

The Bioactive Ingredients in *Niuli Lactucis Agrestibus* Possess Anticancer Effects

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SUMMARY

In the field of medicine, natural treatments are becoming increasingly vital towards the cure of cancer. We investigated the effect of various concentrations of *Niuli Lactucis Agrestibus* crude extract on the survival percentage and proliferation rate of COLO320DM adenocarcinoma cells. Our experimental process consisted of the following methods: a Trypan Blue Cell exclusion assay for viability, a cancer cell migration assay, and a protein expression test. These tests were performed using varying concentrations of crude extract to test the impact of the chemicals in the extract on colorectal cancer cells. Our results for the cell viability test showed that the crude extract significantly increased the death rate of colon cancer cells, but not of healthy cells. Furthermore, the results suggested that increasing the concentration of crude extract inhibited colon cancer cell migration. In addition, we observed that the *Niuli* extract significantly upregulated the expression of p21 and p27, two key proteins in the cell cycle. These results indicate that the presence of bioactive ingredients in *Niuli* lettuce can exhibit anti-colon cancer properties. Further experimentation will be carried out to purify and identify the chemicals found within the crude extract that contain the potential for the treatment of colorectal cancer.

INTRODUCTION

The American Cancer Society reported that colon cancer is the third-most diagnosed cancer in the US with over 100,000 cases expected in 2019 (1). Colorectal cancer affects the lower area of the large intestine around the rectum and may only show symptoms after several years (2). Different colon cancer treatments are available for people in different disease stages, with many treatments involving surgery or other costly procedures. Additionally, if patients are not healthy enough, they may be unable to have the surgery, forcing them to opt for chemotherapy (3). Research has proven that chemotherapy, while killing cancer cells, also has a negative effect on healthy cells, resulting in numerous side effects (4). Some of the side effects include mood changes, fertility issues, hair loss (alopecia), infection, nausea and vomiting, sleep problems, urinary and bladder problems, etc. (5). As another option to the current cancer treatments, which are both dangerous and costly, plant extracts have gained

increased interest as a source of bioactive molecules for cancer treatment.

For instance, certain substances found in the bark of *Lafoensia* can be composed into a mixture capable of killing proliferating cancer cells (6). Another study stated that incorporating spinach, broccoli, lettuce, carrots, celery, greens, etc, and fruits rich in lutein, reduces the risk of developing colon cancer. In addition, frequent consumption of lettuce, decreases the risk of breast cancer (7). Similarly, one study found not eating salad and other leafy greens significantly increased the risk of cancer (8).

After further research, two cyclin-dependent kinase inhibitor proteins exhibited great potential to aid in the battle against cancer: p21 and p27. Firstly, the tumor suppressor protein, p21, is known for its ability to mediate the cell cycle by inhibiting several cyclin and cyclin-dependent kinase (CDK) complexes (9, 10). p21 also has a role in DNA repair through its interaction with the proliferating cell nuclear antigen (PCNA) (11). Furthermore, p21 and p27 have a crucial role in determining the fate of cancer progression. Although they are known to be tumor suppressor proteins, in some cases they also display oncogenic behavior. Recent research has found the subcellular localization of p21 and p27 to be the predominant factor influencing the dual properties of p21 and p27 (12). Nuclear accumulation of p21 and p27, through various mechanisms, leads to increased effectiveness of cancer treatments. When localized in the nucleus, p21 and p27 inhibit CDK complexes at the G1 or G2/M checkpoints, and therefore promote cell cycle arrest (13).

As hypothesized for centuries, recent research has shown that plants may be a much-needed substitute for our current cancer treatments (14, 15). Natural products shows great promise for the future of medicine and cancer treatment. We tested the extract of *Niuli Lactucis agrestibus*, a type of lettuce, for its ability to inhibit colon cancer cell proliferation. We aimed to investigate whether an increased dosage of the *Niuli* lettuce crude extract inhibits proliferation, increases the distance traveled during cell migration, and enhances the activity of tumor suppressors. We hypothesized that the chemical extract of *Niuli* lettuce would hinder the proliferation of colon cancer cells and that it would stimulate increased p21 and p27 protein expression. This increase in protein expression is hypothesized to be a deterrent to the colon cancer cell growth.

Our results demonstrate that *Niuli* lettuce extract

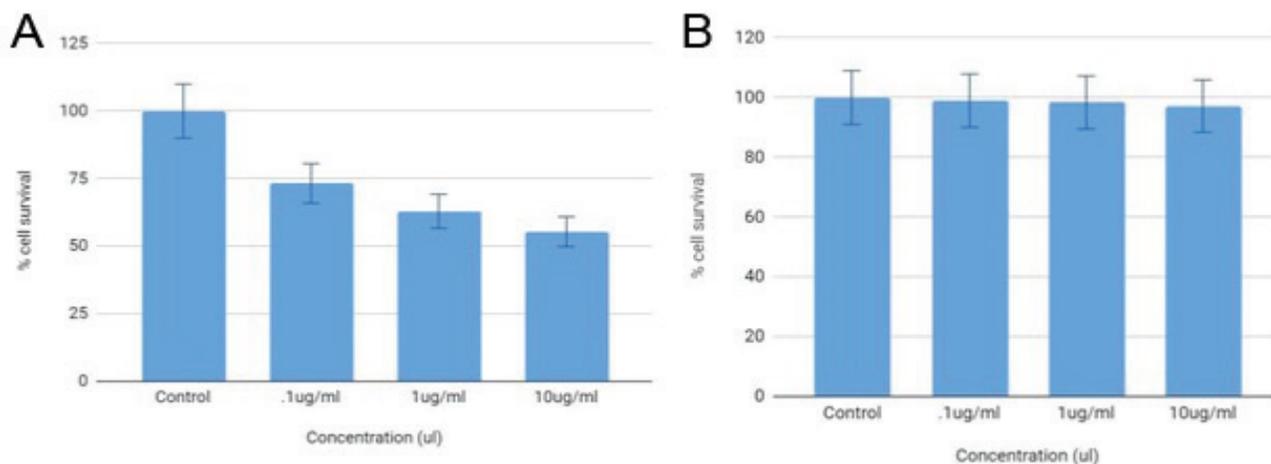


Figure 1: Assessing cell viability of normal and cancerous colon cells treated with *Niuli* crude extract. A) Average cell viability of untreated, 0.1 µg/mL, 1 µg/mL, and 10 µg/mL-Niuli extract treated Colo320 cells (n = 10). **B)** Average cell viability of untreated, 0.1 µg/mL, 1 µg/mL, and 10 µg/mL-Niuli extract treated normal colon cells (n = 10). Error bars depict standard deviation and asterisks indicate statistical significance (student's t-test, p -value < 0.05).

inhibited cancer cell migration and proliferation. Additionally, the crude extracts upregulated p27 and p21 expression. These results emphasize the potential of natural products on the treatment of cancer. With further testing, this extract could be a legitimate and natural way to prevent the formation and growth of colon cancer cells in the body.

RESULTS

The Trypan blue exclusion assay can be used to test for cell viability among cancerous and non-cancerous colon cells after treatment of the extract. This experiment will allow us to determine if the extract is able to decrease the viability of colon cancer cells without harming the non-cancerous ones. After incubating the colon cells with different concentrations of the *Niuli* crude extract, we observed that crude lettuce extract significantly (student's t-test, p -value < 0.05) decreased the percentage of colon cancer cell survival in a dose-dependent manner, relative to the control (**Figure 1A**). The control cell groups were grown without the addition of extract to assess the effect of the extract treatments. At the highest dosage 10 µg/mL, the colon cancer cell survival was reduced by over 50% compared to the control. However, non-cancerous cells also needed to be tested to determine if the extract impacted non-cancerous cell viability. The extract did not significantly affect non-cancerous cell viability even at the highest concentrations tested (**Figure 1B**). The results indicate that an increase in the concentration of crude *Niuli* lettuce extract reduced the survival of colon cancer cells, without significantly affecting the healthy colon cells.

We also wanted to perform the cell migration test to see if the extract was able to prevent the migration of the cancerous colon cells. This would be very beneficial towards stopping the spread of colon cancer cells within a patient's body. Increasing the concentration of the lettuce extract inhibited colon cancer cell migration after 24 hours of incubation. The

results show a directly proportional relationship between the dosage and inhibition of migration (**Figure 2A**). This trend indicates that as the dosage of crude extract increased, the distance between the colon cancer cells also increased. We observed the gap between the two main bodies of cells widened after each consecutive concentration (**Figure 2B**). The largest distance was observed when the concentration was at 6 µg/mL (**Figure 2A**). Therefore, the lettuce extract reduced colon cancer cell migration in a dose-dependent manner (student's t-test, p -value < 0.05), in respect to the control group.

p21 and p27 are important proteins involved in cell cycle regulation (16). Therefore, we wanted to determine if the lettuce extract influenced the abundance of p21 and p27 in cancer cells. To do this, we performed an ELISA assay to measure p21 and p27 in cell lysates from treated and non-treated cells. The results suggest that the effect that the crude extract had on two key proteins in the cancer cell cycle, p21 and p27. In the graphs, there is a clear direct relationship, indicating that an increase in the dosage of crude extract increased the protein expression of both these cyclin-dependent kinase inhibitors (**Figure 3A and B**). In both cases, the cells were incubated for 24 and 48 hours. Significance was determined by taking the levels of p21 and p27 expressed by the cells and comparing them to those in the control. The levels of p27 were slightly higher compared to the levels of p21 at the same concentrations; however, in both cases, significance was exhibited (student's t-test, p -value < 0.05). The subcellular location of p21 and p27 are exceptionally significant in determining their role in cancer (17). Nuclear p21 and p27 have several anticancer properties, while other forms of the proteins can exhibit oncogenic behavior (18). Targeting nuclear accumulation of p21 and p27 could be important in increasing the efficacy of current cancer treatments.

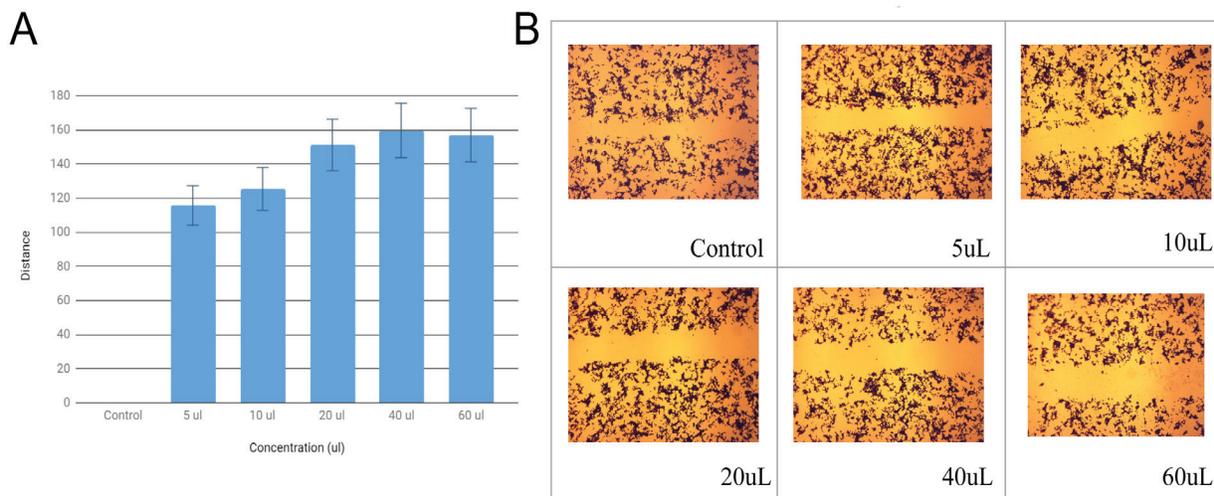


Figure 2: Increasing concentration of *Niuli* crude extraction decreased cancer cell migration. A) Average cell migration of untreated, 5 µL, 10 µL, 20 µL, 40 µL, 60 µL-*Niuli* extract treated Colo320 cells (n = 9). Error bars depict standard deviation and asterisks indicate statistical significance (student's t-test, p-value < 0.05). **B)** The photos visually show the average cell migration of untreated, 5 µL, 10 µL, 20 µL, 40 µL, 60 µL-*Niuli* extract treated Colo320 cells (n = 9).

DISCUSSION

Our results demonstrate that the *Niuli* lettuce extracts exhibited significant inhibitory effects on the proliferation of colon cancer cells. Increasing the concentration of the lettuce extract significantly increased cancer cell death without affecting healthy cells. *Niuli* lettuce extract also significantly reduced colon cancer cell migration in a dose-dependent manner. Preliminary experimentation indicated bioactive components within this extract are able to regulate key protein (p21 and p27) expression, two important proteins involved in tumor suppression. All the results supported our hypothesis that the chemical extract from *Niuli* lettuce would have anti-cancer effects. Our results demonstrate that there may be many natural options to combat colon cancer. Additionally, the crude extract may be able to prevent the proliferation of colon cancer without the use of painful and expensive treatments. We would also like to implement a wider array of tests, such as an MTT assay which measures metabolic activity of cells, strengthening our results on the cell viability test. Our results provide preliminary evidence that *Niuli* lettuce extract could potentially be tested in other model systems. These experiments have also prompted many questions and future studies, such as elucidating the specific chemical or chemicals found in the extract, and performing tests on the specific chemical that has the anti-cancer properties. In the future, more experimentation should also be done to identify the specific pathway at which the extract is able to regulate p21 and p27 expression. Nuclear accumulation of p21 and p27, through various mechanisms, leads to increased effectiveness of cancer treatments (19). Future research can be conducted to discover additional methods through which nuclear accumulation of p21 and p27 can assist cancer treatments.

In summary, we conducted three major experiments

to test the effects of the crude extract on colon cancer cell proliferation prevention. At a cellular level, our results demonstrate that *Niuli* lettuce extract exhibited notable inhibitory effects on cancer cell proliferation and migration. Additionally, the crude extracts upregulated p27 and p21 expression, two important factors involved in tumor suppression. Our study provides significant evidence that the extracts from *Niuli Lactucis Agrestibus* could be an effective and natural way to combat colon cancer already in the body and prevent its initial formation.

METHODS

Cell culture

The COLO320DM (COLO) colorectal cancer cell line (ATCC, New York, NY) was grown in RPMI 1640 media supplemented with 10% fetal bovine serum (Invitrogen, USA). The CCD 841 CoN (CCD) human normal colon epithelial cell line (ATCC, New York, NY) was grown in RPMI 1640 media supplemented with 10% fetal bovine serum (Invitrogen, USA). Cells were incubated at 37 °C in 95% O₂/5% CO₂.

Preparation of crude extract

To separate the supernatant from the *Niuli* leaf, 10 g of fresh leaves were placed in a 50% ethanol solution, ground using a Kinematica PCU Polytron, and centrifuged for 15 minutes at 4000 rpm. Once a density gradient was established, the supernatant was obtained and further purified using Whatman filter paper. The supernatant was stored in Eppendorf tubes and ethanol and water were dried by Labconco centrivap console (Cryostar industries Inc USA). The dry weight of the extract was measured by subtraction of the empty tube. The dried tube was then suspended with 1 mL of distilled water for further experimentation.

Trypan blue cell exclusion assay

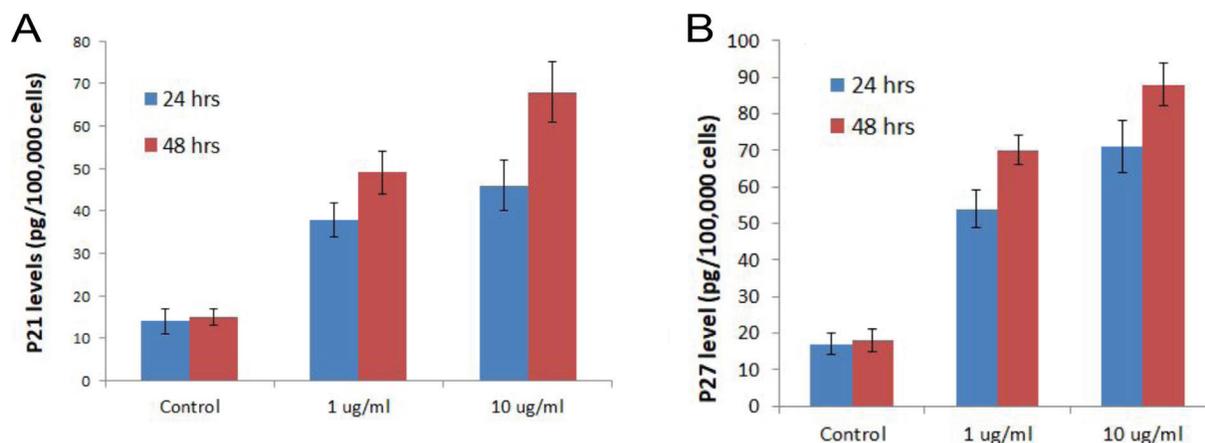


Figure 3: Crude extract treatment increases p21 and p27 expression. **A)** Average levels of p21 expression of untreated, 1 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$ -*Niuli* extract treated Colo320 cells for both 24 and 48 hours ($n = 2$). **B)** Average levels of p27 expression of untreated, 1 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$ -*Niuli* extract treated Colo320 cells for both 24 and 48 hours ($n = 2$). Error bars depict standard deviation and asterisks indicate statistical significance (student's t-test, p -value < 0.05).

First, 100 μL of medium containing suspended colon cancer cells for testing were placed in a microcentrifuge tube and 10 μL of 0.4% trypan blue dye was added to the suspended colon cancer cells. The suspension was mixed by pipetting up and down and incubated for two minutes. Next, a coverslip was placed on top of a hemocytometer and 10 μL of the cell suspension was used to gently fill the indent. The hemocytometer was placed on the stage of a light microscope and the cells were counted, both clear (alive) and blue (dead), in the hemocytometer and the results were documented.

Cell migration assay

COLO320DM cells were detached using 0.25% Trypsin-EDTA, and these cells were centrifuged in a 15 mL conical tube at 1,000rpm for 4 minutes at 20 $^{\circ}\text{C}$. Then the supernatant was aspirated, and the cells were resuspended in the culture media. Approximately 6,000 cells were plated into each well of the 24-well plate. After 24 hours, crude extract was added to the wells in the following volumes: 0 μL , 5 μL , 10 μL , 20 μL , 40 μL , and 60 μL , with the back-side of each well in a 24-well plate was labeled accordingly. Manually, a small wound or scratch was made over the body of cells using a sterile pipette tip. The culture media was inserted against the well wall to cover the bottom of the well and the plate was placed in an incubator. After 24 hours, the plate was observed from under the compound light microscope and snapshot pictures were taken on the computer. To analyze the results of snapshot pictures, the distance of one side of the wound to the other was measured using a scale bar.

ELISA Assay

p21 (CDKN1A) and p27 (CDKN1B) in vitro SimpleStep ELISA[®] (Enzyme-Linked Immunosorbent Assay) kit (abcam USA) was used for quantitative measurement of target proteins in COLO320DM cells. Before lysing, the cell incubation media is removed and rinsed with distilled water.

Later, 1mL of water was added and kept at -80 $^{\circ}\text{C}$. The cells were lysed by undergoing the freeze-thaw cycle (-80 $^{\circ}\text{C}$ to room temperature) 4 times. The SimpleStep ELISA[®] employs an affinity tag-labeled capture antibody and a reporter-conjugated detector antibody, which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards were added to the wells, followed by the antibody mix. According to the manufacturer's instructions, the wells were washed to remove unbound material. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate is added and during incubation it is catalyzed by horseradish peroxidase (HRP), generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the absorbance is measured at 450 nm.

Statistical analysis

A variety of one-way ANOVAS and t-tests were run to test for statistical significance. One-way ANOVAS were used to compare multiple treatments with one another, while t-tests were run for experiments with only two variables. All data were analyzed on SPSS where significance was defined as p -value < 0.05 . * p -value < 0.05 , ** p -value < 0.01 , *** p -value < 0.001 . Error bars show standard deviation.

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