**RESEARCH ARTICLE**

# Chlorophyllin Suppresses Growth, MMP Secretion, Invasion and Cell Migration of Fibrosarcoma Cell Line HT-1080

**Authors**
M Waheed Roomi, Bilwa Bhanap, Aleksandra Niedzwiecki, Matthias Rath

**Affiliations**
Dr. Rath Research Institute

**Correspondence**

Aleksandra Niedzwiecki
Dr. Rath Research Institute
author@drrath.com

**Abstract**

Fibrosarcoma is an aggressive and highly malignant cancer of connective tissue with the lungs being the most common site of metastasis. Surgery, chemotherapy, and radiation are the mainstay of treatments, yet the prognosis is very poor. A number of plant-based phytochemicals are increasingly being used as important treatment methods of cancers. Chlorophyll is a natural pigment that imparts the green color on plants. Chlorophyllin is a water soluble mixture of sodium-copper salts derived from chlorophyll. Chlorophyllin has been studied for its antioxidant potential. In the current study we tested the effects of chlorophyllin in fibrosarcoma HT-1080 cells on cell proliferation by MTT assay, modulation of matrix metalloproteinase (MMP) expression by zymography, cell invasive potential through Matrigel, cell migration by scratch test and morphology and apoptosis by H&E staining. Human fibrosarcoma cells HT-1080 were cultured in the media and were treated with chlorophyllin concentration at 10, 25 and 50 µM. The HT-1080 cell proliferation was significantly decreased at 50 µM dose of chlorophyllin. Expression of both, MMP-2, and MMP-9 decreased in a dose dependent manner. Both the MMPs were significantly inhibited at 25 µM and virtually undetectable at 50 µM. Cell invasion through Matrigel and cell migration was also reduced with the increasing concentrations of chlorophyllin with total inhibition of invasion at 50 µM. H&E staining at 10 µM of chlorophyllin showed a few cellular changes characteristic to apoptosis, while significant changes pertaining to apoptosis morphology were observed with increasing doses of chlorophyllin. Our results suggest that chlorophyllin may be a new chemotherapeutic strategy for fibrosarcoma patients and deserves further investigation as a potential agent in the treatment of this malignancy.

***1. Introduction:***

Fibrosarcoma is a tumor of the soft tissue of mesenchymal origin and begins in fibrous tissues which hold to the bones, muscles, and other organs. Fibrosarcoma is a very aggressive and highly metastatic cancer primarily developing in metaphyses of the long bones and the most common site of metastasis is the lungs. It predominantly affects children, adolescents, and young adults.[1] According to the American Cancer Society, approximately 13,040 new cases of soft tissue sarcomas, including fibrosarcoma, are estimated to be diagnosed in 2018, and close to 5,150 people are expected to die due to this disease.[2] Although the exact cause of fibrosarcoma is unknown, genetic mutations may play a role in causation. The most common mutation includes allele loss, point mutations, and chromosomal translocations.[3, 4]

Surgery, chemotherapy, and radiation are the main modalities of treatment for fibrosarcoma, however, overall this cancer has poor prognosis.[5] Prognosis in fibrosarcoma patients depends on pathologic grading and it worsens progressively with increasing grade fibrosarcoma. However, even when diagnosed and operated on early, the probability of cancer recurrence of fibrosarcoma at metastatic sites is greater than 70% after surgery.[1] The average 5-year survival rates range from 50-80% for moderate to low-grade fibrosarcoma, and drops to 30% for high-grade fibrosarcoma. Due to resistance to chemotherapy drugs and limitation of other current treatment modalities, there is an urgent need for a safe and effective treatment approach.

A number of plant-based phytochemicals are increasingly being used as important treatment methods of cancers, due to their antioxidant, chemo preventative, and antitumor actions. Chlorophyll is a natural pigment that imparts the green color on plants. Plants and some algae use chlorophyll to trap light for the process of photosynthesis. Chlorophyllin is a water soluble mixture of sodium-copper salts derived from chlorophyll.[6,7] Chlorophyllin has been studied for its antioxidant potential.[8, 9]

Anticanrcinogenic properties of chlorophyllin have shown to block the damage caused by free radicals derived from smoking,[10] certain heterocyclic amines from processed and grilled food,[11] and aflatoxin-B1.[12] In the current study we tested the effects of chlorophyllin in fibrosarcoma HT-1080 cells on cell proliferation, modulation of matrix metalloproteinase (MMP) expression, cell invasive potential, apoptosis and cell morphology. Our results suggest that chlorophyllin may be a new chemotherapeutic strategy for fibrosarcoma patients and deserves further investigation as a potential agent in the treatment of this malignancy.

## *2. Materials and Methods:*

*2.1 Cell Culture and chlorophyllin:*

The test compound chlorophyllin was obtained from Sigma-Aldrich Corp. St. Louis, MO, USA.

Human fibrosarcoma cells, HT-1080 were obtained from ATCC (Rockville, MD) and grown in MEM medium supplemented with 10% fetal bovine serum, penicillin G sodium (100 U/ml), streptomycin (100 µg/ml) and amphotericin (0.25 µg/ml) in 24-well tissue culture plates (Costar, Cambridge, MA). Cells were incubated with 1 ml of media at 37°C in a tissue culture incubator equilibrated with 95% air and 5% CO. At near confluence the cells were treated with chlorophyllin (obtained from Sigma Aldrich, St. Luis, MO, USA) 10, 25, and 50 µM in triplicate at each dose. The plates were then returned to the incubator.

*2.2 MTT Assay:*

Cell proliferation was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good index of mitochondrial activity and thus of cell viability. After 24 hours of incubation, the cells were washed with phosphate-buffered saline (PBS) and 500 µl of MTT (Sigma Catalog No. M-2128), and 0.5 mg/mL in media was added to each well. The plates were covered and returned to the 37°C incubator for 2 hours, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the well, the formazan product was dissolved in 1 mL DMSO, and absorbance was measured at 570 nm in a Bio Spec 1601 Shimadzu spectrometer. The OD570 of the DMSO solution in each well was considered to be proportional to the number of cells. The OD570 of the control (treatment without supplement) was considered to be 100%.

*2.3 Gelatinase Zymography:*

MMP secretion in conditioned media was determined by gelatinase zymography. Gelatinase zymography was performed in 10% polyacrylamide precast Novex gel, sodium dodecyl sulphate (Invitrogen Corp.), in the presence of 0.1% gelatin under non-reducing conditions. Culture medium (20 µl) was loaded and SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed with Tris-Glycine SDS buffer as described by the manufacturer (Novex). Samples were not boiled before electrophoresis. After electrophoresis, the gels were washed with 5% Triton X-100 for 30 minutes at room temperature to remove SDS. The gels were then incubated at 37°C overnight in the presence of 50 mM Tris-HCl, 5 mm $CaCl_2$, 5 µM $ZnCl_2$ at pH 7.5, stained with Coomasie Blue R 0.5% for 30 minutes, and destained. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

*2.4 Matrigel Invasion Studies:*

Invasion studies were conducted using Matrigel (Becton-Dickinson) inserts in 24-

well plates. Suspended in medium fibrosarcoma cells HT-1080 were supplemented with chlorophyllin as specified in the design of the experiment and seeded on the insert in the well. Thus, both the medium on the insert and in the well contained the same supplements. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% $CO_2$ for 24 hours. After incubation, the media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were stained with hematoxylin and eosin and visually counted under the microscope.

*2.5 Morphology:*

Morphology of cells cultured for 24 hours in the test concentrations of chlorophyllin were evaluated by H&E staining and observed and photographed by microscopy.

*2.6 Cell Migration:*

To study cell migration, a 2 mm wide single uninterrupted scratch test was made from the top to the bottom of the culture plates of HT-1080 cells grown to confluence. The culture plates were washed with PBS, incubated with chlorophyllin in the medium and tested with 0, 10, 25, and 50 µM in triplicate at each dose for 24 hours. The cells were washed with PBS, fixed and strained with H&E, and photomicrographs were taken.

*2.7 Statistical Analysis:*

The results were expressed as means ± SD for the groups. Data was analyzed by independent sample "t" test.

## *3. Results*

*3.1 Cell Proliferation Study (MTT Assay):*

Figure 1 shows the effect of different doses of chlorophyllin on cell proliferation of fibrosarcoma HT-1080 cells. Although 10 and 25 µM of chlorophyllin did not show any effect, the cell proliferation was significantly decreased at the higher dose of 50 µM. Only 70% of HT-1080 cells were seen viable at 50 µM as compared to the control.

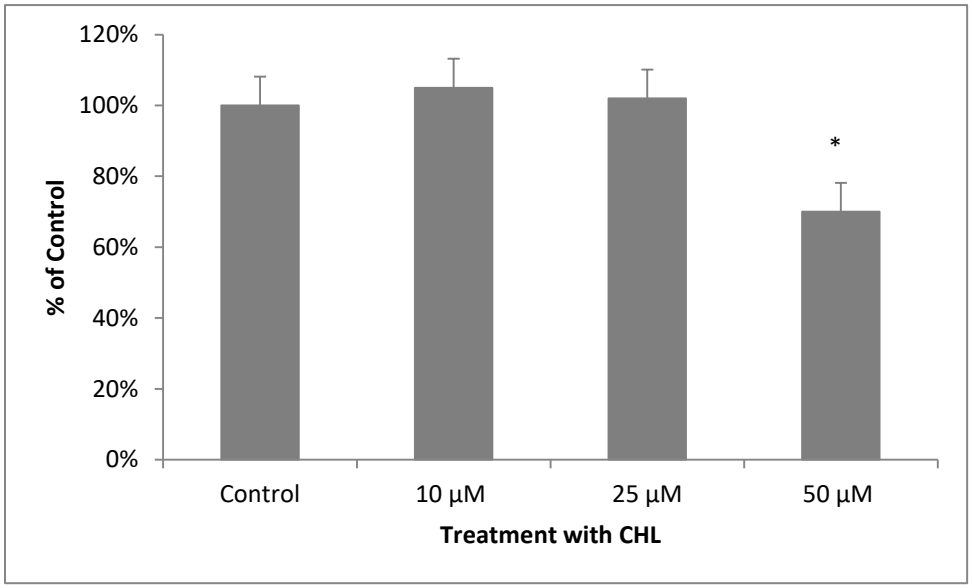



**Figure 1:** Effects of chlorophyllin on cell proliferation of fibrosarcoma HT-1080 (*-*p<0.001*)

*3.2 Gelatinase Zymography Study:*

Gelatinase Zymography study shows two bands corresponding to MMP-2 and MMP-9. Chlorophyllin inhibited the expression of both MMP-2 and MMP-9 of untreated and PMA-treated fibrosarcoma cells in a dose dependent fashion. The expression of both the MMPs was significantly inhibited at 25 µM and virtually undetectable at 50 µM of chlorophyllin. (Figure 2)


**Figure 2:** Effect of chlorophyllin on expression of MMP-2 and MMP-9 by fibrosarcoma HT-1080 cells (Lane 1- Control, Lanes 2-4- 10, 25, and 50 µM chlorophyllin)

*3.3 Invasion Study:*

Chlorophyllin significantly reduced the invasion of fibrosarcoma HT-1080 cells through Matrigel in a dose dependent fashion, as seen in Figure 3A, with 5% inhibition at 10 µM, 15% inhibition at 25 µM, and 98% inhibition at 50 µM. Photomicrographs of the invasion study of fibrosarcoma HT-1080 cells show dose dependent reduction in the invasion through Matrigel. (Figures 3B through 3E)

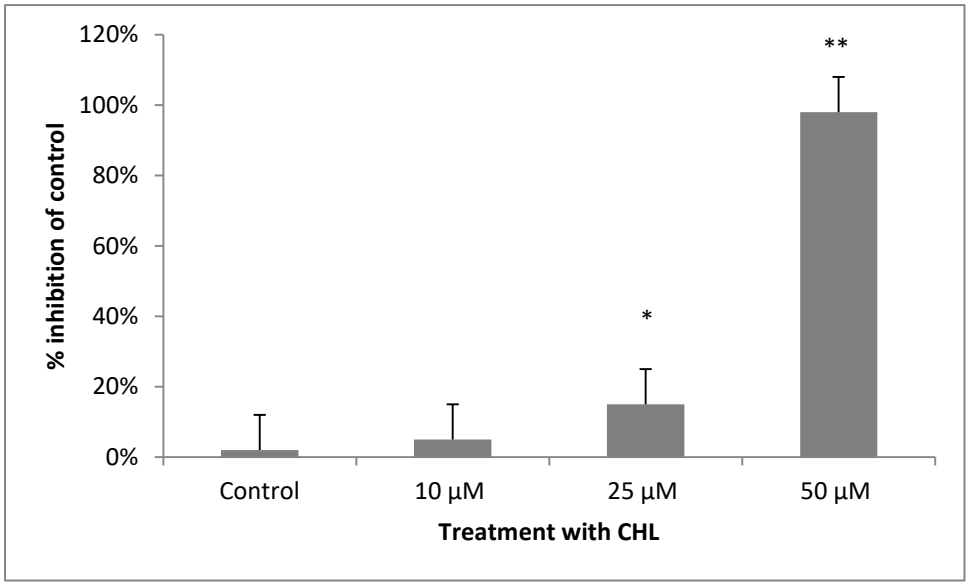
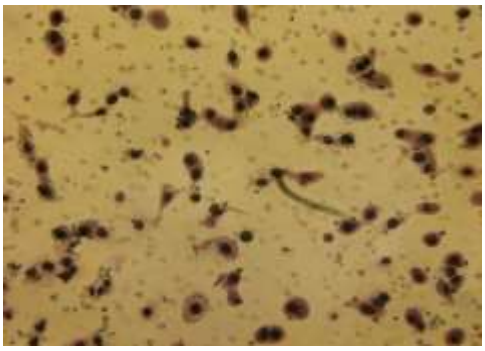
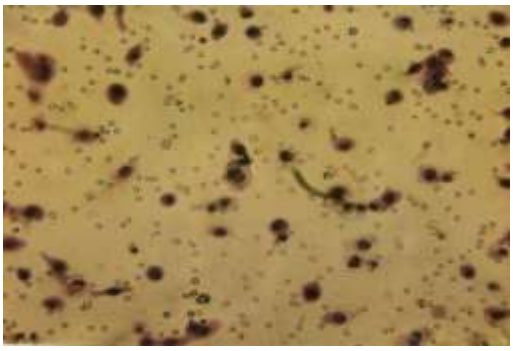



**Figure 3A**: Effect of chlorophyllin on fibrosarcoma HT-1080 cells invasion through Matrigel (* - *p>0.01; **- p>0.001*)

3B- 3E- Photomicrographs of the HT-1080 cell invasion through Matrigel

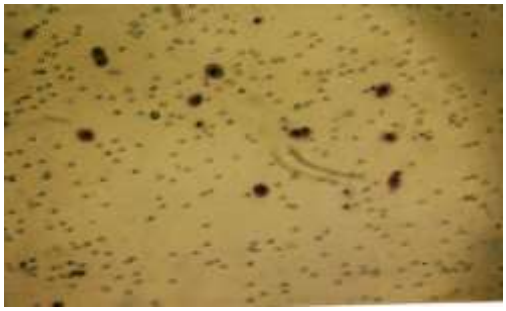


3B-Control



3C- CHL- 10 µM

3D- CHL-25 µM

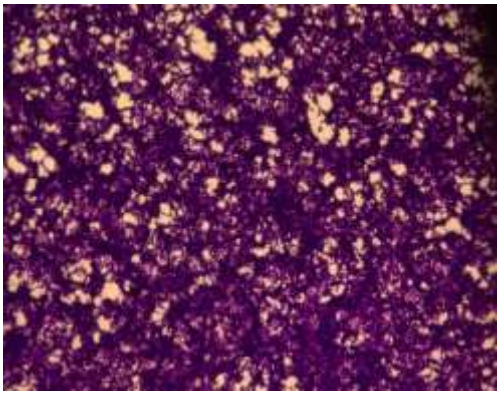


3E- CHL-50 µM

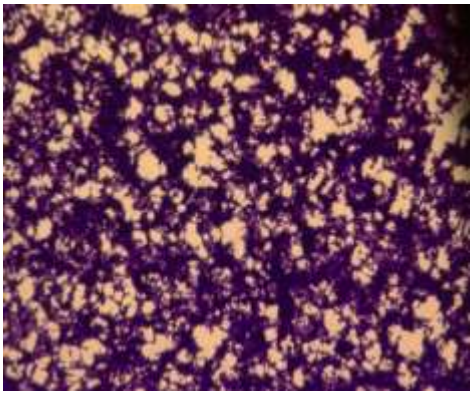
*3.4. Morphology and Apoptosis Study (H&E staining):*

H&E staining revealed significant apoptotic changes in dose dependent fashion in HT-1080 fibrosarcoma cells treated with moderate apoptotic changes at 10 µM, and significant changes noticed at 25 and 50 µM of chlorophyllin doses. These included characteristic morphological changes such as shrinkage of cytoplasm and darkly stained and condensed nuclei with strongly acidophilic cytoplasm (Figures 4A-4D).

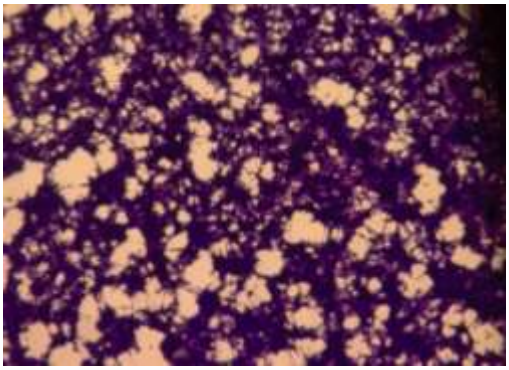
Figure 4: Effect of chlorophyllin on human Fibrosarcoma HT-1080 Cell morphology showing apoptotic studied by H&E staining



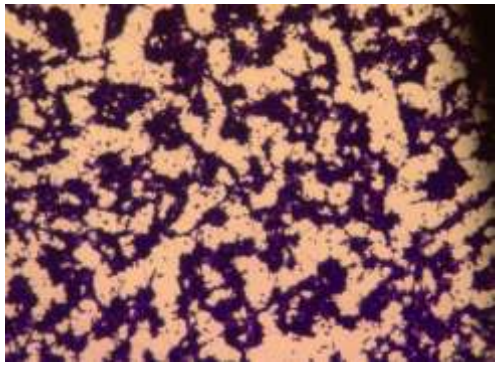
4A- Control



4B- CHL-10 µM



4C-CHL-25 µM

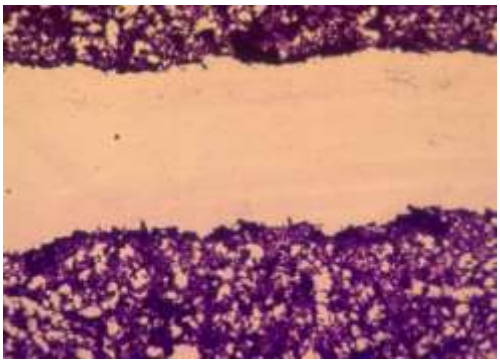


4D-CHL-50 µM

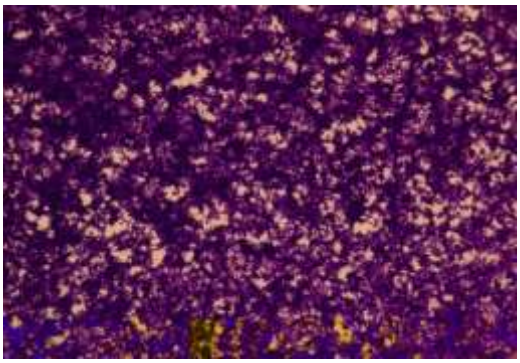
*3.4. Cell Migration by Scratch Test:*

Chlorophyllin reduced cell migration in a dose dependent manner with a complete block of fibrosarcoma HT-1080 cells at 50 µM. Photomicrographs of the results of the scratch test are shown in Figures 5A-E.

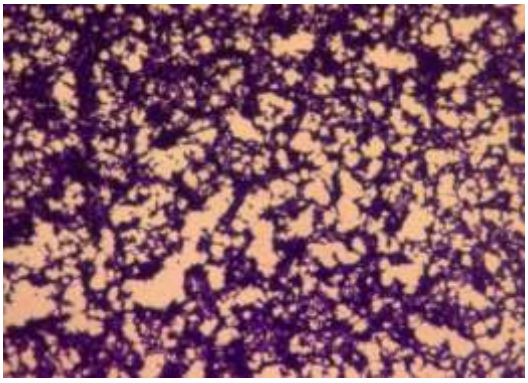
**Figure 5-** Effect of chlorophyllin on invasion of human fibrosarcoma HT-1080 cells by scratch test



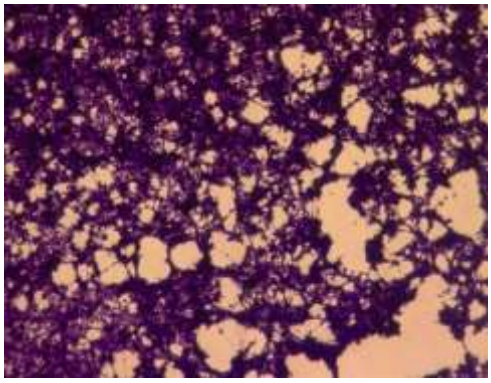
5A-Control (Just Scratch)



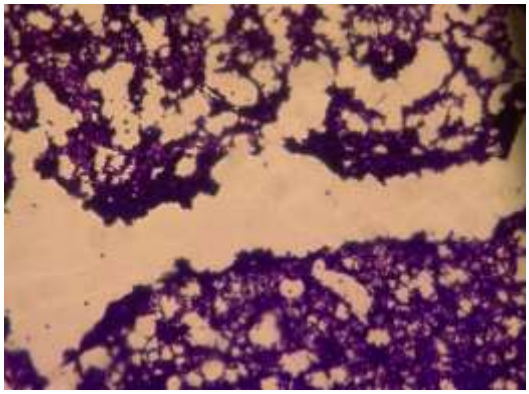
5B-Control (24 hours)



5C-CHL-10 µM (24 hrs)



5D-CHL-25 µM (24 hrs)



5E-CHL-50 µM (24 hrs)

## *4. Discussion*

Invasion of cancer or metastasis is a multistep process that includes tumor growth, cancer cell invasion, and movement of cancer cells into surrounding and distant areas. Degradation of extra cellular matrix (ECM) by the MMP enzymes is a critical step in invasion. It is important to restrict this step to stop the progression of highly

aggressive cancer by tumor encapsulation. Cancer cells that secrete higher amounts of MMP are associated with poor prognosis and decreased patient survival.[13] Therefore, control of ECM degradation provides an opportunity to address common mechanism of metastasis and tumor growth.

In the present study, we investigated effects of chlorophyllin on human fibrosarcoma HT-1080 cell line. The results indicate that chlorophyllin effectively inhibits the HT-1080 cell growth and cell invasion through Matrigel in dose dependent manner. In addition, chlorophyllin decreased the expression of MMP-2 and MMP-9 fibrosarcoma HT-1080 cells in dose dependent fashion. Exposure of HT-1080 cells to chlorophyllin in increasing concentrations also altered their morphological characteristics of apoptosis and inhibited cell migration. MMPs are produced by the cancer cells as well as surrounding stromal cells and are tumor specific.[14] Human fibrosarcoma cells express several MMPs, and of that, MMPs -2 and -9 are of particular importance in tumor invasion, metastasis, and angiogenesis.[15, 16] In an earlier in vivo study and in vitro studies, we have demonstrated that a specific micronutrient mixture significantly inhibited cell growth, invasion and MMP expression in vitro. Using xenograft athymic mice, we also demonstrated that the nutrient mixture inhibited the tumor growth and tumor burden of human fibrosarcoma HT-1080 cells.[17]

Chlorophyll is a plant pigment that gives them the green color and is used to trap the sunlight for photosynthesis, and chlorophyllin is a semi synthetic mixture of sodium and copper salts derived from chlorophyll. Chlorophyllin is said to be one of the most potent antioxidants and protects against free radicals, heterocyclic amines generated from grilled or processed meats, smoking, and Aflatoxin-B1 (AFB1) generated by a fungus growing on grains and nuts. It is well known that the free radical injury plays a key role in cancer initiation and progression. Chlorophyllin has been shown to be effective in blocking the effects from heterocyclic amines and aflatoxin. [8, 9] Chlorophyllin protects against oxidative damage by inducing the expression of heme-oxigenase-1.[18]

Chlorophyllin is an effective inhibitor of numerous mutagens, including AFB1, polycyclic
aromatic hydrocarbons (PAHs), heterocyclic amines, direct acting compounds and complex mixtures.[19] AFB1is a known carcinogen and is a major risk factor for hepatocarcinoma. Breinholt, et al., [20] studied chlorophyllin as an antimutagenic agent and showed that chlorophyllin inhibits actions of AFB1 and may prevent progression to cancer. Chlorophyllin acts as an inceptor molecule and blocks the action of carcinogens by interfering with their absorption by forming a reversible complex.

Simonich, et al., [21, 22] proved that chlorophyllin is an effective anticancer agent in rats as well as in rainbow trout claiming that it has a species-independent mechanism of action. An in vivo study by Priyadarshini, et al., [23] showed that

chlorophyllin effectively reversed the expression of 104 genes associated with cell adhesion, cell-cell communication, and invasion and TGF-b signaling in a hamster carcinogenesis model.

Radiation therapy and surgery are the mainstays of treatment of fibrosarcoma. Radiotherapy has wide spread damaging effects from skin changes, damage to the lungs and other organs, and DNA damage that may lead to future cancers. Occasionally, chemotherapy is used especially in fibrosarcoma with bone lesions. Studies show that chlorophyllin is also effective in protecting the chromosomal damage caused by gamma-radiation, chemotherapeutic drug cyclophosphomide, and carcinogenic agents such as N-nitroso-N-ethylurea and urathane.[24] In vivo and in vitro studies by Kumar SS, et al., show that chlorophyllin offers protection against whole body irradiation in BALB/c mice and chlorophyllin increased the intracellular antioxidant enzymes such as superoxide dismutase and glutathione peroxidase.[25] Furthermore, Boloor, et al.,[26] showed that chlorophyllin not only is effective in protection against cell damage caused by radiation, but it offers a higher degree of protection as compared to other antioxidants, ascorbic acid and glutathione.

Chlorophyllin has been shown to work in association with indol-3-carbinol in carcinogenesis of colon cancer in rats by altering the uptake or metabolism of chemical carcinogens.[27] It has also been studied as a promising anti-carcinogen in human breast cancer cells.[28] Lawrence, et al.,[29] showed that chlorophyllin can induce cell cycle arrest and apoptosis in breast cancer cells by deactivating a family of mitogen –activated protein kinases. Furthermore, Diaz, et al.,[30] showed that chlorophyllin effectively blocks the initiation of carcinogenesis, and induces apoptosis in colon cancer cells.

Due to the aggressiveness of fibrosarcoma, surgery, radiotherapy and chemotherapy have not been able to show adequate promise. A higher rate of recurrence contributes to the poor prognosis. Therefore, it is important to look into other methods of cancer prevention and cure. Our studies suggest that chlorophyllin is an excellent candidate for treatment in fibrosarcoma by inhibiting MMP expression, and Matrigel invasion while avoiding potential side effects commonly associated with chemotherapy and radiation.

**Acknowledgements**

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