that of [100], reported an increase in weight of rats treated with 200 mg/kg of L-methionine for 21 days. Also, the study of [101] and [102] showed an increase in the weight of mice treated with 400 mg/kg of L-methionine for 21 days. However, the work of [89] indicated a significant decrease in the weight of mice treated with 200 mg/kg of L-methionine for 21 days. Given the contradictory results, we can not conclude that there is a relationship between hyperhomocysteinemia and the weight of mice.

- **Plant and L-Methionine group (group P/M)**

The weight of mice group (P/M) during the first 10 days was (25,65 g ± 0,65), (22,95 g ± 0,35) in the second 10 days, (26,13 g ± 0,69) in the third 10 days and (32, 7 g ± 0,88) in the fourth 10 days.

- **Plant group (group P)**

The weight of mice group (P) during the first 10 days was (28,3 g ± 0,65), (29,61 g ± 0,64) in the second 10 days, (32,70 g ± 0,71) in the third 10 days and (25,02 g ± 0,54) in the fourth 10 days.

The results showed an increase in weight for the group PM between the first 10 days and the fourth 10 days (Figure 8). Our results are in agreement with those of [101], [102] and [89] who indicated an increase in weight of mice treated with L-methionine and olive oil,

extract of medicinal plants *Stachys mialhesi* (50 mg/kg), and (500mg/kg) during 21 days respectively and in the group treated only with the medicinal plants . However in the group P the weight of mice is decreased between the first 10 days and the fourth 10 days.Ours result is not agrees with those of [89], [101] and [102] who indicated an increase in weight of mice treated with the extract of medicinal plants.but our result it is in agreement with those of [103] who reported that the weight of mice treated with olive oil for 21 days decreased but not significantly.

This results indicate that the duration of time used in this experiment and the extract of *Vitis vinifera* decreased the weight of animals.

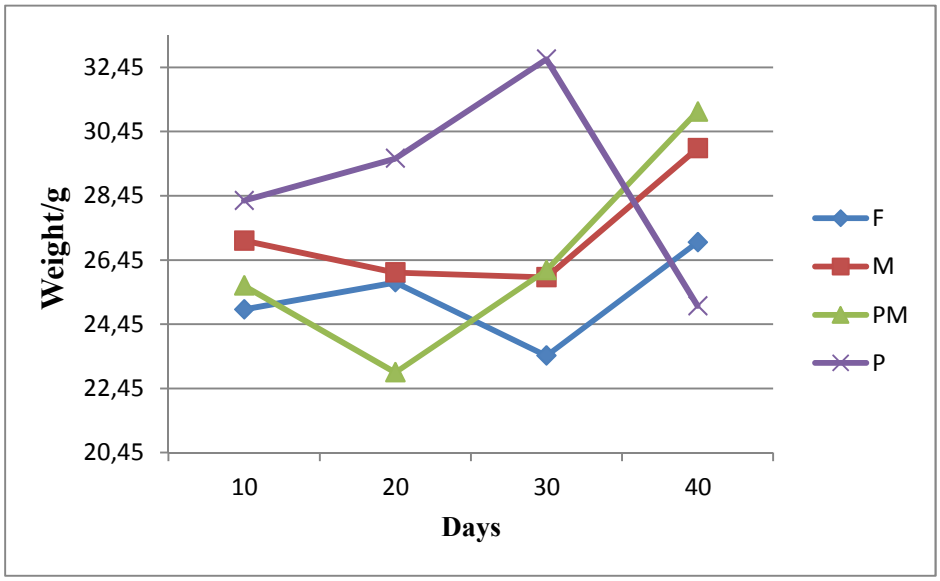

Figure 8: The effect of L-methionine and *Vitis vinifera* intake on mice weight during 40 day

2. Biochemical results

2.1. hs-CRP measurement

The present data show that there isn't any significant difference in the means for the plasma hs-CRP concentration between groups (P > 0,05), the figure 9 showed that plasma hs-CRP concentration in group M (2,7 ± 1,5 mg/L) decreased but not significantly when compared to the control group (3,16 ± 1,61 mg/l) and the groups PM and P (8,3 ± 7,64 mg/L) and (5,03 ± 1,12 mg/L) respectively.

However the group PM revealed a higher plasma hs-CRP level than the control group. This difference is performed by the fact that *Vitis vinifera* compounds could posses anti and proinflammatory properties.

In our results we obtained that the treatement with the extract of *Vitis vinifera* for long time could induce an inflammation, this results is confirmed by [91] who reported that *Vitis vinifera* has aphrodiasiac compound (Photo 2). The study of [92] confirmed that there isn't any toxicity or mortality at the concentration of 2 g/kg. Our result is not agrees with the work done by [104] who obtained that the concentration of hs-CRP decreased in mice treated with *Vitis vinifera* (500mg/kg) for 12 days.

Also our finding is disagree with the study of [93], who reported that the long term consumption of *Vitis vinifera* leaves might be more beneficial for people who suffer from chronic diseases especially diabetes, kidney failure, liver and heart diseases [93].

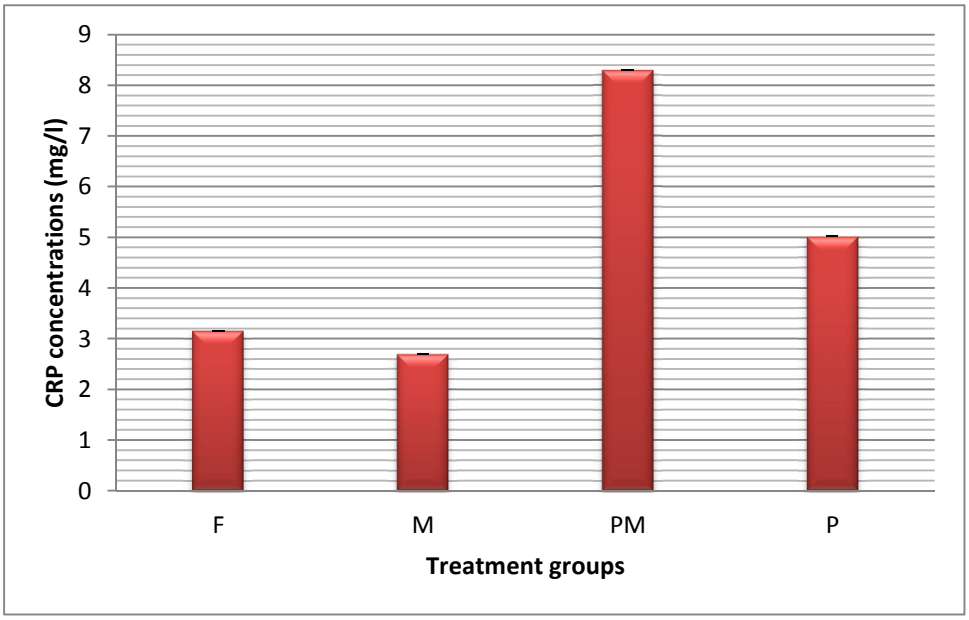

Figure 9: The concentrations of CRP in mice administered with methionine (500 mg/Kg) and treated with *Vitis vinifera* (200mg/Kg).

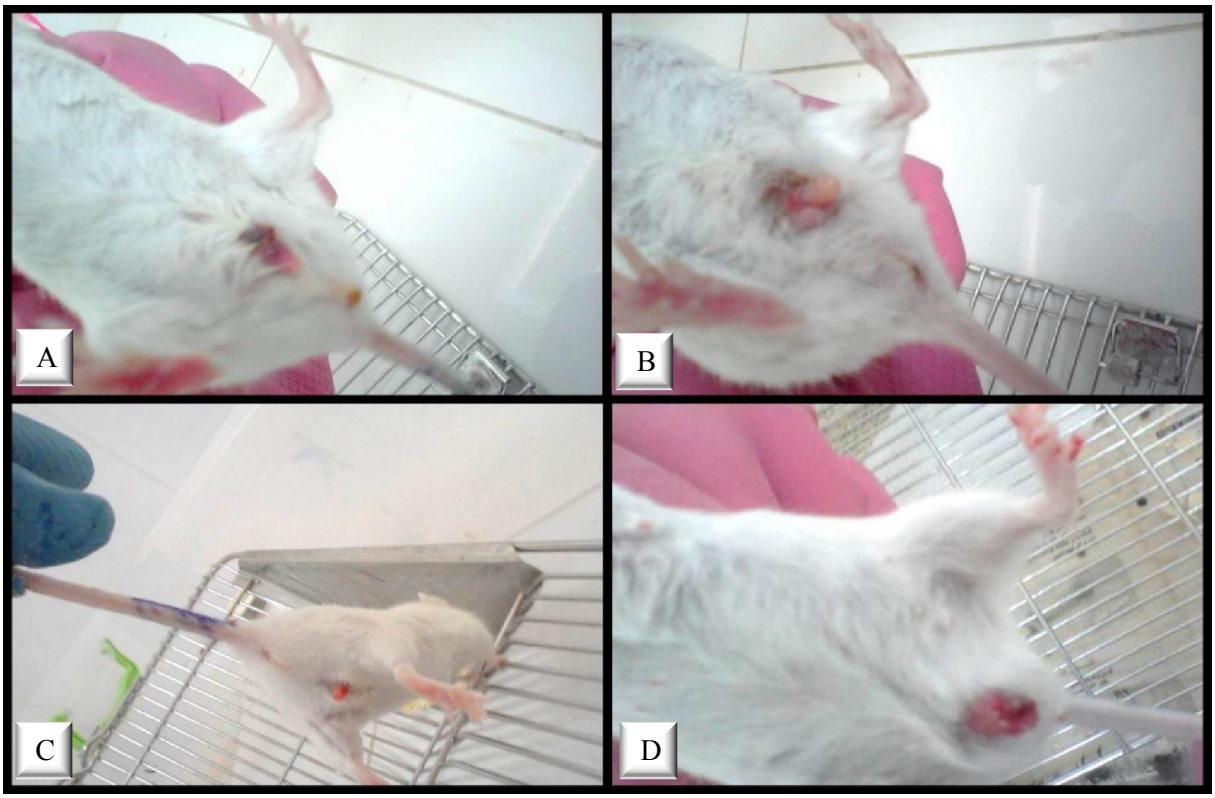

Photo 02: inflammation caused by *Vitis vinifera* on the sex organ

A, B : Group (P/M), **C, D :** Group (P

2.2. Glutathione assay

The concentration of glutathione reduced in groups F, M, PM and P were (50 ± 0,04 nmol/mg protein), (40 ± 0,01 nmol/mg protein), (50 ± 0,02 nmol/mg protein), (50 ± 0,01 nmol/mg protein) respectively.

Figure 10 showed that the concentration of GSH in the liver decreased in the group M when it is compared to the other groups, but not significantly ($p>0,05$), and the concentration of GSH in the group PM and P were reached the same level of concentration of the group F .

This results is not agrees with the study of [94] and [95] who reported that the extract of *Vitis vinifera* reduced the glutathione levels, but is in agreement with other study of [96] who reported that a significant elevation of GSH level in rats treated with *Vitis vinifera* extracts. Which indicates that the extract can either increase the biosynthesis of GSH or reduce the oxidative stress leading to less depletion of GSH, or have both effects [96].

Many studies showed that the hyperhomocysteinemia induce the stress oxydant in different cells, in particular the endothelial cells [97], by inhibition of the antioxydant enzymes, such as the glutathione peroxidase [98].

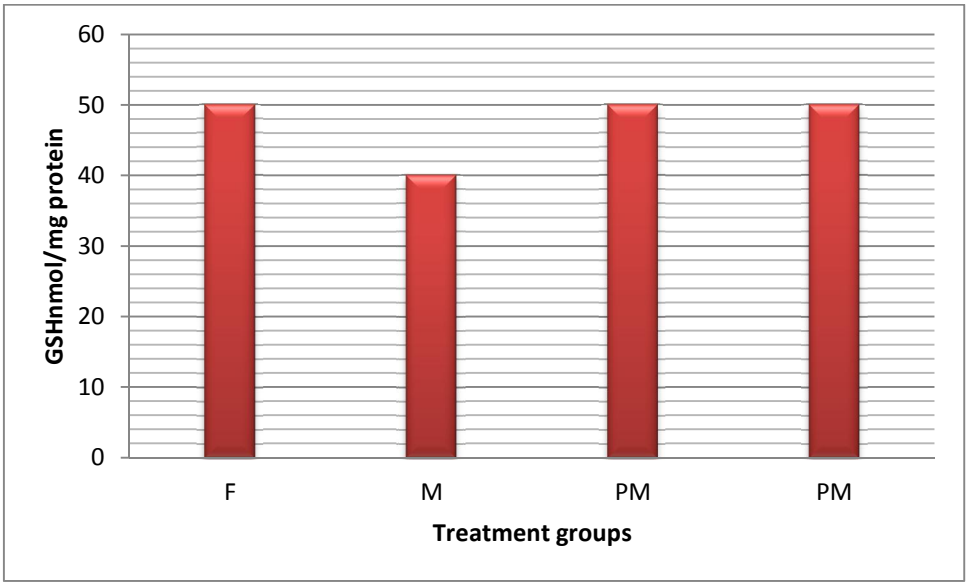

Figure 10: The concentrations of GSH in mice administered with methionine (500 mg/Kg) and treated with *Vitis vinifera* (200mg/Kg).

3. Histological studies

In the group M, which had been fed with 500 mg/kg L-methionine, the segmoid colon showed degeneration, membrane destruction, necrosis and goblet cell dilatation of the crypt (Figure 15 and 16).

Our result is agreed with the study of [104] who observed necrosis into the sigmoid colon in mice treated with 400mg/kg of L-methionine during 12 days.

However the segmoid colon of the group PM that had been fed with 500 mg/kg of L-methionine, and 200 mg/kg of *Vitis vinifera* extract, showed intact crypt with intact goblet cell (Figure 18 and 19). And the sigmoid colon in the group of mice treated with only *Vitis vinifera* was intact (Figure 21 and 22).

The [99] reported that depletion of mtGSH or ATP into very low levels may cause a bioenergetic catastrophe changing the mode cell deth from apoptosis to necrosis.

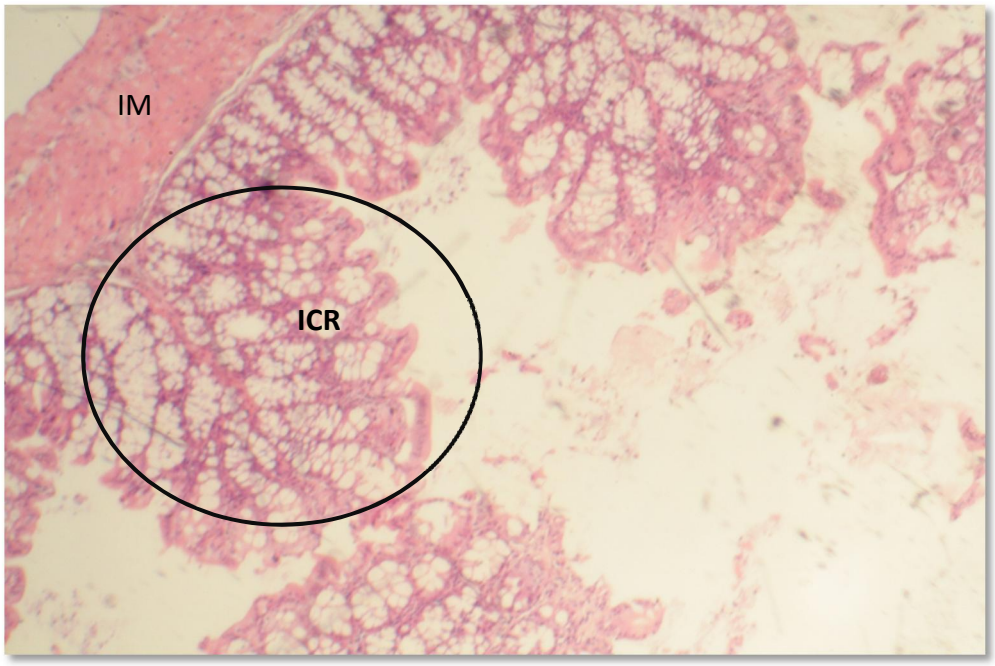

Figure 11 : Histological section of sigmoid colon 40 days flour application

H. E. staining (x40)

IM: Intact Muscularis **ICR:** Intact Crypt

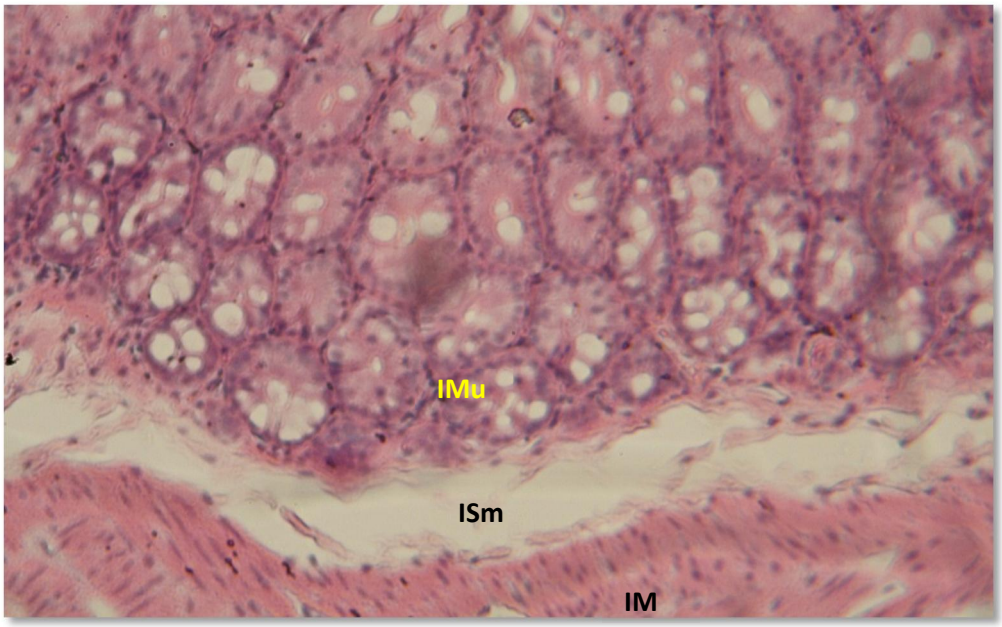

Figure 12 : Histological section of sigmoid colon 40 days flour application .

H. E. staining (x100)

I**M**: Intact Muscularis, **ISm**: Submucosa, **IMu**: Mucosa

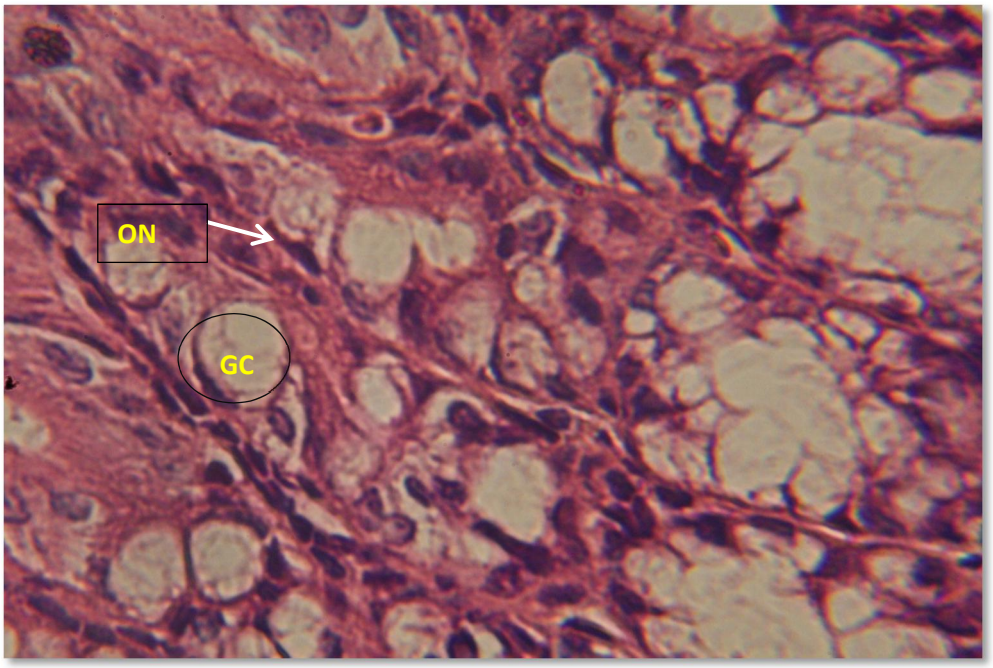

Figure 13 : Histological section of sigmoid colon 40 days flour application

H. E. staining (x400)

GC: goblet cell **ON:** Oval nucleus

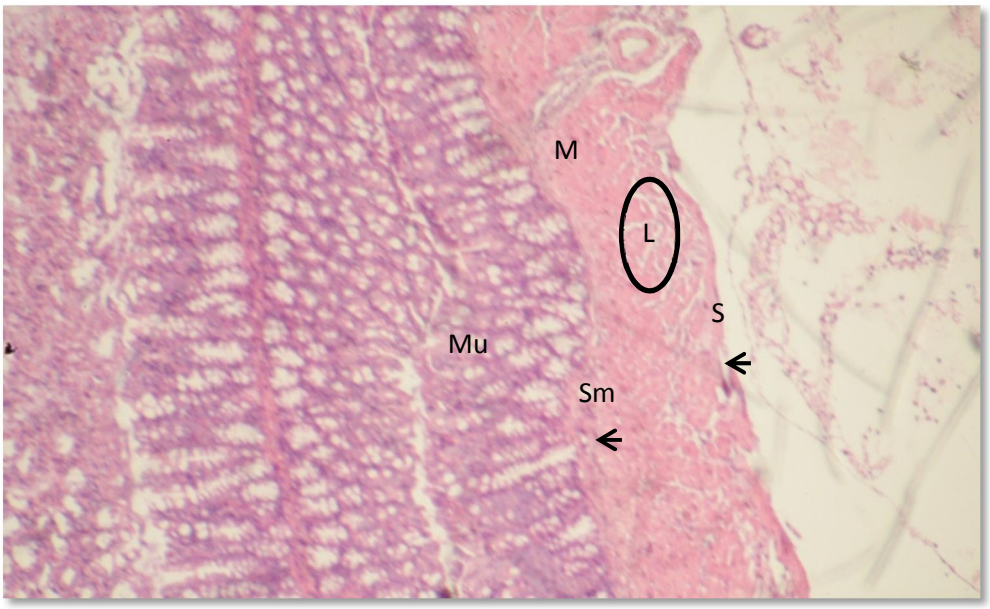

Figure 14: Histological section of sigmoid colon 40 days L-methionine application .

H. E. staining (x40)

M: muscularis, **S**: serosa, **Sm**: submucosa, **Mu**: mucosa L: Lysis

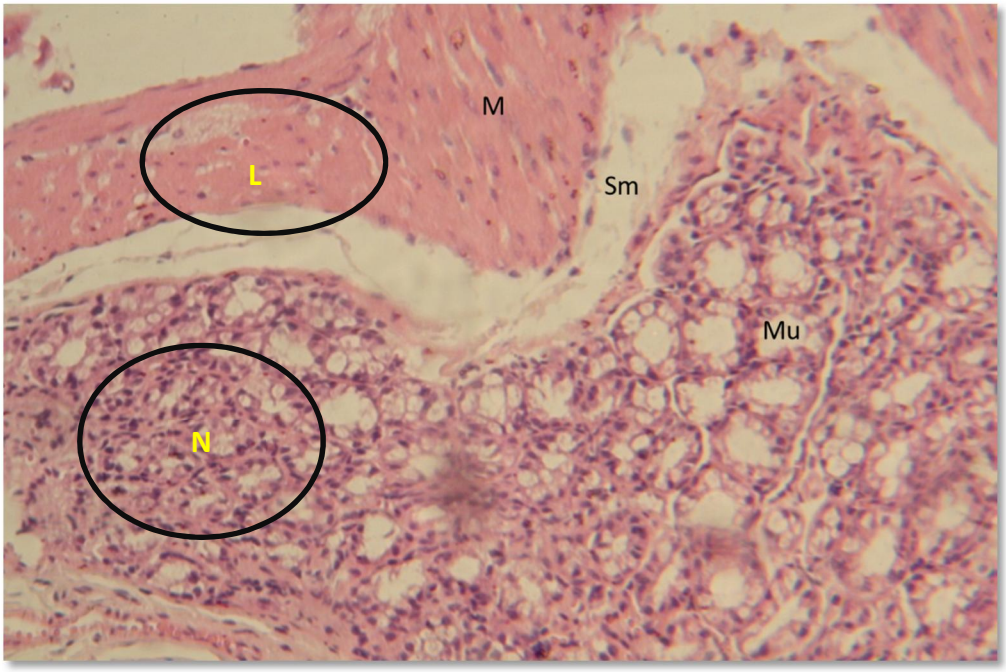

Figure 15: Histological section of sigmoid colon 40 days L-methionine application .

H. E. staining (x100)

M: muscularis, **Sm**: submucosa, **Mu**: mucosa ,L: Lysis , **N:** necrose

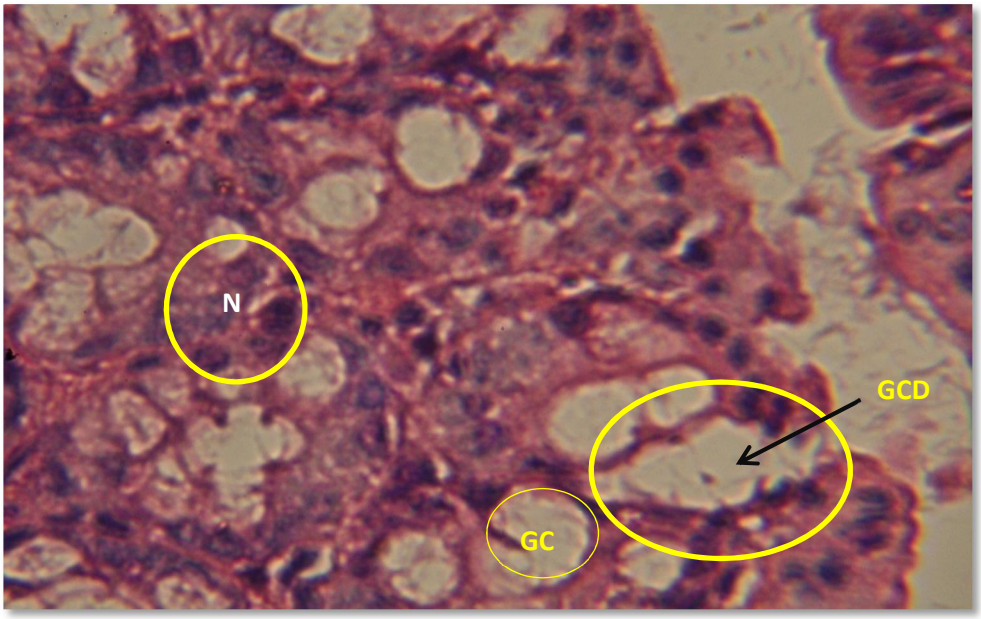

Figure 16: Histological section of sigmoid colon 40 days L-methionine application .

H. E. staining (x400)

GC: goblet cell, **GCD:** Goblet Cell Dilatation N: Necrosis

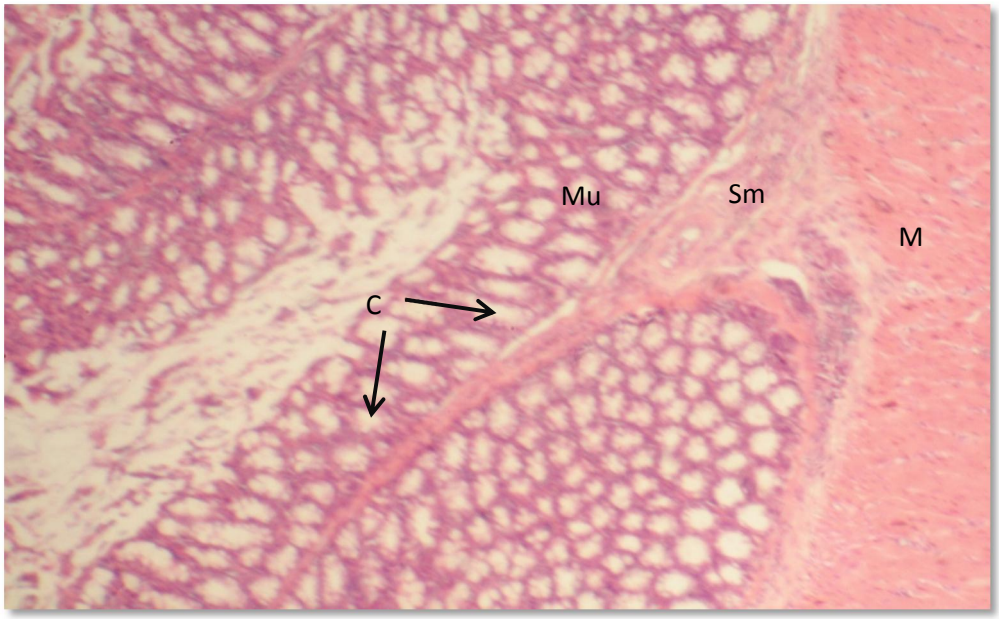

Figure 17 : Histological section of sigmoid colon 40 days L-methionine + *Vitis vinifera* application.

H. E. staining (x40)

M: Muscularis, **Sm**: Submucosa, **Mu**: Mucosa , **C:** Crypt

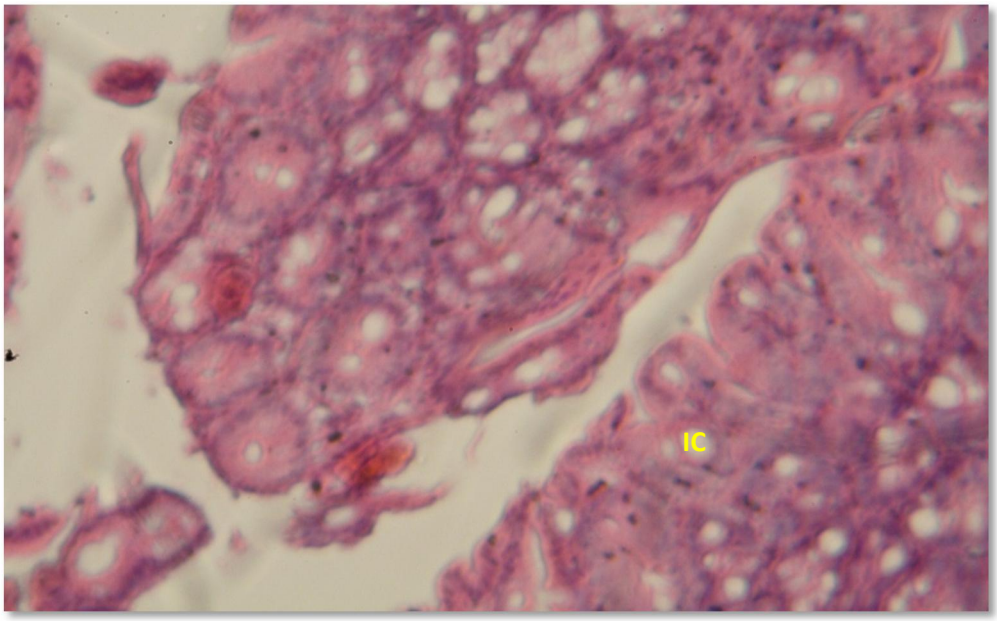

Figure 18 : Histological section of sigmoid colon 40 days L-methionine +*Vitis vinifera* application .H. E. staining (x100)

IC : Intact Crypt

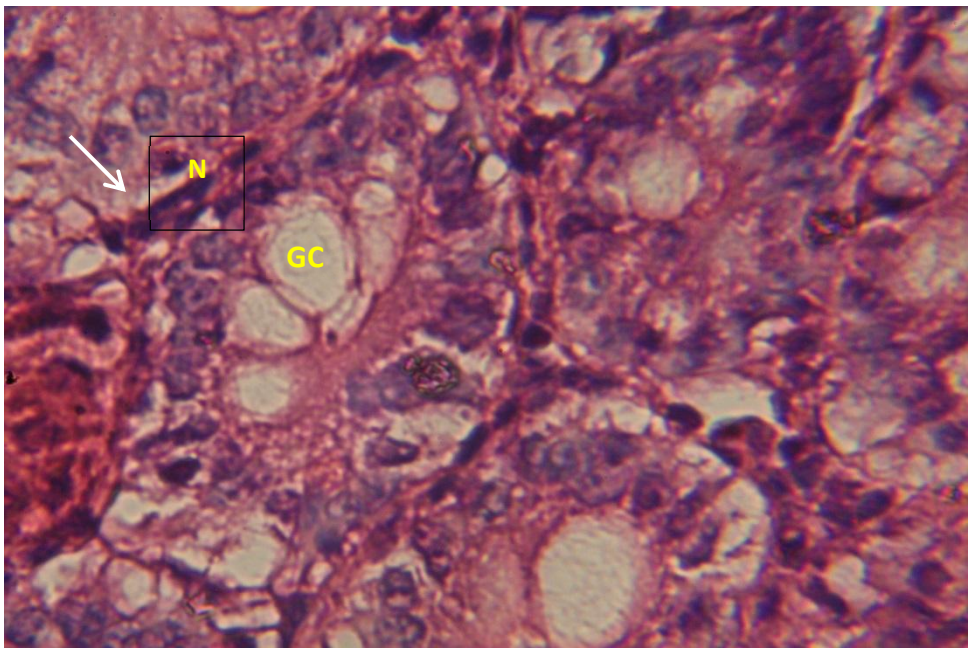

Figure 19: Histological section of sigmoid colon 40 days L-methionine + *Vitis vinifera* application .

H. E. staining (x400)

N: Necrosis **GC:** Globlet Cell

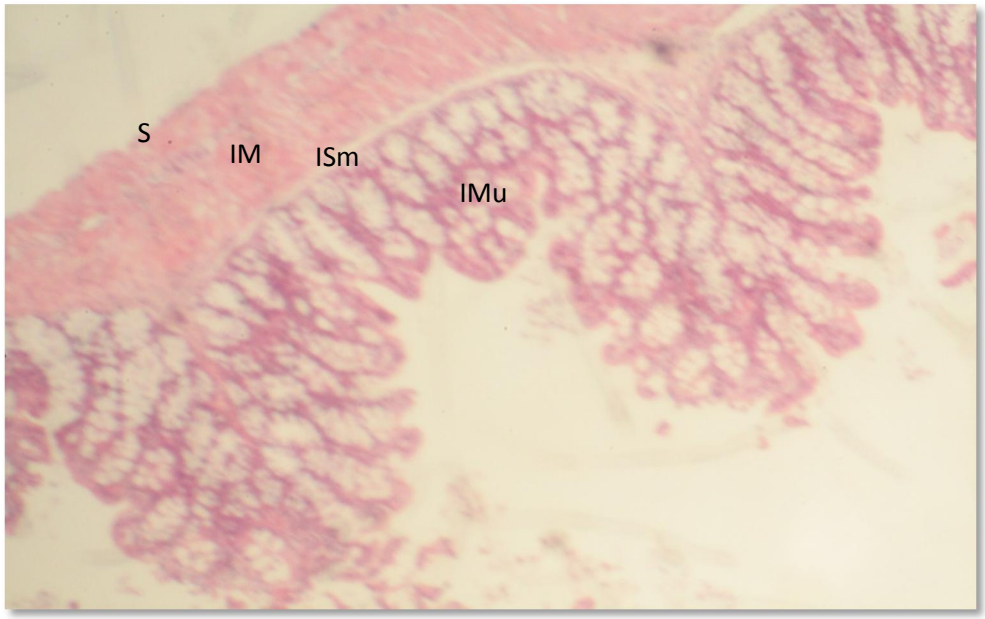

Figure 20: Histological section of sigmoid colon 40 days *Vitis vinifera* application .

H. E. staining (x40)

I**M**: Intact muscularis, I**Sm**: Intact submucosa, I**Mu**: mucosa, **S:** serosa

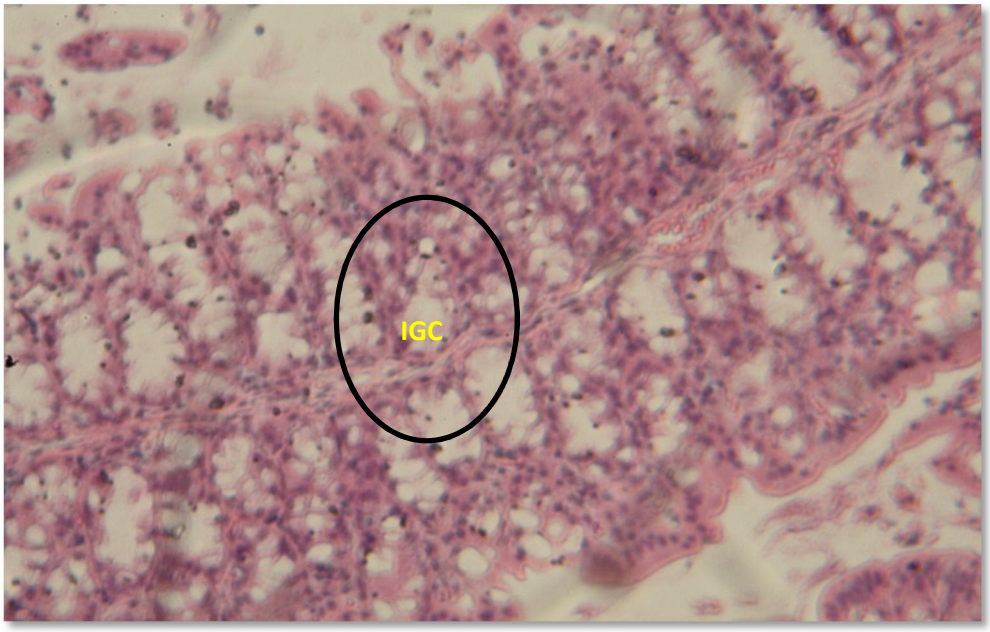

Figure 21: Histological section of sigmoid colon 40 days *Vitis vinifera* application .

H. E. staining (x100)

IGC: Intact Crypt

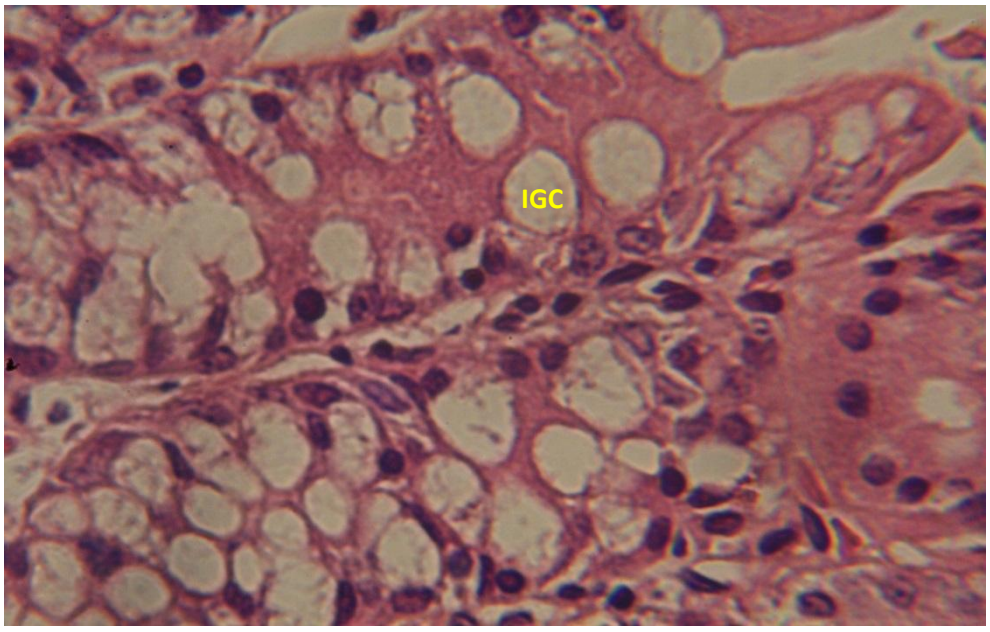

Figure 22: Histological section of sigmoid colon 40 days Vitis *vinifera* application.

H. E. staining (x400)

IGC: Intact Crypt

Conclusion

In this study we evaluated the role of the extract of *Vitis vinifera* on the inflammatory bowel disease induced by hyperhomocysteinemia.

The results obtained showed that *Vitis vinifera* has an anti-obesity effect on body weight, and it has a biphasic effect : an anti-inflamamtory effect when it is used for short period and an inflammatory effect when it is used for long term. Also we have obtained that the *Vitis vinifera* has an anti-oxidant effect when it is used as treatment with hyperhomocysteinemia.

For futur work we need to carry some experiments as shown below :

1 : Identification and purification of bioactive moleculs from *Vitis vinifera.*

2 : We will treat the mice and rats with the bioactive moleculs during different periods.

3 : Determination of glutathione reduced, glutathione oxidized and antioxydants enzymes such us catalase, superoxide dismutase and glutathione peroxidase.

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Summary

Summary

Homocysteine is an intermediate metabolite of methionine. Homocysteine level in plasma depends on several factors of which the most important are folate, vitamins B_6 and B_{12} status and genetic polymorphism of the enzymes involved in its metabolism. Folic acid and vitamin B_{12} can lower homocysteine levels. Studies suggest that moderate hyperhomocysteinemia may play a significant role in the development of cardiovascular diseases and colorectal cancer.

In the present work we have studied the relationship of hyperhomocysteinemia and Inflammatory Bowel disease. The inflammatory process is evaluated by a determination of hs-CRP which was increased in groups PM and P and decreased in group of mice treated with 500mg/kg during 40days of treatment.

The use of the extract of *Vitis vinifera* leaves during long term will induce aphrodisiac effect and anti-inflammatory effect during short periode.

The glutahion reduced (GSH) in treated mice with L-methionine decreased when it is compared to the other proups. *Vitis vinifera* is a good anti-oxidant source which protects from inflammation encountered in intestinal inflammation.

We considered that the extract of *Vitis vinifera* leaves has a natural phytotherapy in IBD but during short period of treatment.

Key words: Homocysteine, methionine, hyperhomocysteinemia, folate, vitamin B6, vitamin B12, IBD, *Vitis vinifera*.

Résumé

L'homocystéine est un acide aminé intermédiaire dans le métabolisme de la méthionine. Sa teneur dans le plasma dépend de nombreux facteurs, parmi lesquels le statut en folates et vitamines B_{12} et B_6 et le polymorphisme génétique de la méthylène tétrahydrofolateréductase jouent un rôle crucial. Des traitements simples (apport oral de folates et de vitamine B_{12}) permettent de réduire le taux plasmatique d'homocystéine. Des études épidémiologiques tendent à montrer qu'une élévation modérée de l'homocystéine est impliquée dans la genèse des maladies cardiovasculaires du cancer colorectal.

Dans le présent travail nous avons tenté d'étudier la relation homocystéine et maladie inflammatoire chronique intestinal MICI. Le processus inflammatoire , peut être évalué par un dosage de la CRP-hs et du glutathion réduit .

Les résultats obtenus ont montré que les concentrations plasmatiques de CRP -hs étaient élevées dans les groupes PM et P est diminué chez les souris, traitées par la L- méthionine à des doses élevés (500mg/kg) pendant 40 jours. L'utilisation de l'extrait de *Vitis vinifera* à long terme va induire effet aphrodisiaque et effet anti-inflammatoire à courte terme .

On n'a observé une diminution dans la concentration du GSH dans le groupe de methionine par rapport aux autres groupes. *Vitis vinifera* est une bonne source anti-oxydant qui protège l'inflammation rencontrée dans d'inflammation intestinale.

Nous avons considéré que l'extrait de *Vitis vinifera* a une phytothérapie naturelle dans les MICI mais pendant courte période de traitement.

Mots clé: Homocystéine, méthionine, hyperhomocysteinémie, folate, vitamine B6, vitamine B12, MICI, *Vitis vinifera*.

ملخّص:

الهوموسيستيين ناتج من ميتابوليزم المثيونين ، و مستويات الهوموسستيين في البلازما تتوقف على عوامل عديدة ، منها حمض الفوليك، الفيتامين B6 و B12 و تعدد أشكال الـ MTHFR .

تناول حمض الفوليك و فيتامين B 12 يمكنها أن تقلل من مستويات الهوموسيستين في البلازما.

تشير الدراسات الوبائية إلى أن ارتفاع تركيز الهوموسيستين يتسبب في أمراض القلب ' الشرايين و سرطان القولون و المستقيم.

في هذا العمل حاولنا دراسة العلاقة بزيادة الهوموسيستين البلازمي و مرض التهاب الأمعاء المزمن، بحيث ظاهرة الالتهاب تم تقييمها بواسطة تقدير(hs-CRP).

هذا الأخير تم ارتفاعه في المجموعتين P و PM و انخفضت عند مجموعة الفئران التي تلقت جرعات عالية من الميثيونين 500ملغ/ كلغ لمدّة 40 يومًا.

استخدام مستخلص كرمة العنب (*Vitis vinifera*) على المدى الطويل، مثير للشهوة الجنسية، كما لها تاثير مضاد للالتهابات إذا ما استُخدمت على المدى القصير. أيضا كان هناك انخفاض في تركيز الغلوتاثيون (GSH) في مجموعة الميثيونين، مقارنة بـالمجموعات الأخرى. ولهذا يعتبر مستخلص كرمة العنب مصدرًا جيّدا لمضادات الأكسدة التي تقي من الالتهاب الناتج من التهاب الأمعاء.

استنتجنا أن مستخلص كرمة العنب هو علاج طبيعي لالتهاب الأمعاء المزمن إن تمّ استعماله لمدّة قصيرة.

كلمات مفتاحيّة: هوموسيستين، ميثيونين، ارتفاع الهوموسيستين، حمض الفوليك، فيتامين B6 ، فيتامين B12 ، التهاب الأمعاء المزمن، مستخلص كرمة العنب.

Annex

1. Calculation of doses

1.1. Methionine dose

0,5 g ⟶ 1000g

X g ⟶ mice's weight g

$$\frac{0{,}5\text{g} \times \text{mice's weight g}}{1000\ \text{g}}$$

1.2. Plant dose

0,2 g ⟶ 1000g

X g ⟶ mice's weight g

$$\frac{0{,}2\text{g} \times \text{mice's weight g}}{1000\ \text{g}}$$

IX. Preparation of *Vitis vinifera* :

The leaves were dried under shade and powdered to a dark green powder.

Photo 3 : dried *Vitis vinifera*

Photo 4: powdered *Vitis vinifera*

2. Preparation of solutions

2.1. Dissection

- **NaCl**

Dissolve 0.9 g of NaCl in 100 ml of distilled water

- **Alcoholic bouin :**

- Picric acid solution 1% (diluted in 95% ethanol) : 45ml

- Formaldehyde : 26ml.

- Acetic acid : 7ml

- Distilled water

3. Preparation of the homogenate

- **TBS solution (Tris 50 mM, NaCl 150 mM)**

The quanitity of the amount :

1 mole ⟶ molar mass (g)

N mole ⟶ x (g)

Product amount : = $\frac{\text{n}\times\text{molar mass}}{1}$

Dissolve 6,057g of tris (121,14g/mole) and 8,766 of NaCl (58,44g/mole) in 1 liter of distilled water, then adjust the pH into 7,4 with HCl.

4. Glutathione assay :

- **Solution of tris (0,4M), EDTA (0,02M) and pH 9,6**

Dissolve 12,114 g of tris, and 1,871 g of EDTA in 250 ml of distilled water, and adjust pH into 9,6 by adding HCl or NaOH.

- **Solution of DTNB (0,01M)**

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

- **Solution of sulfo-salicylique acid 0,25%**

Dissolve 250 mg of sulfo-salycilique acid in 100 ml of distilled water.

5. Dosage of total proteins

- **BSA solution (1mg/ml)**

Dissolve 5 mg of BSA in 5 ml of distilled water.

- **Bradford reagent**

Dissolve 100 of Coomassie Brilliant Blue (G250) in 50 ml of ethanol (95%), agitate (shake) by the agitator for 2 hours, then add 100 ml of ortho-phosphoric acid (85%) and 850 ml of distilled water (to get 1 L of solution).

This reagent can be filtered and and conserved for 1 month, at a temperature of 4°C protected from light.

X. Protein determination :

Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as standard. The procedure is based on the formation of a bleu complex between the coomasie brilliant blue G-250 dye, and protein in solution. The amount of absorbtion is proportional to the protein present.

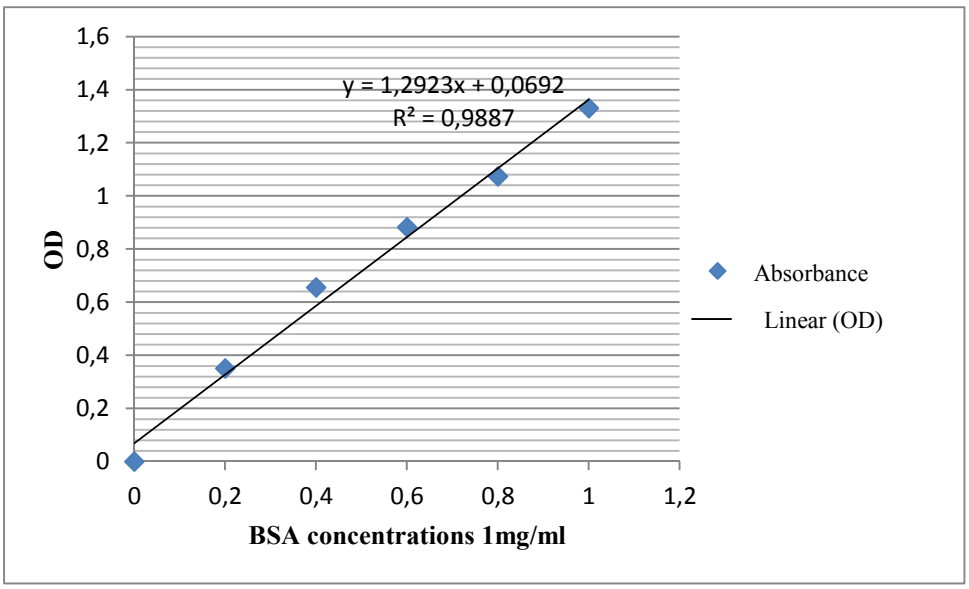

Figure 23 : Calibration curve

Presented by : MOHAMMEDI Sarah & MAKHLOUF Meriem **2014/2015**

Thesis submitted for the degree of Master

Option : Immuno-Oncology

Title :

The effect of *Vitis vinifera* on inflammatory bowel disease caused by hyperhomocysteinemia

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