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Anti carcinogenic Effect of Roman nettle Against chemical Induced Colon cancer in Sprague–Dawley Rats

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Abstract

Colon cancer is an aggressive cancer and it is reported to be one of the most treatment-resistant human cancer. This article describes the anti-carcinogenic activity of Roman nettle (Urtica pilulifera) in comparison to garlic (Allium sativum) against 1,2,-dimethylhydrazine (DMH) induced colon cancer. Histopathological study showed that DMH induced different grades of dysplasia, hyperplasia, neoplastic proliferation and malignant glands invading muscles. Compared to DMH treated rat, the rats received nettle or garlic extracts with DMH formed fewer and smaller tumours. On the molecular level, the results show that nettle extract induces high level of p21 protein suggesting that this extract plays its role by the induction of cell cycle arrest. Furthermore, both garlic and nettle extracts decreased Bcl-2 anti apoptotic protein level indicating that both plants may activate apoptosis as a mechanism of prevention against DMH induced colon cancer. Together these findings suggest that nettle and garlic consumption may play an important role of colon cancer prevention.

Keywords:
Colon Cancer,
Nettle , Garlic,
Cell cycle arrest,
Apoptosis

1. Introduction:

Colorectal cancer is the third most commonly diagnosed cancer worldwide with nearly 1.4 million new cases and 693,900 deaths reported in 2012 1. Enormous efforts have therefore been invested to cope with this problem, but unfortunately limited success has been achieved with most of the current therapeutic strategies. As the conventional cancer therapies failed to completely fulfil the criteria for a successful cancer therapy, the use of naturally developed anticancer agents has evolved as an alternative safe, low-cost and convenient one. Plants with known therapeutic potential have long been used to cure a wide range of diseases. Historically, researchers have been investigating plants, herbs, vegetables and fruits for identifying naturally occurring chemo preventative agents which could be used in combination with other therapies 2.

Roman nettle also known as Urtica pilulifera, a facultative wetland plant, is common in many parts of the world. It is a member of the urticaceae family which is considered as an invasive plant. Members of the genus Urtica, including Urtica massaica, Urtica parviflora, Urtica pilulifera, Urtica urens, and Urtica dioica are known for the stinging sensation caused by the injection of histamine, acetylcholine, and Serotonin into the skin by fine, needle-like projections found on the leaves and stems of the plant 3. Despite this deterrent, many of these nettle species have been used for generations in the preparation of herbal medications 4. Extracts of Urtica dioica were shown to have an antiproliferative effects against

several cancer cells 5. Such effects were determined by testing methanolic and aqueous extracts from, milled dried leaves, stinging nettles or roots 3–5. The antitumor activity of Urtica members was attributed to its chemical constituents of flavonoids and aromatic compounds with antioxidant properties. In an ethno-botanical survey carried out in the west bank, Palestine to evaluate the most popular traditional plants, Urtica pilulifera was reported as a popular traditional plant used as aphrodisiac, diuretic, and fresh young leaf are eaten to treat kidney stone rheumatism and bleeding 6. Another survey conducted in the west bank and Negev desert, of Palestine concluded that Urtica pilulifera is a traditional plant widely used in the treatment of cancer. It was suggested that a standard decoction be prepared from 50 g plant leaves in one litter and taken orally, 150 ml, 3-4 times/day until the condition improves 7.

In this study, we investigated the anti-carcinogenesis activity of the Urtica pilulifera (nettle) extract compared to garlic extract against colon cancer induced by (DMH). Several studies showed that DMH specifically induces colon cancer in rats similar to the human colon cancer 8,9. Therefore, this model has been used extensively to evaluate and compare the efficacy of several agents, natural and synthetic, in preventing colon cancer occurrence.

Our data show that nettle and garlic extracts inhibit the growth of colon cancer and this was accompanied by increasing levels of p21 and decreasing levels of Bcl-2. These observations indicate that both nettle and garlic extract may activate cell cycle arrest and apoptosis in colon cancer cells.

2. Materials and Methods

2.1 Chemicals

DMH was purchased from Sigma–Aldrich and 4 mg/ml solution was freshly prepared by re suspending DMH in saline solution and buffered to ph7 by NaOH [8,10].

2.2 Plant extracts preparation

Twenty grams of grounded nettle leaves or peeled garlic cloves were soaked in 80 ml distilled water (20% wt/v) overnight and crushed in a blender for 30 sec at room temperature. The extract was then passed through a 0.22 µm filter and the filtrate was used fresh [2,11].

2.3 Animals

Female Sprague-Dawley rats at the age group of 45-48 d were procured from the Islamic University -Gaza. The animals were housed in well ventilated large spacious polypropylene cages and had 12 h light and dark cycle throughout the experimental period. The animals received a balanced diet of commercially available pellet rat feed and water.

2.4 Experimental design

The rats were divided into six groups and each group consisting of six animals. Group 1; garlic extract only 0.5 ml/100 gm daily, group 2; garlic extract as in group 1 + DMH 20 mg/kg weekly, group 3: nettle extract 0.5 ml/100 gm only (daily), group 4: nettle extract as in group 3 + DMH 20 mg/kg weekly, group 5: DMH 20 mg/kg weekly and group 6 received DMH vehicle only (daily). Animals in group 1-4 received plant extracts for two weeks before starting the DMH treatment of animals in groups 2,4 and 5. Group 2, 4 and 5 received DMH doses in 0.5 ml saline given orally and group 6 received 0.5 ml saline weekly.

Animal weight was monitored weekly for the period of twenty weeks after 1st DMH injection. At the end of the experiment rats were anesthetized using chloroform and then dissected for colon samples collection. All tumors in each rat were weighed for further statistical analysis.

2.5 Pathological Evaluation

After dissection, colons were open longitudinally, and fixed overnight in 4% buffered formaldehyde (Sigma–Aldrich) at 4 Co, washed twice in PBS and stored in 70% ethanol at 4 Co. For histopathological determination tumours or Gross lesions where present, were sectioned and paraffin embedded. When no evident lesions were present, the whole colon was paraffin embedded. Sections were hematoxylin and eosin (H/E) stained and evaluated by a pathologist.

2.6 Protein Extraction and Western Blotting

For immunoblotting, proteins from each group (three tumours from three animals per group) were homogenized in called PBS/TDS 12 and extracted in whole cell lysis buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol and 0.02% bromophenol blue) 13. Samples boiled for 10 minutes and proteins were resolved by SDS/PAGE (8 and 15% gels) as required and transferred to Hybond ECL membranes (Amersham Biosciences). The membranes were incubated with primary antibodies against p21 (sc-756) (sc 752) (Santa Cruz, California, USA), BCL-2 (#2876) from Cell Signaling (Boston, MA, USA) and the p38 antibody (M0800) was from Sigma (St. Louis, MO,

USA). After the primary antibody incubation, the membranes were incubated with appropriate HRP-conjugated secondary antibodies (1:5,000) (Biorad) and antibody-reactive proteins were visualised using the chemiluminescence reaction (ECL) detection system (Thermo Scientific, Hudson, NH, USA). Membranes were washed again with 1XPBS/T and visualised by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

2.7 Statistical analysis

Data presented are mean ± SEM (Standard error of the means) of three independent experiments or samples. Statistical significance was assessed between the animal groups using the Student's t-test. A value of $P < 0.05$ was accepted as statistically significant.

3. Results

3.1 Roman nettle protects against colon cancer

To investigate the prevention effect of nettle extract against the colon cancer, colon tumour model induced by DMH was used and garlic extract was used for comparative purposes in the study. While the DMH only group showed a rapid tumor growth (Figure 1a and b), both garlic and nettle feeding attenuated tumor development as measured by total tumor weight (Figure 1c). Indeed nettle treatment led to more than 40% reduction in tumours weight and garlic treatment led to more than 50% tumours weight reduction.

Histopathologically, H/E stained sections from gross lesions or whole colons were evaluated and tumours from each experimental group were analyzed. Macroscopically, the neoplasm induced by DMH treatment was mainly of protrudent type to lumen side of the colon. Different grades of dysplasia, hyperplasia, neoplastic proliferation and malignant glands invading muscles were observed in the colon of DMH treated rats (Figure 2). While all DMH treated animals developed colon tumours, all control groups (received DMH vehicle only, Roman nettle only or garlic only) were healthy and free of tumours. Interestingly, two animals of DMH + garlic didn't show any tumours and the other four animals in the same group showed small size and little number of tumours. However, it's important to note that garlic treatment was associated with an inflammatory reaction in the wall of the colon. Importantly, Roman nettle significantly delayed colon cancer progression treated animals as judged by the less and small size of DMH induced tumours. More interestingly, Roman nettle treatment didn't show any visible or histological side effect. In addition, body gain weights (245.4-291.5 g) were similar among untreated group, garlic only and Roman nettle only groups suggesting that these plants had no adverse effect on the animal growth. However, by the end of the experiment DMH treated animals showed about 40% weight loss in comparison to the control group. Importantly, both DMH +garlic and DMH +Roman nettle groups showed about 20 % weight loss in comparison to the control group.

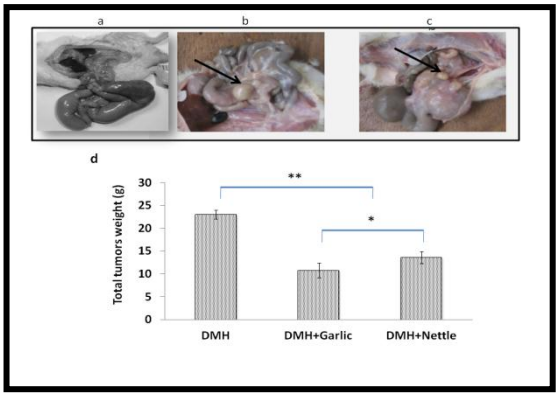

Figure 1. Garlic and nettle consumption inhibit tumor growth in vivo. Top panel, photographs show morphological features of colon tumours in DMH-treated rats in comparison to normal rat. Macroscopically appearance: a normal, b black arrow indicate sessile and c black arrow indicate pedunculate or polypoid tumours. d a graph shows total tumors weight (g) for each group measured 6 weeks after DMH treatment *P < 0.05, **P < 0.01.

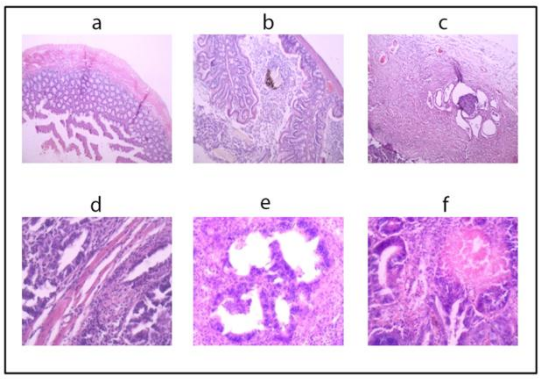

Figure 2. Histological features of rat colons. The morphology of rat colon tissues using paraffin-embedded sections stained with haematoxylin and eosin (H&E). a untreated , b nettle only shows normal colon features, c garlic only shows some necrotic features, d DMH treated, e DMH + garlic treated and f

DMH nettle treated. a, b, and c show normal colon features at magnitude X100 and d, e, and f show malignant features of rat colon tumours at magnitude (×400).

3.2 Nettle extract activates p21 and down regulates BCL-2 in colon tumors

The next set of experiments were performed to determine how Roman nettle exerts its anticancer effect. In these experiments protein was extracted and pooled from three tumour samples per group and subjected to western blotting with antibodies to the cell cycle regulator p21 and anti apoptotic Bcl-2 protein. Our results showed that both Roman nettle and garlic treatment significantly increased p21 and decreased Bcl-2 protein (Figure 3). However this effect was more noticeable in Roman nettle treated group which may indicate that this plant exerts its anticancer effect by inducing cell cycle arrest and enhancing of apoptosis. Taken together, these results clearly demonstrate the protective effect of Roman nettle consumption against colon cancer.

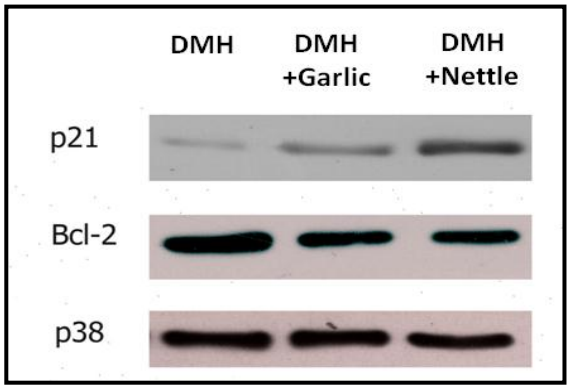

Figure 3. Nettle inhibits colon tumor growth in rats by induction of cell cycle arrest. Western blotting of proteins from each group (three tumours from three animals, per group) shows Bcl-2 and p21 levels. p38 was used as a loading control.

4- Discussion

Colon cancer is the third common cancer among men and women and its incidence is increasing in Gaza strip and worldwide 14. Lately, there has been improvement in the treatment strategies of colon cancer, which has resulted in prolonged survival of patients with chronic cancer disease. However there is a growing need for additional means of cancer therapy, in the form of both prevention and curative treatments. The strategies available today, are sophisticated, and are only able to affect 50 to 60% of cancer patients, while the others will eventually die from the disease 2,15,16. Arabic tradition is particularly rich in medical plants that have been used by pioneer Arabic physicians to establish the basis for modern therapies. Of these plants, garlic (Allium sativum) and Roman nettle (Urtica pilulifera) are traditionally used as a popular medication 3,17. Several studies have shown a decrease in cancer incidence with high consumption of raw and cooked garlic and that garlic offers potential protection against cancer development 11,18,19. On the molecular level, other studies have demonstrated that garlic extract exerts anti carcinogenic activities through inducing apoptosis in certain cancer cells in vitro 10,11,20,21. More recent research has shown that crude garlic extract inhibits cell proliferation and induces cell cycle arrest and apoptosis of cancer cells in vitro 2. According to the same study garlic extract induces G1 cell cycle arrest and sub-G1 peak (cell death) in cancer cells. Similarly our study showed that garlic extract inhibits colon tumour growth by inducing both apoptosis and cell cycle arrest in colon cancer cells as indicated by the increasing levels of p21and decreasing levels of Bcl-2. It is important to note that nettle extract showed a superior effect on the p21 level indicating that nettle might work mainly through this mechanism. These results are also in parallel to previous data showed that garlic constitutes induce G1 cell cycle arrest in MCF7 breast cancer cells 17. However, the same study revealed that garlic constitutes have some cytotoxic effect on MCF12 normal cells indicating some side effects of high dose of garlic treatment in vitro.

On the other hand several studies showed that different species in the Urticacae family have anti proliferative activities 22,23. Root extracts from Urtica dioica inhibited prostatic hyperplasia growth by more than 27% through inhibition of the Na, K ATPase activity 22. Similar effects were determined by testing methanolic aqueous extracts from deride milled stinging nettles root 24. Lymph node carcinoma of the prostate cell (Lncap), and human primary culture of the prostate stromal compartment (hpcps) were cultivated without extract (control) or treated with various extract concentrations at different time intervals. The results demonstrated that the methanolic- queous extract treated Lncap cells are reduced in proliferation in

comparison to the untreated controls, while hpcps remained unchanged.

More relevant to this study, intraperitoneal injection of Urtica pilulifera crude extract reduced Ehrlich Ascites Carcinoma growth in mice 17. This effect was attributed to the high content of bioactive components such as polysaccharides which were shown to reduce sialic acid and phospholipids in cancer cell membrane 25.

In agreement with these results, we have observed a potent anti tumor activity a combined with minor necrotic lesions in garlic treated rat colons. Significantly, we didn't observe any necrotic lesions in colons of nettle extract treated rats. In conclusion, this study shows that both nettle and garlic extracts inhibited colon cancer growth in vivo and the extract may induce its effect through inducing cell cycle arrest and apoptosis, however further experiments are required to confirm this.

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الملخص باللغة العربية:

سرطان القولون هو أحد أخطر السرطانات وهو احد أكثر السرطانات مقاومة للعلاج. هذا البحث يصف نشاط مضاد لسرطان القولون المستحث كيميائيا من نبات القريص الروماني بالمقارنة مع الثوم. وأظهرت الدراسة النسيجية أن المادة المسرطنة و تسمى اختصارا DMH أحدثت سرطان من مختلف الدرجات وتضخم، وانتشار الأورام والغدد الخبيثة غزو العضلات. بالمقارنة مع الفئران المعالجة DMH فقط، فإن الفئران التي تلقت مستخلصات نبات القريص أو الثوم مع DMH قد كونت أورام أقل عددا وأصغر حجما. على المستوى الجزيئي فإن الدراسة تبين أن مستخلص نبات القريص استحث مستوى عال من البروتين P21 مما يشير إلى أن هذا المستخلص يلعب دوره من خلال احداث وقف لدورة الخلية. و بشكل مشابه فان مستخلص الثوم ونبات القريص أدى الى انخفاض بروتين BCL−2 المضادة لموت الخلية مشيرا إلى أن كلا من النباتين قد ينشط وقف الخلية عن الانقسام وموتها كآلية للوقاية من سرطان القولون. و بالإجمال فإن هذه النتائج تشير إلى أن تناول نبات القريص والثوم قد يلعب دورا هاما في الوقاية من سرطان القولون.