PREVENTIVE EFFECT OF HELIX ASPERSA CRUDE EXTRACT AGAINST CHEMO-INDUCED COLONIC DAMAGES IN RATS

Meriem Kouachi \(^1\), \(^2\), Dalila Naimi \(^*\), \(^1\), \(^2\), Djalal Djadouri \(^2\) and Ismahane Bendahra \(^2\)

Higher National School of Biotechnology \(^1\), Taoufik Khaznadar – nouveau pôle universitaire Ali Mendjli BP E66, 25100 Constantine - Algeria.
Laboratory of Microbiological Engineering and Applications \(^2\), University Frères Mentouri - Constantine, molecular and cellular biology and physiology team, route de Ain el bey Constantine 25000 - Algeria.

**ABSTRACT:** The present work aimed to explore in vivo the effect of Helix Aspersa Crude Extract (HACE) on the chemo-induced damages occurred in rat colon specially on the histology of the crypts foci in liberkhun glands. The toxicity of HACE was evaluated with different HACE concentrations. With the concentration of 160 mg, animals showed the best gain weight, stayed normal and all animals survived. Therefore, this concentration (5g/kg of body weight) was chosen for the rest of the experiences. 30 males rats used in our experiment were divided in 5 groups and colitis was induced by transrectal administration of 4% acetic acid. At the end of experiments, blood was collected for Biochemical assessments including aspartat aminotransferase (AST), alanine aminotarnsferase (ALT), gamma glutamyltarnsfersase (GGT). Thiobarbituric acid reactive substances (TBARs) and CRP measurements were also registered. Colon was examined macroscopically and microscopically after standard hematoxylin-eosin staining. We sought the changes in the crypt foci (CF) structure of the Lieberkuhn glands because foci damages were regarded as precursor lesions of colo-rectal cancer in human. HACE showed not only a significant improvement of certain parameters affected by experimentally induced colitis: enzymes TBARs, CRP, but also a protection against structure injuries in distal colon. The Liberkhun glands injuries observed in animal induced colitis appear to be protected by snail crude extract. The HACE could probably play a healing role on the colonic mucosa and also a preventive role against possible changes of CF to Aberrant Crypt Foci ACF and could avoid possible malignant transformation.

**INTRODUCTION:** In the Worldwide, 2.5 million people suffer from inflammatory bowel disease (IBD). These chronic inflammations of the colon and gastrointestinal tract are the result of an inadequate intestinal immune response against the bacteria of normal intestinal flora. Feeling unjustly attacked, it will trigger the inflammation of the intestinal mucosa. In the absence of aggressors, the immune system (the GALT) engages defense mechanisms more harmful than protective.\(^1\),\(^37\)

Usually, the diagnosis can be confirmed by endoscopic or sometimes radiological studies, as well as inflammation markers assay.\(^2\)

On the other hand, experimental studies on animal models are often interested in furthering the use of carcinogenic to check for damages in ACF (Aberrant Crypt Foci).

Previously, no study was interested to seek these changes in chemo-induced inflammation in animal model.
The colon has no villi but only crypts or glands called «Lieberkühn glands». In some cases and unpredictably, chronic inflammation of the colon may progress to cancer; It’s why we try in this present work to explore the histological changes in Lieberkuhn glands because changes of crypt foci CF to aberrant crypt Foci ACF could lead to malignant transformation.

Aberrant crypt foci were described by Bird as lesions consisting of large, thick crypts in methylene blue–stained specimens of colon from mice treated with a carcinogen: azoxymethane 3.

Few studies have explored the Lieberkuhn glands damages in chemo induced inflammation in animal models. Many studies have identified in rat colon the ACF appearing a few weeks after treatment with a carcinogen and becoming larger with time, with more marked nuclear atypia or dysplasia 4, 5, 6.

Many works reported that aberrant crypt foci are not only morphologically but also genetically distinct lesions and are precursors of adenoma and cancer 7, 8. Increased proliferative activity and KRAS mutations of aberrant crypt foci were also demonstrated 9, 10.

However, the studies mainly analyzed surgical specimens from patients with colon cancer in early stage or dissected colonic tissues obtained at autopsy with stereoscopic microscopy 11.

Unfortunately, data are completely lacking on normal subjects or patients suffering of chronic IBD and/or with adenoma. Such data could provide essential information about the relation of aberrant crypt foci to colon cancer and also could elucidate the progression of inflammation to early stage of cancer 11.

*Helix aspersa* is a kind of terrestrial snail which consumed by human. In the last decade, many snail farms have been established in the world, on one hand to compensate for the decrease in natural population in certain countries and on the other hand in order to produce good quality snails for consumption 12. Snail farming is a very lucrative agricultural activity given the fact that snail’s meat provides high level quality of proteins. Also, terrestrial Snails are an important source of microelements (salt, copper, iron, phosphor).

Deficiency of certain microelements can be responsible for an increased risk of many diseases, such as: cardiovascular diseases, several forms of cancer, immunodeficiency, allergies and inflammation. Many studies confirmed in human nutrition, the potential quality of snails protein content. However, its importance in Human therapeutics is poorly documented.

In the rat model, the formation of aberrant crypt foci was enhanced by cancer promoters (such as chenodiol) and suppressed by chemo preventive agents (docosahexaenoic acid and aspirin) 4, 5. Aberrant crypt foci similar to those in rodents have also been reported in colonic mucosa in humans 13, 14.

Paying particular attention to the histological progression of CF to ACF, we tried to explore *in vivo* the effect of *Helix aspersa* crude extract (HACE) on the chemo-induced damages occurred in rat colon and also we investigated the impact of these damages on the CF and their possible transformation to ACF. And then, we evaluate the chemopreventive effect of the HACE on this possible progression.

**MATERIELS AND METHODS:**

**Preparation of HACE (*Helix aspersa* Crude Extract):** Snails are collected from farms located at the east of Algeria. The shell is removed carefully 15. Just the feet end the head are recovered and used in this study. Feet and head are then immersed in a NaCl solution (12%) to remove any trace of the slime.

After that, they are rinsed with tap water, then distilled sterile water and drained, ground, homogenized and filtered. Fresh HACE obtained was weighed and administered to animals.

Before experimental treatment of animals the composition and the toxicity of the HACE were performed.

**Characterization of the HACE:** The fresh HACE, was lyophilized; 2.5 g of the obtained lyophilizate were added to 100 ml of a mixture of chloroform and hexane (2/1 v/v), the solution was centrifuged at 8000g for 10 minutes at 4 °C 16.
Lipids assay: After centrifugation, the supernatant was used for determination of lipid. In hot and sulfophosphoric media, lipids develop with vanillin an assayable pink coloring spectrophotometer, based on a range of sunflower oil standard (concentrations from 0 to 5 mg / ml).

The pellet (after evaporation of chloroform and hexane) was added to 100ml of ultrapure water and left to macerate for 24 hours at 40 °C with stirring. After 24 hours, the solution was centrifuged at 8000 g for 10 min at 4 °C. The supernatant was used for the determination of proteins, total sugars, uronic acids, sulfated sugars and polyphenols.

Total protein assay: The protein content was determined using the method of Bradford using Coomassie blue G250 and bovine serum albumin as standard.

Total sugar assay: The total sugar assay was performed according the method of Dubois and al. 1956.

Uronic acid assay: The composition of uronic acid was determined through the use of the method developed by Blumenkrantz and Asboe-Hansen (1973) modified by Filisetti-cozzy et al., (1991).

Sulfated sugar assay: The sulfated sugar content in the medium was determined by the colorimetric assay developed by JAQUES, L.B., et al., (1968).

Polyphenols assay: The phenolic compounds from the HACE are determined according to the method of Sinleton and Rossi (1965) using the Folin-Ciocalteu reagent.

Assessment of the HACE Toxicity: The guidelines for use and care of all experimental animals were faithfully respected (according to the guide for the care and use of laboratory animals).

Assessment of the HACE toxicity was performed according to the OECD guideline standard methods. The animals are randomly selected, marked to permit individual identification. An oral toxicity test for the HACE was carried out according to the protocol of Amiard, 2011.

The animals were kept 1 week under careful control and observation before beginning treatments and have free access to food and water. The light /dark cycle was on 12 H basis with lights on at 06.00 a.m. and off at 06.00 p.m.

Three mice groups (10 animals /group and 5 animals / cage) were respectively submitted to 120mg, 140mg and 160mg/body weight on alternate days during 15 days where we recorded the temperature, weight, and animal behavior. Very few changes have been observed and all animals survived.

Experimental design: According to the protocol of Shalaby and Shatta modified by us, 30 males rats (150-200gr) used in our experiments were divided in 5 groups (6 animals/group) as following:

Control group: C group, received orally distilled water during 7 days and transrectal administration of distilled water on the 8th.

AA group (Acetic Acid group) received orally distilled water during 7 days and on the 8th day, transrectal administration of 1ml of 4% acetic acid followed by transrectal administration of 2 ml phosphate buffer solution with pH 7.2.

HACE/AA group received the crude extract (5g/kg) during 7days and on the 8th transrectal administration of 1ml of 4% acetic acid followed by phosphate buffer.

ASA group received 5-ASA (5-aminosalisylic acid acts against inflammatory bowel disease) as standard drug during 7 days and on the 8th they received transrectal administration of 1ml of 4% acetic acid followed by phosphate buffer.

HACE group received orally the crude extract during 7 days and on the 8th rectal administration of 1ml of distilled water.

At the end of the experiments, and after 12H fasting, blood was collected for Biochemical assessments including aspartat aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltarnsferase (GGT), and C-reactive proteins (CRP).
Enzymes were measured by commercial spin react kits. Levels of malondialdehyde MDA were measured in cytosolic fractions from 1g of frozen liver according to the method of Iqbal et al., 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 anesthetized by ether inhalation and immediately dissected for harvesting colon and then sacrificed by overdose of ether.

Colon was washed three times with PBS. 10 cm from the distal colon were collected and examined macroscopically. Then it was cut, fixed in Formalin, and embedded in paraffin, stained by routine technique with hematoxyline-eosine for microscopic investigation using a photomicroscope (OPTECH).

**Statistical analysis:** The statistical analysis was performed using the one way analysis of variance technique (ANOVA), completed by the test of turkey and fisher. p < 0.05 was considered as significant.

**RESULTS:**

**Composition of HACE:** Table 1 provides a brief determination of the nutrients in the HACE. The rate of proteins, lipids and polyphenols appears appreciable. Although the composition of the various foods has been known for a long time, it was relatively little information on the nutritional components of the snail right to consume. Snails have a low fat content and a high content of substances dietary minerals, essential amino acids and fatty acids useful

<table>
<thead>
<tr>
<th>Nutriments</th>
<th>Concentrations (mg/ml)</th>
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<tbody>
<tr>
<td>Lipids</td>
<td>1.778</td>
</tr>
<tr>
<td>Proteins</td>
<td>3.433</td>
</tr>
<tr>
<td>Polyphenols</td>
<td>0.132</td>
</tr>
<tr>
<td>Total sugar</td>
<td>3.218</td>
</tr>
<tr>
<td>Uronic acids</td>
<td>0.0371</td>
</tr>
<tr>
<td>Sulfates</td>
<td>0.0053</td>
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In the present work, we focused on polyphenols content since these compounds are considered to have anti inflammatory and anti tumor actions; and on the other hand few studies have focused to search polyphenols in gastropods tissues.

Dietary habits are estimated to contribute to, at least, one third of all human cancers, showing that dietary components can exacerbate or interfere with carcinogenesis.

The analysis Snail fat is useful because it provides our bodies of omega-3 fatty acids that the human body cannot synthesize itself and therefore must be obtained through food. This is very useful for health as they block arteriosclerosis and thrombosis and have an anti-inflammatory effect that can prevent allergies, depression and other nervous system diseases. Snail meat is a good source of calcium and phosphorus, two very important components for the growth of bone, as well as magnesium, sodium and phosphorus. Also, uronic acids may be complexed with toxic molecules for the organism and allow their elimination.

Carbohydrates in the diet are an important source of food energy with a range of chemical, physical and physiological effects. Some vegetables showed high content of polyphenols as mentioned in the Table 2.

<table>
<thead>
<tr>
<th>Vegetables</th>
<th>Total Polyphenols (mg GAE / 100g)</th>
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<tbody>
<tr>
<td>Artichoke</td>
<td>321.3</td>
</tr>
<tr>
<td>Parsley</td>
<td>280.2</td>
</tr>
<tr>
<td>Brussels sprouts</td>
<td>257.1</td>
</tr>
<tr>
<td>Shallot</td>
<td>104.1</td>
</tr>
<tr>
<td>Broccoli</td>
<td>98.9</td>
</tr>
</tbody>
</table>

Assessment of the HACE toxicity: With the concentration of 160 mg, animals showed the best gain weight and a slight decrease in temperature just at the beginning of the experiment. The behavior of all animals stayed normal. Therefore, this concentration (5g/kg of body weight) was chosen for the rest of the experiences (Fig. 1a, 1b).
HACE was no toxic, this means that the increase of CRP is related to chemo-induced colitis, CRP is a good marker of inflammation (Table 3). The chemo induced colitis obtained in this work showed changes in different biochemical parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyltransferase (GGT), confirming the alterations of liver function. The increase of ASAT and ALAT enzymes in ACT group suggest an hepatolysis. The increase of GGT enzyme informs of a possible cholestasis (Table 3).

Macroscopic examination of distal colon showed a rich vascularization in chemo-induced inflammation group comparatively to control one and HACE+ ACT group appeared healthier than the ASA group (Fig. 2). This suggested that the HACE could exert better effect on the induced damages.

A. Control group showed normal colon no macroscopic abnormalities were found in the in distal colon

B. ACT group has a rich vascularization (blue arrow) which clearly appears
Mucosal architecture is well organized in control group (A). Extensive necrosis (red arrows) in ACT group (B1) showed slight enlargement, irregularity (yellow arrows B1 B2), and elongation of the ducts (black arrows) findings consistent with the previously reported features of aberrant crypt foci but without dysplasia or hyperplasia. Histologic examination revealed a loss of polarity, hyperchromatism of the nuclei, and stratification of the nuclei of crypt epithelium, findings in agreement with the previously reported features of dysplastic aberrant crypt foci. Histological investigations showed normal and well organized mucosa architecture of colon in control group and reported the previously features of aberrant crypt foci but without dysplasia or hyperplasia in ACT group (Fig. 3 A, B). ASA seemed to be less protective against the chemo-induced damages than the HACE (Fig. 3 C, D, E).

**FIG. 2: MACROSCOPIC EXAMINATION OF DIFFERENT PARTS OF COLON**

A. Control group  
B1. ACT group  
B2. ACT group  
C. ASA+ACT group  
D. HACE+ ACT group the distal colon appears healthier than in the ASA group
DISCUSSION: The aim of this study is to find an animal model that allows us to detect in vivo early changes of the colonic mucosa that might induce inflammation, to see if this inflammation would affect the crypts of Lieberkühn gland and ultimately to test the healing effect of HACE and/or restorer the functions of the crypts foci.

ACT group showed an increase in all the parameters. HACE seems to restore these parameters. The chemo induced colitis showed changes in different biochemical parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyltransferase (GGT), confirming the alterations of liver function. The increase of ASAT and ALAT enzymes in ACT group suggest an hepatolysis. The increase of GGT enzyme informs of a cholestasis. Also, changes in thiobarbituric acid reactive substances (TBARs) and CRP levels confirmed the repercussion of the inflammation on the liver function and suggest an increase in free radical production, followed by low antioxidant Defense, leading to inadequate intestinal immune response of the GALT.

These changes appear to be corrected by the HACE. We focus in this chemo induced colitis, on the changes in the crypt foci (CF) structure of the Lieberkühn glands. We looked for changes in these CF, because foci damages were regarded as precursor lesions of colo-rectal cancer in human. Many studies reported positive correlation
between aberrant crypts Foci (ACF) multiplicity and the onset of colon tumors at the late stage.

The untreated inflammation can evolve over time to a chaotic proliferation of the cells of Lieberkühn crypts glands and could lead to the installation of malignant nodules. Our hypothesis was: if HACE could inhibit the evolution of a normal crypte to aberrant crypts Foci?

According to many results on polyphenols effects, we suggest that the HACE polyphenols could exert a healing effect on colon mucosa and restore the bacterium flora, which is important in immune response of mucosa.

In recent years, a large number of studies have attributed a protective effect to polyphenols and foods containing these compounds as fruits and vegetables (quercetin, rutin, myricetin, chrysin, epigallocatechin-3-gallate, epicatechin, catechin, resveratrol, and xanthohumol); however, these studies do not discuss clearly about the polyphenols bioavailability.

In the present work, we are interested specially to know about polyphenols content. These compounds have been reported to interfere with cancer initiation, promotion, and progression, acting as chemopreventive agents. In cell culture models, these compounds inhibited cell growth, by inducing cell cycle arrest and/or apoptosis, inhibited proliferation, angiogenesis, and/or metastasis and exhibited anti-inflammatory and/or antioxidant effects but it is difficult to extrapolate these results to humans.

The effects of these micronutrients on health depend on the consumed amount and bioavailability which varies from one to another polyphenol. This probably explains why the most abundant polyphenols are not necessarily those with the greatest biological activity in target organs and not necessarily those exerting protective effects to health. To date, no daily amount of antioxidants was recommended, but the Aprifel (fruits and vegetables Agency) gives us a value of about 1 gram of polyphenols per day. 20

Polyphenols found in HACE could provide very important biological activity and bioavailability too.

Although small amounts of polyphenols are present in snails, they could instead provide high bioavailability to the cells and target tissues. The metabolism of polyphenols needs many other micronutrients as vitamins (especially niacin or vitamin PP oxydo-reducing cofactor) and microelements as (salt, copper, iron, phosphor).

All these elements are found in Helix aspersa extract 12 15. Recently our team showed that the extract of Helix aspersa induces necrosis which is associated with BcL2 down regulation in Human breast cancer Hs578T cell line 29. Many other works performed by our team showed the antiproliferative and anti-inflammatory effects of Helix aspersa extract 31, 32, 33.

In this case, it seems that the apoptotic process has been prevented and the anti tumour effect of H.aspersa extract may be resulted from the action of the polyphenols that have anti inflammatory, antioxidant and anti carcinogenic activities, or from the action of peptides, lectins or polysaccharides that bind to some surface molecules. This research is being experimented.

As reported by many authors, the identification of novel predictor classifiers for inflammatory bowel disease by gene expression profiling before and after HACE treatment seems very interesting to be investigated in this animal model.

The assessment of Immunomodulatory activity of the HACE was also performed in vivo by using carbon clearance assay 36; preliminary results seem interesting and need to be confirmed.

We plan to study many other biological activities of the HACE in the aim to explore its effect in enhancing anti-cancer immunity and this work is in experimental progress 37, 38.

The HACE could probably play a healing role on the colonic mucosa and also a preventive role against possible changes of CF to ACF. (by regulation of pathway signaling)

HACE could also adjust the natural bacterial flora to avoid self-destruction of the colon which may leave room for the inflammation 39. In IBD, it is common to need to use immunosuppressive drugs. These drugs, promoting a reduction in the immune
system, are likely to promote the development of cancer and in particular lymphoma. However, multiple follow-up studies in IBD have shown no increased risk of cancer, particularly lymphoma among patients treated with immunosuppressive drugs and the general population.

It be aware that, in patients who are not suffering from IBD, colorectal cancer develops most often from a polyp, that is to say, a benign tumor that occurs at the mucosal colon. After a number of years at the mucosa of the polyp, architectural abnormalities appear, it is called dysplasia.

Abnormalities of the same type can be observed in ulcerative colitis on a flat mucosa and are the cause of cancer. It is this type of abnormality that is being sought by systematic multiple biopsies performed as part of the prevention of colon cancer in IBD.

HACE at the concentration used, showed preventive effect against chemo induced damages by acetic acid in the rat at the distal colon.

We consider that this model has been a good model to induce damages in CF in experimented rats compared to controls but we did not observe severe dysplasia or hyperplasia. We just found slight enlargement, irregularity and elongation of the ducts; findings consistent with the previously reported features of aberrant crypt foci but without dysplasia or hyperplasia.

We can suggest that this stage is the first one around which these changes must be detected in patients suffering of colitis.

We must pay particular attention to patients with repeated transit disorders of colon, because these disorders may leave room for the inflammation which in turn may develop into ACF.

We will soon investigate. Since the Helix aspersa snail has a high quality of protein and are an important source of microelements, and contains polyphenols, it would be interesting to associate it in a diet for patients in the early stages of hypertrophic crypts Foci in order to prevent and avoid possible malignant transformation.

The Helix aspersa crude extract may contain bioactive molecules with anti-inflammatory, immunostimulant, antioxidant and anti-tumor effects.

It remains to identify these molecules and study their chemical structure to better understanding the interactions with cellular components in the prevention against various pathologies. This work is currently under experimentation.

CONCLUSION: In this study, we developed an in vivo examination of a possible evolution of the CF to ACF in Lieberkühn glands, in chemo-induced colitis in rat. The present study revealed damages in crypt foci of Lieberkuhn glands. CF have an aberrant appearance but without hyperplasia and dysplasia. These first changes could be considered as the origin of the first inflammatory focus. These changes must be sought in patients with repeated transit disorders of colon. These damages should be detected at the early symptoms and the Patients should be monitored seriously to protect them against a possible evolution of this first inflammatory focus; we should not wait until the inflammation settles.

If these damages are early detected, it becomes possible to subject the patients to rich diets in polyphenols. These preventive procedures could be better than the immunosuppressive drugs treatments which could induce malignant progression of CF to ACF. In this context, it would be interesting to associate Helix aspera and other nutriments rich in polyphenols in a diet for patients at the early stages of hypertrophic crypts Foci, in order to prevent and avoid possible malignant transformation.

CONFLICT OF INTEREST: The authors declare they have no conflict of interest.

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