Sabbatical Report
Spring, 2017
Nitika Parmar
Biology Program

Purpose: The primary goal of my sabbatical leave taken in spring, 2017 was to complete a comprehensive research project that I had initiated in 2011-2012. This was a line of experimentation that I had started back then with several undergraduate students. Although I was successful during that period in gathering preliminary data which provided implications for a novel approach to study adipogenesis, due to my teaching commitments during the academic semesters in subsequent years, as well as other research projects undertaken in the summer sessions, this project had to be indefinitely shelved. The sabbatical leave allowed me to revive this project and conduct experimentation in a detailed manner so as to explore answers for the research question that I was addressing.

Research questions: Can plant extracts be screened for compounds that exhibit anti-adipogenic activity? Can novel compounds be isolated from plant extracts that are capable of regulating adipogenesis?

Background: Over the last two decades people have become obsessed with weight. The epidemics of obesity and anorexia have become an increasing problem. Research has shown that many health issues arise from obesity and being overweight, likewise the obsession of needing to have no fat or having to be super skinny have equal consequences. Obesity is one of the major problems prevailing in society today directly contributing to diseases such as diabetes, stroke and heart disease. 66% of American adults are overweight or obese and in 2005 more than 600,000 people died of heart disease. There is a critical need to develop a new approach to the treatment or prevention of obesity. Adipogenesis is the study of fat formation and involves the formation of fat cells (adipocytes) from precursor cells via a differentiation process. Treatments that regulate both size and number of adipocytes may provide a better therapeutic approach for treating obesity. Medicinal plants and plant extracts represent the oldest form of medication and previous research has identified a number of factors from plant extracts that have inhibited adipogenesis such as fermented blueberry juice, berberine, hibiscus, mint, pepper and grape seed extracts. In addition, ginseng extracts have been found to enhance adipogenesis. In the proposed study, natural extracts of various plants such as cactus, acai berry, ginger oil and tumerone, as well as chemically conditioned plant extracts were tested to determine if they regulate the process of adipogenesis.

My project was focused on identifying anti-adipogenic compounds using a chemical screen of plant-derived extracts. I used the mouse pre-adipocyte cell line- 3T3L1 as the model system for adipogenesis. 3T3L-1 cells are a well-established pre-adipocyte model which can be induced to differentiate into adipocytes in cell culture. Fat formation in these cells can be easily monitored by visual and biochemical means. These cells undergo differentiation upon stimulation with adipogenic ligands. Screening of natural extracts using 3T3L-1 cells will provide an idea of how

plant-derived compounds, including antioxidants, may play a role in inhibition or activation of adipogenesis.

Previous Research: Previous research has investigated a huge scope of inhibiting factors for adipogenesis including anti-oxidant producing agents like fruits, vegetables, and herbs/plants to help alleviate different health related issues. Voung et al., (2007) studied the effects of fermented blueberry juice on the uptake of glucose, adipogenesis and the signaling pathways that regulate glucose transport in muscle cells and adipocytes. They concluded that when 3T3L-1 cells are exposed to fermented blueberry juice triglyceride levels were dramatically inhibited. Other work regarding the activation of the AMP-Activated Protein Kinase and its beneficial metabolic effects in diabetic and insulin-resistant states suggested that a natural plant product berberine decreased the levels of lipid formation in 3T3L-1 cells. Kim et al., (2003) studied the effects of hibiscus extracts on 3T3L-1 cells and found an inhibitory effect on both lipid accumulation and expression of adipogenic transcription factors. Similar experiments have been done using other compounds such as mint, rapamycin, grape seed extracts, black soybeans extracts, tannic acid, and many Traditional Chinese medicine (TCM) herbs showing inhibitory effects on differentiation and adipogenesis. The opposite effect has been shown by Shang et al., (2007) who studied the effects of Ginseng extracts and found them to have an enhancing effect, increasing adipogenesis and triglyceride (TG) accumulation. Other compounds such as Thiazolidinediones (TZD's), rosiglitazone, and some TCM extracts also show an enhancement in adipogenesis. An interesting study was done using pepper extracts and derivatives of those extracts. A mix of outcomes showed that while many of the pepper compounds enhanced adipogenesis, capsaicin, one of the most common compounds used today, had an inhibitory effect. Capsaicin is the spice found in all types of chili peppers.

My main goal was to isolate compounds which are capable of inhibiting this differentiation. I proposed to screen compounds such as cactus extracts, acai berry extracts, ginger oil, curcumin, and other known potential anti-cancer compounds. Cactus is a very significant plant that has been used for medical purposes in Mexico for thousands of years. It is believed that the cactus has the ability to decrease sugar levels in blood and increase insulin levels as well as increasing the insulin sensitivity and sugar tolerance (Kim, 2005). Since insulin is critical to the process of adipogenesis I hypothesized that cactus extracts will reduce sugar levels and therefore decrease the amount of insulin, and the process of adipogenesis.

Experimental procedures:

(a) Culture of 3T3-L1 cells: 3T3-L1 cells acquired from American Type Culture Collection, Rockville, MD was used. 3T3L-1 cells are a well-established pre-adipocyte model which can be induced to differentiate in cell culture. The most common method of differentiation is exposure to insulin. Cells were grown in Dulbecco's modified Eagle's medium (DMEM), 10% Bovine calf serum (BCS), in a standard growth condition of 5% CO_2, 95% air and at 37°C. Cultures were seeded to a density of ~50,000 cells per 30mm plate and allowed to grow for 6-9 days using a pre-adipocyte medium that contained DMEM, high glucose, HEPES buffer, 10%

BCS, Penicillin, Streptomycin, and Amphotericin B. A differentiated cell (adipocyte) can be distinguished by its high content of lipids and triglycerides. Adipocytes can be identified by staining cells using Adipored fluorescent reagent or Oil "O" Red.

(b) Differentiation: Cells were seeded on a 24-well plate with a density of ~10,000 cells per well. Confluent 3T3-L1 cells were treated with insulin to induce transition to adipocytes. Differentiation process can be accelerated by treating the cells with methylisobutylxanthine and dexamethasone. This was done for 2 days and after this period the medium was replaced and only insulin was used. After waiting for 3-5 days, 85% of the cells should be differentiated.

(c) Plant extracts: Several plant extracts as well as a large number of fractions of chemically conditioned ginger oil, argan oil and curcumin (from turmeric) extracts were screened. Fractions of the plant extracts were provided in individual vials as powders. Stock concentrations of each fraction at 10mM were prepared in 5% DMSO and stored at -20^{o}C under light sensitive conditions. Fractions which are insoluble at 10mM in 5% DMSO were prepared at lower concentrations (1-5mM). Each sample was carefully catalogued and maintained. The extracts screened are indicated below-

1. Soy Protein Concentrate
2. Whey Powder
3. Pappy's Green Tea Concentrate -
4. Ginger Oil
5. Anise Oil
6. Beet Powder
7. Garlic Powder
8. Henna Powder
9. Mushroom Powder
10. Pumpkin Powder
11. Red Wine Vinegar
12. Spinach Powder
13. Tomato Powder
14. Yogurt Blend Powder
15. Wasabi Powder
16. Horseradish Powder
17. Eden Safflower Oil, Organic
18. Almond Oil
19. Black Walnut Oil
20. Castor Oil Apothecary
21. Clove Oil - Stove
22. Menthol- Eucalyptus Oil
23. Nutmeg Oil
24. Cinnamon Bark Oil

(d) Treatment of cells with the plant extracts and Cell Viability Assays: In the preliminary screen, cells were seeded in 96-well flat bottomed plates at a density varying from 1-2 x 10^4 cells/well. 18-24h post seeding, the cells were treated with the extracts in quadruplicate wells. Ten extracts were tested at a time. Each extract was tested at a concentration range varying from 0 to 20μM. Control cells were treated with DMSO alone. Cell viability was analyzed using the MTT assay 24h, 48h and 72h post treatment using a plate reader (Biotek Instruments) whereby the absorbance at 550nm will directly correlate with the number of viable cells. Each experiment was repeated twice. Dose and time-dependent profiles for each extract were prepared and analyzed carefully to identify extracts which have the maximum inhibitory effects on cell viability.

(e) Preparation of cell lysates: Cell suspensions obtained from the 24-well plates were lysed in RIPA lysis buffer containing phosphatase inhibitors and lysates were subject to immunoblotting.

(f) Immunoblotting: Cell lysates were fractionated on a 6.5% SDS-PAGE gel and western blotting was performed using antibody against phospho-mTOR (Ser2448). Phosphorylated S6K and 4E-BP1 levels were also analyzed by running lysates on a 12% gel using anti-phospho S6K (Thr389) and anti-phospho 4E-BP1 (2855) antibodies as probes. Detection was done by chemiluminescence ECL method (Amersham) and/or ABC peroxidase method (VECTASTAIN). Signal intensities were quantitated by density-scanning using a molecular imager (ChemiDoc XRS, Biorad). Antibodies were purchased from Cell Signaling.

Induction and Differentiation

- Seeded a 48 well plate with 30,000 cells/well and grow to confluency (90-100%)
- Induced Differentiation via MDI media (Day 0)
- Changed media to Insulin containing Media (Day 2)
- Changed media to 10% FBS DMEM high glucose (Day 4)
- Changed media every 2 days until Day 10

Quantification

- Fixed cells with 10% Formalin and stain cells with Oil Red O
- Air dried and dissolve lipids with 100% isopropanol
- Quantified colorimetric changes @ A500 and A540nm

Known compounds:

1: MAP Kinase inhibitor
2-4: p38 Kinase Inhibitors
5: Curcumin
6: Astaxanthin
7: Lycopene
8: Lycophyll

Results: My screen consisted of 24 plant extracts and chemically-conditioned extracts. Differentiation of 3T3L1 pre-adipocytes to adipocytes was done by treating them with an induction/differentiation cocktail (insulin, dexamethasone and IBMX) for 8-10 days and fat cell formation was subsequently monitored by Oil-Red staining using absorbance at 540nm as a direct read-out. Cells not exposed to the differentiation cocktail were used as controls. Preliminary observation involved visualization of the stained fat cells by microscopy. In my initial screen, I followed two treatment protocols: cells were treated with the compounds either at the time of induction or five days post-induction. Interestingly, varying results were obtained depending upon when the treatment was done. A significant effect on adipogenesis was observed if cells were already primed toward adipogenesis (five days post induction and showing accumulation of fat droplets) and then treated with the compounds versus treating them concurrently at the point of induction (treatment on the same day as induction when fat droplets have not formed). Although my aim was primarily to isolate anti-adipogenic compounds, I have identified a few extracts which had strong adipogenic as well as anti-adipogenic effects when used at a concentration of 1μM. I subsequently analyzed these compounds in detail using a dose and time dependent response curve. I also intended to follow up my study by investigating the levels and activation of proteins involved in the adipogenic signaling pathway such as members of the CCAAT/enhancer binding protein (C/EBP) and peroxisome proliferator activated receptor (PPAR) families but was unable to do so due to lack of quality time. These experiments will be completed in the future.

Data and Figures:

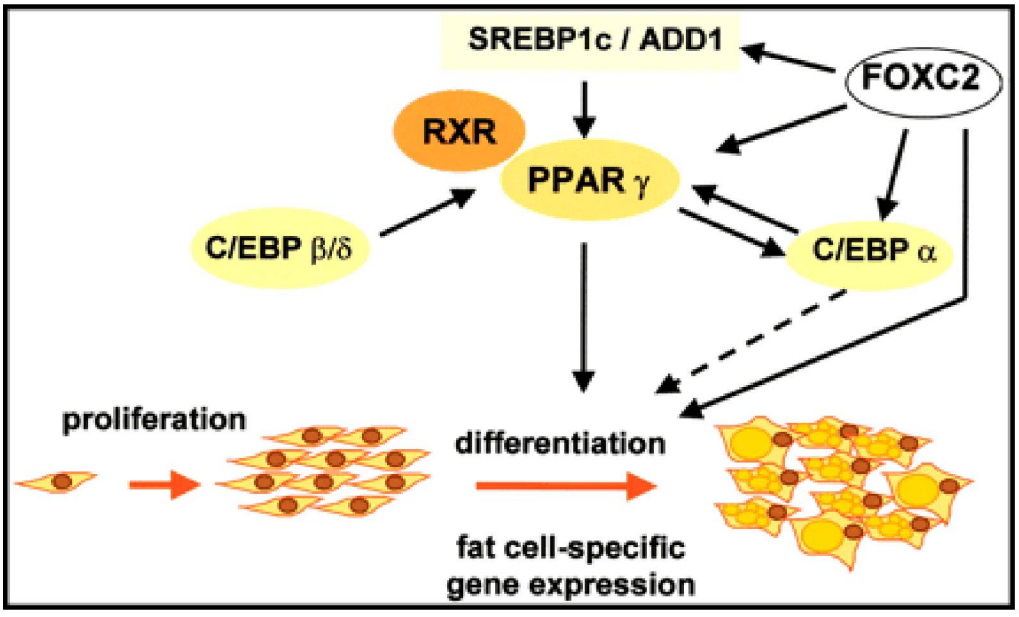

Fig. 1: Current Model of Adipogenesis: The differentiation of preadipocytes into adipocytes is promoted by several families of transcription factors

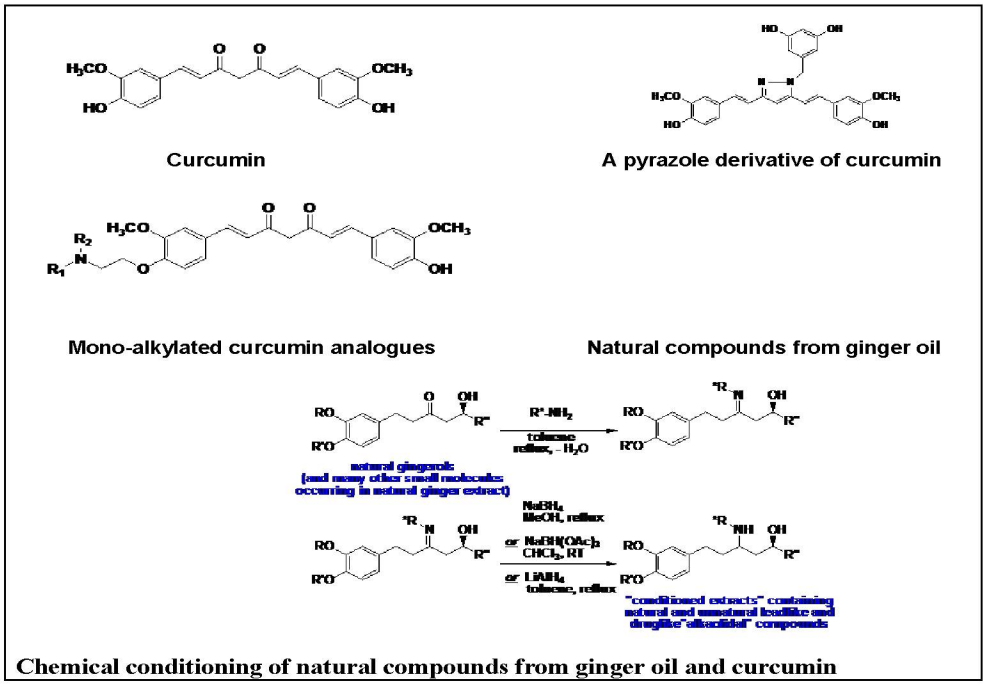

Fig. 2: Chemically conditioned extracts of curcumin and ginger oil

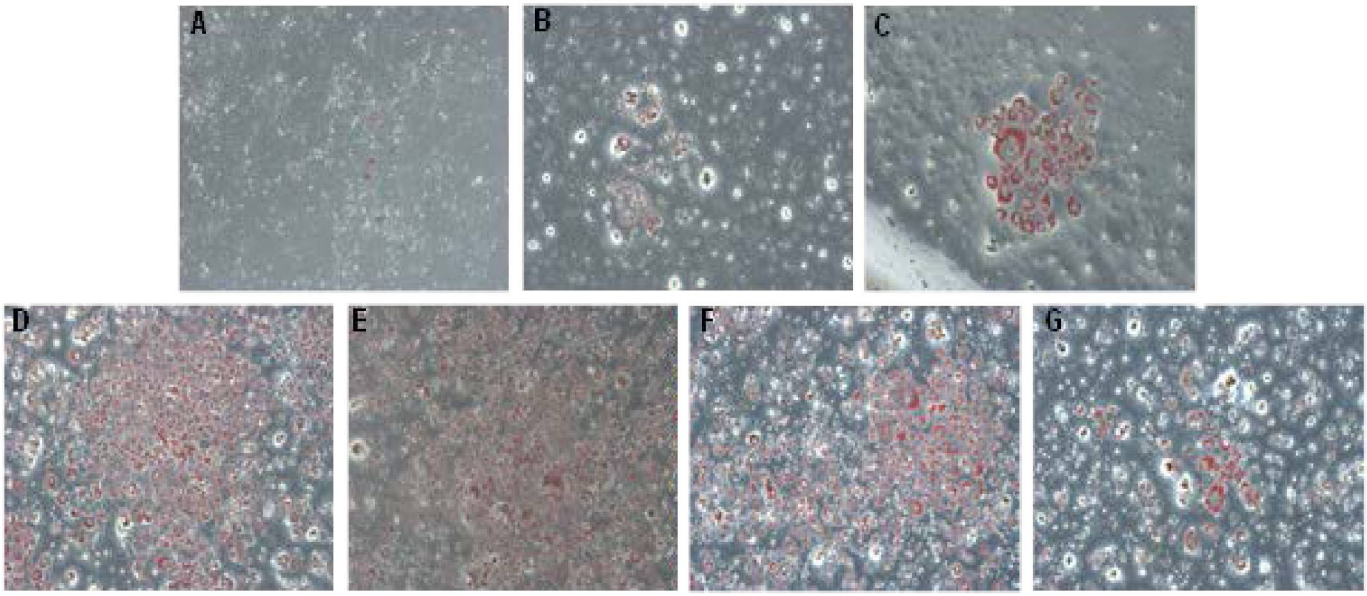

Fig. 3. Adipogenesis and Insulin Dependence A) control: Calf Bovine Serum (CBS) media only, no induction, B) control: Fetal Bovine Serum (FBS) media only, no induction, C) control: Insulin media, only no induction, D) Induced with 1:500 Insulin conc., E) Induced with 1:1000 Insulin conc., F) Induced with 1:2000 Insulin conc., G) Induced with 1:5000 Insulin conc.

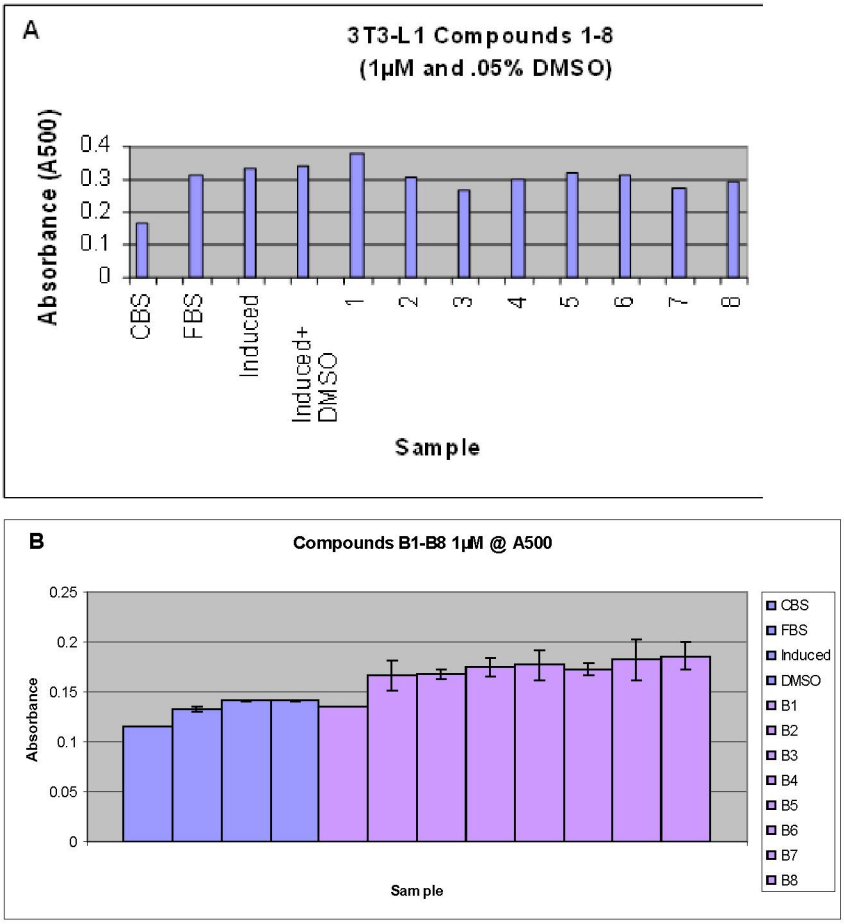

Fig. 4: (A) Positive and negative regulation of adipogenesis; (B) Screen of eight chemically conditioned plant extracts on adipogenesis (8 days post induction) (dark blue are controls, light purple are chemically conditioned compounds)

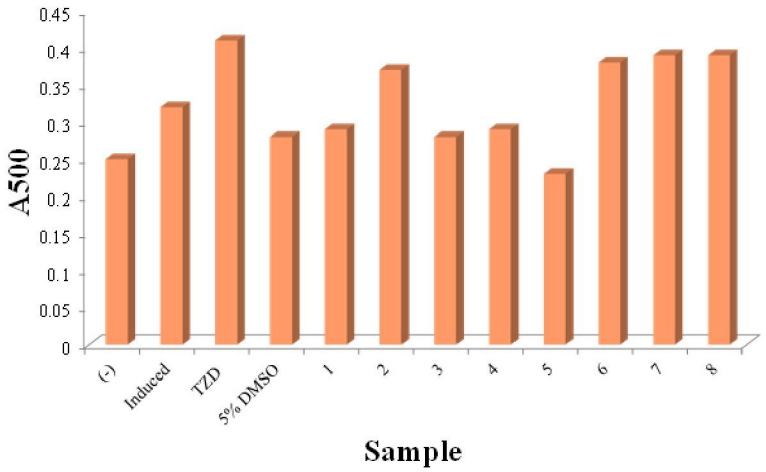

Fig. 5: Effect of known compounds on adipogenesis: Cells were induced and the compounds were added at the time of induction. Estimation was done 10 days post induction.

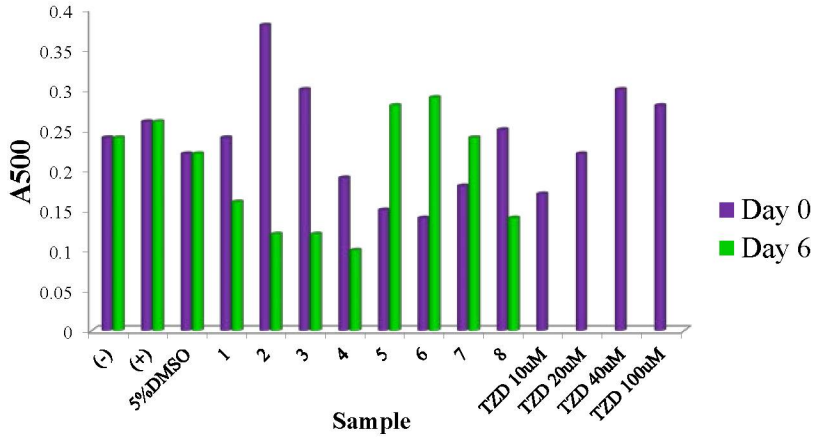

Fig. 6: Effect of time of treatment on adipogenesis: Cells were induced and the known compounds were added either on the day of induction (Day 0) or 6 days post induction (Day 6) Estimation was done 14 days post induction

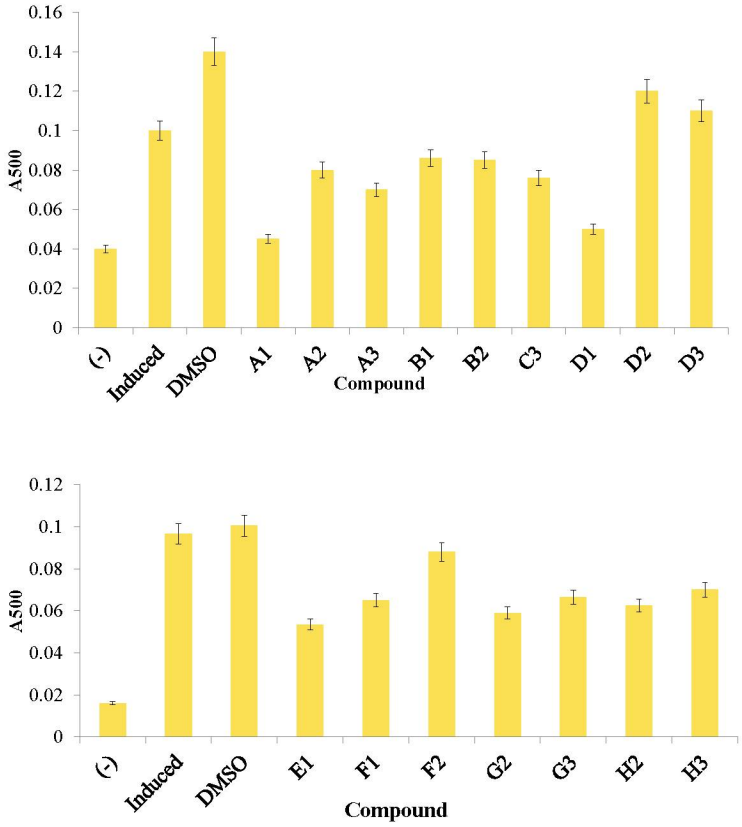

Fig. 7: Screening modulators of adipogenesis: Chemically conditioned plant extracts (1mM) were added to the cells on the day of induction and estimation

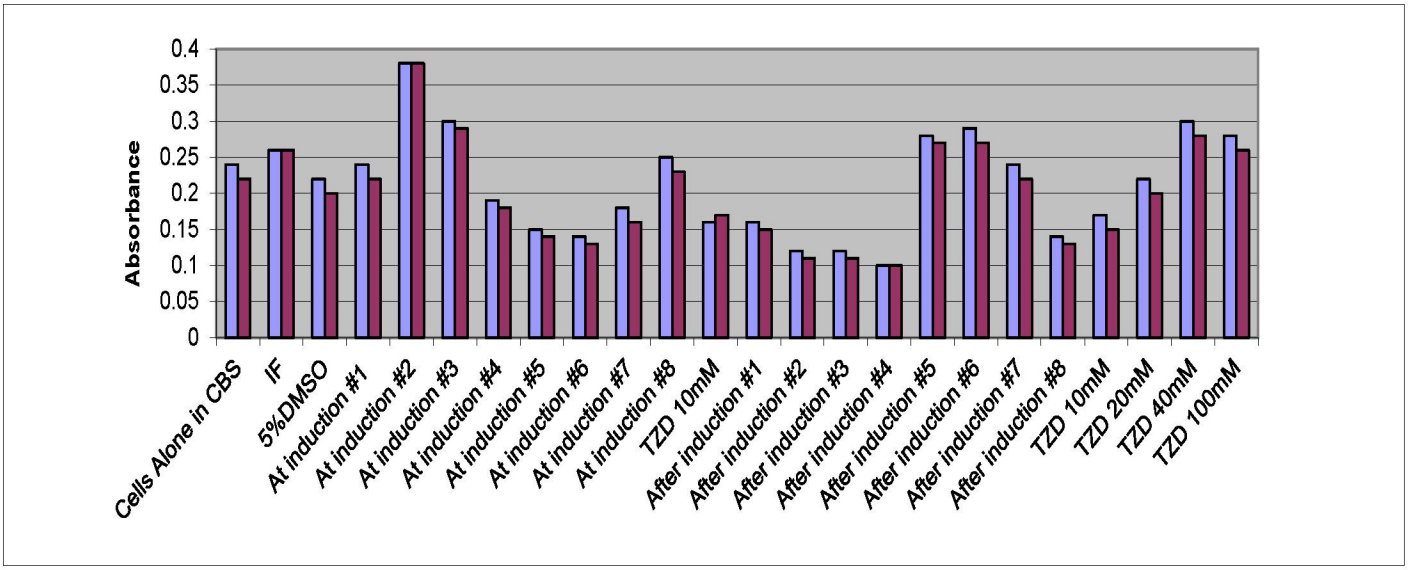

Fig. 8: Differentiation of 3T3-L1 cells under a variety of conditions.

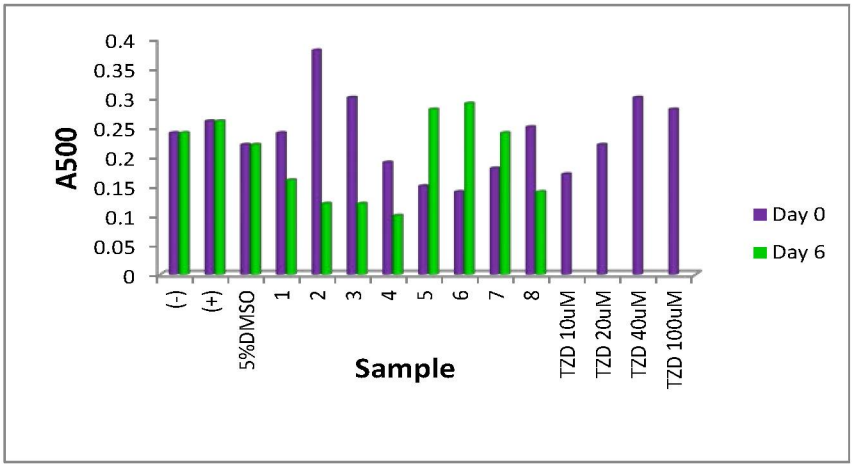

Fig. 9: Comparison of effects of plant extracts on the differentiation of 3T3-L1 cells relative to treatment with TZD.

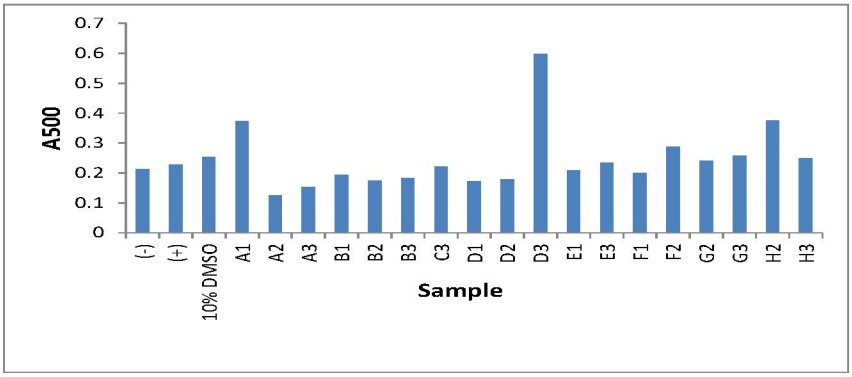

Fig. 10: Effect of chemically conditioned extracts on adipogenesis.

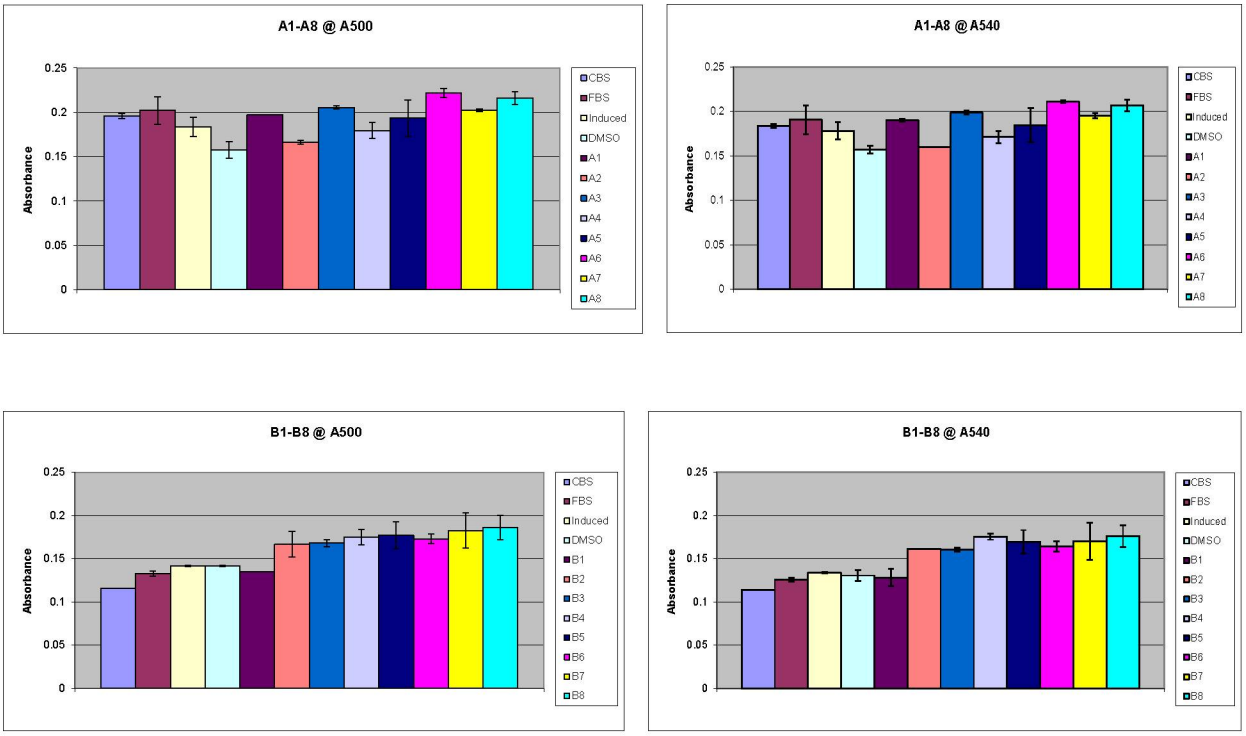

Fig. 11: Effect of chemically conditioned extracts on adipogenesis.

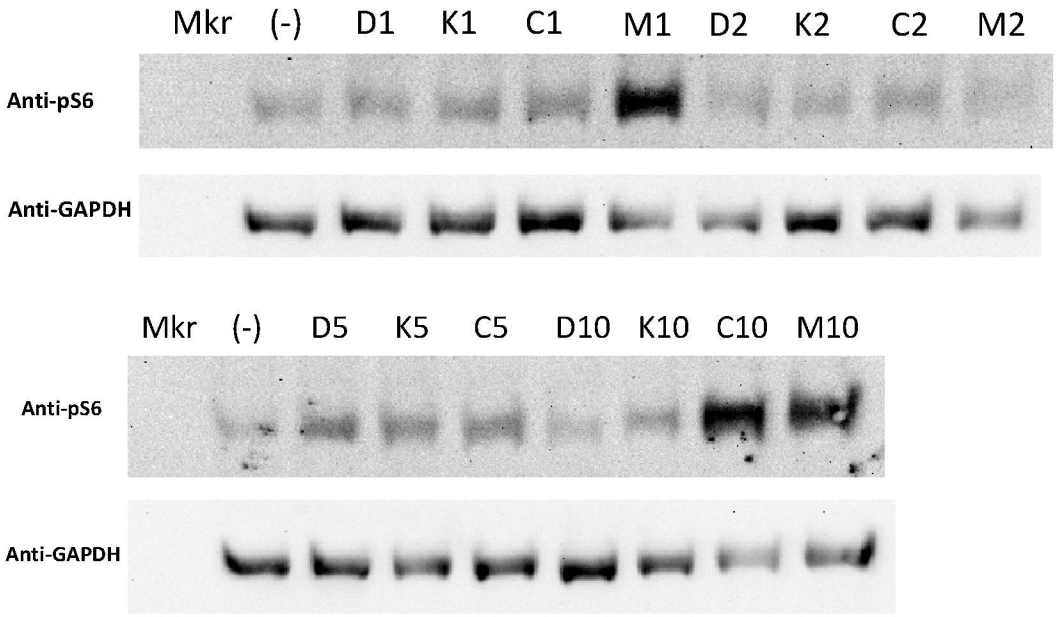

Fig. 12: Effect of menthol-eucalyptus oil (K), cineole (C), menthol (M) on the phosphorylation of pS6 protein.

Conclusions:

- Differentiation of 3T3-L1 cells is insulin and glucose dependent
- Spontaneous differentiation can occur without induction due to over growth and glucose in the media
- Amongst the known compounds, curcumin has an inhibitory effect on adipogenesis
- Effect on adipogenesis is dependent on the time of treatment of the cells (at the pre-adipocyte stage- Day 0 or the adipocyte stage- Day 6 onwards)
- Amongst the unknown compounds tested several candidates were found to inhibit adipogenesis

Future Directions:

- Establish dose response profiles in detail
- Extend time course trials
- Test compounds at lower concentrations.
- Screen new compounds for possible leads.
- Test derivatives of additional lead compounds
- Immunodetection of activated cellular proteins for mTOR pathway
- Effects of the compounds on induced oxidative damage in cells.

References:

- Ross E. Sarah, Nahid Hemati, Kenneth A. Longo, Christina N. Bennett, Peter C. Lucas, Inhibition of Adipogenesis by Wnt Signaling, Science; New Series, Vol. 28, No.5481,(2000), 950-953.
- Kaiden, Adina, Robert Y. Hsu, M. Daniel Lane, Induction of Fatty Acid Synthetase Synthesis in Differentiating 3T3-L1 Preadipocytes, The Journal of Biological Chemistry, Vol. 255, No. 10, (1980), 4745-4750.
- Qi-Qun, Tang, Tamar C. Otto, M. Daniel Lane, Mitotic Clonal Expansion: A Synchronous Process Reuired for Adipogenesis, Proceedings of the National Academy of Scences of the USA, Vol. 100, No.1, (2003), 44-49.
- Morrison, F. Ron, Stephen R. Farmer, Hormone Signaling and Transcriptional Control of Adipocyte Differentiation, Journal of Nutrition, (2000), 3116s- 3121.
- Voung, Tri, Louis C. Martineau, Charles Ramassamy, Chantal Martar, Pierre S. Haddad, Fermented Canadian blueberry juice stimulates glucose uptake and AMP-activated protein kinase in insulin-sensitive cultured muscle cells and adipocytes, Canadian Journal of Physiology and Pharmacology, Vol 85, (2007), 956-965.
- Kim MS, Kim JK, Kim HJ, Moon SR, Shin BC, Park KW, Yang HO, Kim SM, Park R. Hibiscus extract inhibits the lipid droplet accumulation and adipogenic transcription factors expression of 3T3-L1 preadipocytes. J Altern Complement Med. (2003) 9(4):499-504.
- Shang W, Yang Y, Jiang B, Jin H, Zhou L, Liu S, Chen M. Ginsenoside Rb1 promotes adipogenesis in 3T3-L1 cells by enhancing PPARgamma2 and C/EBPalpha gene expression. Life Sci. 2007, J80 (7):618-25.

OVERALL OUTCOMES OF SABBATICAL LEAVE:

As a result of this sabbatical leave I was able to complete a research project that was pending for several years. The new data that has been gathered through comprehensive analyses is currently being analyzed for a potential publication in a peer-reviewed journal. Although one key experiment still remains, I am confident that the current level of data obtained warrants a publication.

New findings that have emerged from this project will allow me to propose a few mini-projects that I could offer to students as independent research assignments in the coming terms. Overall, this study has revealed important implications in the area of adipogenesis, particularly with regard to effects shown by a variety of plant derived natural extracts.