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Preventive effect of *Helix aspersa* slime against experimentally chemo-induced colitis in rat

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ABSTRACT

To evaluate the effect of the slime of Helix aspersaon acetic acid induced colitis in male Wistar rats, the slime of Helix aspersa was prepared by using the simplest method in heliciculture. Toxicity test was done by using DL50 guidelines. Wistar rats were divided into five groups (n=6). Seven days before induction of colitis, Group C and ACT received NaCl 0.9%; Group ASA+ACT received 5-aminosalisylic acid (5-ASA) 100 mg /kg. Group Slime and Slime+ACT received Helix aspersa slime 20 ml/Kg. Colitis was induced by trans-rectal administration of 4% acetic acid on the 8th day. Blood was withdrawn for C-Reactive Protein (CRP) analyses and all animals were sacrificed after seven days of colitis induction, the colon and the liver was dissected. Colon was analysed macroscopically and microscopically. Biochemical assessment of tissue total proteins and malondialdehyde (MDA) was done in liver tissue homogenate. Helix aspersa showed significant reduction in MDA, macroscopic and microscopic lesions showed improvement compared to Group ACT. The slime of Helix aspersa showed significant amelioration of experimentally induced colitis, which may be attributed to its anti-inflammatory and antioxidant property. In conclusion, treatment by the slime offered protection against colon inflammation, thus the slime is a good immune stimulant and could also contain natural antioxidant molecules.

Key words: *Helix aspersa*, Slime, Anti-inflammatory, Antioxidant, Colitis.

INTRODUCTION

Inflammatory bowel disease (IBD) comprises Crohn's disease (CD) and ulcerative colitis (UC) which are defined as chronic and relapsing inflammations of the gastrointestinal tract caused by variable pathophysiological mechanisms characterized by clinical manifestations including diarrhoea, blood in the stool, abdominal pain, and weight loss [1]. Despite the fact that aetiology of IBD still remains poorly understood, complex interactions among genetic, environmental, immunological and reactive oxygen species (ROS) have been implicated in the pathogenesis of IBD [2-3]. In many studies, it has been reported that antioxidants show beneficial effects on experimental colitis [4], [5].

Snail mucus has lots of hidden properties, many of them have been discovered even in early history and in recent years' scientific researchers have demonstrated that mucus-derivate drugs can be used in a large variety of therapies. For example, it is used in creams to ease skin abrasions and scars, to cure respiratory diseases, heartburn and at last scientists discovered unexpected and previously unknown properties. At the beginning of twentieth century, a new interest about gastropods has grown up for their possible employment in medicine and industries. Scientists started

paying attention to the ability of snails in crawling on rough surfaces without hurting themselves and being resistant to pollutants and bacteria [6].

The objective of our work is to evaluate probable potential anti-inflammatory and antioxidant effects of the slime of *Helix aspersa* snail in experimental induced acute colitis in thirty male Wistar rats.

MATERIALS AND METHODS

Preparation of the "*Helix aspersa* slime":
To extract the slime, we used the simplest means in heliciculture, this allowed us to have a pure fresh slime, i.e. with a sterile wooden rod stimulate the snail, by rubbing the rod on its muscular foot, this makes the snail to secret more slime; the slime was collected and kept in a sterile container which was then preserved at (- 30 °C) until use. At the time of the use, the slime is placed in conical tubes, then it's left at the room temperature centrifuged at 5000 tr/min for 10 min, the supernatant is collected by pouring it into a new sterile container and it's ready to be used in the experiment.

Experimental design Protocols

- **Treatment of the animals before induction of inflammation:**

The guidelines for use and care of all experimental animals were faithfully respected [24, 25] (according to the guide for the care and use of laboratory animals).
The experiments were carried out in male Wistar rats, weighing 150–200 g, obtained from the breeding farm of Pasteur's Institute in Algiers and the breeding farm of the University of Constantine 1. Also in male mice of the species *Mus musculus*, weighing on average 30 g for toxicity studies, obtained from the Institute of Pharmacy in Constantine. The animals were acclimatised for 15 days under laboratory conditions. They were fed with standard diet, and water was provided ad libitum.

- **Toxicity Studies**

An oral toxicity test for the slime extract of the snail *Helix aspersa* was carried out according to the protocol of [7, 25], in six groups of ten mice of the species *Mus musculus*, weighing on average 30 g. This study lasted fifteen days in the manner as follows:

The mice received the slime by forced feeding the amounts: 3.33 ml/Kg (Group 1), 6.66 ml/Kg (Group 2), and 9.99 ml/Kg (Group 3), and the Groups 4, 5 and 6 respectively received 19.98 ml/Kg, 26.64 ml/Kg and 33.3 ml/Kg. This gives us 100 µl (Group 1), 200 µl (Group 2), and 300 µl (Group 3), and the Groups 4, 5 and 6 respectively received 600 µl, 800 µl and 1000 µl per mouse.

The mice were under surveillance every day after forced feeding for thirty minutes, one hour, five hours and twenty-four hours, as well as the temperatures and weights of mice were recorded, during 15 days of the study
.

- **Experimental studies in rats**

Thirty healthy male Wistar rats, weighing 150 – 200 g, were used in the study and were divided into five groups of six rats. They received treatment of NaCl 0.9% (Sodium Chloride), 5-ASA (commercialized anti-inflammatory drug) and the pure slime of *Helix aspersa* snail for the first seven days of the experiment. The groups are as follows:
Group C: (negative control group) which received 1ml NaCl 0.9% by forced feeding for one week.
Group ACT: (positive control group) which received 1ml NaCl 0.9% by forced feeding for one week.
Group ASA+ACT: this group received an amount of 100 mg of 5-ASA/Kg of animal weight/day for one week by forced feeding.
Group Slime: this group received an amount of 20 ml of slime/Kg for one week by forced feeding, according to our toxicity study of the slime, the allowed amount was of 20 ml/kg, which makes us 5 ml/rat.
Group Slime+ ACT: received an amount of 20 ml of slime/Kg for one week by forced feeding.

- **Induction of the inflammation with 4% Acetic acid (ACT) on the 8th day of the experiment:**

All the rats were put under the same conditions: they fasted for 36 hours before the induction, this was done on the 8th day of the experiment (23)
Group C: received 1 ml of NaCl 0.9% by rectal injection.
Group ACT: received 1 ml of ACT (4%) by rectal injection.
Group ASA+ACT: received 1 ml of ACT (4%) by rectal injection.
GroupSlime: received 1 ml of NaCl (0, 9%) by rectal injection.
Group Slime+ACT: received 1 ml of ACT (4%) by rectal injection.

Lastly the treatment of the animals after the inflammatory induction, it is the same as before the induction for the last seven days of the experiment. The whole experiment has lasted for fifteen days.

- **Biochemical analyses**

After fifteen days of the experiment, all animals were anaesthetized with chloroform for 2 minutes before withdrawal of blood samples; a capillary was inserted in the cavernous sinus of the animal and blood obtained was directly collected in dry tubes for the analyses of C-reactive proteins (CRP). All tubes undergone centrifugation at 5000 turns/min for ten minutes. The supernatants were collected and conserved at – 30 °C until use. CRP serum levels were measured by an Immunoturbidimetric method using commercial Randox kit (UK) with standards provided by the same firm.

After that, all animals were sacrificed by chloroform overdose for the MDA assay and histological analyses.

Immediately after animals' sacrifice, the abdomen was opened, the livers and colons were exposed. The livers and colons were excised, preserved in NaCl 0.9% and conserved on ice for the study of Tissue Total Proteins, Malondialdehyde (MDA) assay and Histological analyses for the colons.

- **Tissue Total Proteins level:**

A 0.5 g of liver samples was homogenized with TCA (Trichloroacetic) for the extraction of proteins of the liver, according to method of Bradford [9].

- **Malondialdehyde (MDA) level:**

The level of MDA in the liver was determined by the technique [10].100 Mg of liver samples were crushed in 0.9 ml of KCl (1.15%). The reactional medium contains 0.2 ml of the homogenate obtained, 0.2ml of SDS (Dodecyl Sodium Sulphates) (8.1%) solution, 1.58ml of acetic acid at 20% (pH 3.5) and 1.5 ml of TBARS (Acid Thiobarbituric) (0.8%). The final volume of the reactional medium was adjusted to 4 ml of distilled water. Incubate in the bain-marie at 95C° for sixty minutes. After cooling, 1 ml of distilled water and 5 ml of N-butanol-pyridine are added in the reactional medium. Then it's mixed with vortex. Centrifuge at 5000 turns/min for ten minutes. Estimate the level of MDA with a spectrophotometer at λ = 532 nm, according to the standard curve of MDA.

Histological analyses of the colon

The abdomen was opened and the colons were exposed. The colons were excised and opened by longitudinal incision. After washing with saline water, macroscopic observation of the colons was carried out. For the Group C (1) the colon appears to be normal, round and healthy, comparing Group ACT with Group C, Group ACT (2) presents nodules and it is rich of vascularization. For Group ASA+ACT (3) the colon is irregular (flat), with the absence of vascularization and it has a milky color. The colons of the Group Slime and Group Slime+ACT are shiny and clean compared to the three previous Groups, the Group Slime (4) kept a regular round shape, the Group Slime+ACT (5) shows very weak vascularization and it is thin. Formalin fixed tissue samples were embedded in paraffin and stained with Haematoxylin-Eosin stain. The microscopic observation was done using a photo-microscope (OPTECH®) and images were taken, whereby the histological damages and improvements were observed.

Statistical analyses

All the values are expressed in the form of mean ±SEM. The results were analysed by ANOVA with a factor followed by a test of LSD Fisher [11]. A value of $p < 0.5$ is considered statistically significant.

RESULTS

- **Toxicity study results**

No sign of toxicity or mortality was recorded during the experiment as well as the weights and temperatures of the mice were stable during the fifteen days.

Table 1: Effect of *Helix aspersa* Slime in induced Colitis

Groups	CRP (mg/Lserum)	Tissue Total Proteins (g /mL)	MDA (µM /g tissue)
C	1,407±0,19	2,52±0,27	1,61±0,68
ACT	6,68±0,74*****	4,45±0,49*****	2,49±0,57*****
ASA+ACT	3,95±0,41****,c**	2,58±0,55****, c**	2,12±0,65****, c**
Slime	2,22±0,57*****	4,65±0,49*****	1,21±0,65*****
Slime+ACT	2,18±0,4******	3,05±0,32******	1,80±0,65******
ANOVA (p<0.5)	**p<0,016**	**p<0,007**	**p<0,021**

*Value expressed as mean ±SEM (n=6). *p: in comparison to normal control;*
***p: in comparison to experimental control; c: in comparison to standard group.*

- **Macroscopic observations**

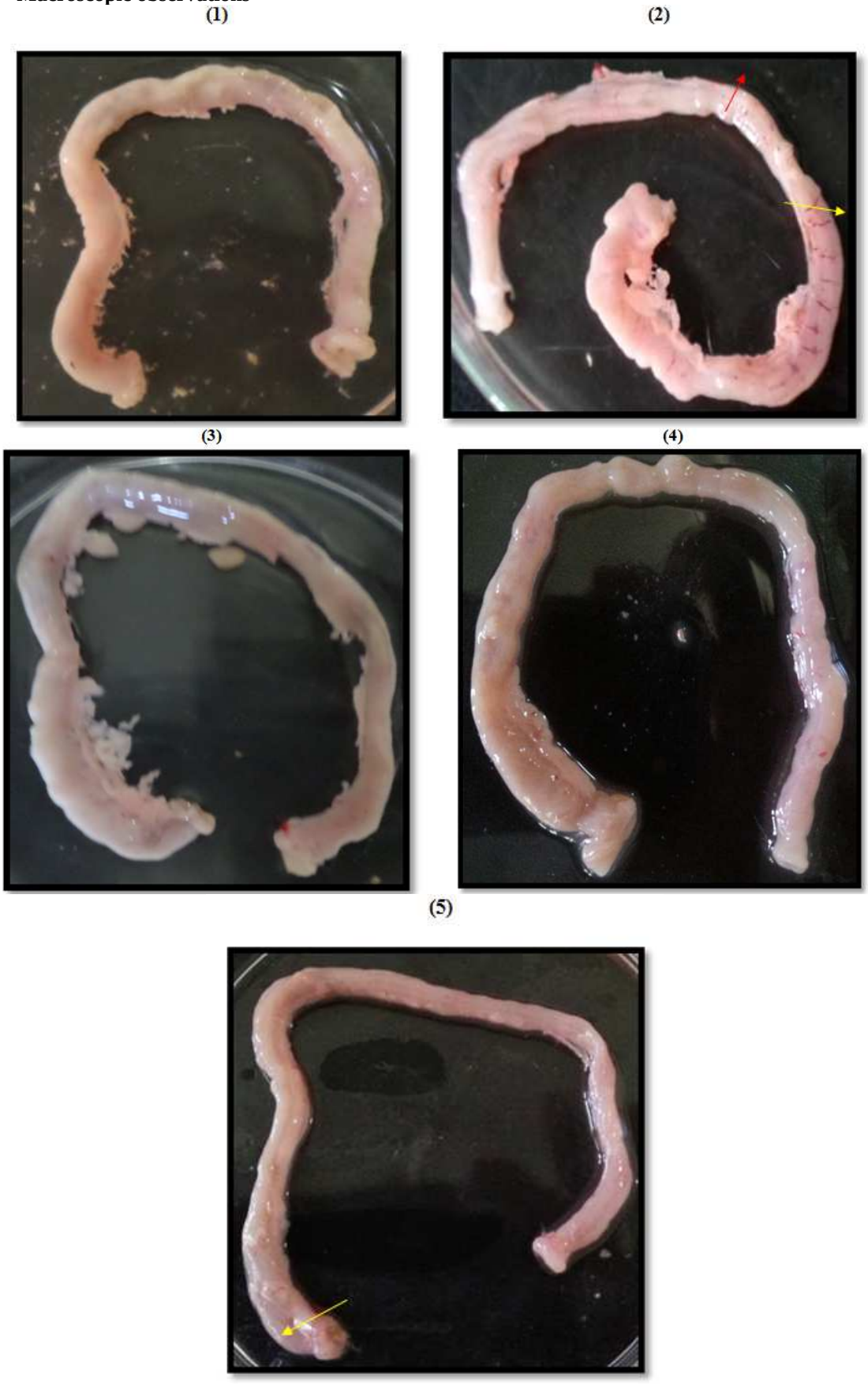


Figure 2: Group ASA+ACT (3): The colon is flat instead of round, no vascularization and with a milky colour; Group Slime (4): Regular round shape and shiny; and Group Slime+ACT (5): Shows very weak vascularization (yellow arrow), thin and shiny.

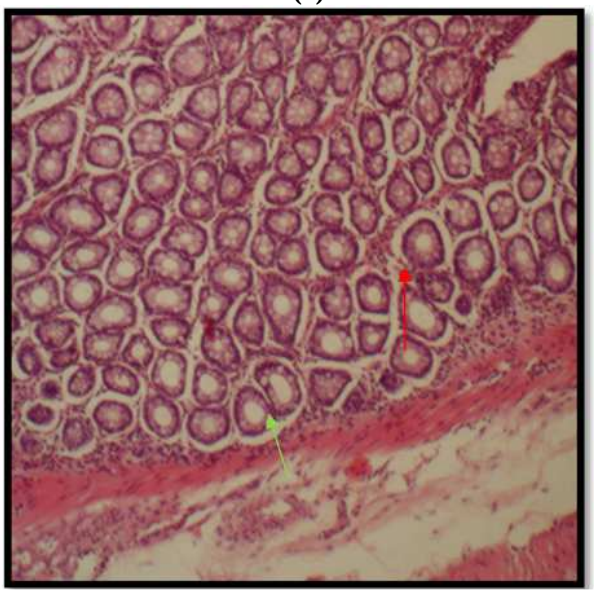
Table 1 Shows that Group ACT induced a significant increase of CRP in the serum 6,68±0,74Mg/L but also Group ASA+ACT shows a slit increase, while Groups Slime and Slime+ACT shows an increase respectively compared to Group C. The averages are significantly different.

It also Shows that Group ACT induced a significant increase of MDA with 2,49±0,57µM/g, but also the Groups ASA+ACT, Slime+ACT and C show an increase respectively contrary to Group Slime. The averages are significantly different.

Group Slime induced a significant increase of proteins in the liver 4,65±0,49g/ml but also the Groups ACT and Slime+ACT shows an increase contrary to the Groups ASA+ACT and C respectively. The averages are significantly different.

- **Microscopic observations**

(1)



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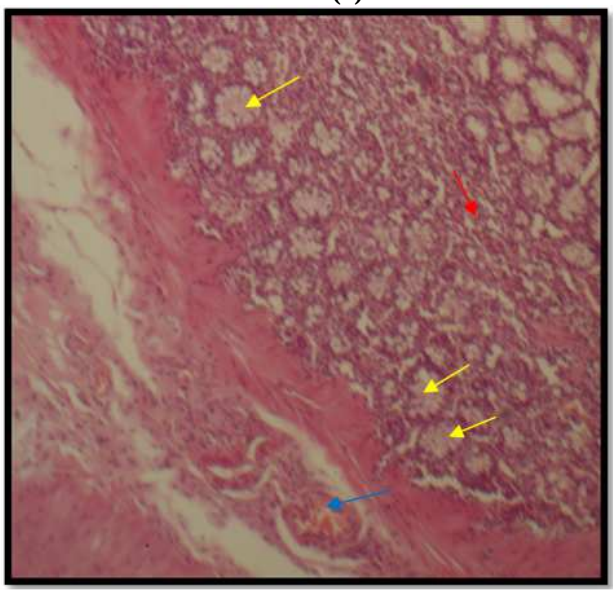

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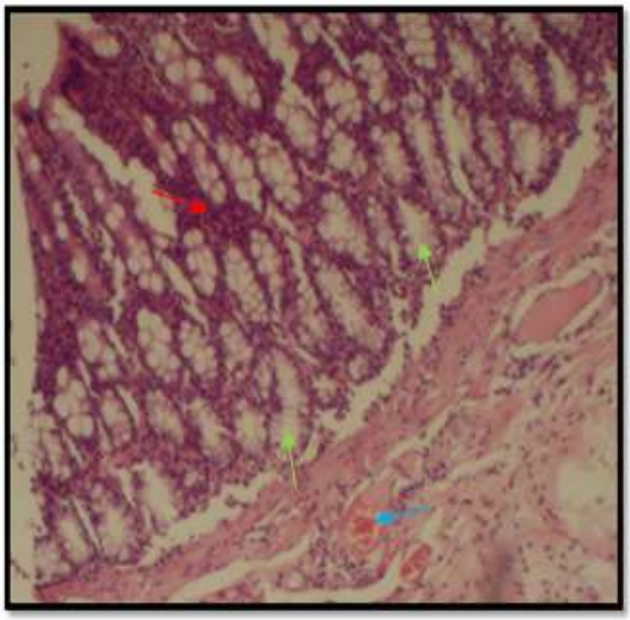
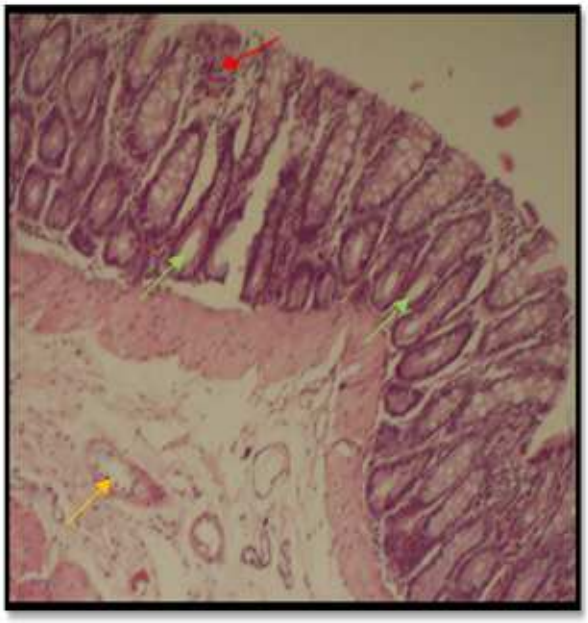


(4)



(5)

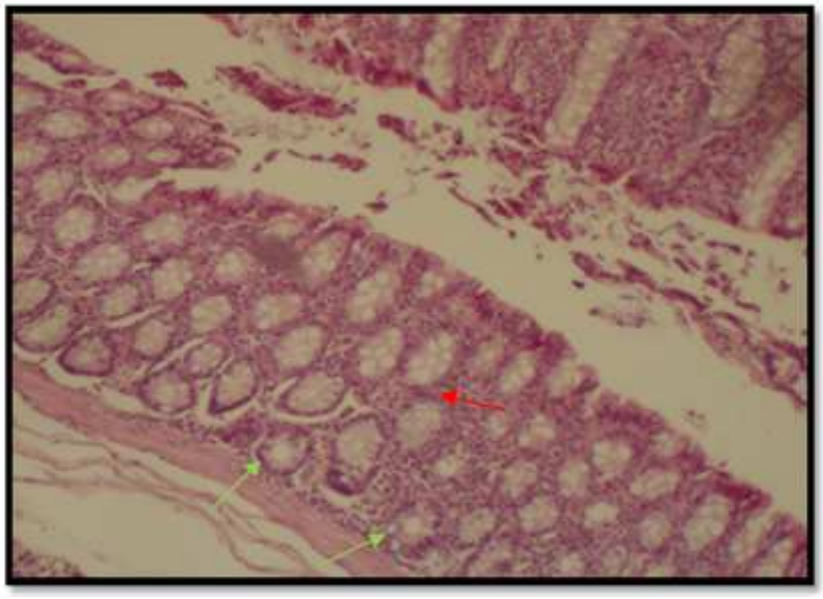


Figure 4: Group ASA+ACT (3): There is a reduction of the ACF, an organization and preservation crypts (green arrows), the lamina propria is reduced contrary to Group ACT meaning less neutrophil infiltration of the mucosa (red arrow) and with a vascularization in the submucosal layer (blue arrow). That shows 5-ASA, has protected and prevented the mucosal layer against the ACT. Group Slime (4): Shows well-organized crypts (green arrows), the lamina propria is normal (red arrow) and a submucosal layer rich in lymphatic vessels (orange arrow). Group Slime+ACT (5): Some crypts have been preserved and are regular (green arrows), reduced ACF (lysis of some mucosal epithelial cells) and a lamina propria which is reduced than in Group ACT meaning less neutrophil infiltration of the mucosa (red arrow).

All that shows that the treatment by the slime of Helix aspersa has reduced the inflammation caused by ACT.

DISCUSSION

Acetic acid induced colitis model is similar to human ulcerative colitis in terms of histological features. It affects the distal colon portion and induces non-transmural inflammation, massive necrosis of mucosal and submucosal layers, mucosal oedema, neutrophil infiltration of the mucosa and submucosal ulceration. The protonated form of the acid liberates protons within the intracellular space and causes massive intracellular acidification resulting in massive epithelial damage. Inflammation is the pathogenesis of IBD, and several pathways are associated with inflammatory response in IBD due to mucosal intestinal flora [12]. The inflammatory response initiated by acetic acid includes activation of cyclooxygenase and lipoxygenase pathways [13], [14].

The results showed that the slime extract of *Helix aspersa* has got a significant protective activity against experimental colitis in rats, as indicated by macroscopic, microscopic, serological and biochemical evaluations.

Evidence is mounting supporting the idea that aberrant crypt foci (ACF) are colon cancer precursors [15], whose size and numbers directly correlate with the risk of developing colon cancer. Aberrant crypt foci were first discovered by Bird in 1987 [16]. ACF formation accompanies changes in the morphology of colonic crypts in both benign diseases of the bowel and colon cancer [17]. The induction of colitis with acetic acid has induced the formation ACF in rats of the Groups (ACT, ASA+ACT and Slime+ACT) were induction was done. Where in Group ACT the ACF are very well visible considering it's a positive control, comparing it to Slime+ACT shows that the treatment by the slime of *Helix aspersa* has reduced the ACF and inflammation caused by ACT in this Group. This slime might have preventive and protective properties against the formation of ACF.

CRP is one of the most important proteins that is rapidly produced by hepatocytes during an acute-phase response upon stimulation by IL-6, TNF-α, and IL-1-β originating at the site of inflammation or pathology. CRP is therefore a good marker of measuring disease activity in CD [18]. This work has shown a significant increase of CPR in Group ACT, meaning that the inflammation in this group persisted because of no treatment in this group. But Group Slime+ACT compared to Group ACT and ASA+ACT, its CRP is very low. This indicates that the slime might have anti-inflammatory properties.

MDA is considered as an important indicator of lipid peroxidation [19], which is found to be increased in rats treated with acetic acid. This might be due to lipid peroxidation. Rats treated with *Helix aspersa* slime showed

protection against lipid peroxidation characterised by significant decrease in MDA level. Meaning the slime contains natural antioxidant molecules. Oxidative stress is believed to play a key role in the pathogenesis of IBD-related intestinal damage [20]. Intestinal mucosal damage in the IBD is related to both increased free radical production and a low concentration of endogenous antioxidant defence [21]. The antioxidant enzymes, mainly superoxide dismutase and catalase are first line defensive enzymes against free radicals and, ascorbic acid is also known to control oxidative damage [22]. In the present study it was observed that the *Helix aspersa* slime significantly increases level of total proteins (meaning it contains some of those antioxidant enzymes) in colitis induced rats. This shows that the *Helix aspersa* slime can reduce reactive free radicals that might lessen oxidative damage to the tissues.

The slime of *Helix aspersa* is rich in vitamins A, C and E, which have nourishing, anti-inflammatory and antioxidant effects. Also composed of a large amount of mucopolysaccharides components rich in glycine, proline and glutamic acid, these components plays a role in repairing ulcers [23].

CONCLUSION

Our study was only an initiation in research on immunology to evaluate the anti-inflammatory, antioxidant and antitumor effects of the slime of *Helix aspersa* snail on the chemically induced colitis. This work showed that a treatment by the slime offered protection against colon inflammation, thus the slime is a good immune stimulant and could also contain natural antioxidant molecules.

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