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AMELIORATIVE EFFECT OF *HELIX ASPERSA* CRUDE EXTRACT AGAINST COLONIC DAMAGES INDUCED BY HYPERHOMOCYSTEINEMIA IN RATS

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ABSTRACT: The present study was designed to evaluate the effect of *Helix aspersa* crude extract on colonic injury resulting from hyperhomocysteinemia induced by feeding rat's high methionine diet. Wistar albino rats (200-250g) were divided into four groups. Hyperhomocysteinemia (Hhcy) was induced by methionine treatment (1g/kg/day) for 15 days. M group received only Methionine, MH and MP received Methionine and *Helix aspersa* Crude Extract (8g/kg/day) or Prednisolone (4 mg/kg/day) respectively at the 8th day until the end of experimentation. Prednisolone was used as a standard drug. At the end plasma homocysteine and C-reactive protein were evaluated, followed by colon histopathological investigations. Methionine treatment induced significant increase (P <0.001) in homocysteine and CRP levels in plasma as compared to control rats. *Helix aspersa* crude extract induces significant decrease of homocysteine (P <0.001) and CRP (P <0.01) levels as compared with experimental control group. The results were comparable to those obtained with prednisolone, a standard anti-inflammatory drug. Histological assessment showed colonic tissue damages in methionine treated group compared to control (P <0.001) as mucosal inflammation, crypt damage and hyperemia. Crude extract markedly reduces the damage induced by the Hhcy and preserved the histo-architecture of colonic tissue compared to M group (P <0.001). This study provides clear evidence that *Helix aspersa* crude extract treatment decrease the major inflammatory markers homocysteine and CRP. Also it acts as a potent protective agent of colon against the inflammatory state induced by elevated level of plasma homocysteine.

INTRODUCTION: Inflammation is a natural defensive process that a living body initiates against local tissue damage or the presence of inflammatory stimulants [1]. There are two types of inflammation: acute inflammation which can be defined as a regulated form and chronic inflammation which can be defined as a dysregulated form of inflammation [2].

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Acute inflammation, an immediate and early defensive response in the host to all forms of injuries, helps to heal wounds and promotes tissue regeneration. However, when this inflammation process is not controlled properly via competent negative feedback, a chronic low-grade inflammatory state could result [1].

Previous research has shown that nutrients as natural bioactive compounds in fruits, vegetables, grains, legumes and tea, influence inflammation [3, 4]. Homocysteine (Hcy) is a metabolite of the essential amino acid methionine [5]. Elevation of circulating homocysteine is associated with inflammation [6]. As a risk factor, the risk of Hhcy is not limited to heart disease but can be to include other inflammatory

diseases such as cardiovascular disease, Alzheimer's dementia, pregnancy complications neural tube defects and osteoporotic fracture [7]. It has been reported that in patients with inflammatory bowel disease (IBD) the Hcy levels in plasma and colonic mucosa are increased [8, 9, 10]. It should be noted that Hhcy not only is produced from inflammation, but the oxidative stress generated from Hhcy will again promote inflammation [4].

Circulating homocysteine is an inflammatory marker and a risk factor of life-threatening inflammatory diseases. C-reactive protein (CRP) also is considered among major inflammatory markers [4].

Humans have always considered snails as a source of numerous therapeutic properties [11]. The *Helix aspersa* is a species of land snail, its meat is characterized by a high dietetic value and excellent nutritious traits. Research shows that it is rich in protein at the same time being low in lipids [12].

Snail extracts have been increasingly used in numerous therapeutic conditions and recent literature attributes healing, soothing and anti-aging properties to them [13]. Snails have been used with success against most inflammations, and especially against bronchitis, asthma, influenza, lung diseases such as pneumonia and pulmonary phthisis, and inflammatory skin diseases [11] but no reports have been yet published about the effects of snail extract on colitis, also no research was done on the pro-inflammatory effects of Hhcy on colon histological integrity. In this context, the aim of the present study is to evaluate the ameliorative effect of *Helix aspersa* crude extract on colonic morphological and histological changes resulting from Hhcy induced by height dietary methionine supplementation in albino rats.

MATERIALS AND METHODS:

Drugs and chemicals: L-methionine and prednisolone were purchased from Sigma chemical company (St. Louis, MO, USA).

Preparation of crude extract: Ninety snails (*Helix aspersa*) were bought from commercial farmer from the region of Mila, East Algeria. Animals were kept in plastic cages and were fed with fresh leaves of lettuce and carrots for two weeks under laboratory conditions (25 – 26 °C). This time was allowed to ensure temperature acclimation. At the end of the second week adjustment period, the shell was removed using hooks and the pedal mass was separated from visceral mass. The head was removed then specimens were weighted. The tissue is then homogenized on ice using a T_{25} Ultra-Turrax homogenator. The dose of 8g/kg/day of Helix aspersa crude extract was considered after toxicity study on mice (data not shown but available).

Animals: Eight weeks old male albino Wistar rats (n= 24) weighing 200 to 250g used were received from Pasture Institute, Algiers, Algeria. Animals were housed in plastic cages in a light and temperature controlled room on a 12 to 12 h light–dark cycle, in which the temperature (25 °C) and relative humidity (65 to 70%) were kept constant. All the rats were weighted every day.

The experimental groups were fed the same diet as the control and access to water was allowed ad libitum. The standard diet is composed of 30mg proteins and 4.5mg methionine per 200g of diet. The animal studies were conducted after obtaining clearance from Institutional Animal Ethics Committee.

Experimental design: After two weeks of acclimation, the animals were divided into four groups with six animals in each group as follows: Control (C); to induce Hhcy treated groups (M, MH and MP) received L-methionine 1g/kg/day, p.o. 17 [14]. MH received *Helix aspersa* crude extract (8g/kg/day, p.o.), MP received prednisolone (4mg mg/kg/day p.o.) [15]. Treated animals received methionine daily for 15 day while crude extract and prednisone treatment begin at the 8th day until the end of experimentation. The rat body weight was monitored daily during the experiment.

Biochemical assays: At the end of experimentation blood was collected for biochemical assessment. Plasma homocysteine levels were determined using the Bio-Rad high-performance liquid chromatography (HPLC) kit (Bio-Rad, Hecules CA, USA) [16]. CRP serum levels were measured by an Immuno-turbidimetric method using commercial Randox kit (UK) with standards provided by the same firm, and expressed in mg/L.

Assessment of disease activity index: The disease activity index (DAI) was determined using three parameters (the ratio of colon weight to length, macroscopic score and microscopic score). Disease activity index is used as a parameter to assess the degree of tissue edema and reflects the severity of colonic inflammation [17].

Assessment of Colonic Damage: All the animals were sacrificed at the end of experimentation by ether overdose. Abdomen was opened and colons were exposed. Distal 8 cm of colon was excised and opened by a longitudinal incision. After washing the mucosa with saline solution, colons were imaged and weighted. Weight of distal colon is used as a marker for inflammation and tissue edema. Then mucosal injury was assessed macroscopically using the scale of Morris *et al* [18]. (**Table 1**)

TABLE 1: CLASSIFICATION OF MACROSCOPIC ALTERATIONS IN COLONIC MUCOSA

Score	Macroscopic changes
0	no damage
1	Mucosa erythema only
2	Mild mucosal edema, slight bleeding, or slight erosion
3	Moderate edema, bleeding ulcers, or erosion
4	Severe ulceration, edema, and tissue necrosis

A small cross section of colon was fixed in 10% formaldehyde and embedded in paraffin, and 5µm sections were prepared. Tissues were stained with hematoxylin and eosin and observed under light microscope Optech Optical Tecnology®. All reagents were purchased from Sigma Aldrich, Algeria. Colonic tissues were scored for histological damage using the criteria of Ackerman *et al* [19] (depth of necrosis, extend of necrosis, degree of inflammation, extent of inflammation and fibrosis).

The scores for each category examined were calculated for each specimen in the different groups then added to obtain the total score, which was then divided by the number of rat colons examined in each group to obtain the average histologic score for the group.

Statistical analyses: The data were expressed as Means ± Standard deviation (SD). Data analyses and presentation of graphs were performed using Pearson's correlation and one way analysis of variance (ANOVA) with the statistical package for social science (SPSS) version 20 for windows followed by Newman-keuls post hoc test. *P* values <0.05 were considered as significant.

RESULTS AND DISCUSSION:

Body weight: Treated groups showed significant ($p<0.001$) weight loss during all days of the experiment compared to control rats. However *Helix aspersa* crude extract seems to prevent reduction of body weight compared to experimental control ($p=0.0014$). For the control group there was a gain of weight. (**Fig. 1**)

A decrease in food intake was observed progressively from d 4 to 15, with a minimum intake that reached 11.60% the initial intake on d11 to 15 for methionine group. *Helix aspersa* crude extract group has shown increase in food intake from d13 to 15, with a maximum intake that reached to 10.75% the initial intake. At day 15 MP group has shown no significant increase in food intake (0.72%).

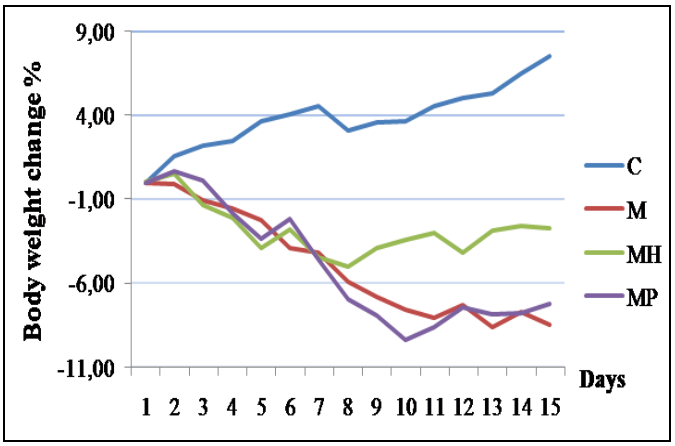

FIG. 1: TREATED RATS WEIGHT LOSS COMPARED TO CONTROL GROUP

Measurement of homocysteine and CRP (inflammatory marker): Methionine supplementation was found to increase plasma levels of homocysteine (**Fig. 2**), and protein reactive c (**Fig. 3**), in all treated groups compared control group ($p<0.0001$). Compared to experimental group, *Helix aspersa* crude extract induced significant decrease of Hcy ($p<0.001$) and CRP ($p<0.01$) levels.

However MP group showed significant decrease of Hcys ($p<0.05$) and no significant decrease of CRP ($p=0.52$). CRP is used mainly as a marker of

inflammation. Statistical analysis shows positive correlation between Hcy and CRP but not significant (r=0.778, p=0.068).

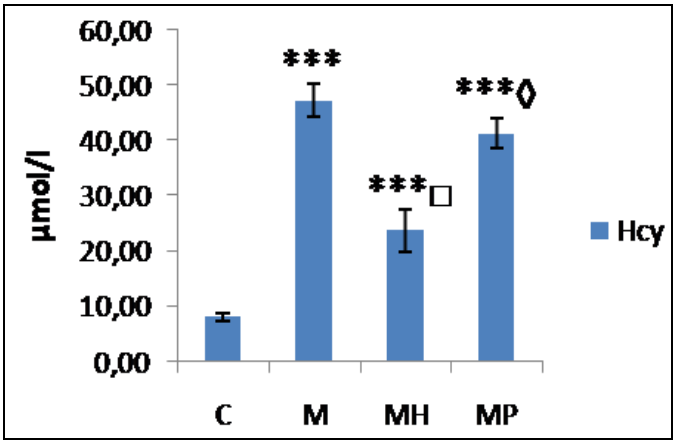

FIG. 2: EFFECTS OF L-METHIONINE SUPLLEMENTATION AND *HELIX ASPERSA* CRUDE EXTRACT ON HCY LEVELS IN RATS. Data are expressed as means± SEM (n= 6). ***P< 0.001 when compared to control; ◊ p< 0.05 and □ p< 0.001 when compared M.

Song *et al* demonstrated that methionine supplementation induces hyperhomocysteinemia in rats [20].

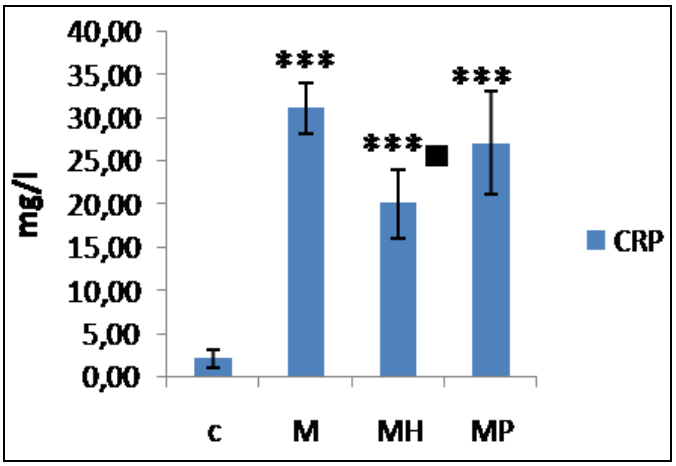

FIG. 3: EFFECTS OF L-METHIONINE SUPLLEMENTATION AND *HELIX ASPERSA* CRUDE EXTRACT ON CRP LEVELS IN RATS. Data are expressed as means± SEM (n= 6). ***P< 0.001 when compared to control; ■p < 0.01 when compared M.

Recent studies supported the fact that elevation of circulating homocysteine is associated with inflammation. Level of circulating homocysteine can be effectively reduced by the administration of anti-inflammatory medications [21]. Current data have suggested the possible pathogenetic implications for Hhcy in inflammatory bowel disease (IBD) suggesting that Hcy may act as a pro-inflammatory and immuno-stimulating molecule [6].

C-reactive protein is released by the body in response to acute injury, infection, or other inflammatory stimuli. It is a pro-inflammatory factor that has been implicated in the pathogenesis of autoimmune diseases as IBD [22].

Evaluation of macroscopic and microscopic damages: Macroscopic damage parameters of colon after methionine treatment revealed significant changes (p<0.001) (colonic mucosal hyperemia, moderate edema and erosion) in M and MP groups compared to control group more marked in M. *Helix aspersa* crude extract group showed significant changes (p<0.01). However, compared to M group, *Helix aspersa* crude extract reduced significantly the intensity of scores (p<0. 01). Prednisolone treated group showed no significant improvement of macroscopically score when compared to methionine group (**Fig. 4, Table 2**).

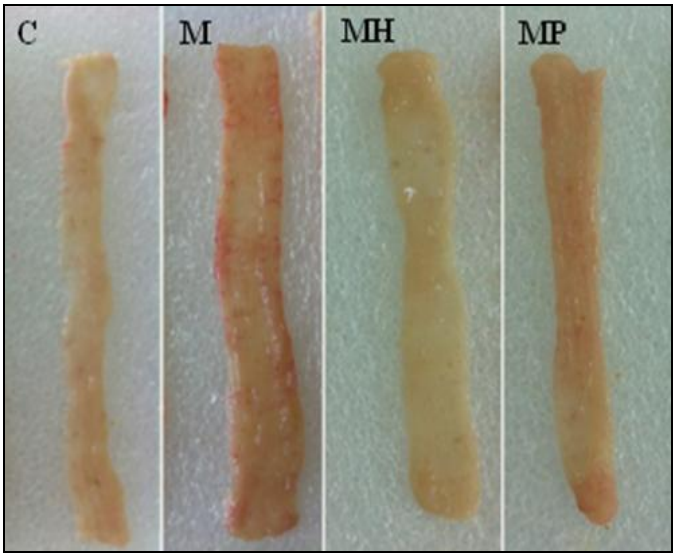

FIG. 4: MORPHOLOGICAL REPRESENTATION OF RAT'S COLON

As standard drug, prednisolone activity against colonic inflammation was not better than that of *Helix aspersa* crude extract with regard to all parameters (p<0.05). We observed significant increase (p<0.001) in DAI for M group compared to C group. *Helix aspersa* crude extract and prednisolone induced decrease of DAI compared to experimental control (p<0.001). (**Table 2**)

TABLE 2: EFFECT OF HEIGH DIET METHIONINE AND CRUDE EXTRACT OF *HELIX ASPERSA* ON COLON

Groups	Macroscopic score	Colon Weight(g)/length(cm)	Microscopic score	DAI
Control	0±0.0	0.12±0.01	0±0	0.04±0.00
M	2.0±0.0***	0.15±0.03*	3.0.7±0.22***	1.58.±0.07***
MH	1.13±0.58**◊	0.12±0.01◊	1.87±0.89***□	0.99±0.18***□
MP	1.33±0.44***	0.11±0.01◊	1.80±0.53***□	1.04±1.21***□
ANOVA				
F	19.285	3.683	24.629	62.206
Df	3.098	3.098	3.098	3.098
P	<0.0001	0.0292	<0.0001	<0.0001

Data are expressed as means± SEM (n= 6). *P<0.5, **p<0.01, ***p< 0.001 when compared to control; ◊ p< 0.05, ▪p < 0.01 and □ p< 0.001 when Compared to M.

Microscopic illustration (**Fig. 5**) of colon tissue in control rats group showed normal tissue; crypts and mucosal layers are intact and inflammatory infiltration is absent. Methionine treated group (M) showed mutifocal areas of ulcers and regions of loss of crypts (mucosal layers destruction; red arrow) and inflammatory infiltration (white arrow) including edema in submucosa (black arrow). MH group showed improvement of intestinal mucosa compared to experimental control and more goblet cells (yellow arrow), hyperchomatic nuclei, little ulcer and inflammatory cells infiltration. Finally, MP group showed attenuated cell damage with less goblet cells slight inflammatory infiltration. One of the possible mechanisms by which Hhcy is implicated in colon inflammation disease is the fact that it leads to endothelial dysfunction [23] which facilitate immune cells infiltration in colonic tissue by an increase of leukocyte adhesiveness and leukocyte diapedesis [24]. Also oxidative stress plays a key role in pathophysiology of Hhcy, indeed several studies showed an association between Hhcy and reactive oxygen species (ROS) production (such as superoxide anion, hydrogen peroxide and hydroxyl radicals) which leads to proteins and cells damage [25].

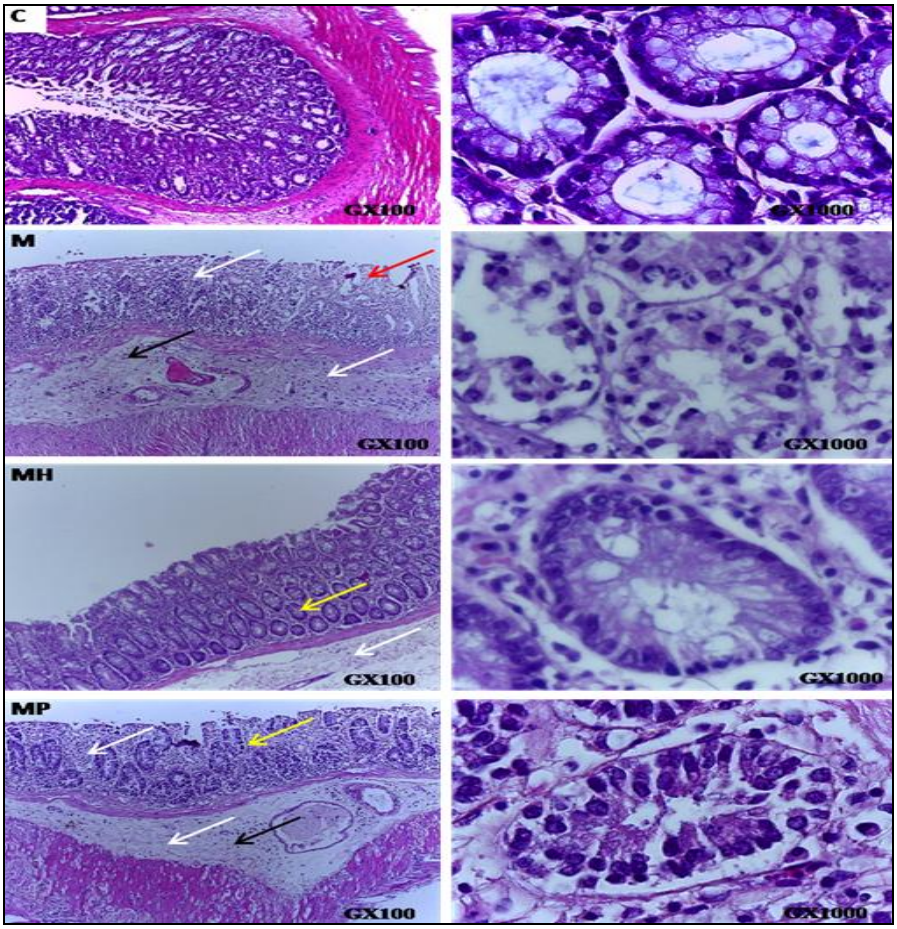

FIG. 5: PHOTOMICROGRAPHS OF COLON SECTIONS OF RATS STAINED WITH H&E

It has also been reported that the oxidative stress derived from hyperhomocysteinemia will again induce acute and chronic inflammation via the regulation of NF-κB transcription factor [6]. Ding *et al* speculated that Hcy-mediated pro-inflammatory responses and elevation of expression of MMPs might be involved in the pathogenesis action of Hhcy in IBD. [24] *Homocysteine* was also found to stimulate IL-1beta production by human peripheral blood monocytes and TNF-alpha production by monocyte [26, 27]. TNFα and IL-1 proinflammatory cytokines have important roles in the pathogenesis of chronic inflammatory diseases such inflammatory bowel disease (IBD) [28].

Immune cells found in mucosa and submucosa are eosinophils, mast cells and lymphocytes (**Fig. 6**). Lampinen et al demonstrated that the inflammatory process in IBD involves many inflammatory cells, such as lymphocytes, macrophages, mast cells, neutrophils, and eosinophils. They indicated that eosinophils are proinflammatory agents thus producing effects such as inflammation, tissue destruction, formation of fibrosis [29, 30].

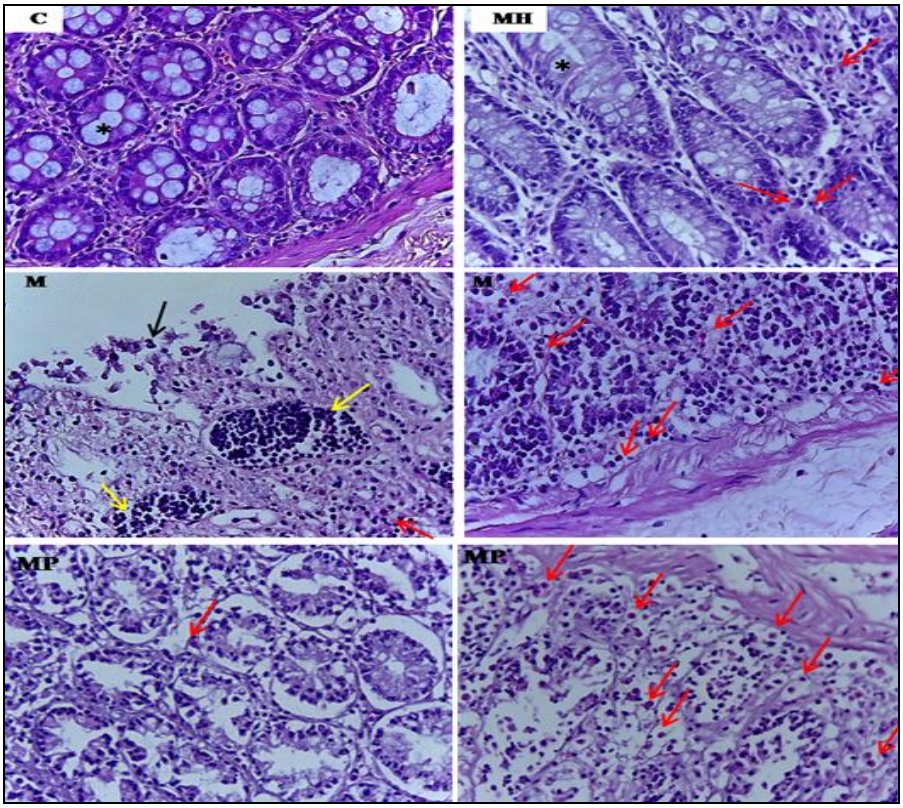

FIG. 6: IMMUNE CELLS INFILTRATION IN COLONIC MUCOSA WITH H&E (x 400) Mast cells (red arrow); lymphocytes (yellow arrow); necrosis (black arrow); goblet cell (*).

Our results showed colonic wall recovery after treatment of hyperhomocyseinemic rats with crude extract of *Helix aspersa* compared to experimental control. Heling properties of snails are due to the highly nutritious value of mollusc meat owing to its content of essential amino acids, high proteins quality, low fat, rich vitamins and minerals [31]. More than the nutritious value, Citil *el al*, reported that *Helix aspersa* contains high levels of polyunsaturated fatty acids of the omega 6 series and especially omega 3 series (eicosapentaenoic acid, linolenic acid, …) [32]. These essential biochemicals are important components of the cell membrane and they give many other compounds involved in the regulation of blood pressure and inflammatory responses. More and more evidence suggests that omega -3 fatty acids exert an anti - inflammatory effect [33].

Recent study found that *Helix aspersa* contains antioxidant superoxide dismutase (SOD) and Glutathione-S-Transferase (GST) activities which protect cells from the damage caused by reactive oxygen species and reported that the snail slime

induced cellular regeneration by stimulating fibroblast proliferation, extracellular matrix assembly and the regulation of metalloproteinase activities [34].

CONCLUSION: The results of this study have shown that crude extract of *Helix aspersa* has got a good potential to reduce severity of colon inflammation induced by homocysteine in rats, as indicated by macroscopic, microscopic and biochemical evaluations.

CONFLICT OF INTEREST: Authors declare they have no conflict of interest

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