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Proteomic Evaluation and Cytotoxicity of Red Maple (*Acer rubrum*) Leaves

A Thesis by

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Irvine, CA

School of Pharmacy

Submitted in partial fulfillment of the requirements for the degree of

Master of Science in Pharmaceutical Sciences

August 2019

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August 2019

Proteomic Evaluation and Cytotoxicity of Red Maple (*Acer rubrum*) Leaves

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Saud O. Alshammari

Dedicate this thesis to my wife, son, and parents

For their prayers and support

ABSTRACT

Proteomic Evaluation and Cytotoxicity of Red Maple (*Acer rubrum*) Leaves

by Saud O. Alshammari

Red maple (*Acer rubrum*), also known as swamp, water or soft maple, is endemic to eastern and central North America and was widely used as traditional medicine by the first peoples. Commercially, its well-known products include maple syrup and high-quality lumber. The potential medicinal benefits of phenolic compounds extracted from the red maple plant, such as glucitol-core containing gallotannins, include antioxidant, and antiglycation effects as well as their importance in cosmetic applications. Plant-derived proteins and peptides are important biomolecules; however to date, there is no published data on the identification of proteins/peptides from red maple leaves. Therefore, the present study focuses on the activity guided purification of proteins from red maple leaves collected in spring and fall seasons. In addition, the focus of this project was in the evaluation of maple leaves employing bottom-up proteomics and De Novo protein profiling by PEAKS Studio-X and Gene Ontology Bioinformatics. The red maple leaves were grounded, defatted in hexane and proteins extracted in 25 mM sodium phosphate buffer pH 6.5. The extracted crude proteins were recovered by precipitation in 80% ammonium sulfate. The first-dimensional chromatography of crude extracted proteins was performed on a gel filtration column (HiLoad 16/600 Superdex200). The separation of crude extract and the partially purified gel filtration fraction was conducted by reversed-phase HPLC. The crude and eluted fractions were analyzed by SDS-PAGE gel electrophoresis. The extract was screened for cytotoxicity activity on Michigan Cancer Foundation-7 breast cancer (MCF-7), M.D. Anderson Metastasis Breast (MDA-MB-231) cancer cell lines and anti-

inflammatory activity on murine macrophage (RAW 264.7) cell line from American Type Culture Collection (ATCC). The drug Doxorubicin was used as a positive control whereas untreated cells as a negative control in these experiments Preliminary data revealed that active protein fractions were eluted at two different regions of gel filtration chromatography both in spring and fall leaves. Bottom-up proteomics of crude and active fractions by PEAKS Studio-X and MASCOT bioinformatics database identified over 54 proteins. The Gene Ontology Annotation classified these proteins involved in the biological processing, cellular compartment, and molecular functions.

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Chapter 1

INTRODUCTION

1.1. Herbal Medicine

An herb refers to a plant or any part of a plant such as leaves, flower, bark, seeds, and fruit that is used for its medicinal, aromatic, or flavoring properties. Herbal medicine is the use of unpurified plant extracts or plant components for their therapeutic activity (Kunle et al., 2012). Many civilizations have used the plants for their medicinal benefits such as Egyptian, Chinese, Greek, Indian, and Mediterranean for over 4,000 years as remedies for various ailments. In the 19th century, medicinal plants led to the start of a new era of discovery, substantiation, and isolation of many plant-based herbal medicines such as alkaloids from poppy, ipecacuanha, strychnos, quinine, and pomegranate. However, scientific pharmacy began with the isolation of glycosides. Later, the advanced techniques have facilitated discoveries of new substances from the medicinal plants, such as tannins, saponosides, etheric oils, vitamins, and hormones (Petrovska, 2012).

1.2. Importance of Herbal Medicine

The herbal remedies are used for more than 80% of various diseases in the population of developing countries (WHO, 2003). In the underdeveloped countries, many people used herbal medicine because of limited access to advance medicine as well as poverty (Kunle et al., 2012).

Whereas, the developed countries, the emphasis is on the discovery of pharmacologically active chemical entities which limited the uses of traditional herbal medicine in healthcare (Pirotta et al., 2000; Suchardet et al., 2004). However, in the past few years, there is tremendous growth in the global demand for herbal products as supplements. According to the recent report (March 27, 2019) by Market Watch (<https://www.marketwatch.com/press-release/herbal-supplements->

[market-2019-global-analysis-share-trends-application-analysis-and-forecast-to-2024-2019-03-](#)

[27](#)), there is a tremendous growth in global herbal supplements market accounted for \$49.1 billion in 2016 and forecasted to reach about \$86.7 billion by 2022 with the compound annual growth rate of 9.9% from 2016 to 2022.

1.3. Herbal Medicine in Cancer Therapy

Cancer is one of the major diseases that cause an increase in mortality in the world population. According to the National Center for Health Statistics in 2019, the number of deaths from cancer estimated as 606,880 and 1,762,450 as new cases in the United States (Siegel et al., 2019). Conventional medicine of cancer, such as surgery, chemotherapy, and radiotherapy is the primary methods to treat cancers.

Plant-derived natural products have been in use for several years. Many of these approved anti-cancer agents isolated from plants (Dholwani et al., 2008). Some of the key plants derived natural products approved as anti-tumor agents in the USA are Vinblastine, Vincristine, Taxol, and Camptothecin (Demain & Vaishnav, 2011). Vinblastine and vincristine are alkaloids from the Madagascar periwinkle plant (*Catharanthus roseus*). Herbal formulations are typically used in Chinese medicine. According to Chinese herbal clinicians, the herbal formulations, such as PHY906 (KD018) and flavonoids derived from *Scutellaria baicalensis* are affecting cancer in multiple ways: (1) Protecting body normal cells and tissues from the side effects of chemo/radiotherapy; (2) maximizing the effect of chemo/radiotherapy; (3) minimizing infectious and inflammatory complications in tissues located around carcinoma; (4) enhancing body

resistance and immunity; (5) improving the patient quality of life and prolonging life span (Hsiao & Liu, 2010).

According to the GLOBOCAN 2012 report, colorectal cancer is the third most common reason for death by cancer diseases (Ferlay et al., 2015). One novel peptide called BG-4 was isolated, purified, and characterized from *Momordica charantia* plant has shown activity against HCT-116 and HT-29 human colon cancer cells as shown by trypsin inhibitor assay (Dia & Krishnan, 2016). Also, another protein extracted from the leaves of *Morinda pubescens* has shown cytotoxic activity against A549 human cancer cell line (Thomas et al., 2017). In another study, proteins extracted from *Gynura procumbens* showed anti-cancer activity in breast cancer cell line, MDA-MB-231 as shown by lactate dehydrogenase (LDH) cytotoxicity assay (Hew et al., 2013). This information revealed the significant role of extracted bioactive proteins and peptides in the treatment of several cancer conditions (Hernández et al., 2013).

1.4. Herbal Cosmeceuticals

However, nowadays the herbal products not only used as remedies but also used for cosmetic purposes. With that increase in demand for plant resources as cosmetics, the cosmetics industry is focusing now on traditional herbal cosmetic knowledge. It is becoming an essential resource for economic development and innovation (Evariste et al., 2017). In the 1990s, the pharmaceutical company began to use the term cosmeceuticals for any pharmaceutical products that intended to enhance the beauty and health (Vasisht & Kumar, 2002). Also, R.E Reed, in 1962, defined the cosmeceutical term in four points. (1) A cosmeceutical is a scientifically designed product intended for external application to the human body (2) A cosmeceutical produces a useful, desired result

(3) A cosmeceutical has desirable aesthetic properties; and (4) A cosmeceutical meets rigid chemical, physical and medical standards (Reed, 1964). The cosmetics industry has discovered many plants as sources of innovative ingredients with specific pharmacological properties such as anti-inflammatory, anti-allergy, anti-carcinogenic, moisturizing, anti-aging, and anti-hyperpigmentation (Dorni et al., 2017).

1.5. Red Maple (*Acer rubrum*)

The red maple, also known as swamp, water, or soft maple, is located in eastern North America, as shown in **Figure 1**. The people of eastern North American region use it as an herbal medicine for multiple conditions, including diarrhea, abdominal pain, eye diseases, and several skin disorders (Lans et al., 2007). Recently, researchers from the University of Rhode Island investigated the phytochemical products extracted from red maple leaves for medical purposes and cosmetic applications. A new gallotannin were isolated from red maple leaves have shown a potent α -glucosidase inhibitory effect. Also, the gallotannin are active against melanin overproduction in B16F10 cells. The accumulation of abnormal melanin may cause several skin hyperpigmentation disorders, such as freckles, age spots, post-inflammatory hyperpigmentation, as well as melanoma. The Maplifa^{TM} is a new product that extracted from red maple leaves which contains 40–45% ginnalin A (GA) and other phenolic compounds. GA decreases the levels of reactive oxygen species (ROS) and melanin content in B16F10 melanoma cells, which means the GA can inhibit the formation of glycation end products (AGEs) (Ma et al., 2017).

Figure 1. Picture of Red maple leaves and its distribution in North America. Photos retrieved from https://en.wikipedia.org/wiki/Acer_rubrum

Nevertheless, there are no studies conducted on the proteins and peptides extracted from red maple leaves. Therefore, this collaborative research focused on the proteins and peptides of Red maple leaves. The processes including isolation, purification, and characterization of proteins and peptides employing bottom-up proteomics. Furthermore, to a better understanding of their biological effects and therapeutic applications, cell-based anticancer assays were conducted using MCF7, MDA-MB-231 cell lines. Whereas the anti-inflammatory assay was conducted on murine macrophage (RAW 264.7) cell line.

1.6. Proteomics

In 1995, proteome term was used for the first time by Marc R. Wilkins to describe the complement of a protein genome (Wasinger et al., 1995). The proteome first book was published in 1997 (Wilkins et al., 1997). Leigh Anderson defined proteomics as “the use of quantitative protein-level measurements of gene expression to characterize biological processes (e.g., disease processes and drug effects) and decipher the mechanisms of gene expression control” (Anderson & Anderson, 1998). The significant role of proteins as direct bio-functional molecules in the living organisms spark the ambition of the researchers and scientists to discover and investigate their therapeutic effects for several diseases.

The advanced technologies, such as mass spectrometry and bioinformatics data analysis, promoted the progress of in the isolation and characterization of peptides/proteins from various plants. The bottom-up proteomics is the analysis of a mixture of peptides, derived from proteolytic digestion of intact proteins by LC-MS/MS. To identify the peptide tandem mass spectra and match it with theoretical tandem mass spectra that have been generated from *in silico* digestion of protein databases, including PeptideMass and PeptideCutter at ExPASy, MS-Digest at Protein Prospector, and Protein Digest at ISB. Also, the theoretical molecular weights and functional annotations of the proteins can be determined by using Gene Ontology (GO) terms (Lu et al., 2008; Y. Zhang et al., 2013). On the other hand, a different approach called ‘top-down’ proteomics, which performed to characterize the intact proteins. This method has some advantages for post-translational modifications (PTM) as well as protein isoform identification. Furthermore, top-down is useful to characterize protein up to 200 kDa (Han et al., 2006), but it has critical limitations that represent difficulties in protein fractionation, ionization, and fragmentation in the gas phase.

In proteomics, a new technique named *De novo sequencing* now can be performed using bioinformatics software package, PEAKS Studio-X. It is used in the characterization of the amino acid sequence, which is not present in available protein sequence databases (NCBI and ExPASy). This advance bioinformatics software uses a new algorithm model to obtain the best tryptic fragmented peptide sequence ions from the interpretation of the peaks in the LC-MS/MS quadrupole-time-of-flight (Q-TOF) spectrum. The result of the PEAKS software gives the identified amino acid sequences a confidence scores to each sequence to validate the results called false discovery rate (FDR). The FDR is used to measure the accuracy of the outcomes to obtain the percentage of the false identifications above the score threshold (Zhang et al., 2012).

Chapter 2

METHODS

2.1. Materials

All chemicals used in this project were purchased from Thermo-Scientific, Bio-Rad, Sigma-Aldrich, or Acros. The reagents and biological materials include sodium phosphate dibasic and monobasic, hexane, Tris (hydroxymethyl) aminomethane, hydrochloric acid (HCl, 37%), ammonium sulfate, acetic acid, trifluoroacetic acid (TFA), acetonitrile (ACN), ammonium persulfate (APS), acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), bisacrylamide, urea, glycerol, 2-mercaptoethanol, brilliant blue G-pure, 10x Tris/Tricine/SDS, sodium dodecyl sulfate (SDS), trypsin, tosyl-phenylalanyl-chloromethyl-ketone (TPCK) treated, dithiothreitol (DTT), iodoacetamide, phosphate-buffered saline (PBS), bovine serum albumin (BSA), trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT), fetal bovine serum (FBS), 1% penicillin-streptomycin, Dulbecco's Modified Eagle Medium (DMEM), dimethyl sulfoxide (DMSO), doxorubicin hydrochloride. Hiload 16/600 Superdex 200 pg column, Vydac C18 (2.1x150mm) column. Dialysis tubing (3500 D MWCO), Amicon® ultra centrifugal filters (3000 D MWCO). MDA-MB-231 (ATCC® HTB-26™) and MCF-7 (ATCC® HTB-22™).

2.2. Protein Extraction

Fall and Spring Red maple leaves were collected from Rhode Island in the United States. Leaves were immersed in n-hexanes for 24 hr separately. Hexane was removed by filter paper, and the solid mass recovered dried at room temperature for 2 days in the chemical hood. Dried defatted leaves were blended within three different conditions: i. 20 mM Tris/HCl pH 8, ii. deionized water, and iii. 25 mM sodium phosphate pH 6.5 buffers and stirred for 4 days at 4°C. The samples were filtered through cheesecloth. The recovered filtrate was centrifuged at 14,000 rpm using Thermo

Scientific™ Sorvall LYNX 4000 Superspeed Centrifuge for 30 min. The supernatant collected and precipitated with 80% ammonium sulfate at 4°C and stirred for 24 h. Precipitated proteins were recovered by centrifugation at 14,000 rpm for 10 min. The pelleted proteins were dissolved in deionized water and dialyzed by using dialysis tubing (MWCO 3.5 kD) for 2 days at 4°C. The dialyzed samples were lyophilized by using lyophilizer (Domžalska et al., 2016). The samples were stored at 4°C for further experiments. The schematic workflow adopted in this project is presented in **Figure 2**.

Figure 2. Schematic of workflow of Red maple leaves proteomics project.

2.3. Gel Filtration Chromatography

The crude extracted proteins from both seasons (spring and fall) leaves were fractionated by using ÄKTAStart (GE Healthcare) fast protein liquid chromatography (FPLC). The system is equipped with a 280 nm detector and an automated fraction collector. The dried crude proteins were dissolved in the same extraction buffer and loaded into the column. The gel filtration column HiLoad™ 16/600 Superdex™ 200 pg was pre-equilibrated with sodium phosphate pH 6.5, and the sample was eluted in isocratic mode at 1 mL/min flow rate. The absorbance was monitored at 280 nm.

2.4. Reverse Phase High-Performance Liquid Chromatography (RP-HPLC)

The purification process was continued to purify proteins and peptides from red maple leaves by using the RP-HPLC technique. The Hitachi Elite LaChrom HPLC system equipped with pump L-2130, Diode Array Detector L-2455, Refrigerated auto-sampler L-2200, and software package EZChrom Elite (version 3.3.2 SP2) was used. The crude proteins extracted in three different buffers from different seasons (spring and fall) leaves were loaded onto a reversed-phase column. The column Vydac C18 (2.1x150mm) equilibrated in 0.1% TFA-water. The samples were eluted by a gradient 0-60% B (0.1% acetonitrile) in 85 min at 1 mL/min flow rate and absorbance at 280 nm.

2.5. Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the spring and fall crude proteins, as well as FPLC separated protein fractions. The tris/tricine SDS-PAGE technique was used (Schägger & Jagow, 1987). The 10% separating gel and 4% stacking

gel were prepared as listed in **Table 1**. The separating gel solution was poured in gel cassette, and then, bubbles were removed by the spraying of 70% ethanol on the top of the separating gel and waited for 20-30 minutes until the gel polymerized. The stacking gel was prepared, and before the addition of stacking gel, ethanol was removed. The comb was placed immediately before stacking gel polymerization. The samples were prepared by mixing 50% of protein and 50% tricine loading buffer containing 2% of 2 β -Mercaptoethanol, and boiled at 95°C for 10 min. The samples at 10, 20, and 30 μ L were loaded into the stacking gel and 10 μ L of standard protein (ladder). The gel was separated under electrical current of 200 V for 55 minutes using the 1x-Tris/tricine/SDS running buffer. BioSafe™ Coomassie solution was used to stain the gel for 2 hr and then destained using water overnight. Gels were imaged using ChemiDoc™ Imaging system (Bio-Rad).

Table 1. List of gel casting solution for 10% separating and 4% stacking gels.

Gel Casting Solution	10% Separating gel	4% Stacking gel
40% Acrylamide/Bis (29:1)	3.75 mL	400 μ L
Water	3.75 mL	775 μ L
3 M Tris/HCl pH 8.45 containing 0.3% SDS	2.62 mL	1950 μ L
50% Glycerol	1.125 mL	N/A
TEMED	15 μ L	10 μ L
30% APS	15 μ L	10 μ L

2.6. Bottom-up Proteomics

The shotgun proteomics approach, which refers to bottom-up proteomics too, used to characterize in-solution tryptic digest of crude maple leaves and FPLC fraction 14 (F14). The mass spectrometer was equipped with Q-TOF Model Impact II (Bruker) connected to the UltiMate 3000 UHPLC Systems (Thermo-Fisher Scientific).

2.6.1. In-Solution Digestion

The lyophilized fall crude maple leaves proteins 40 mg and lyophilized active fraction F14, were solubilized in 400 μ L of 6M urea prepared in 50 mM tris and mixed with 20 μ L of reducing agent 200 mM dithiothreitol (DTT). The 200 mM DTT was prepared in 50mM Tris pH 8.0. The samples were vortexed and incubated for 1 hr at room temperature. Then, 80 μ L (200 mM) of the alkylating agent iodoacetamide was added to the mixtures and incubated in the dark for 1 hr at room temperature. The iodoacetamide 200 mM was prepared in 50mM Tris pH 8.0. After Iodoacetamide was added, 80 μ L of DTT was mixed with the samples again and incubated for 30 min at room temperature. Then, the pH was monitored while adding 50mM Tris pH 8.0 until the pH adjusted at 8.0. The trypsin was prepared in 50 mM tris/HCl pH 8.0 and was added in ratio 1:30 (Trypsin:Protein). The mixture was incubated overnight (<16 hr). On the next day, the digestion was stopped by adding acetic acid until pH 4.0 reached.

2.6.2. LC-MS/MS Q-TOF

The tryptic digest of crude maple leaves proteins and fractions F14 were analyzed by using Impact II™ UHR-QqTOF (Ultra-High Resolution Qq-Time-of-Flight) mass spectrometry instrument along with Phenomenex BioZen 2.6 μ m Peptide XB-C18 (pore size 100 Å, dimension 150×2.1)

column. The mobile phase used was solvent A: 0.1% formic acid in water and solvent B: 0.1% formic acid in ACN at a flow rate of 0.3 mL/min. The elution gradient was 0-55% B in 45 min.

2.6.3. MS/MS Data Analysis

In these experiments, Mascot and *De novo* sequencing by PEAKS bioinformatics software were used. The data obtained from MS/MS called mascot generic files (mgf). These data were used to search in SwissProt and NCBI nr- taxonomy Viridiplantae database. The search parameters selected is illustrated in **Table 2**. Whereas, *De novo* sequencing PEAKS search parameters are illustrated in **Table 3**.

Table 2. The parameters used in Mascot search.

Search Parameters	
Fixed modification	Carbamidomethylation of cysteine
Variable modification	Oxidation of methionine
Peptide mass tolerance	± 1.2 Da
Fragment mass tolerance	± 0.6 Da
Enzyme	Trypsin
Max Missed Cleavages	1
The false discovery rate	1%
Probability	99% for the crude 95% for F14

Table 3. The parameters used in *De novo* sequencing (PEAKS) search.

Search Parameters	
Search Engine Name	PEAKS
Parent Mass Error Tolerance	50.0 ppm
Fragment Mass Error Tolerance	0.5 Da
Precursor Mass Search Type	Monoisotopic
Enzyme	Trypsin
Max Missed Cleavages	2
Digest Mode	Semi specific
Fixed Modifications	Carbamidomethylation: 57.02
Variable Modifications	Oxidation (M): 15.99 Acetylation (Protein N-term): 42.01 Pyro-glu from E: -18.01 Pyro-glu from Q: -17.03
Max Variable PTM Per Peptide	3
Database	Viridiplantae-reviewed
Taxon	All
FDR Estimation	Enabled
Merge Options	No merge
Precursor Options	Corrected
Charge Options	No correction
Filter Charge	2 - 6
Process	True
Associate chimera	Yes
De novo ALC Score	$\geq 50\%$
Protein -10lgP	≥ 20

2.6.4. Gene Ontology Annotation

In this search, the UniProt Knowledgebase (UniProtKB) gene ontology (GO) project was used to obtain the annotation of gene ontology from the identified proteins (<https://www.uniprot.org/uniprot/>). The proteins were categorized based on their biological process, molecular functions, and cellular compartment.

2.7. Cytotoxic Evaluation of Protein Extracts

2.7.1 Protein Estimation

The Coomassie (Bradford) protein assay kit (Thermo Scientific) was used for the estimation of total protein concentration. PBS was used as a diluent for BSA standard as following concentrations: 2,000, 1,500, 1,000, 750, 500, 250, 125, 25 $\mu\text{g/mL}$, and blank. The protein extracts in sodium phosphate buffer were desalted and concentrated by centrifugal filters (3,000 Da MWCO). Using 96-well plate, 2 μL of protein extract, and an equal amount of BSA were mixed and added in triplicates. Then, 250 μL of Coomassie Plus Reagent solution was added to each sample and incubated in the dark for 10 min. The samples were analyzed by using SpectraMax M5 UV/VIS Plate Reader to measure the absorbance at 595 nm. The concentration was obtained from calculating the average reading of the triplicate results.

2.7.2. MTT Assay

The potential cytotoxic activity of the proteins extracted from red maple was evaluated by performing MTT assay (Hansen et al.,1989). Human breast cancer MCF-7 (ATCC HTB-22^{TM}) and MDA-MB-231 (ATCC® HTB-26^{TM}) cell lines were cultured and maintained in DMEM medium supplemented with 10% (v/v) of fetal bovine serum (FBS) and 1% (v/v) penicillin-

streptomycin in a humidified incubator at 37°C containing 5% CO₂. The cells were cultured in tissue culture treated flasks (T-75 flask) of 75 cm² at confluency. After the medium was removed, 7 mL of PBS was used twice for cell washing. The cells were detached from the flask surface by adding 1 mL of trypsin and incubated in a CO₂ incubator at 37°C for 3 min. Then 4 mL of the medium was added, and cells were transferred into 15 mL tube. The cells were centrifuged at 1,000 rpm for 5 min. The supernatant was removed, and 1 mL of the medium was mixed with the pellet. In cells counting process, trypan blue was mixed with 10 µL of the cell suspension and counted the cells under a microscope by using hemocytometer. In order to prepare the cells for the treatment, the cells were diluted at a density of 3x10⁴/mL in 96 well plate in 200µl of volume (10,000 cells in each well) and incubated in 5% CO₂ incubator at 37°C for 24 hr. The medium was removed from the flask, and cells were treated at different concentrations of F14 (in triplicate). The concentrations were used as following 4 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL, 0.12 µg/mL, and 0.06 µg/mL. The negative control in this experiment was untreated cells, whereas the positive control was doxorubicin in different concentrations as following 0.6 µM, 0.3 µM, 0.15 µM, 0.07 µM, and 0.03 µM. After 48 h, the medium containing the samples were removed, and 0.5 mg/mL of MTT dye was prepared in 200 µL medium was added to each well and incubated for 4 h in 5% CO₂ incubator at 37°C. The medium containing MTT dye was removed after incubation, and the formazan crystals were solubilized in 100 µL of DMSO in each well. The absorbance was measured at 550 nm using SpectraMax M5 UV/VIS Plate Reader. The percentage of cell inhibition was calculated using the formula:

$$\% \text{ Inhibition} = \frac{OD \text{ of untreated cells} - OD \text{ of treated cells}}{OD \text{ of untreated cells}} \times 100$$

2.8. Anti-Inflammatory Assay

The potential anti-inflammatory activity of the proteins extracts from Fall and Spring red maple leaves were evaluated by performing the cell-based assay. In this experiment, RAW 264.7 Murine Macrophages cell line was used at a density of 2.5×10^5 and seeded in a 96-well plate and incubated overnight. Next day, lipopolysaccharide (LPS) $1 \mu\text{g/mL}$ and $200 \mu\text{g/mL}$ of the protein samples were added to cells and incubated for another 24 hr. The Nitric oxide (NO) production was measured by using Griess Reagents. The NO production measures the anti-inflammatory activity of the samples. If the cells produced less amount of NO, it means the protein samples possess anti-inflammatory activity. The crude protein extracts from Fall and Spring were used, as well as the Fall and Spring FPLC fractions (F12, F13, F14, F15, F19, and F20-21), (S4-8, S9-10, S11-13, S14, and S15-18), respectively. The positive control in this assay was lipopolysaccharide (LPS).

Chapter 3

RESULTS AND DISCUSSION

3.1. Protein Extraction and Precipitation

Fatty acid removal process before the protein extraction was achieved by immersion of Fall and Spring Red maple leaves in 100% hexanes for 24 hr. The protein extraction was performed in three different buffers; 25 mM sodium phosphate pH 6.5, deionized water, and 20 mM Tris/HCl pH 8.0. Proteins precipitation were successfully achieved using 80% ammonium sulfate. This technique is preferred because the ammonium sulfate has properties such high solubility in aqueous buffers, low density and relatively inexpensive as compared to other protein precipitation technique employed organic solvent acetone or acidified acetone with 2% HCl. At the concentration of 60%, ammonium sulfate extracted proteins recovered by precipitation based on “salting out”. The denatured proteins are recovered can be pushed back into their native conformations. This step helped to get rid of small molecules and non-protein soluble components from the extracts. The extraction conducted in phosphate buffer resulted in comparatively more protein quantity than deionized water and Tris/HCl, respectively.

3.2. Gel Filtration Chromatography

The partial purification of protein extracted from Red maple leaves was accomplished by using gel filtration chromatography on HiLoad™ 16/600 Superdex™ 200 pg column using ÄKTA start FPLC system. This column was used as it has a wide protein separation range from 10-600 kD. The gel filtration was used as the first-dimensional purification step which separates proteins based on their molecular weight in elution order from high molecular weight to low molecular weight proteins/peptides. The proteins extracted in 25 mM sodium phosphate pH 6.5, eluted into two major peaks marked as F12-13 and F14-15. were observed in the separation profile of spring leaves

protein extract, as shown in **Figure 3**. In contrast, using the same chromatography conditions Fall leaves extract separated into four major peaks, namely F12-13, F14, F19, F20-21as shown in **Figure 4**.

Figure 3. Gel filtration chromatography elution profile of crude protein from Red maple spring leaves. Column: HiLoad™ 16/600 Superdex™ 200 pg, buffer: 25 mM sodium phosphate pH 6.5, flow rate at 1 mL/min, and absorbance at 280 nm.

Figure 4. Gel filtration chromatography elution profile of crude protein from Red maple fall leaves. Column: HiLoad™ 16/600 Superdex™ 200 pg, buffer: 25 mM sodium phosphate pH 6.5, flow rate at 1 mL/min, and absorbance at 280 nm.

3.3. Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

The RP-HPLC is most widely use chromatography technique for the separation of proteins/peptides. It separate proteins based on their hydrophobicity properties from low to high hydrophobicity. In this project, proteins extracted in three different conditions were evaluated by RP-HPLC. The crude protein extracts from Spring and Fall were loaded onto a reversed-phase column, Vydac C18 (2.1x150mm) column. The elution profiles showed that 25 mM sodium phosphate pH 6.5 was the best extraction buffer, as compared to DI water and Tris/HCl, which extracted comparatively more proteins from Red maple leaves. The elution profile observed using all three extraction conditions for Spring and Fall leaves are shown as **Figures 5-7**.

Figure 5. RP-HPLC elution profile of Red maple crude proteins from Spring and Fall in 25 mM sodium phosphate buffer. Column: Vydac C18 (2.1x150mm), gradient: 0-60% acetonitrile in 85 min, flow rate 1 mL/min and absorbance at 280 nm.

Figure 6. RP-HPLC elution profile of Red maple crude proteins from Spring and Fall in deionized water buffer. Column: Vydac C18 (2.1x150mm), gradient: 0-60% acetonitrile in 85 min, flow rate 1 mL/min and absorbance at 280 nm.

Figure 7. RP-HPLC elution profile of Red maple crude proteins from Spring and Fall in 20 mM Tris/HCl pH 8 buffer. Column: Vydac C18 (2.1x150mm), gradient: 0-60% acetonitrile in 85 min, flow rate 1 mL/min and absorbance at 280 nm.

3.4. Electrophoresis

The tris/tricine gels SDS-PAGE were successfully performed for the crude protein samples extracted from Spring and Fall leaves as well as active (based on anticancer and anti-inflammatory assays) gel filtration fraction F14. The SDS-PAGE electrophoresis which separates proteins based on the molecular weight from high molecular weight to low molecular weight (from top to bottom). The most frequently employed electrophoresis technique for proteins is Tris/tricine gels. Instead, in our project, tris/tricine gels were preferred. This method of gel electrophoresis resolved the small molecular weight proteins better than Tris/glycine gels. The electrophoretic profile observed at different loading concentrations, in Fall leaves crude protein (**Figure 8**) and gel filtration fraction F14 (**Figure 9**) several protein bands within a range of ~150-25 kDa, and relatively very weak bands from 25-5 kD range. However, the Spring leaves crude protein showed smeared bands in the gels, as shown in **Figure 8**. The smearing observed while keeping all electrophoresis conditions identical can not be answered at this time. It can be assumed that in Spring leaves abundant proteins are expressed ranging from 37-20 kD, which overshadowed low abundant proteins in the electrophoretic profile.

Figure 8. Tris/tricine gel electrophoresis profile with reducing agent for the Spring (S) and Fall (F) crude protein of Red maple in different concentrations.

Figure 9. Tris/tricine gel electrophoresis profile with reducing agent for the Fall F14 FPLC fraction protein of Red maple.

3.5. Proteomics of Maple Leaves

3.5.1. *LC-MS/MS Q-TOF*

Plant proteomics is a rapidly evolving field in the biochemistry with a focus on comparative or differential protein expression profiling. In a proteomic evaluation of proteins, two main strategies are in use known as top-down proteomics and bottom-up proteomics. The bottom-up proteomics is most common and approaches for protein profiling studies where a limited quantity of crude sample is available. Without going into exhaustive protein purification, peptides are generated typically by enzymatic cleavage by trypsin and infused directly to electrospray LC/MS. The mass spectrometric data than analyzed using bioinformatics tools. We successfully employed a bottom-up proteomic approach using common LC/MS/MS mass spectrometric technique to characterize the amino acid sequence from tryptic peptides of crude protein of Fall leaves. Due to the limited quantity of Spring leaves protein, it was not proceeded further protein profiling. The characterization of Fall leaves crude protein, and FPLC fraction F14 were achieved successfully. The representative mass spectrometric profile of the fraction F14 LC/MS base peak chromatogram (BPC) red in color, which show the most intense peaks at any one time in the mass spectra, while the total ion chromatogram (TIC) green in color resulting from the summing up intensities of all mass spectral peaks in the same scan as shown in **Figure 10**.

Figure 10. Tryptic digest profile of **fraction F14** LC/MS profile of Fall leaves protein extracted in FPLC. Column: Phenomenex BioZen 2.6 μ m Peptide XB-C18 (pore size 100 Å, dimension 150×2.1), Mobile phase: A: 0.1% formic acid in water and solvent B: 0.1% formic acid in acetonitrile at 0.3 mL/min flow rate, and gradient 0-55%B in 45 min.

3.5.2. Evaluation of MS/MS Data by Mascot

The mass spectrometric data is typically analyzed first by using search engine Mascot. It identifies proteins from peptide databases (Perkins et al., 1999). Mascot uses a probabilistic scoring algorithm adopted from the MOWSE (for MOlecular Weight SEarch) algorithm. The database used was SwissProt, a manually annotated and reviewed database. We also used the NCBI nr (non-redundant) database, compiled by the National Center for Biotechnology Information (NCBI), which contains non-redundant sequences from GenBank. The Mascot search engine used to analyze the mass list obtained from LC-MS/MS data; a total of 25 proteins were identified in Fall leaves crude protein extract by using the SwissProt database (**Table 4**). Identified proteins are from

7 to 81 kDa. The highest score of 104 and the highest protein matches of 15 observed for protein Ribulose biphosphate.

The Fall leaves partially purified gel filtration fraction F14, two proteins identified are Chaperone protein ClpC, chloroplastic (*Pisum sativum*) and protein Accelerated cell death-6 (ACD6) from *Arabidopsis thaliana* by SwissProt database using the ID threshold score =27 (**Table 5**). The Chaperone protein ClpC protein involved in the degradation of denatured proteins in the chloroplast. The ACD6 is a novel ankyrin protein which is a dose-dependent activator of the defense response against virulent pathogens, including bacteria, fungi, and oomycetes. The NCBI nr database search revealed the identity of Phosphatidylinositol 3-/4-kinase (*Macleaya cordata*) and unnamed protein from *Coffea canephora* using the ID threshold score =50 (**Table 6**). The Phosphatidylinositol 3-/4-kinase is a signaling molecule in stomatal responses to environmental signals. The highest protein Score 34 observed for Protein accelerated cell death-6 and by SwissProt while the highest score of 56 for Phosphatidylinositol 3-/4-kinase (*Macleaya cordata*) by NCBI nr.

Table 4. Proteins in crude Fall leaves by Mascot search engine using SwissProt database. A total of 25 proteins identified with a protein score above 44.

Protein accession NO.	Protein description	Protein score	Protein mass	Protein matches	Protein sequence	Peptide No. matches
RBL_AILAL	Ribulose biphosphate carboxylase large chain (Fragment) OS=Ailanthus altissima OX=23810 GN=rbcL PE=3 SV=1	104	52126	15	GGLDFTK	3
RBL_PHYPA	Ribulose biphosphate carboxylase large chain OS=Physcomitrella patens subsp. patens OX=3218 GN=rbcL PE=1 SV=2	96	53185	10	QVTLGFVDLLR	7
RBL_ACRAU	Ribulose biphosphate carboxylase large chain (Fragment) OS=Acrostichum aureum OX=29594 GN=rbcL PE=3 SV=1	91	46596	10	DTDILAAFRMTPQPGVPAEEAGAAVA AESSTGTWTVVTDGLTLLDR	6
RBL_CAPBA	Ribulose biphosphate carboxylase large chain (Fragment) OS=Capsicum baccatum OX=33114 GN=rbcL PE=3 SV=1	87	52511	8	MSGGDHIIHAGTVVGK	5
RBL_PTYGA	Ribulose biphosphate carboxylase large chain (Fragment) OS=Ptychomitrium gardneri OX=61565 GN=rbcL PE=2 SV=1	82	49049	9	AEFARELGAPIVMHDY LTGGFTANTTLAHYCR	4
RBL_ADOMO	Ribulose biphosphate carboxylase large chain (Fragment) OS=Adoxa moschatellina OX=4208 GN=rbcL PE=3 SV=1	81	52159	10	ALRMSGGDHIIHSGTVVGK	7
RBL_MARPO	Ribulose biphosphate carboxylase large chain OS=Marchantia polymorpha OX=3197 GN=rbcL PE=1 SV=1	80	53271	10	MSGGDHIIHAGTVVGK	5
RBL_AFRGR	Ribulose biphosphate carboxylase large chain OS=Afrocarpus gracilior OX=56897 GN=rbcL PE=3 SV=1	75	53203	11	EVTLGFVDLLRDDFIEK	4
RBL_BOROF	Ribulose biphosphate carboxylase large chain (Fragment) OS=Borago officinalis OX=13363 GN=rbcL PE=3 SV=1	68	49540	9	ALRMSGGDHIIHSGTVVGK	7
RBL_ANGEV	Ribulose biphosphate carboxylase large chain OS=Angiopteris evecta OX=13825 GN=rbcL PE=3 SV=1	67	53138	5	EGNEIIREASK	5
RBL_ABIMA	Ribulose biphosphate carboxylase large chain OS=Abies magnifica OX=3320 GN=rbcL PE=3 SV=1	64	53147	9	GIFFTQDWVSMGPVLPVASGGIHVWHMPA LTEIFGDDSVLQFGGGTLGHPWGNAPGAAANR	2
RS27A_DAUCA	Ubiquitin-40S ribosomal protein S27a (Fragment) OS=Daucus carota OX=4039 PE=2 SV=2	86	16517	10	KECPNGECGAGTFMANHFDR	7

Protein accession NO.	Protein description	Protein score	Protein mass	Protein matches	Protein sequence	Peptide No. matches
G3PC2_ARATH	Glyceraldehyde-3-phosphate dehydrogenase GAPC2, cytosolic OS=Arabidopsis thaliana OX=3702 GN=GAPC2 PE=1 SV=1	84	37004	8	GILGYTEDDVVSTDFVGDNR	7
G3PC1_ORYSJ	Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic OS=Oryza sativa subsp. japonica OX=39947 GN=GAPC1 PE=1 SV=1	54	36561	3	AGIALNDNFVK	8
G3PC1_HORVU	Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic OS=Hordeum vulgare OX=4513 GN=GAPC PE=2 SV=1	55	36605	2	VVISAPSK	5
ENO_ALNGL	Enolase OS=Alnus glutinosa OX=3517 GN=PGH1 PE=2 SV=1	83	47796	8	YGQDATNVGDEGGFAPNIQENK	7
ENO_MESCR	Enolase OS=Mesembryanthemum crystallinum OX=3544 GN=PGH1 PE=2 SV=1	76	48661	7	YGQDATNVGDEGGFAPNIQENK	7
ALFP1_ARATH	Fructose-bisphosphate aldolase 1, chloroplastic OS=Arabidopsis thaliana OX=3702 GN=FBA1 PE=1 SV=2	82	43075	4	YAAISQDSGLVPIVEPEIMLDGEHGIDR	3
ALFP_ORYSJ	Fructose-bisphosphate aldolase, chloroplastic OS=Oryza sativa subsp. japonica OX=39947 GN=ALDP PE=1 SV=2	83	42208	4	TVVSI PN GPSELAVKEAAWGLAR	5
ALFP2_ARATH	Fructose-bisphosphate aldolase 2, chloroplastic OS=Arabidopsis thaliana OX=3702 GN=FBA2 PE=1 SV=2	44	43132	3	YAAISQDSGLVPIVEPEILLDGEHDIDRTYDVAEK	3
RCA_ARATH	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic OS=Arabidopsis thaliana OX=3702 GN=RCA PE=1 SV=2	74	52347	5	LVNSR	2
H42_WHEAT	Histone H4 variant TH091 OS=Triticum aestivum OX=4565 PE=3 SV=2	69	11460	4	DNIQGITKPAIR	5
ATPAM_PHAVU	ATP synthase subunit alpha, mitochondrial OS=Phaseolus vulgaris OX=3885 GN=ATPA PE=3 SV=1	68	55595	8	EVAAFAQFGSDLDAATQALLNR	4
RL37_SOLLC	60S ribosomal protein L37 (Fragment) OS=Solanum lycopersicum OX=4081 GN=RPL37 PE=2 SV=1	66	7372	1	EGTEAAPR	7
RUB1_BRANA	RuBisCO large subunit-binding protein subunit alpha, chloroplastic (Fragment) OS=Brassica napus OX=3708 PE=2 SV=2	63	57714	7	LAGGVAVIKVGAATETELEDNR	6

Table 5. Proteins identified in Fall leaves gel filtration fraction F14 by Mascot search engine using SwissProt database.

No.	Taxonomy	Protein Description	Unique Peptide Sequence	Protein Score	Peptide Expect
1	<i>Pisum sativum</i>	Chaperone protein ClpC, chloroplastic <i>Pisum sativum</i>	VSKAVSSRR	33	0.0044
2	<i>Arabidopsis thaliana</i>	Protein ACCELERATED CELL DEATH 6 <i>Arabidopsis thaliana</i>	SGLRAR	34	0.059

* ID threshold score = 27

Table 6. Proteins identified in Fall leaves gel filtration fraction F14 by Mascot search engine using NCBI nr database.

No.	Taxonomy	Protein Description	Unique Peptide Sequence	Protein Score	Peptide Expect
1	<i>Macleaya cordata</i>	Phosphatidylinositol 3-/4-kinase [<i>Macleaya cordata</i>]	SAASLMAR	56	0.03
2	<i>Coffea canephora</i>	unnamed protein product [<i>Coffea canephora</i>]	AAQVGLVSGK	50	0.039

* ID threshold score = 50

3.5.3. MS/MS Data Search by *De novo* sequencing by PEAKS® software

The identification of proteins by mass spectrometry is dependent on the availability protein sequences in mass spectrometry database. The classical Edman degradation technique is typically utilized to establish the amino acid sequence. This technique is independent of the database to identify the protein. The PEAKS® X is a new *De novo* peptide sequencing bioinformatics solution for proteomics developed by Bioinformatics Solution Inc. It performs analysis and statistics utilizing LC/MS/MS data using *De novo* sequencing and database search. A total of 54 proteins were identified using PEAKS Studio- *De novo sequencing* database search from Fall maple leaves, gel filtration fraction F14 as listed in **Table 7**. In this analysis, identified 54 proteins have at least one unique peptide listed in the column as #Unique (**Table 7**). The statistically significant data required to have a $-\log P$ ($P < 0.05$) values above 13. The analysis further revealed that four proteins identified have at least two unique peptides $-\log P$ values above 25. The presence of at least two unique peptides sequences found are highlighted in green color are from *Cuscuta reflexa*, *Arabidopsis thaliana*, and *Chlamydomonas reinhardtii* (**Table 7**). The location and the amino acid sequence of these peptides in the protein are shown in **Figures 11, 12, and, 13**, respectively.

Table 7. Proteins in Fall leaves gel filtration fraction F14 by *De novo sequencing* using PEAKS Studio-X software. A total of 54 proteins identified.

Protein ID	Accession	-10lgP	Coverage (%)	#Unique	Avg. Mass	Identified Protein
12646	Q04979 LO NP2_SPIOL	34.53	1	1	98272	Lon protease homolog 2, peroxisomal Spinacia oleracea
8915	A2ZAI7 RO C3_ORYSI	33.14	1	1	94725	Homeobox-leucine zipper protein Oryza sativa subsp. indica
8863	A2WY50 M RS2F_ORY SJ	32.12	3	1	48356	Magnesium transporter MRS2-F Oryza sativa subsp. indica
6660	Q8GYU3 TY Q_ARATH	30.94	1	1	162425	Transcriptional elongation regulator MINIYO Arabidopsis thaliana
3263	F4J7T2 EAF 1B_ARATH	30.08	1	2	206411	Chromatin modification-related protein EAF1 B Arabidopsis thaliana
3264	F4J7T3 EAF 1A_ARATH	30.08	1	2	211665	Chromatin modification-related protein EAF1 A Arabidopsis thaliana
5210	A9L9E2 TI2 14_LEMMI	29.91	1	1	228108	Protein TIC 214 Lemna minor
33720	Q9SJF1 LSG 12_ARATH	29.61	2	1	66743	GTPase LSG1-2 Arabidopsis thaliana
33722	Q9C9W9 A DO3_ARAT H	29.49	2	1	69063	Adagio protein 3 Arabidopsis thaliana
33727	B9GE13 KN 12F_ORYSI	29.06	1	1	317312	Kinesin-like protein KIN-12F Oryza sativa subsp. japonica
33732	P0C587 HE M1_ORYSI	28.32	2	1	58419	Glutamyl-tRNA reductase, chloroplastic Oryza sativa subsp. japonica
33733	A2Z928 HE M1_ORYSI	28.32	2	1	58435	Glutamyl-tRNA reductase, chloroplastic Oryza sativa subsp. indica
33741	Q9FPP7 FA C2B_CALO E	27.3	2	1	43365	Fatty acid conjugase FAC2 Calendula officinalis
33742	Q7XL03 CL PD2_ORYSI	27.15	1	1	101483	Chaperone protein ClpD2, chloroplastic Oryza sativa subsp. japonica
3427	A7M9B2 TI 214_CUSRE	26.53	1	2	207375	Protein TIC 214 Cuscuta reflexa
7216	Q41382 RH7 _SPIOL	25.71	2	1	73085	DEAD-box ATP-dependent RNA helicase 7 Spinacia oleracea
33759	P43213 MP AP1_PHLPR	25.36	4	1	28457	Pollen allergen Phl p 1 Phleum pratense
33760	Q8H7T4 EX B10_ORYSI	25.36	4	1	28937	Expansin-B10 Oryza sativa subsp. japonica
33761	Q50EK5 C7 2B2_PINTA	25.34	2	1	56222	Cytochrome P450 720B2 Pinus taeda
33763	Q43316 HE M3_ARATH	25.29	2	1	41043	Porphobilinogen deaminase, chloroplastic OS=Arabidopsis thaliana OX=3702 GN=HEMC PE=1 SV=1
6755	Q32065 YC X9_CHLRE	25.04	1	2	341667	Uncharacterized 341.7 kDa protein in psbD-psbC intergenic region Chlamydomonas reinhardtii
9299	P10290 MY BC_MAIZE	24.99	5	1	28751	Anthocyanin regulatory C1 protein Zea mays
33771	P58051 C71 BE_ARATH	24.37	2	1	56900	Cytochrome P450 71B14 Arabidopsis thaliana
33767	Q9S7C0 HS P7O_ARAT H	24.26	1	1	91750	Heat shock 70 kDa protein 14 Arabidopsis thaliana
33768	F4HOD4 HS P7P_ARAT H	24.26	1	1	91681	Heat shock 70 kDa protein 15 Arabidopsis thaliana
5682	F4JSZ5 BIG 1_ARATH	23.22	1	1	187536	Brefeldin A-inhibited guanine nucleotide-exchange protein 1 Arabidopsis thaliana
33776	Q9LVR3 U4 96G_ARAT H	22.69	3	1	45597	UPF0496 protein At5g66670 Arabidopsis thaliana

33777	Q4888I1TPS I BRANA	22.61	2	1	56045	Thiamine biosynthetic bifunctional enzyme BTH1, chloroplastic Brassica napus
33779	Q9SZH4IPE P ARATH	22.5	3	1	54025	RNA-binding KH domain-containing protein PEPPER Arabidopsis thaliana
33704	Q8HOT4IUP L2 ARATH	22.41	0	1	403618	E3 ubiquitin-protein ligase UPL2 Arabidopsis thaliana
33730	Q8S2G5IGP DH2 ORYS I	21.64	3	1	51584	Probable glycerol-3-phosphate dehydrogenase [NAD (+)] 2, cytosolic Oryza sativa subsp. japonica
33793	Q9SXE1IGS OX3 ARAT H	21.55	2	1	52688	Flavin-containing monooxygenase FMO GS-OX3 Arabidopsis thaliana
2997	Q14FG5IRP OB POPAL	21.38	1	1	120521	DNA-directed RNA polymerase subunit beta Populus alba
3218	Q2L8Z9IRP OB GOSHI	21.38	1	1	120559	DNA-directed RNA polymerase subunit beta Gossypium hirsutum
3041	A4QLS5IRP OB NASOF	21.38	1	1	121076	DNA-directed RNA polymerase subunit beta Nasturtium officinale
3038	A4OKI4IRP OB CAPBU	21.38	1	1	121085	DNA-directed RNA polymerase subunit beta OS=Capsella bursa-pastoris OX=3719 GN=rpoB PE=3 SV=1
3042	P50546IRPO B ARATH	21.38	1	1	121059	DNA-directed RNA polymerase subunit beta Arabidopsis thaliana
3040	A4OKS3IRP OB CRUW A	21.38	1	1	120979	DNA-directed RNA polymerase subunit beta OS=Crucihimalaya wallichii OX=78192 GN=rpoB PE=3 SV=1
3036	A4QIS5IRP OB OLIPU	21.38	1	1	120991	DNA-directed RNA polymerase subunit beta OS=Olimarabidopsis pumila OX=74718 GN=rpoB PE=3 SV=1
3037	A4QLI1IRP OB DRANE	21.38	1	1	120871	DNA-directed RNA polymerase subunit beta Draba nemorosa
3294	A4QLI6IRP OB LOBM A	21.38	1	1	121059	DNA-directed RNA polymerase subunit beta Lobularia maritima
33794	A4QJ11IRPO B AETGR	21.38	1	1	120945	DNA-directed RNA polymerase subunit beta OS=Aethionema grandiflorum
33796	Q9FEB5IDS P4 ARATH	21.32	3	1	42626	Phosphoglucan phosphatase DSP4, chloroplastic Arabidopsis thaliana
6733	Q10PC5ITT A1 ORYSJ	21.15	2	1	78389	Protein TITANIA OS=Oryza sativa subsp. japonica
33797	Q7EZD5IY8 577 ORYSJ	21.06	4	1	30720	Putative B3 domain-containing protein Oryza sativa subsp. japonica
10085	F4JSV3IED A40 ARAT H	21.05	1	1	81479	Probable E3 ubiquitin-protein ligase EDA40 Arabidopsis thaliana
8990	F4JVN6ITPP IL ARATH	20.88	1	1	152367	Tripeptidyl-peptidase 2 Arabidopsis thaliana
33800	P83181POA L KALTU	20.87	21	1	4654	Pollen allergen Sal k 1 (Fragments) Kali turgidum
33803	A2ZVG7IFT SH9 ORYSJ	20.79	2	1	85793	ATP-dependent zinc metalloprotease FTSH 9, chloroplastic/mitochondrial Oryza sativa subsp. japonica
4099	A8J666ICFA 54 CHLRE	20.71	1	1	317779	Cilia- and flagella-associated protein 54 Chlamydomonas reinhardtii
33817	Q94BZ7IGY RBM ARA TH	20.37	1	1	81053	DNA gyrase subunit B, mitochondrial Arabidopsis thaliana
33783	Q94H98IP2C 34 ORYSJ	22.27	3	1	41755	Probable protein phosphatase 2C 34 Oryza sativa subsp. japonica
33784	P27759IPLY 5 AMBAR	22.26	4	1	42709	Pectate lyase 5 OS=Ambrosia artemisiifolia
33785	Q6AVI8ICD PK9 ORYSJ	22.25	2	1	63978	Calcium-dependent protein kinase 9 Oryza sativa subsp. japonica

1	MIVNNMYNLC	PKIINSVIVV	GLYYGFMTAL	SIKPSHIFLI	RALLEKETN	KNKGVAETK	KKVAATTGFI	MGQFIRLISI		
81	YVGPLYVALG	RPHTITILAL	PYLLIHLFWN	TDKSFFAYDS	NKLNLSIRNLE	IYCVFLNHFA	LQLLNSCILP	NSTLARLVSI		
161	YMFRCNNKIL	FLTSSFFAWF	IGQLFILNCF	ELVLVWIRKK	NSIRSTFRNY	LLRNSIFVIF	LNCIFGSLLF	LLSIQCLGRI		
241	PSPIPTQKLS	EVSKIEQRER	ERLQKEEERG	VEKKEQSTEE	DPSLFLEEKA	GWDKEKEPDY	KFPDSELEIL	QKKKIKNQEF		
321	EKHLAALLFD	YKRWTRPFRY	IKNNHLEQAL	RNEMSQYFFD	TYQSDGKNRL	SFTHPISLSA	FLKMIKPKIP	LLLVEKNTFN		
401	SNSLDNGWVY	RNKKQMNLYR	IDFLNRVKNL	DKAKAVALPR	IEFETTRTQL	CIHNDEKQE	YLPENFDPLL	NGPYRGRIKK		
481	GLLPINDTLS	EHLRETVMNL	RLHALVLLNT	NSKNSNQKMS	TFGKKPLEIC	GFSTFNLNLM	DSELKTEVLV	NPIETHDLNF		
561	LKKYSTIEEI	SKKVPWRSYK	LITELEQISY	YKNPPDDHDI	RSRKGISVVI	FDPNKEATTT	NSKTNTTKDT	NLETKKESES		
641	DEDKLVVIRY	PQQSDFRQGL	IKDSMRNQRR	KIIIWELFNA	NVHSPLFFDR	LTIVFSFPRL	KQLFINLSAR	HVFGISKSTD		
721	KKRGKTKKEE	KRENKQREQK	ERLEIGEAWD	VFPVAQIIRG	FLLLNQTFIR	KKIILPSLII	GKNIGRILLF	QIPEWSEDLR		
801	ELNRETHIKK	TYNGIPLSEK	EFFENWLTEG	IQIKILFPFC	LKPWHYPKPK	TSHYDFCFLT	VWGRETEQPF	GHPKTPSPFF		
881	EPVLQELDKK	IVNINIKARI	FSKVKINLFK	RFSKEKDFQI	SNQIINESFQ	QIEEIPGCTN	SSLIEKMQNM	AHRTSTIKKE		
961	IERVTEEKRR	VTLERYICFY	KRSYRLALAK	NIFKKVKVKV	TKNRLICKFF	FFKKLFNQRI	YNNIFLETIY	ICRITTQLFL		
1041	ESTKKLIYKY	IFNYERNKKR	IDINKETKNK	FNLISKLTKY	NHCKKNSYLS	CDLSNLSQAY	VFYKIPQTV	LVNCKKLISAL		
1121	QQNGIPSFYK	TQIKDSFHTQ	GICKYELIQK	KLQWPKNQW	KNWLRVNSEY	DLSHILWFSL	ISQKQKWRNR	VEQYHRSKEK		
1201	YLNKRNSRGN	YR LSDSKQN	VPK PVSDNYK	KCYQYDLSY	KSINYAKKSA	SVISRSTPKG	QAISYNDNML	QNIPGKIKRL		
1281	YITYIPYIGK	TLDRKYLIWK	NIHFYLRKKV	DIESWVAVNT	SSDKDSTIGT	YNYQLIDQID	KKEKELFSIP	IRQNTENRPF		
1361	NSTNSLVDWM	GMNEQILNRP	ITNLELWFFF	EFVWFFNVYK	TKPWIIPSKI	LILNSNLSET	DSKQKSETDS	KQKSETDSKQ		
1441	KSETDSKQKS	ETDSKQKSET	DSKQKSETDS	KQKSETDSKQ	KSETDSKQKN	NAEIQKDLDE	DSTKSDKKNK	KEKETELELF		
1521	AKKYFLFQLR	GDPTFKKSFF	KNIQIYCLLL	RLTNRRKMTL	SCIQRKFNFL	RIMPTMTNLF	NVPEFLKMTG	LVMDPLPLLI		
1601	KTNGKFLLYQ	IVGISLVHKS	KHQTNTQYRK	RIIIRAGMTN	LENNHLDVLV	LENILSSRCR	REFRTLICLN	YKNWNGVNTN		
1681	SIFCSKNCNQ	FWEERKPQYN	EKRELIQKFL	WPNYRLEDLA	CVNRYSDIT	NGSRFSLLRF	HMYLPWKIHG			
A7M9B2 T1214_CUSRE										
Peptide	Uniq	-10lgP	Mass	Length	ppm	Fraction	Scan	Source File	Start	End
N.VHSPLFFDR.L	Y	19.03	1116.5715	9	21.1	2	3880	FML_F14_998.mzXML	682	690
R.LSDSKQNVPK.P	Y	15	1242.6931	11	-47.4	2	6653	FML_F14_998.mzXML	1213	1223
total 2 peptides										

Figure 11. Two unique peptides from fall leaves protein FPLC F14 matching to *Cuscuta reflexa* by using *De novo sequencing* (PEAKS) software.

1	MHGSVSGYLL	VNAEVDMSGG	VIDSGGGIGV	KTSPPRTAIE	KAQAEALRQY	DVREERRREL	EFLEKGGNPL	DFKFGIATSH		
81	SVQSTSLTDQ	QAEHFVNSEV	KDSFALTASP	HGDSVESSGR	PGVPTISEPN	TADNLLLFD	ENKSVEGERN	LRHPNRQNR		
161	SESERSKKAH	TNQTKETED	SAIFRPYARR	NRSKISRDP	RSSSTDVLQN	RGGLATSISI	RRGSVEGKGC	IPEAANQKDM		
241	HTTSVSCPVF	ANSNGNIVPK	NRVSSNSLNT	KVDGEPVVR	STAGSKTSL	KDEADISYSK	SSAYLPVGES	GLAGEKAQLV		
321	STGGSPKAAT	IAGQKNSSTQ	LNGLRDSTVE	EESLTNRGAT	GTNGLESESS	HANNVEVND	NERDLYKVDK	LDSDEISMOK		
401	TLRVEGLLDQ	TVGEMTKTKI	EDETQGSTTI	ISECIPBECM	QMSVKIENQ	SHRSTAEMQT	KEKSETEKRI	LQDGLVVLEN		
481	DSKVGSILSE	NPSTLCSGI	PQASVDTSSC	TVGNSLISGT	DIEALKHQPS	SDAVMLDTVK	EDATLEEAR	IQAKKRIAE		
561	LSCGTAPVEV	REKSQWDFVL	EEMAWLANDF	AQERLWKMTA	ATQICHRAVAL	TCQLRFEERN	QHRKLKKIAS	VLSYAILQFW		
641	SSVEAEVPGE	LEETSLGIVK	ETCQESNCLN	GIRCLAAGVK	EYASRFLKYN	NSSISYHSAA	LSTPDNMCDP	EILDISMVDQ		
721	LTEASLFYSV	PSGAMEVYLK	SIESHLTRCE	KSGSSMQEEV	DTSAYDTAGD	IGYNVTAFFE	DEGETSTYYL	PGAFESSRSF		
801	NISHKKRKNL	MKSHSARSYD	LGDDLPHYNN	TGGSNSSSLI	VKRPDSNINA	GSVPTRRVRT	ASRHRVVSFF	GCATTGNLFP		
881	PSKTDASSGD	TSSFQDEYSS	LHGGSAVQKG	TEVESSVNEF	KLLPYDMAET	SGKPKKKKKK	HQGSAYDQTV	HLNPSVHVEQ		
961	KDHWWKRPEN	NFDMNGLYGP	HSAKKQKTTK	QLVENNFDMA	IPHTGSIPSP	AASQMSNMNS	PNKSIKFIGG	RDRGRKIKGL		
1041	KISPGQHSGS	NPWSLFEDQA	LVVLVHDMGP	NWELISDAMN	STLKIKYIYR	NPTECKDRHK	ILMDKTAGDG	ADSAEDSGNS		
1121	QSYPTSLPGI	PKGSARQLFQ	RLQGPMEEDT	LKSHFEKICL	IGKKLHYRKT	QNDGRDPKQI	VPVHNSQVMA	LSQVFPNNLN		
1201	GGVLTPLDVC	DASTSGQDVF	SLNPGLPLML	NQGTPLVPTS	GAHPSTPGSS	GVVLSNNLPT	TSGLQSASVR	DGRFNVPGRS		
1281	LPLDEQHRQL	QFNQTLSGRN	LQQPSLSTPA	AVSGSDRGHR	MVPGGNAMGV	SGMNRNTPMS	RPGFQGMASA	AMPNTGNMHT		
1361	SGMVGIPNTG	NIHSGGGASQ	GNSMIRPREA	VQHMMRMQAA	QGNSPGIPAF	SNLSSSGFTNQ	TPPVQAYPGH	LSQQHQMSFP		
1441	SHVLGNSHHF	HLQSPSQATG	AQQEAFAIRQ	RQIHQRYLQZ	QQQQFFPASGS	MMPHVQZPQG	SSVSSSSQNS	PQTQPPVSPQ		
1521	PLSMFPVSPS	PNINAMAQQK	PQKSQALHGL	LGRSPQSGTS	GVNNQAGKQR	QRQLQQSARQ	HPHQRPQPTQ	QQLNKQLKGM		
1601	GRGNMIHQNI	TVDQSHLNGI	TMPQGNQATE	KGEIAVSVRP	DQQSSVGTIT	STDLSQSKPFV	SPLSSNHSQQ	LPKSFPGALS		
1681	PSFQQQMQLH	SDNSIQGQSS	GATPCNLTSL	SSLSIAPAVA	PSNHQHLIHL	QKQRNQVQST	AQRVVQHNNH	GNSLSKKSQ		
1761	AECMPRVQPS	VNTNTQTASM	GTTKGMPQAS	NDLKNIKAVG	STAVPALEPP	SCVASVQSTA	SKVVNSNTSD	SAGNDPVSTP		
1841	NQGLAQKHGI	KGVTQRQQQS	LPSEKRPKPL	PEKPTVQNQK	HLASEEQPHL	EEAQELSSSK	PPDTKVE			
F4J7T3 EAF1A_ARATH										
Peptide	Uniq	-10lgP	Mass	Length	ppm	Fraction	Scan	Source File	Start	End
K.VDGEPPVVR.E	Y	21.66	869.4606	8	-15.8	2	5179	FML_F14_998.mzXML	272	279
K.EDATLEEAR.I	Y	16.84	1044.5087	9	-27.8	2	5977	FML_F14_998.mzXML	541	549
total 2 peptides										

Figure 12. Two unique peptides from fall leaves protein FPLC F14 matching to *Arabidopsis thaliana* by using *De novo sequencing* (PEAKS) software.

1	MTFLNHYTYL	FSIPEKQADK	VSGILRLAQA	RPIETLQNER	INKQLNAFLK	TYKFEKLITN	YKMQSFIPN	NSLNGNKTNS		
81	STNKLYATSL	NVFPENPPLM	VRKAVSDEAD	KFSKFTYSKV	QVVTNNLNNG	MNSKEFIKAN	NLKPSLRAAE	SLVLNHLTYN		
161	KFKENLYFKT	NNIQPTKSXS	TSLFFLNILS	NSKPRTCSDF	LSSPKIRKTW	FRNTAWSLQT	QQHRSSNGIN	LSLQLPYALG		
241	PSVPAGASGQ	NMYELFVAQS	SSRFGTYYFL	QKLLSKYLDV	WNASADNGSV	LSNSENIKLN	FSMVSLLDK	MAIQTPNSLY		
321	FVFTQLNQKT	FLSYWLLPVA	GLALLTPTLL	TLTGQSVSVQ	KFNSFINKKT	DMMVLSNTEM	PSKSFGTPTL	FGTSVEIYLP		
401	NSYMPKGEGE	SGINRVNSSI	NAVKKNTVTA	NLVLDSESQE	VATSFQNDLI	SIKYCFNNLY	NYISNKTALS	TKNLFLFSAI		
481	KSNATKHKRT	QSFFSVENTT	TLGNNSNFVK	GHFKSSINAF	SSYLPSTNVH	SMIPLTSLPY	LKAISPLYSK	FMIDHSLKFI		
561	TPKTTLLKLLQ	HKLNKSQKQM	YTKTQNFQGL	RDLRALNSFS	FGQVNFRTNH	FLHSNSRPLN	HYNQALKLIN	GYEQYKNNLQ		
641	INCNKTLDLN	TKNKLQYQVH	KSHLFNQKCS	QIVYKQSLYN	RDLCTIRGTG	TKVVDYFSGH	DKLSNKNQIV	LDYFVYSNLL		
721	FDNKTNTIIN	KDQKQNTIKL	KLNLTKTTPV	EKTLIKKYTS	INSLVANEQT	RNNLNLGLIH	FNGHLSVSVN	ANLLTGRPVK		
801	FIYYKFDKRL	NSYLIYVNQN	LKKFIQLNNS	FLKPKPLSHQ	KNKPVEDFNQ	YATNNSPFPK	TNVFEKSFEV	DSSLRKPLTS		
881	LRGSKQFLNS	LTILFKHQKM	FKKKTILKAH	WHSQTQGFIR	KHTNSSFGSA	NFSNGPEESS	LSTRLHIQKK	RKAKKQRLT		
961	RRQKKRTRFF	PRPVWLRSRM	FLNFLTERNK	YYLNSTITKQ	GFSLPSKDVV	TTKLDWLKED	MRPSSLGAYQ	YKSLLTQKAG		
1041	NKFQRQSFTV	VVSTMEYING	IHKALNNSIF	NKIVRKSLLS	SSQNPLKLRL	VANYSKMQFM	HRVKLPFYRT	LKHSEGTKNL		
1121	ANKKQNLRLD	KIKANYNNFK	SQKANNQPPQ	NDKDKDKD	FRDFVWWSYN	NTQTNAFNQN	LWWLLPNLTT	KQSNLEFLT		
1201	TYPTAKETQR	AKEEIHGNSI	PTASKNQIAL	IRLNWALNKT	NINTFTDYSK	RNNLWTTQKL	RNQSKNNKTK	SLEKQFITNW		
1281	EKFFLNKNLN	IFSKKIISKV	KQKKQKLNMY	TSYLVNQSEH	NVKIFHNSWW	THLNIKNLVN	NQDMVIPVRE	GYFSVGNFNS		
1361	EFINSAAIKS	INNKTLVENY	VYSPSSEKET	MQLLLMSSSI	LLHLCAIISL	VSISQVRCFV	KPHLILLYKL	SNVYNAILNQ		
1441	LSNKLQKNLP	IYNNINKLNS	RYFYMNHQKS	QIKQRKKLLT	YFSLTLLKKQ	FVTVKPLQIR	NFASIKNQSS	NNSNLTYTDM		
1521	LPLSLRANKF	RGSKYDISIR	EEEGQSAHIK	PSKSMYAKLN	ILSLKTIFLK	QLLMNKKPSA	LPSNVGLKSN	RETQKSQLIQ		
1601	RIKTKELQIS	LKKNIIGFSK	VTKNHILKIL	FNVIEWFQTA	VRNISSFFEK	PAEFTTTWIA	YGFLVEWSSD	FITIIPENV		
1681	IYIWNVFSKI	YRTIPLSFIS	TTLGPASTVF	DPVTNSTIPI	QMGNFNYQKM	VAFPILLSLS	HLLHRRILYL	FDTLFSTITQ		
1761	PDTDLIARQE	KGTLEFWDIWA	DFLVTAADYY	NUNVAALSTI	KAEQNSLIEN	ISNDFDNLTM	SSKKPFFMPN	KGVSNIKNI		
1841	WKKKLKEPQL	PESIVQNREV	FVRERKRTLK	GLFNIIYAPQE	ETLWNNPTSP	KNLSDEKISF	KLFNQNLNLQ	FAEKNKIKPY		
1921	FEAYFSTTQQ	KTNIQMOSAFP	EANLNRWSVN	QFITYQSWHS	HNGSNNSNGD	LFIDYHPPKT	FSHIPALKYN	SILQQPIGSL		
2001	VCQIYSGLFN	KQISKNILLV	NPKTTSNNLV	DYNVLLIQAL	AGETEMKIIT	DNAQRYALVN	RGFAIGIKLL	REVFDAIALN		
2081	TPCIFLLEDI	HAIGERRPML	ISDFGGGMSD	DNGSFKEDFF	GSQRDEVHEK	NQVVYQLTRH	AITHYKKPFK	GDYSLAIPTN		
2161	LYVTDLFLKL	PTQSIISNLN	VENHNLSIKN	KIQHNGTQSL	TETKRNLGSD	INKNSYLQLT	QFTKTLAPPS	TSPFSVLLLK		
2241	EKRLKPNKI	VEELPWTSLP	GEQLATKPRT	SYSVRAKVAM	LAELSLSNLS	AKLDMITDLL	VIIIDSVRSNK	GFVVFATTDI		
2321	PHVLDPALRR	PGRLDETICL	PNIHTSNILN	FTKNYEIFKS	AKDTSNFGKK	IILNEMQNLT	TTSTQORDMYL	SCLPTNNQTH		
2401	KTKREGVLTM	NLKDYNNILLN	QVYFAEGTGG	ILNSQMHKDS	LQK SLNFALI	SHSK KLKELN	VSKLIGSNGT	VSQGNVDQLG		
2481	VFAQGIVNKQ	KKSLQQHLPN	SKKSFKKKYK	DKAIIYYEVG	KFVLNYFLNN	QLTQSSIIDK	PVSVTNKQTN	DITIFGNDFL		
2561	NLKTINYLSL	YNSKNKILLQ	LMILIFGGKIS	QLLSKKNLVK	SLKQASINSY	MVEEESGSI	SAGMPLGQTH	LLPKALSVLA		
2641	KPMIFSDGYN	NQNLKTATTL	LLSFIHKRYL	YRKNLIVPKL	LSFADGNILD	EPPSPPFSSS	LIPAKRFENY	KRFRDRTLGT		
2721	DKMGQRKSQI	TLLEKLQYHM	QLRSIKQLNA	TFSSQENLDF	QSNAALTSQK	LDTLMSLSTN	NLLQNPTNIN	WYYQNRIKLR		
2801	HGQYLTNQWW	NGQLSEHNAE	TVFLSDIDWR	SSFINKKNIN	ITKSKNLYRL	TQQKNNTDGL	DVLLDFPDPTD	QYYNPKRRRW		
2881	LLNNGSWNFW	FNFDKLYSEE	IVTTWILESL	IQTYKYLHKN	TELLDFVTNK	FITLGYIAPE	NANLQNISGF	PSQSELLSTK		
2961	EIILTNSFKR	F								

Q32065 YCX9_CHLRE										
Peptide	Uniq	-10lgP	Mass	Length	ppm	Fraction	Scan	Source File	Start	End
K.SLNFALISHSK.K	Y	17.12	1215.6611	11	-39	2	6754	FML_F14_998.mzXML	2444	2454
K.LNFSMVSLLDK.M	Y	15.84	1352.7009	12	-1.1	2	7308	FML_F14_998.mzXML	299	310
total 2 peptides										

Figure 13. Two unique peptides from fall leaves protein FPLC F14 matching to *Chlamydomonas reinhardtii* by using *De novo sequencing* by PEAKS Studio-X software.

3.5.4. Gene Ontology Annotation

The gene ontology (GO) is a computational model of biological systems by GO Consortium <http://geneontology.org/>. This tool provides a set of hierarchical vocabularies such as biological process, molecular function, and cellular component https://www.uniprot.org/help/gene_ontology. Based on GO annotation tool, the Fall leaves proteins are characterized in the category of biological process 47% and 41% were involved in the cellular process and metabolic process, respectively, as shown in **Figure 14**. Whereas, in cellular compartment categorization, most of the proteins were found to be localized in chloroplast, membrane, and nucleus (34%, 21%, and 19%, respectively) as in **Figure 15**. However, binding activity (48%), catalytic activity (36%), and oxidoreductase activity (9%) were found in the category of molecular function, as in **Figure 16**.

Figure 14. Fall leaves protein extracts of Red maple based on biological process categorization by using UniProt GO annotation.

Figure 15. Fall leaves protein extracts of Red maple based on cellular compartment categorization by using UniProt GO annotation.

Figure 16. Fall leaves protein extracts of Red maple based on molecular function categorization by using UniProt GO annotation.

3.6. Cytotoxic Evaluation of Protein Extracts

3.6.1. Protein Estimation

The Fall leaves protein extracted in 25 mM sodium phosphate buffer from gel filtration fraction F14 was successfully estimated by using Bradford assay. The total protein was estimated as 250 µg/mL to prepare several protein concentrations for MTT assays.

3.6.2. MTT assays

The gel filtration chromatography peak fraction number 14 (F14) was screened at different doses (4, 2, 1, 0.5, 0.25, 0.12, and 0.06 µg/mL) for their anti-proliferative effect on human breast cancer cell lines (MCF-7 and MDA-MB-231). In a dose-dependent manner, F14 showed a potent inhibitory effect in MCF-7 as well as MDA-MB-231 cell lines as illustrated in **Figures 17 & 18**. In MCF-7 cell line, the growth and proliferation were inhibited by 92% at 4 µg/mL and 88% at 2 µg/mL concentration. Further, in MDA cell line, the F14 showed almost the same inhibitory effect for the growth and proliferation by 90% at 4 µg/mL while 86% at 2 µg/mL concentration. The IC₅₀ values were calculated for F14 in both experiments. In MCF-7, the IC₅₀ value was 0.40 µg/mL ± 0.01 while in MDA-MB-231, the IC₅₀ value was 0.43 µg/mL ± 0.02. These experiments revealed that the fraction F14 was very potent against human breast cancer MCF-7 and MDA-MB-231 cell lines even with low protein concentrations. Doxorubicin was used as a positive control in different concentrations (0.6, 0.3, 0.15, 0.07, and 0.03 µg/mL) whereas untreated cells as a negative control (**Figure 19 and 20**).

Figure 17. Percentage of cell inhibition of MCF-7 human breast cancer cell line treated with different concentrations of Fall leaves extracted protein from FPLC (F14) ranging from 0.06 to 4 µg/mL. Data expressed as a mean of triplicates $\pm$ SD for each dose.

Figure 1811. Percentage of cell inhibition of MDA-MB-231 human breast cancer cell line treated with different concentrations of Fall leaves extracted protein from FPLC (F14) ranging from 0.06 to 4 µg/mL. Data expressed as a mean of triplicates $\pm$ SD for each dose.

Figure 19. Percentage of cell inhibition of MCF-7 human breast cancer cell line treated with different concentrations of doxorubicin ranging from 0.03 to 0.6 µg/mL. Data expressed as a mean of triplicates $\pm$ SD for each dose.

Figure 20. Percentage of cell inhibition of MDA human breast cancer cell line treated with different concentrations of doxorubicin ranging from 0.03 to 0.6 µg/mL. Data expressed as a mean of triplicates $\pm$ SD for each dose.

3.7 Anti-Inflammatory Evaluation of Protein Extracts

All crude extracted proteins from Spring and Fall leaves as well as gel filtration chromatography fractions were successfully evaluated against RAW 264.7 Murine Macrophages cell line. The Fall maple leaves crude protein extract, and gel filtration fractions F14 and F19 were active/ The Spring maple leaves crude protein extract, and gel filtration pooled fraction (F11-13) and fraction F14 were active extracted proteins from Spring maple leaves as illustrated in **Figure 21**. The Fall leaves gel filtration chromatography (F14), found to have significant activity in comparison to the positive control ($P < 0.05$) by decreasing nitric oxide production (**Figure 22**). These marked differences observed could be due to the change in the expression of active proteins affected by two different seasons.

Figure 21. Activity Evaluation of extracted proteins from Red maple leaves against RAW 264.7 Murine Macrophages cell line by calculating NO production level.

Figure 22. Active and inactive extracted proteins from Red maple leaves using RAW 264.7 Murine Macrophages cell line.

Chapter 4

CONCLUSION

This study has provided preliminary data on proteomics profile of red maple leaves using bottom-up proteomics. Bioinformatics tools such as Mascot search engine based on LC/ MS/MS data identified 34 proteins in fall leaves crude extract. De novo sequencing identified 54 proteins by PEAKS software. GO Annotation, a computational model of biological systems further advanced our knowledge about the proteins identified from Red maple leaves that grouped into three categories involved in biological process, cellular compartment, and molecular functions. The analysis of extracted proteins from Fall leaves crude and fraction (F14) in cell culture-based bioassays revealed that Red maple has anti-cancer activity in MCF-7 and MDA-MB-231 human breast cancer cell lines. Additionally, the extracted proteins (crude, fraction F14, and F19) from Fall leaves as well as Spring leaves crude and fraction (F11-13 and F14) showed anti-inflammatory activity against RAW 264.7 Murine Macrophages cell line. Future research to evaluate the activities of purified peptides and their synthetic analogs could have a numerous impact to develop anti-cancer and anti-inflammatory agents.

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