ANTICANCER EFFECT OF PHYSICALLY FRACTIONATED BIOACTIVE COMPOUNDS OF GREEN COFFEE BEAN IN OVARIAN CANCER CELL

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Anticancer Effect of Physically Fractionated Bioactive Compounds of Green Coffee Bean in Ovarian Cancer Cell

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Ovarian cancer is one of the leading causes of female cancer death worldwide. Current treatment of the disease is by chemically-produced synthetic drugs; However, chemical drugs mostly have toxicity that are harmful for substantial treatment. Physically-extracted green coffee bean (GCB) extract may have anticancer effect to ovarian cancer cells by the substances of caffeine, chlorogenic acids, and specific proteins. In order to validate dose dependent cell killing efficacy of the physically extracted GCB, $3*10^4$ cells of ovarian cancer cell line (A2780) were seeded in a 96 well plate and were treated with the GCB extract in various concentrations from 0.1 μg/mL to 3000 μg/mL for 24 hours. The extract may be used in the development of novel anticancer drugs in future. Distinct and physical extraction method of our research allows higher yield of significant substances than most of chemical extraction method. Our method is also environmentally friendly since the process does not involve chemicals.

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NOMENCLATURE

GCB	Green Coffee Bean
NFRT	Nanofiltration
UFRT	Ultrafiltration
IC50	Half Maximal Inhibitory Concentration
CCK-8	Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.)
A2780	Human Ovarian Carcinoma Cell Line

CHAPTER I

INTRODUCTION

Cancer is a serious problem to be solved in modern medical field and is still one of leading cause of women's death in the United States.[1] Over centuries, mankind has fought with cancer and yet the perfect cure has not been found. Chemotherapy is considered as the most prevalent treatment to fight cancer; however, due to its cytotoxic effect on normal body cells, serious side effects of chemotherapy have been unresolved serious problem for researchers.[2] According to the incidence data provided by the National Center for Health Statistics, approximately 22,240 new cases of ovarian cancer and 14,070 ovarian cancer deaths in the United States occurred. The high mortality rate is due to late stage diagnosis, and therefore, the American Cancer Society suggests improving prevention as key to reduce possibility for late-stage diagnoses.

Coffee consumption has been a significant increase in the last century due to its distinct flavor as well as health-promoting properties. Phenolic compounds such as chlorogenic acid are rich in green coffee bean and has been studied for its potential effect on suppressing growth of cancer cells.[3-5] Most cancer is currently treated by chemically synthesized drugs, and there are many toxic side-effects that limit substantial and prolonged treatment.[6] This research determined anti-cancer effect of a physically extracted natural product, green coffee bean, to ovarian cancer cell line A2780 having higher yield of essential substances such as chlorogenic acids while having less toxic effect than chemical extraction and accurate measurement of anticancer effect of natural product itself.

Anticancer effects of green coffee bean have been found in various cancer cell lines, but its anticancer effects to ovarian cancer cell line A2780 are not yet found.[7] Thus, this research determined anti-cancer effect of a physically extracted natural product, green coffee bean, to ovarian cancer cell line A2780 having higher yield of essential substances such as chlorogenic acids. Moreover, by using physical fractionation in green coffee bean extraction, it involved far less chemicals compared to chemical-based extraction method providing higher yield of extract per crude sample and more accurate analysis of anticancer effects of green coffee itself without the cytotoxic contribution of any other chemical factors.[8-9]

CHAPTER II

METHODS

Membrane-based Fractionation

Green coffee bean used in this study were provided by a local store. By using a commercial grinder, the green coffee bean was sieved into a powder form with 40 mesh screens. A hexane extraction was applied to discard fats from the GCB in a 1 to 4 solid ratio. The defatted GCB meal was treated with 85% phosphoric acid in a 1 to 10 solid ratio to adjust the pH into 3.4 and was agitated for 2 hours at RT. After washing in 5 µm filter bag three times, supernatant was collected from the acid slurries after its separation from the wet cake processed through centrifugation at 4,700 rpm for 30 minutes (Sorvall RC-5C Plus Centrifuge and SLA-1500 rotor, Kendro Laboratory Products, Asheville, NC, USA). The wet cake was washed three times with 85% phosphoric acid. The supernatants from the acid slurries were filtered with a spiral wound composite cross flow system of 5 kDa MWCO (Synder, Vacaville, CA) and the retentate of this filtration process is the UFRT solution. The permeated liquid was further filtered with 150-300 kDa Da MWCO (Synder, Vacaville, CA), and the retentate after this nanofiltration is NFRT solution. Both UFRT and NFRT solutions were freeze-dried to eliminate water and obtain highly purified bioactive compounds. The fractionation diagram is shown in Figure 1.

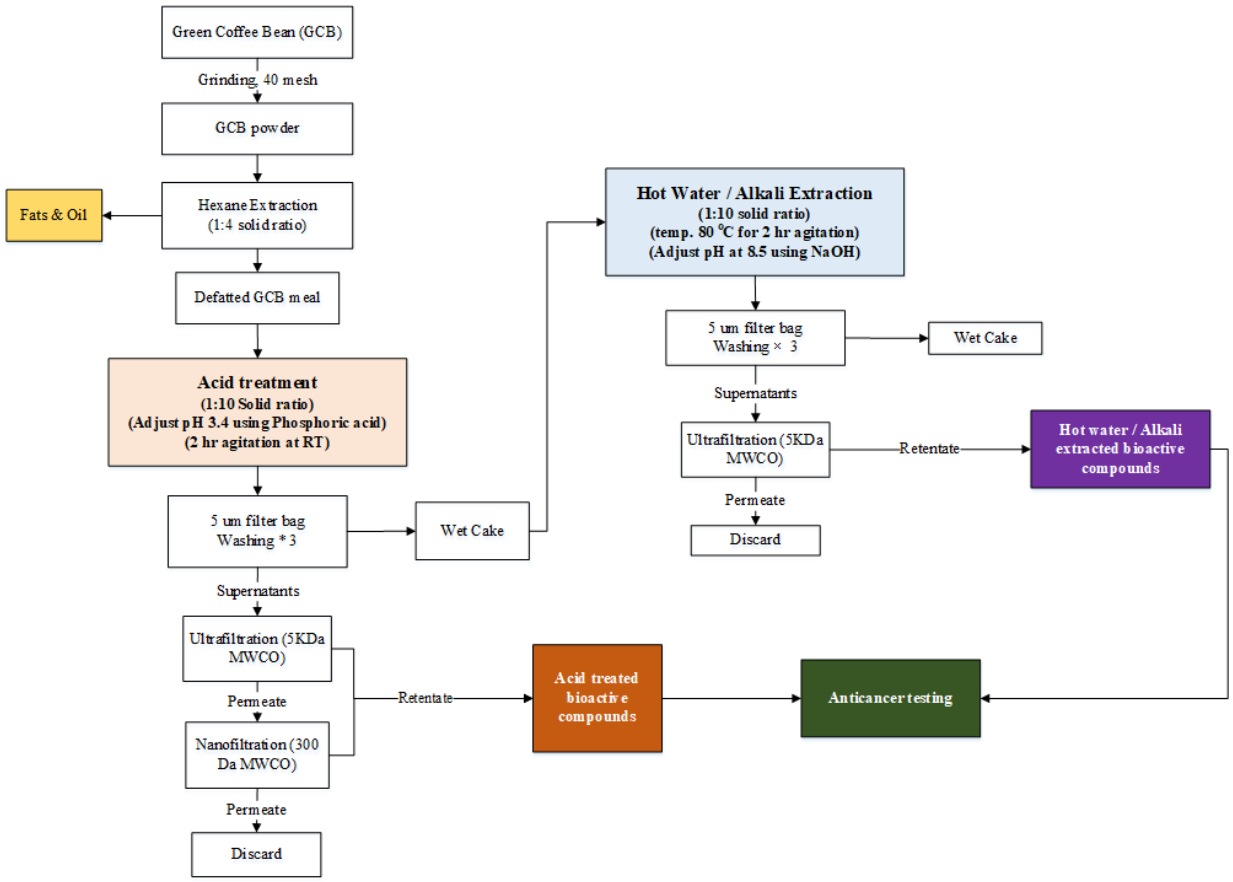

Figure 1: Fractionation Diagram of Green Coffee Bean

Cell Viability Test

A2780 ovarian cancer cell suspension of 3*10^3 cells per 100 microliter were seeded in each well of a 96 clear-bottom well plate. The well plate was pre-incubated until it reaches 100% confluency for incubator condition of temperature at 37 °C, 5% CO2, and 100 % humidity. The fractionated UFRT and NFRT solutions were diluted to 1, 10, 100, 500, 1000, 2000, and 3000 µm/ml in cell medium (RPMI-1640), and each diluted solution were treated to the cell culture in an anticipation of increasing cell killing efficacy in A2780 ovarian cancer cell line. After 24 hours of incubation, 10 microliter of CCK-8 kit solution, provided by Dojindo Molecular Technologies Inc. (Kumamoto, Japan), were added to each well of the 96 well plates. The treated well plates were incubated for an hour in the same CO2 incubator (37 C, 5% CO2), and was

taken out for absorbance measurement under the microplate reader provided by Molecular Devices (San Jose, CA). the absorbance of the solution was measured at 450 nm according to the instruction provided by CCK-8 kit protocol of Dojindo Molecular Technologies Inc.

CHAPTER III

RESULTS

Extraction and Separation of Bioactive Compounds

Table 1: Proximate Compositional Analysis of Green Coffee Bean (GCB) Fractions

Content (%)	GCB	Defatted GCB Meal	Acid Treated UFRT	Acid Treated NFRT	Hot Water/Alkali Treated UFRT
Moisture	6.49 ±1.53[a]	7.01 ± 0.73[a]	95.98 ± 3.43[b]	94.31 ± 5.43[b]	93.41 ± 3.73[b]
Protein (d.b.)	16.25 ± 1.67[a]	23.12 ± 2.23[b]	4.26 ± 0.36[c]	1.61 ± 0.13[d]	7.31 ± 1.33[e]
Lipid (d.b.)	14.33 ± 1.24[a]	0.72 ± 0.12[b]	0.68 ± 0.33[b]	0.63 ± 0.15[b]	0.58 ± 0.13[bc]
Ash (d.b.)	4.06 ± 0.22[a]	4.67 ± 0.45[b]	0.36 ± 0.08[c]	1.47 ± 0.31[d]	0.43 ± 0.02[e]
Carbohydrates & Others[1](d.b.)	65.36 ± 2.32[a]	71.49 ± 4.52[b]	94.69 ± 6.23[c]	96.29 ± 4.13[c]	90.68 ± 4.93[d]
Caffeine(d.b.)	0.82 ± 0.09[a]	0.74 ± 0.03[a]	N.D.	2.81 ± 0.19[b]	N.D.
Chlorogenic Acid(d.b.)	3.75 ± 0.12[a]	2.43 ± 0.03[b]	N.D.	4.75 ± 0.89[c]	0.03 ± 0.01[d]

Data are expressed as mean ± S.D. (n = 3).

[a-e] Means within a row with different letters are significantly different ($P < 0.05$)

[1] Including also the bioactive compounds such as polyphenols

N.D. Not detected

Among the grinded green coffee bean powder, more than 99% of overall mass were retained during the drying process. After discard of fatty molecules through hexane extraction process, 85% of initial dry weight remained. By acid treatment and centrifugation, a wet cake having 54% of the original weight was separated from its supernatant. About 11% of protein-rich fraction from UFRT was obtained from the supernatants. Shown in the proximate compositional analysis (Table 1), the UFRT fraction retained 4% of proteins after the collecting most of the

proteins as a wet cake through acid treatment and 95% of carbohydrates and others such as simple sugars and polyphenols.

Remarkably, Caffeine and chlorogenic acids were not detected in UFRT fraction. The supernatants that passed through the UFRT membrane were further filtered through NFRT membrane and the retentate were found containing 5% of chlorogenic acid and 3% of caffeine which is a much higher percentages in comparison to the percentage it originally had in either standard GCB powder or defatted GCB meal (Table 1).

Cytotoxic Effect of Physically Fractionated Green Coffee Bean

The acid treated UFRT green coffee bean fraction significantly reduced the viability of ovarian cancer cell line A2780 by 50% at approximately 2 mg/mL (IC50: 2024±304) dosing at 37 ºC for 24 h incubation (Figure 2). The acid treated NFRT fraction significantly reduced the viability of ovarian cancer cell line A2780 by 50% at approximately 1 mg/mL (IC50: 1020±182) under the same condition with the UFRT fraction (Figure 3).

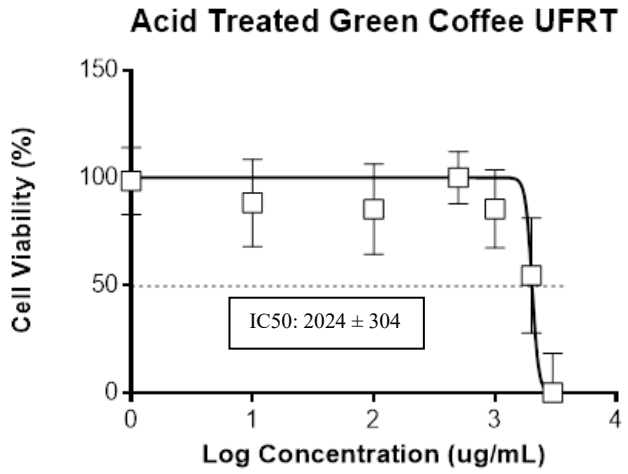

Figure 2: Cytotoxic Effect of Physically Fractionated GCB by Ultrafiltration (UFRT)

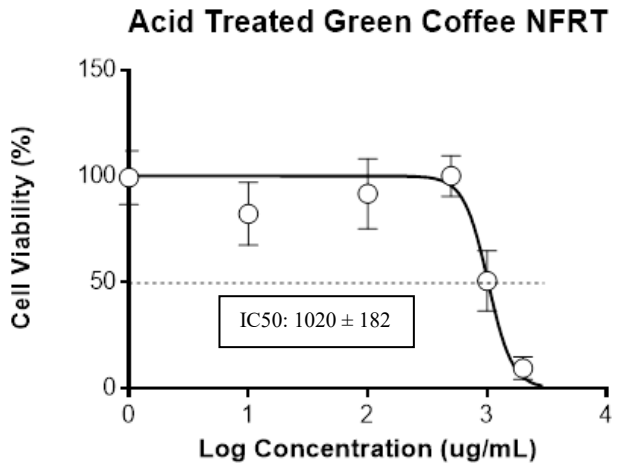

Figure 3: Cytotoxic Effect of Physically Fractionated GCB by Nanofiltration (NFRT)

NFRT Fraction Analysis

Supported by the proximate compositional analysis shown in Table 1, NFRT fraction contains approximately 2.81% of caffeine and 4.75% of chlorogenic acid after the acidic treatment and nanofiltration. The NFRT fraction produced a significant cytotoxic effect on ovarian cancer cell line A2780 reducing the cell viability by 50% dosing at 1020 ± 182 (IC50). The cytotoxic effect of caffeic acid and chlorogenic acid to various cancer cell lines (liver[10], gastric[11,12], colon[11,13], prostate[10,14], breast[15,16]) has been summarized as a review by Rocha et. al; however, the cytotoxic effect of these phenolic compounds to an ovarian cancer were yet to be determined.[7] By using the membrane-based fractionation method to separate these phenolic compounds, the bioactive compounds of GCB caffeine and chlorogenic acid produced the anticancer effect to the ovarian cancer cell line A2780. Moreover, Castro-Muñoz et al. demonstrated that the membrane-based fractionation of natural products provides high yield of high-added-value compounds including the caffeic acid and chlorogenic acid, which may be another considerable importance to our membrane-based fractionation of caffeine and chlorogenic acid from GCB.[9] In addition, by using far less chemicals compared to chemical-

based extraction method, the anticancer effect of green coffee itself was more accurately analyzed without the cytotoxic contribution of any other chemical factors.

UFRT Fraction Analysis

Similarly to the NFRT fraction, the UFRT fraction of GCB also produced a cytotoxic effect to ovarian cancer cell line A2780 having an IC50 value of 2024 ± 304. However, unlike the NFRT fraction, the UFRT fraction had non-detectable amount of chlorogenic acid or caffeine as shown in Table 1. The anticancer effect of UFRT fraction must have come from other bioactive component of GCB rather than the chlorogenic acid or caffeine. Researching over the literature studies conducted by other scholars, the bioactive component that produced the anticancer effect can be assumed to be alpha-galactosidase in GCB. Alpha-galactosidase is a bioactive compound that are soluble in acidic environment.[17] Alpha-galactosidase is a multimeric enzyme having a size of 370 kDa, composed of four monomers of 87 kDa.[17] During the fractionation process of this research shown in Figure 1, the defatted GCB meal was acidified with phosphoric acid, and the proteins that are soluble in acidic environment were collected with the supernatant after the centrifugation. All of the insoluble proteins were separated as wet cake, and the supernatant containing the acidic-soluble proteins were physically fractionated through the ultrafiltration (UFRT). The filtrating membrane used in UFRT had a pore size of 5 Kda which is smaller than 370 kDa, the size of alpha-galactosidase, and therefore, it was retentated during UFRT process. The proximate compositional analysis of Table 1also supports the fact that UFRT fractionation contained alpha-galactosidase and other acid-soluble proteins. Since all other components in UFRT fraction – moisture, ash, and carbohydrate - has a low potential for anticancer effect except the proteins, alpha-galactosidase, one of the most abundant acidic-soluble proteins, can be assumed as the potential candidate for the anticancer effect of the UFRT

fraction.[18] According to the research conducted by Isshiki et al., the authors found suppressive effect of coffee beans to the human colon carcinoma Caco-2-cells. Similar to the UFRT fraction, the authors claim that there were no major bioactive constituents such as caffeine and chlorogenic acids to produce the anticancer effect.[19] Therefore, future implication can be conducted to separate and purify alpha-galactosidase and validate for its anticancer effect to the ovarian cancer cell line A2780.

CHAPTER IV

CONCLUSION

Ovarian cancer is one of the leading causes of death of women in United States, and chemotherapy using chemical drugs is the most prevalent treatment to fight against cancer. However, due to the bad side effects of chemotherapy, anticancer effect of natural products, which are more compatible and biological safe, can be an alternative way to combat cancer. As one of the natural products, green coffee bean has several bioactive compounds such as chlorogenic acid, caffeine and caffeic acid that have been studied by many scholars and shown their anticancer effect upon many cancer cell types, but yet to the ovarian cancer cell lines. By utilizing physical fractionation and acidic treatment, this research has determined the anticancer effect of green coffee bean to the ovarian cancer cell line A2780. NFRT fraction, which retentate most of the bioactive compounds, have produced a significant reduction in cell viability. UFRT fraction, which contains non-detectable amount of the essential bioactive compounds also significantly reduced cell viability but not as much as NFRT. Since all other components in UFRT fraction – moisture and carbohydrate - has a low potential for anticancer effect, proteins that were soluble in acidic environment seem to be the most considerable source. As one of the most prevalent acid-soluble proteins, alpha-galactosidase can be a potential candidate for the anticancer effect produced by UFRT fraction. Further research may be conducted to separate and purify alpha-galactosidase in UFRT fraction and validate for its anticancer effect to the A2780 ovarian cancer cells.

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