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Anti-adipogenic effects of *Moringa oleifera*, *Brassica oleracea* and *Ocimum basilicum* on 3T3-L1 (pre)-adipocytes and development of functional foods with anti-obesity properties

Thesis submitted for the partial fulfilment for the award of the degree of

BACHELOR OF TECHNOLOGY
IN
FOOD TECHNOLOGY AND MANAGEMENT
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DECLARATION

I, Abhinav Jain hereby declare that this thesis on the topic of “*Anti-adipogenic effects of Moringa oleifera, Brassica oleracea and Ocimum basilicum on 3T3-L1 (pre)-adipocytes and development of functional foods with anti-obesity properties*”, represents my original work which has been done under the guidance of **Dr. N. ILAIYARAJA**, Scientist ‘D’ from **3rd July 2017 to 3rd January 2018** at the Department of Nutrition, Biochemistry and Toxicology Division, Defence Food Research Laboratory (DFRL - DRDO), Mysore (Karnataka – INDIA) for the partial fulfilment of the award for the degree of Bachelor of Technology in Food Technology and Management at *National Institute of Food Technology Entrepreneurship and Management* (NIFTEM).

It is also declared that, this work has not been previously included in a thesis/dissertation submitted to this or any other institution for a degree, diploma or other qualifications.

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DATE: 3rd January, 2018

PLACE: Mysore, India.

Abstract

Obesity is one of the most chronic and widespread body disorders, and a major risk factor for several chronic disorders like Diabetes, Heart diseases, Stroke, High blood pressure, Hypertension and Cancer. The rising prevalence of obesity has compelled the scientific community to come up with sustainable solutions to address this modern epidemic. Many drugs based treatments have been identified to treat obesity, but all having serious side-effects. This necessitates investigating the prospect of natural substances as alternative and complementary means to deal with the present scenario. Numerous studies on bio-active compounds isolated from plant sources have shown promising results against obesity. Present study was undertaken to investigate the effect of crude extracts of *Moringa oleifera* (*MO*), *Brassica oleracea* (*BO*) and *Ocimum basilicum* (*OB*) on 3T3-L1 pre-adipocytes proliferation and their influence on adipogenesis and also to develop anti-obesity functional foods incorporated with these extracts to tackle obesity with dietary means.

The dried plant powders were used for extraction of phyto-compounds using hydro-alcohol extraction at acidic pH. These extracts were then analysed for phytochemicals (Total phenolic and flavonoid content) as well as anti-oxidant activity. Further, these extracts were evaluated on the cultured 3T3-L1 pre-adipocyte cell line model at different concentrations against their potential cytotoxicity and anti-adipogenic properties. Several assays and analytical methods were utilised to strengthen the claim. Also, the major bioactive compounds responsible for the activity of these extracts were identified using UPLC-HRMS/MS in negative ion mode.

Total phenols and antioxidant potential were found to be highest in *MO* followed by *OB* and *BO*. However, total flavonoid content was highest in *OB* followed by *MO* and *BO*. Major probable bioactive compounds present in the extracts were identified using cutting edge technology (UPLC-HRMS/MS) and were listed in the following report. *BO* and *OB* both were non-cytotoxic and showed insignificant changes in cell viability at physiological and supra-physiological concentrations. *MO* showed significantly high cytotoxicity even at very low concentrations and thus it was not pursued for analysis. *BO* and *OB* extracts exhibited anti-adipogenic effects by inhibiting lipid droplets and triglyceride (TG) accumulation in 3T3-L1 mature adipocytes, dose-dependently. *BO* inhibited TG accumulation by 27% at 3 mg/mL

dosage while *OB* inhibited the accumulation by 46% at a low concentration of 0.75mg/mL. Also both extracts significantly increased the protein expression level of Adipsin in the mature adipocytes in comparison to control cells.

‘Anti-obesity Instant Soup mix powder’ incorporated with these plant extracts developed under this project, showed high overall all acceptances in the sensory panel. Also the analysis of the product sample showed high contents of protein, crude fibre, Vitamin C and Vitamin B complex which can be crucial against obesity. The product was also found rich in polyphenols and thus had the potential to work against obesity.

Although, the present study indicate towards the high effectiveness of *OB* and *BO* extracts against obesity, further investigation including *In-vivo* studies may substantiate the potential use of these extracts as functional foods against obesity.

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ABHINAV JAIN

In the memory of my beloved mother



Late Shrimati Neelam Jain

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Chapter 1

Introduction

Introduction

In this era of technology, automation and gadgets, life had never been much easier and predictable. The 21st century providing the pleasure of convenience and comfort had extreme effects on the traditional human lifestyle. On one hand where technology and science have markedly enhanced the quality of life, the other aspects are much more distressing. This ease of living has influenced the natural human body balance in worst ways. Humans have become prone to such abnormalities and chronic diseases that were never a topic concern in the past centuries. Diabetes, Heart diseases, Stroke, etc. are some of the major lifestyle dependent abnormalities having the highest mortality rates. Obesity is one of these abnormalities that have raised concerns worldwide. It is a serious health problem that is implicated in various diseases including hypertension, type II diabetes, coronary heart diseases, and cancer [2]. More than half of the human adult population is either obese or overweight. According to WHO database ‘in 2016, 39% of adults aged 18 years and over (39% of men and 40% of women) were overweight and overall, about 13% of the world’s adult population (11% of men and 15% of women) were obese.’ The complexity of the situation can be understood by the fact that World Health Organisation has termed Obesity as ‘Modern epidemic’ (WHO, 2000).

At least 2.8 million people die each year as a result of being overweight or obese. The prevalence of obesity nearly tripled in last 45 years. Once associated with western and high-income countries, obesity is now also prevalent in low- and middle-income countries. Also the obesity epidemic is not just an adult problem; over 41 million preschool children being overweight, they are more likely to become obese adults and are much likely to develop diabetes and cardiovascular diseases at a younger age, which in turn are associated with a higher chance of premature death and disability. Figure 1.1 shows the statistics regarding current scenario of overweight and obesity.

1.1 Overview of Obesity

1.1.1 What is Obesity?

Overweight and obesity can be defined as abnormal or excessive fat accumulation in the body that presents a risk to health (WHO). In other terms, excess white adipose tissue accumulation leads to obesity. It can occur due to increased adipocyte cell volume (hypertrophy) or cell number (hyperplasia). [3] Adipose tissue can be termed as energy reservoir for our body. They have the ability to store energy in form of lipids in periods of energy excess and release them in response to energy deficit.

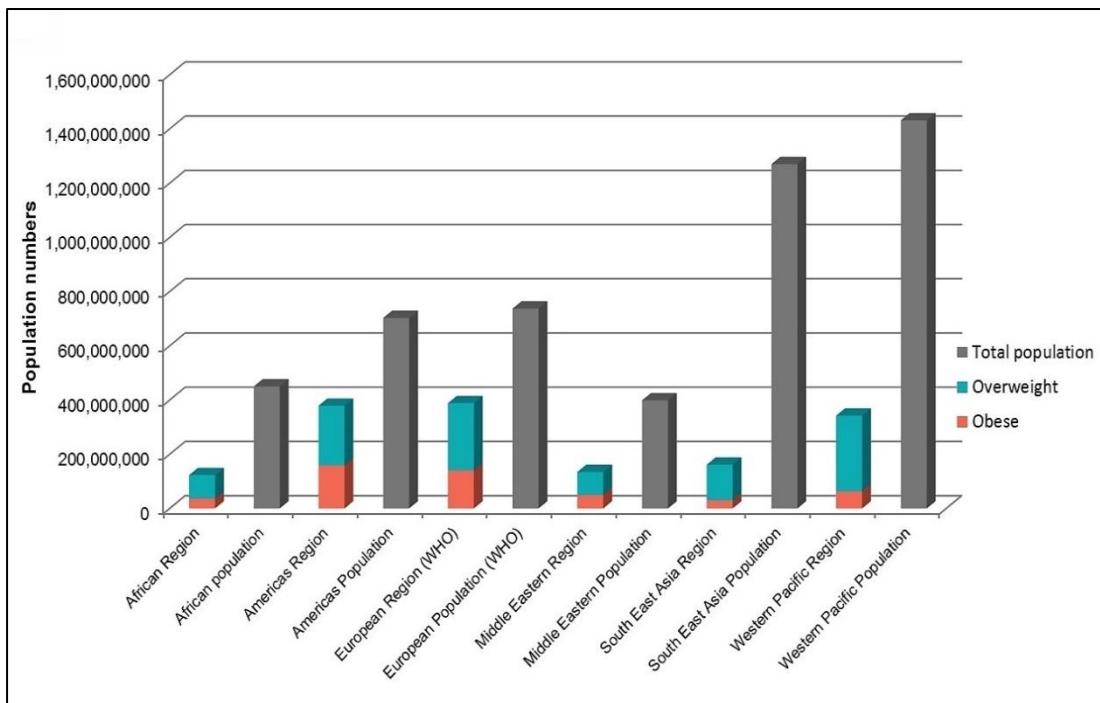


Figure 1.1: Number of Overweight & Obese for individual's ≥ 15 years of age. (Adapted from – World Obesity, London 2016)

1.1.2 Causes of Obesity and Overweight

The fundamental cause of obesity is an imbalance between energy intake and energy expenditure associated with genetic, metabolic and behavioural components. The reason behind energy balance in humans can be complex interactions among neuroanatomical, genetic, endocrinological, pathophysiological, nutritional, physical, psychological, and social-environmental factors [1].

While genes are important in determining a person's susceptibility to weight gain, energy balance is determined by calorie intake and physical activity. Thus societal changes and worldwide nutrition transition are driving the obesity epidemic. Increased consumption of

more processed foods that are full of sugar and saturated fats (energy-dense) and are nutritionally poor, in combination with technology driven sedentary lifestyles with minimum physical activity have led to obesity becoming the most common nutritional disorder worldwide.

1.1.2.1 Genetic Predisposition

Obesity has a strong genetic component like many other medical conditions. Obesity is a complex condition having multifactorial origin and can be explained by monogenic mutations and polygenic condition, which may be affected by a myriad of environmental influences [15]. Genes influence both appetite and body metabolism of an individual. There are significant number of cases with ‘clustering’ of obesity in families, furthermore results from a number of twin studies have demonstrated that there is always a high (>50–70%) concordance rate for obesity in identical twins, even if they are not brought up in a shared environment. [7] Many evidence regarding linkage of Human Single-gene mutations with obesity have also been found, e.g. leptin genes [11], leptin receptors [8], pro-opiomelanocortin (POMC) [10], peroxisome proliferator-activated receptor- γ 2 (PPAR- γ 2) [12], melanocortin- 4 receptor (MC-4R) [9] or dopamine receptors D4 (DR-D4) [13]. Other studies have linked mendelian syndromes with obesity as a clinical feature (Prader-Willi, Wilson-Turner, Bardet-Biedl etc.) [14]. Till date studies have identified over 200 genes that may influence body weight. FTO gene being the most significant is relatively common in obese population. An average of 1.5 kg weight increase is associated with its presence [22]. Table 1.1 summarises the inherited causes of obesity.

Inherited causes of obesity		
Condition	Key features	Genetic defect
• Prader-Willi syndrome	Short stature, small hands and feet, almond-shaped eyes, learning difficulties, hypogonadism	Paternally imprinted gene, chromosome 15
• Bardet-Biedl syndrome	Mental retardation, renal dysplasia, polydactyly, hypogonadism	Several described (chromosomes 4, 11, 15, 16) that affect the function of cilia
• Leptin deficiency	Severe hyperphagia, hypogonadism	Leptin gene (autosomal recessive)
• Leptin receptor mutations	Severe hyperphagia, hypogonadism	Leptin receptor gene (autosomal recessive)
• Pro-opiomelanocortin defects	Moderate obesity, red hair	Pro-opiomelanocortin gene (autosomal dominant)
• Melanocortin-4 receptor defects	Severe early-onset obesity	Melanocortin-4 receptor gene (autosomal dominant)
• Pro-hormone convertase 1 deficiency	Failure to process insulin and pro-opiomelanocortin	Pro-hormone convertase 1 gene (autosomal recessive)
• Neurotropin receptor (TrkB) deficiency	Hyperphagia, impaired speech and nociception	TrkB (autosomal recessive)

Table 1.1: Most common genetic disorders associated with obesity. (Adapted from - John P H Wilding, 2010 [24])

1.1.2.2 Environmental Factors

A healthy environment is important in order to prevent and treat obesity. Genetic predisposition determines a person's susceptibility to weight gain, but obesity can only manifest if the environment is conductive to weight gain. An individual's surrounding environment is likely to contribute towards changes to the quantity or composition of food in the diet, and changes in the amount of physical activity undertaken by the population [16]. Economic status of the population also plays an important role in development of obesity. Generally, energy dense, high fat and high sugar foods are the cheapest options available to a consumer in the market. Reason being fat and simple sugars cheaper than whole grain and vegetable based diets [23]. Thus, the most economically deprived social groups appear to be at greatest risk, particularly for extreme obesity [24].

1.1.2.3 Social Factors

Though, imbalance in energy consumption and expenditure leads to obesity on individual basis. Societal influences play a major role in defining an individual's lifestyle and life choices. From eating habits to the composition of food depends on these social factors. In developing countries women of a high social class were less likely to be obese. No significant differences were seen among men of different social classes. Also the population of high social classes had greater rates of obesity [17]. Other factors like alcohol and smoking have significant effects on an individual's weight. Those who quit smoking will gain an average of 4.4 kg (men) and 5.0 kg (women) over ten years. However, changing rates of smoking have little effect on the overall rates of obesity [18]. Factors operating through gender, ethnic, socio-economic or familial hierarchies provide a powerful determinant of body weight by setting moral and social connotations to body weight and defining attitudes to eating and exercise behaviours [25].

1.1.2.4 Diet

Increased availability and consumption of energy dense foods, soft drinks and alcoholic beverages is one of the major factors behind rising Obesity threat. Some experiments show that consumption of such food leads to less satiety in comparison to less energy dense foods, and thus encourages overconsumption, suggesting that the peripheral and hypothalamic regulatory systems are less sensitive to a high fat diet [16]. Certain eating disorders are also linked to Obesity with Binge-eating disorder (BED) being the most common in adults [19]. BED is characterized by binge eating without subsequent purging episodes and an association with the development of severe obesity [20]. BED is also linked with a type of food addiction.

1.1.2.5 Physical Activity

The major components of energy expenditure can be classified into BMR, thermic effect of food and physical activity [14]. Levels of physical activity have declined dramatically in the recent past. Currently, at least 60% of the world's population gets insufficient exercise. There is greater reliance on the car, there are fewer manual occupations and there is increasing use of labour saving devices [16]. Leisure time physical activity has also declined, in parallel with increased use of television and computer games. Computer games and television raises particular concern amongst children, where rates of obesity are rising in an alarming way [21].

1.1.2.6 Drugs

Certain medications regarding certain physical or mental conditions often increase the risk of obesity. The mechanism behind their influence on weight gain is still unclear, but may involve effects on appetite (neuroleptic drugs) and energy metabolism (oral hypoglycaemic drugs and protease inhibitors) both [24]. Table 1.2 summarises effects of certain classes of drugs in reference to obesity.

Drugs associated with weight gain	
Class	Examples
• Anticonvulsants	Sodium valproate, gabapentin
• Antidepressants	Citalopram, mirtazapine, amitriptyline
• Antipsychotics	Clozapine, chlorpromazine, risperidone, olanzapine
• β -blockers	Atenolol
• Corticosteroids	Prednisolone, dexamethasone
• Insulin	All formulations
• Migraine-relieving drugs (serotonin antagonists)	Pizotifen
• Sex steroids	Medroxyprogesterone acetate, progesterone, combined oral contraceptives
• Oral hypoglycaemic drugs	Glibenclamide, gliclazide, repaglinide, rosiglitazone, pioglitazone
• Protease inhibitors	Indinavir, ritonavir

Table 1.2: Certain drugs associated with Obesity. (*Adapted from - John P H Wilding, 2010 [24]*)

1.1.2.7 Endocrine and Hypothalamus Disorders

Endocrine disorders being the cause of obesity are rare and contribute to a small number of cases. Hypothyroidism being the most significant among this category, weight gain occurs primarily due to decreased energy expenditure in this case. Other disorders that may influence weight gain include Cushing's syndrome, thyrotoxicosis, deficiency of growth

hormone, and sex hormone disorders [24, 25]. Hypothalamic tumours and lesions in this region often damage the ventromedial hypothalamic regions that regulate energy intake and expenditure thus leading to weight gain [24].

1.1.3 Assessment of Obesity

There are several assessments designed to assess obesity and overweight. Body mass index (BMI), Waist to hip ratio (WHR), Skin fold method etc. are some examples of the methods developed to identify those at risk of serious health issues.

1.1.3.1 Body Mass Index

BMI is the easiest and fastest method to gauge obesity. It is a weight to height ratio of a person defined as the weight in kilograms divided by the square of the height in metres (kg/m^2). According to WHO standards an adult with a BMI equal or above $25 \text{ kg}/\text{m}^2$ is defined as overweight and a BMI of $30 \text{ kg}/\text{m}^2$ or above as obese. However, it should be considered a rough guide because it may not correspond to the same degree of fatness in different individuals because it does not distinguish weight associated with muscle from weight associated with fat. Table 1 lists the values of BMI with respect to the body condition according to the WHO published in 2000.

Classifications based on BMI	Body Mass Index (BMI)
Underweight	$< 18.5 \text{ Kg}/\text{m}^2$
Normal Weight	$18.5 - 24.9 \text{ Kg}/\text{m}^2$
Overweight	$25 - 29.9 \text{ Kg}/\text{m}^2$
Obesity (Class 1)	$30 - 34.9 \text{ Kg}/\text{m}^2$
Obesity (Class 2)	$35 - 39.9 \text{ Kg}/\text{m}^2$
Extreme obesity (Class 3)	$\geq 40 \text{ Kg}/\text{m}^2$

Table 1.3: Classification of Obesity based on BMI (Adapted from - The Practical Guide for Identification, Evaluation and Treatment of overweight and obesity in Adult. [42])

1.1.3.2 Waist Circumference

It is most common way to measure ‘abdominal obesity’- the extra fat found around the abdomen which is an important and independent risk factor for cardiovascular disease, type 2 diabetes, dyslipidemia, and hypertension. It is the circumference of the abdomen, measured at the natural waist (in between the lowest rib and the top of the hip bone), the umbilicus (belly button), or at the narrowest point of the midsection. [5] Waist circumference measurement is particularly useful in patients who are categorized as normal or overweight.

According to NHLBI individuals with waist circumference greater than 40 in. (102 cm) in men and greater than 35 in. (88 cm) in women have more than a fivefold greater risk of multiple cardio-metabolic risk factors, even after adjusting for BMI. [4]

1.1.3.3 Waist to Hip Ratio (WHR)

It is similar to Waist circumference and is used to measure abdominal obesity. It's calculated by measuring the waist circumference and the hip (at the widest diameter of the buttocks), and then dividing the waist measurement by the hip measurement. [5] As per WHO, abdominal obesity is defined as a waist to hip ratio above 0.90 for men and above 0.85 in case of women. Individuals with WHR higher than these limits are prone to increased health risk. WHR has been shown to be a better predictor of cardiovascular disease than waist circumference and body mass index. [6]

1.1.3.4 Skinfold Thickness

Skinfold thickness measurement provides an estimated size of the subcutaneous fat deposit (Fat under the skin). Special callipers are used to measure the thickness of skin and the fat beneath it in specific areas of the body (trunk, thighs, front and back of the upper arm, and under the shoulder blade). [5] By estimating the thickness, total body fat can be assessed.

1.1.4 Pathophysiology linked to Obesity

The health consequences of obesity range from increased risk of premature death, to serious chronic conditions that reduce the overall quality of life. It leads to adverse metabolic effects on blood pressure, cholesterol, triglycerides and insulin resistance. Obesity causes fat to build up in the arteries, narrow vessels, and leads to reduced blood flow to the heart. The non-fatal, but debilitating health problems associated with obesity include respiratory difficulties, chronic musculoskeletal problems, skin problems and infertility. The more life-threatening problems fall into four main areas: CVD problems; conditions associated with insulin resistance such as type 2 diabetes; certain types of cancers, especially the hormonally related and large-bowel cancers; and gallbladder disease. The likelihood of developing Type 2 diabetes and hypertension rises steeply with increasing body fatness. Approximately 85% of people with diabetes are type 2, and of these, 90% are obese or overweight.

Raised BMI also increases the risks of cancer of the breast, colon, prostate, endometrium, kidney and gallbladder (WHO 2003). Figure 1.2 describes the overall health risks associated with obesity.

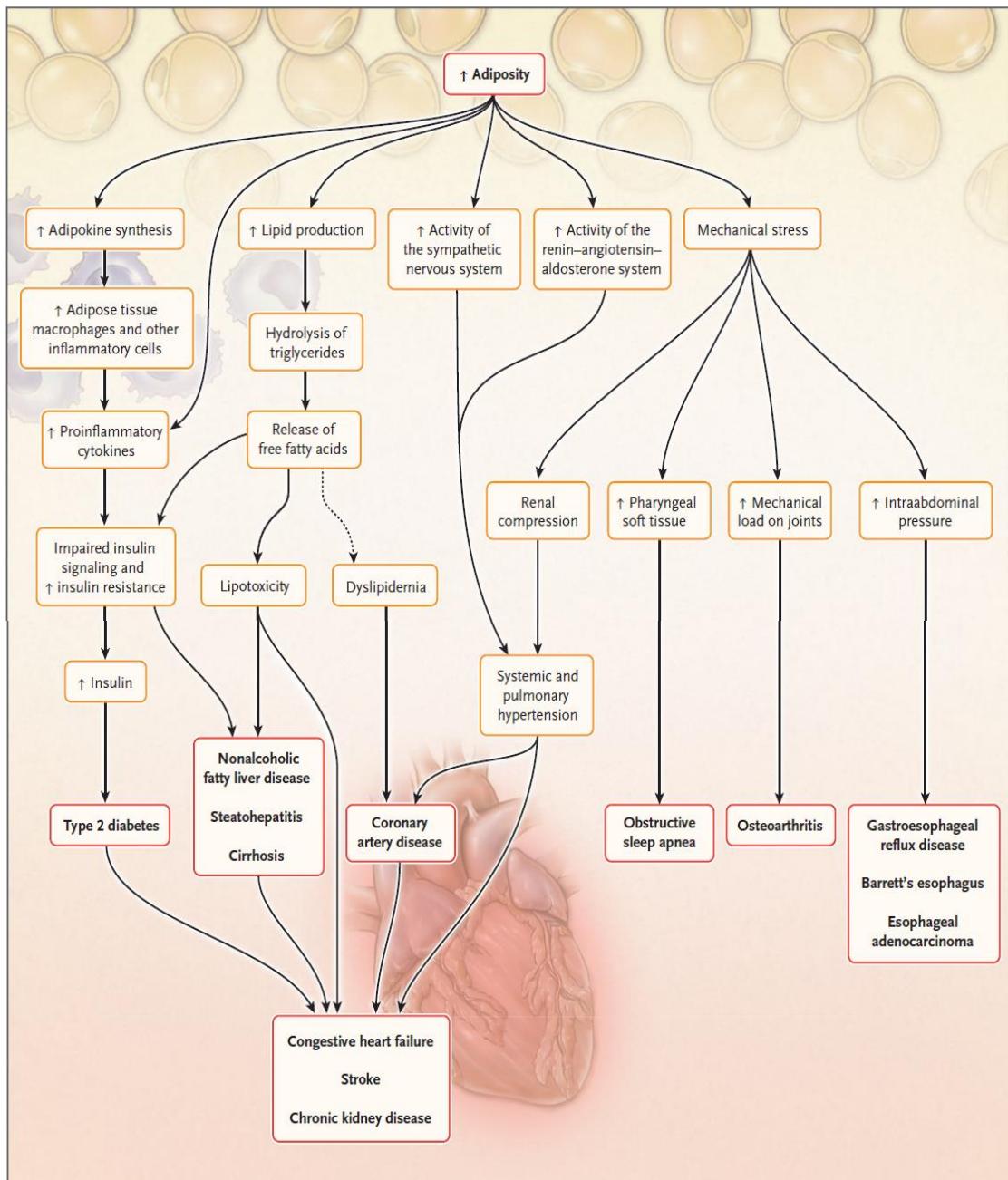


Figure 1.2: Some pathways through which excess adiposity leads to major risk factors and common chronic diseases. (Adapted from - Steven B. Heymsfield et al., 2017[26])

Patho-physiology related to obesity can be classified into two categories:

- Diseases due to increased fat cell size
- Diseases due to increased fat mass

1.1.4.1 Diseases due to increased fat cell size.

Enlarged fat cells contribute towards increased secretion of free-fatty acid and numerous peptides that leads to lipotoxicity, inhibition of lipogenesis and contributes to hypertriglyceridemia that induces serious health impacts.

- **Diabetes mellitus and insulin resistance.**

Type II diabetes or diabetes mellitus is strongly associated with weight gain. The risk of type 2 diabetes mellitus increases with the degree and duration of overweight and with a more central distribution of body fat [29]. As the BMI increases, the relative risk of diabetes increases in a manner such that at a BMI of 35kg/m² the risk increases by 40 times. Over 2/3rd of the cases of Type II diabetes can be attributed to Obesity. Both increased insulin secretion and insulin resistance result from obesity. A greater BMI correlates with greater insulin secretion. Also, due to tissue remodelling in response to adipocyte apoptosis there is an increase in macrophages and other immune cells in adipose tissue [27]. These immune cells secrete pro-inflammatory cytokines, contributing to the insulin resistance in obese patients [26]; this generates compensatory hyper-insulinemia with over stimulation of pancreatic cells and reduction in insulin receptors.

- **Hypertension**

Epidemiological studies have demonstrated that 50 to 60 per cent of patients with hypertension were overweight or obese. Renin-angiotensin system (RAS) – secreting adipokines contributes to vasomotor dysfunction and cause endothelial injury and Hypertension [30]. Overweight and hypertension both interact with cardiac function and when in combination can lead to thickening of the ventricular wall and increased heart volume, resulting in a greater likelihood of cardiac failure [29].

- **Cardiac alterations**

Overweight and obesity is associated with a number of cardiac abnormalities like heart failure, sudden cardiac arrest, angina etc. Increase in body and cardiac weight results in increased cardiac work which may produce cardiomyopathy and heart failure even in the absence of diabetes, hypertension, or atherosclerosis [29]. Dyslipidaemia is an important factor leading to increased risk of heart diseases. With an increased secretion of non-esterified free-fatty acids into the body (lipotoxicity), there is an increase in plasma triglycerides and low density lipoprotein (LDL) cholesterol and a decrease in high density lipoprotein (HDL) cholesterol, causing dyslipidaemia.

- **Cancer**

Obesity is a major risk factor for many forms of cancer, including breast, colon, endometrial, esophageal, hepatocellular, renal, and prostate cancer [30]. According to recent studies endometrial cancer in overweight women may be linked with increased oestrogen release from adipose cells [29] while colorectal cancer may be associated with Diabetes, insulin resistance, and increased body-mass index (BMI) [31]. Mechanisms of carcinogenesis or tumour growth include perturbed cellular proliferation, dedifferentiation and/or apoptosis, angiogenesis, and chronic adipokine-associated inflammation, along with the effects of cancer genes and/or environmental toxins that enhance inflammation [30]. Adipokine secretagogues such as unbound insulin-like growth factor enhance angiogenesis, which promotes cancer growth in general [32].

- **Non-alcoholic fatty liver disease (NAFLD)**

NAFLD is a combined term describing many liver abnormalities that are associated with increased BMI. It includes hepatomegaly, elevated liver enzymes, and abnormal liver histology [33]. Certain biopsy studies on liver suggest the prevalence of steatosis, steatohepatitis, and cirrhosis is approximately 75%, 20%, and 2%, respectively [34].

- **Gallbladder disease**

Gallstones are one of the major biliary tract pathology associated with obesity. The increased risk of gallstones is described by the increased cholesterol levels in an obese patient. Cholesterol is one of the major components of bile, and an increase in its levels results in higher concentration of cholesterol with respect to bile acids and phospholipids. This leads to the increased risk of precipitation of cholesterol gallstones in the gall bladder [35].

1.1.4.2 Diseases due to increased fat mass.

These are the abnormalities associated with the burden of weight and space-occupying effects of obesity. These include serious health issues like enhanced degenerative joint disease, sleep-apnea, renal compression, intra-abdominal pressure etc.

- **Obstructive sleep-apnea**

Obesity can influence respiratory system of an individual as there is a decrease in residual lung volume associated with increased abdominal pressure on the diaphragm in an obese patient [36] that may give rise to pulmonary disorders. Obstructive sleep-apnea may

result from increased neck circumference accumulation of extra adipose tissue in pharyngeal area, which adversely affects ventilation.

- **Degenerative joint diseases and skin abnormalities**

Osteoarthritis or degenerative joint disease is directly related to the trauma associated with the weight-bearing on joints due to increased adiposity and the injurious effects that inflammatory adipokines such as resistin have on joint synovia and muscle function [37, 38]. Several skin abnormalities are also associated with excess weight. Stretch marks, or striae, are common and reflect the pressures on the skin from expanding lobular deposits of fat [29].

1.1.5 Treatments available for Obesity

A number of medical and surgical strategies are available to treat obesity besides the typical combination of diet, exercise, and other behavioural modifications [19] (Yi Zhang et al.). Strategies should be aligned with the severity of overweight, associated coexisting chronic diseases, and functional limitations. Various important guidelines have been made available to assess an individual's health risks and treatment options. The major strategies against obesity based on sufficient evidence can be classified as lifestyle intervention, pharmacotherapy, and bariatric surgery [39] (Apovian CM et al.).

1.1.5.1 Lifestyle Interventions

Lifestyle interventions have been designed to modify eating behaviours and physical activity as the first option for weight management, given their low cost and the minimal risk of complications [40]. These strategies aims at decreasing energy intake and increasing energy expenditure through a balanced dietary and exercise program which is an essential component for any weight management programs [41] (Lau D.C. et al.). The aim for patients who are overweight or obese is to improve health and quality of life by achieving and maintaining moderate weight loss [26] (Steven B. Heymsfield and Thomas A. Wadden). Lifestyle interventions can be classified into two categories - Behavioural therapy and Dietary therapy. Dietary therapy involves reducing the number of calories you eat and learning strategies like how to read nutrition labels and select portion sizes, which types of foods to buy, and how to prepare them. Moderate caloric reduction is the goal for the majority of cases; however, diets with greater caloric deficits are used during active weight loss. The diet should be low in calories, but it should not be too low (≤ 800 kcal/day) [42]. Behavioural therapy involves changing diet and physical activity patterns of an individual that can promote weight control. Specific behavioural strategies include the following: self-monitoring, stress management, stimulus control, problem-solving, contingency management, cognitive

restructuring, and social support. Behavioural therapies may be employed to promote adoption of diet and activity adjustments; these will be useful for a combined approach to therapy [42].

1.1.5.2 Pharmacotherapy (drug therapy)

Pharmacotherapy may be helpful for eligible high-risk patients with $\text{BMI} \geq 30 \text{ kg/m}^2$ or $\text{BMI} \geq 27$ and having an obesity-related condition such as hyper-tension, type-2 diabetes, or dyslipidemia. Pharmacotherapy, approved by the FDA for long-term treatment, can be useful adjuncts to diet and exercise for obese adults who have failed to achieve weight loss with diet and exercise [43, 44]. These drugs should be used only in the context of a treatment program that includes the elements described previously—diet, physical activity changes, and behaviour therapy [42].

To date, four weight loss drugs have been approved by the US Food and Drug Association (FDA): Orlistat (Xenical), Contrave, Qsymia (Phentermine–topiramate), and Lorcaserin (Belviq) [45, 46, 47]. These medicines are divided into two types. Xenical is the only fat absorption inhibitor. Xenical acts as a lipase inhibitor, which decreases the absorption of fats from the human diet by 30%. It is intended for use in conjunction with a healthcare provider-supervised regimen of caloric restriction [48].

Another type, which includes the other three medications, acts on the CNS as an “appetite suppressant.” The newly approved (in 2012) drug Lorcaserin, for example, is a selective small molecule agonist of the 5HT2C receptor. It was developed based on the anorexigenic property of the receptor to mediate weight loss [49]. Activation of 5HT2C receptors in the hypothalamus stimulates pro-opiomelanocortin (POMC) production and promotes satiety. A 5-HT2C receptor agonist regulates appetite behavior through the serotonin system [50]. Use of Lorcaserin is associated with significant weight loss and improved glycemic control in patients with type 2 diabetes mellitus [49]. The other two medications, Contrave and Quexa, target the DA reward system. Contrave is a combination of two approved drugs—bupropion and naltrexone. Either drug alone produces modest weight loss, while the combination exerts a synergistic effect [51]. Qsymia (Quexa) consists of two prescription drugs, phentermine and topiramate. Phentermine has been used effectively for years to reduce obesity. Topiramate has been used as an anti-convulsant in epilepsy patients, but induced weight loss in people as an accidental side effect [50]. Qsymia suppresses appetite by making people feel full. This property is particularly helpful for obese patients because it deters overeating and encourages compliance with a sensible eating plan.

It is also essential to keep in mind that while pharmaceutical agents can help patients achieve clinically meaningful weight loss, the medications must generally be continued to maintain the reduction [52]. Drugs for treating obesity have had a bad track record. All these drugs produced serious side-effects. Orlistat may reduce the absorption of fat-soluble vitamins and nutrients. Sibutramine, an appetite suppressant that is proposed to work via norepinephrine and serotonergic mechanisms in the brain, may increase blood pressure and induce tachycardia, and was thus banned in several countries including India. However, the products on the market now have good safety records, and studies have shown them to be effective. But no products are 'magic bullets', Patients taking drugs still need to work on their diets and physical activity. The decision to add a drug to an obesity treatment program should be made after consideration of all potential risks and benefits and only after all behavioural options have been exhausted. Table 1.4 shows the mechanism of action for FDA approved anti-obesity drugs and their potential side-effects.

Drug	Mechanism of Action	Potential side-effects
Lorcaserin (Belviq)	Decreases appetite, increases feeling of fullness	Headache, dizziness, fatigue, nausea, dry mouth, constipation
Orlistat (Xenical)	Blocks absorption of fat	Intestinal cramps, gas, diarrhoea , oily spotting
Phentermine and topiramate extended release (Qsymia)	Decreases appetite, increases feeling of fullness	Increased heart rate, birth defects, tingling of hands and feet, insomnia, dizziness, constipation, dry mouth.

Table 1.4: Anti-obesity Medications Approved for Long-term Use (Adapted from - Allison DB, et al., 2012 [289])

1.1.5.3 Bariatric Surgery

Surgery is an option for patients with extreme obesity [42]. Multiple studies have demonstrated that bariatric surgery produces substantial and sustained weight loss, and results in amelioration of obesity- related comorbidities, compared with usual care. Bariatric surgery also appears to improve long-term survival and has the potential to dramatically improve a patient's quality of life [53, 54]. Weight loss surgery provides medically significant sustained weight loss for more than 5 years in most patients. Although there are risks associated with surgery, it is not yet known whether these risks are greater in the long term than those of any other form of treatment. Surgery is an option for well-informed and motivated patients who have clinically severe obesity ($BMI \geq 40$) or a $BMI \geq 35$ and serious comorbid conditions. (The term "clinically severe obesity" is preferred to the once commonly used term "morbid obesity.") Surgical patients should be monitored for complications and lifestyle adjustments

throughout their lives [42]. Numerous bariatric procedures are in use and are generally categorized as either restrictive or primarily malabsorptive and represent only current form of treatment for overt obesity with established long-term effectiveness [56]. Restrictive procedures limit the size of the stomach. Examples include adjustable gastric banding (AGB) and laparoscopic sleeve gastrectomy (LSG). Malabsorptive procedures restrict the size of the stomach to some extent but also involve bypassing a portion of the small intestine eg. Roux-en Y gastric bypass (RYGB) [55, 58]. Bariatric surgery also alters the gut hormone profile and neural activity. RYGB is the most frequently performed bariatric procedure, providing significant and sustained weight loss at long-term follow-up [57]. No doubt that the bariatric surgery can be considered as the ultimate weapon against obesity, there are many possible perioperative complications, including the risk of death [59]. Also following surgery, a significant number of patients fail to achieve optimal weight loss and/or regain weight.

Following bariatric surgery, sustainable lifestyle changes including diet and physical activity are essential and must be continually addressed. Physicians should follow up with their patients after bariatric surgery, and should encourage them for maintaining a healthy lifestyle in regard to long-term weight management [60].

1.1.5.4 A new and healthier approach

Due to limited and moderate weight loss associated with lifestyle intervention strategies and various severe side-effects associated with pharmacotherapy and surgery, there is a need to explore new prospective and strategies having promising results without any side-effects. Naturally occurring phytochemicals present an exciting opportunity for the discovery of newer anti-obesity agents. A variety of natural products, including crude extracts and isolated compounds from plants, can induce body weight reduction and prevent diet-induced obesity [64]. Many studies have shown the possibility of plant based bioactive compounds (dietary phytochemicals) as an alternative and complimentary method for treating obesity.

Dietary phytochemicals appear to be able to target different stages of the adipocyte (fat cell) lifecycle. For example, several classes of polyphenols have been implicated in suppressing the growth of adipose tissue through modifying the adipocyte lifecycle. Anti-obesity mechanisms of phytochemicals appear to involve mediation of complex and interconnected cell signalling pathways, therefore the combination of multiple phytochemicals may give rise to synergistic and enhanced anti-obesity effects [61]. However, such synergistic interactions among dietary bioactive compounds acting on adipocytes have received only limited attention [62, 63]. So far these studies have been encouraging with results indicating an enhanced induction of apoptosis and suppression of adipogenesis by

phytochemicals used in combination. Results from such studies suggest that anti-obesity effects could be achieved by consuming lower levels of phytochemicals but in specific combinations [61]. This aspect of alternative treatment for weight regulation will be discussed in detail under literature review.

1.2 Adipogenesis and Obesity

Obesity arises from the imbalance between energy intake and energy expenditure, leading to a pathological accumulation of adipose tissue. This development of adipose tissue is caused by an increase in the number and size of adipocytes differentiated from fibroblastic pre-adipocytes. The size of adipocytes increases because of increased storage of triacylglycerol from dietary sources or endogenous lipogenic pathways.

1.2.1 Adipose Tissue and Adipogenesis

Adipose tissue is necessary for maintaining normal physiology. It is the largest energy reserve in the body. This tissue contains a unique cell type (adipocyte) that is involved in the storage and mobilization of lipids. When energy is needed, triglycerides (TG) stored in the adipocytes are hydrolysed by lipases to produce free fatty acids (FFA), which are then transported via the circulation to tissues that oxidize them in the mitochondria for energy production. In addition, adipose tissue is an active endocrine organ that secretes hormones and cytokines involved in the regulation of appetite and metabolism, energy expenditure, insulin sensitivity, inflammation and coagulation [65]. Adipose tissue is a highly dynamic tissue that can change its metabolism rapidly in response to the body's energy status. Humans have two types of adipose tissue, brown adipose tissue (BAT) and white adipose tissue (WAT). WAT is the most predominant, and in non-obese adult humans, makes up 10-25% of total body weight. It has three major functions: to provide thermal insulation, the major role of the subcutaneous layer; mechanical cushioning by surrounding the internal organs; and acting as an efficient storage site of excess energy. 60-80% of white adipose tissue is made up of lipid, 90-95% of which is triglyceride, the remainder comprising of free fatty acids, di-glyceride, cholesterol, phospholipid and minute quantities of cholesterol ester and mono-glyceride are also present. Water (5-30%) and protein (2-3%) make up the remaining weight of white adipose tissue.

Unlike WAT, BAT develops and differentiates during foetal development. Its primary function is non-shivering thermogenesis, which protects the new born against cold. The amount of BAT declines with age in both humans and rodents [66], although it is always detectable in adults, even at low levels. BAT is located in depots located in the inter-scapular, sub-scapular and cervical regions. BAT derives its colour from rich vascularisation and its

densely packed mitochondria. In hibernating animals and neonates, brown adipose tissue is important for regulating body temperature via non-shivering thermogenesis. The lipids stored in brown adipose tissue release their energy directly as heat [67]. Multiple lipid droplets and uncoupling protein containing mitochondria are found within brown adipocytes, which can be activated to produce heat through sympathetic nervous system stimulation after cold exposure [26]. A new role has emerged for both WAT and BAT as endocrine organs, secreting a range of bioactive polypeptides, known as adipokines.

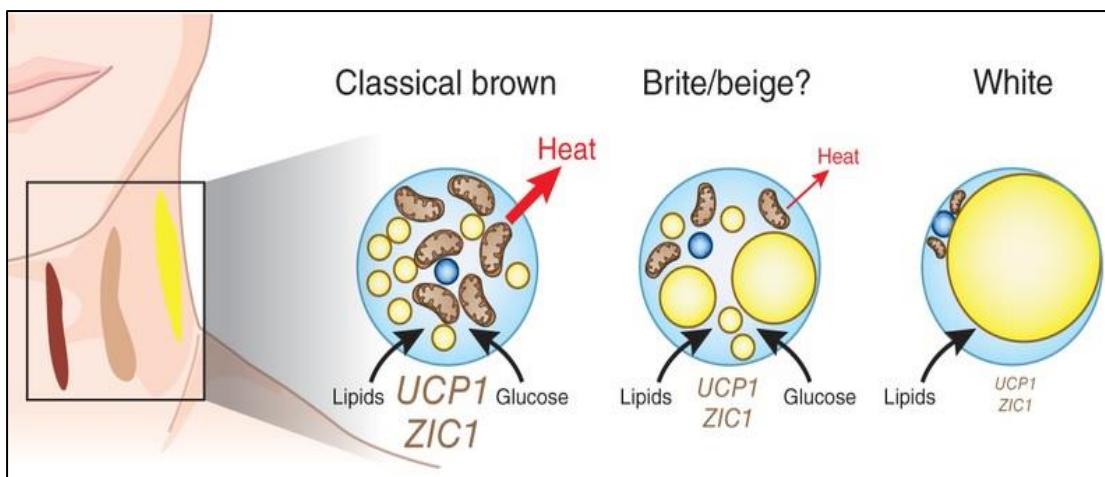


Figure 1.3: Types of Adipocytes (Adapted from – Jan Nedergaard & Barbara Cannon, 2013 [290])

Adipocytes originate from pluripotent mesenchymal stem cells (fibroblast-like precursors) that commit to pre-adipocytes and either stay dormant or, differentiate into lipid-laden and insulin-responsive mature adipocytes [68, 71]. This phenomenon is termed as adipogenesis or adipocyte differentiation. During terminal differentiation, the fibroblast-like pre-adipocytes undergo a series of morphological and biochemical changes to eventually accumulate lipid droplets [67]. The size of adipose tissue is a function of both an increase in adipocyte size (hypertrophy) and an increase in adipocyte number (hyperplasia) caused by lipid accumulation and thus resulting in obesity [69, 70].

Adipogenesis occurs in two phases, determination (or commitment) and terminal differentiation. During the determination process, pluripotent stem cells undergo a multistep process, which results in restriction to the adipocyte lineage, although they do not express any adipocyte markers yet. At this point, the pluripotent stem cells convert to pre-adipocytes, but remain morphologically indistinguishable. At the same time, the cells lose their pluripotent ability. There are many factors that can trigger the process of commitment; these factors may be secreted by cells within the stromal vascular population, such as macrophages and adipocytes undergoing hypertrophy.

In the second phase, terminal differentiation, pre-adipocytes are exposed to differentiation inducers, which trigger two rounds of mitotic clonal expansion. This leads to a 4-fold increase in cell number. Subsequently, the pre-adipocytes differentiate into mature adipocytes, which exhibit the characteristics associated with mature adipocytes such as lipid transport and synthesis, insulin sensitivity and the production of adipokines. Overall, adipogenesis is a sequential and temporally defined process, which involves multiple signalling cascades. Unlike terminal differentiation of pre-adipocytes to adipocytes, which is well studied, the specific details of the commitment phase, when the pluripotent stem cells become restricted to the adipocyte lineage, are not known. The process of adipogenesis has been reviewed in detail in various articles [72, 73].

1.2.2 Regulation of Adipogenesis

Adipogenesis occurs under the regulation of several transcriptional factors, such as sterol regulatory element binding protein (SREBP), CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor γ (PPAR- γ) [76]. These factors are involved in the sequential expression of adipocyte-specific proteins such as GLUT4, LPL, SCD-1, and ap2, which mediate fatty acid or glucose uptake into adipocytes, triglyceride hydrolysis, and lipogenesis [74, 75].

Peroxisome proliferator-activated receptor γ is the most adipose specific and has been shown to be enough to induce growth arrest or adipogenesis initiation besides playing a critical role in the regulation of adipocyte differentiation. Peroxisome proliferator-activated receptor α has been also reported to be capable of inducing adipocyte differentiation, but is less adipogenic than PPAR- γ . Finally, peroxisome proliferator-activated δ is not adipocyte specific, however is highly expressed in adipose tissue [75].

C/EBP family was among the first transcription factors confirmed to perform an important role in adipocyte differentiation. Isoforms of C/EBP metabolize lipid and cholesterol-related compounds and are expressed in tissues such as liver [77]. Although C/EBP- α is not adipocyte specific, is expressed before the transcription of most adipocyte-specific genes has started, and in some instances is sufficient to induce differentiation. C/EBP- α , C/EBP- β , and C/EBP- δ are isoforms; each one has a distinct sequential and spatial expression during adipocyte differentiation. C/EBP- α express relatively late in differentiation, meanwhile β and δ are present in pre-adipocytes, and their levels increase during differentiation [78].

The role of PPAR isoforms in adipogenesis is not completely understood. The PPARs form heterodimers with the retinoid X receptor (RXR) and regulate transcription through this

binding. The expression of PPAR- γ is directly induced by the co-expression of C/EBP- β and δ ; although C/EBP- α and PPAR- γ appearance rises intensely during adipocyte differentiation. This suggests that an increase in C/EBP- β above a certain level induces the expression of PPAR- γ . The ligand activation of PPAR- γ with C/EBP- α leads to full adipocyte differentiation [78].

1.2.3 Hormonal Signalling Transduction

A combination of hormones such as insulin, glucocorticoids and cAMP generating agents have been demonstrated to effectively promote the terminal differentiation of cultured pre-adipocyte cells. The role of insulin is to increase the percentage of cells that differentiate and increase the amount of lipid accumulation in each fat cell [79]. Insulin affects differentiation by cross-activating the IGF-1 receptor. Activation of the IGF-1 receptor is essential for pre-adipocytes to acquire their adipocyte morphology and accumulate lipid droplets [80].

IGF-1 and insulin also activate several distinct downstream signal transduction pathways including ras and protein kinase B, which may mediate their adipogenic effects [79]. Glucocorticoids, usually in the form of dexamethasone, are used to activate the glucocorticoid receptor. The transcriptional targets involved are unclear, but induction of CCAAT/enhancer binding protein-8 (C/EBP8) may be a possible mechanism. IBMX is a phosphodiesterase inhibitor, which can elevate intracellular levels of cAMP. Thiazolidinedione (TZDs) have also been shown to influence adipocyte differentiation as they are PPAR- γ agonists.

1.2.4 Adipocyte Metabolism

Preventing or alleviating excess lipid storage is a strategic approach in the fight against obesity that involves understanding how adipocytes regulate the ebb and flow of lipid synthesis and breakdown.

The primary role of adipocytes is to store energy, in the form of triglyceride, for times of need, such as during exercise and fasting. Circulating free fatty acids are readily taken up and stored as triglyceride, while excess energy in the form of glucose is synthesized into triglyceride through the process of *de novo* lipogenesis. When needed, adipocytes release this stored lipid into the blood stream through the process of lipolysis, in which triglyceride is broken down into free fatty acids (FFA) and glycerol that are released into the bloodstream [81]. Another fate of stored lipid is β -oxidation, in which the adipocyte itself uses free fatty acids to generate energy. Investigating ways to manipulate lipolysis and β -oxidation is an

approach to reduce the amount of lipid stored in hypertrophied adipocytes and thus alleviate the metabolic demand they face.

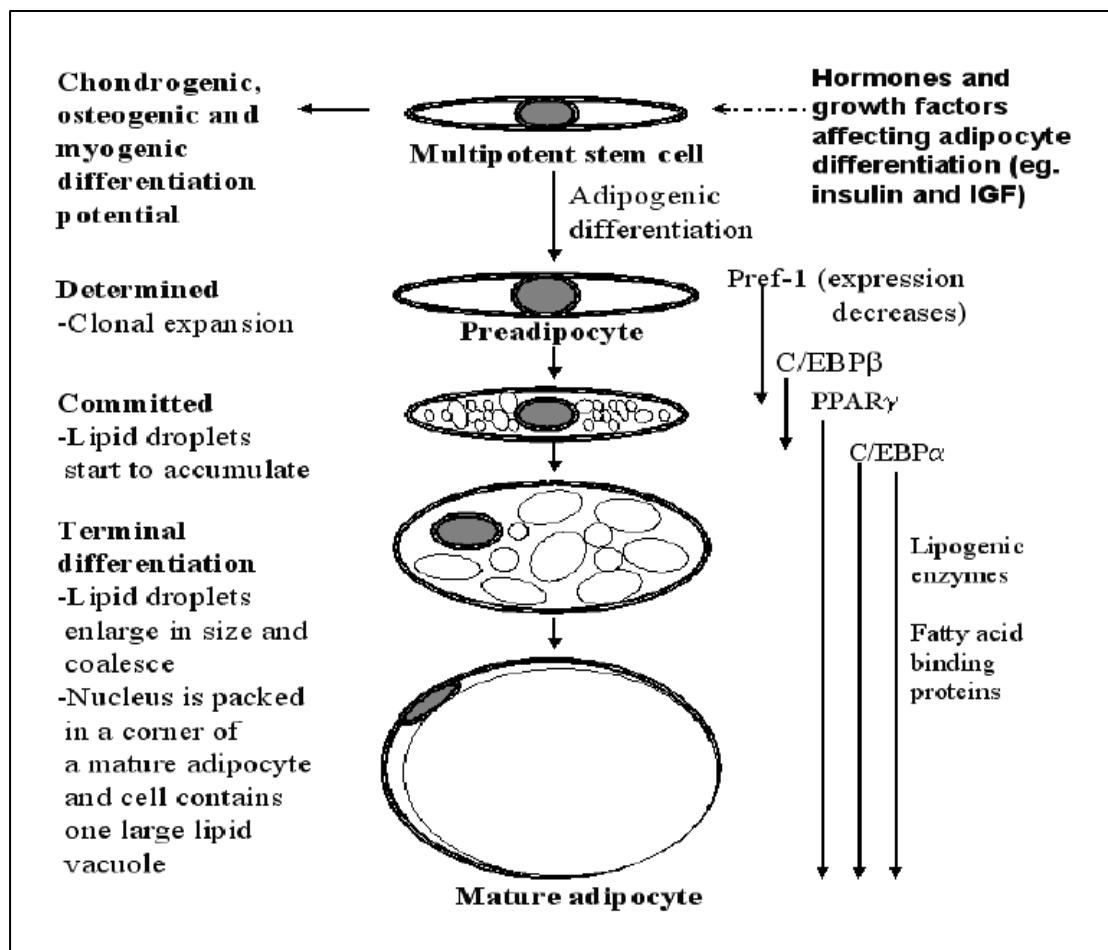


Figure 1.4: Adipogenesis process and expression of certain transcriptional factors (Adapted from - Niemelä et al., 2008 [291])

1.2.5 Adipocyte Clearance

Similar to many other cell types, adipocytes have a half-life of about 4-5 years [82]. Different mechanisms are involved in adipocyte clearance and include programmed cell death (apoptosis) or inflammation (necrosis). These pathways are regulated by different factors such as diet [83].

1.2.5.1 Necrosis

Death of adipocyte cells occurs primarily through necrosis [84]. Necrosis is an irreversible process that leads to cell death due to factors such as injury, radiation or chemicals. This type of cell death is associated with inflammation and is closely tied to the metabolic complications of obesity. Due to the release of cellular contents into the

extracellular matrix, necrotic cell death is associated with macrophage accumulation in WAT [85], which leads to induction of “crown-like structures” which consist of a dying adipocyte surrounded by macrophages. Since the macrophages are also responsible for removing the lipid droplets of the dead adipocyte; lipid accumulates in the macrophages, resulting in the formation of foam-like cells [86], which are specifically found in subcutaneous and omental fat depots of obese humans [87]. Cinti et al. (2005) [85] reported that obese humans and mice have a 30-fold increase in necrosis-like cell death in WAT compared to their lean counterparts.

1.2.5.2 Apoptosis

Another type of cell death that adipocytes can undergo is apoptosis. Apoptosis is a type of programmed cell death that is observed in physiological processes involved in the removal of selected cells in an efficient manner, without releasing their contents into the surrounding extracellular space [88]. Apoptosis can be initiated by many pathways, all of which trigger an energy-dependent enzymatic cascade that leads to the breakdown of DNA, lipids and other macromolecules [89]. There is evidence that suggests PPAR γ is involved in the induction of apoptosis in large adipocytes [90].

1.3 Adipokines

In addition to storing excessive energy, adipose tissue is a dynamic endocrine organ releasing various bioactive substances called “adipocytokines” or “adipokines”, into the bloodstream which impact directly the regulation of food intake, insulin sensitivity, energy metabolism, immune response, inflammation, hypertension, cell adhesion, adipogenesis and bone morphogenesis, cell and tissue growth and the vascular micro-environment [91]. There are over 600 well known and less well known adipokines and bioactive peptides that contribute to normal physiological processes and pathological conditions, including adiponectin, leptin, resistin, adipsin, chemerin, vasfatin, omentin, vaspin, lipin, angiotensinogen, various prostaglandins, plasminogen activator inhibitor-1, interleukin (IL)-17D among a long list of interleukins, TNF- α , BMP-7, IGF-1, fibronectin, monocyte chemoattractant protein-1 (MCP-1) and macrophage migration inhibitory factor [92]. The amount of adipokine production depends on both the origin of the adipocytes (subcutaneous or visceral adipose) and the size of the adipocytes as illustrated in two recent reviews [94]. The situation worsens when adipocytes release excessive amounts of cytokines/ adipokines as TNF- α , resistin, IL-6, PAI-1, MCP-1 and others; which circulate via the vascular system to insulin target tissues such as liver, muscle and islet cells inducing insulin resistance [93]. Figure 1.5 describes the relation between adipokines and obesity.

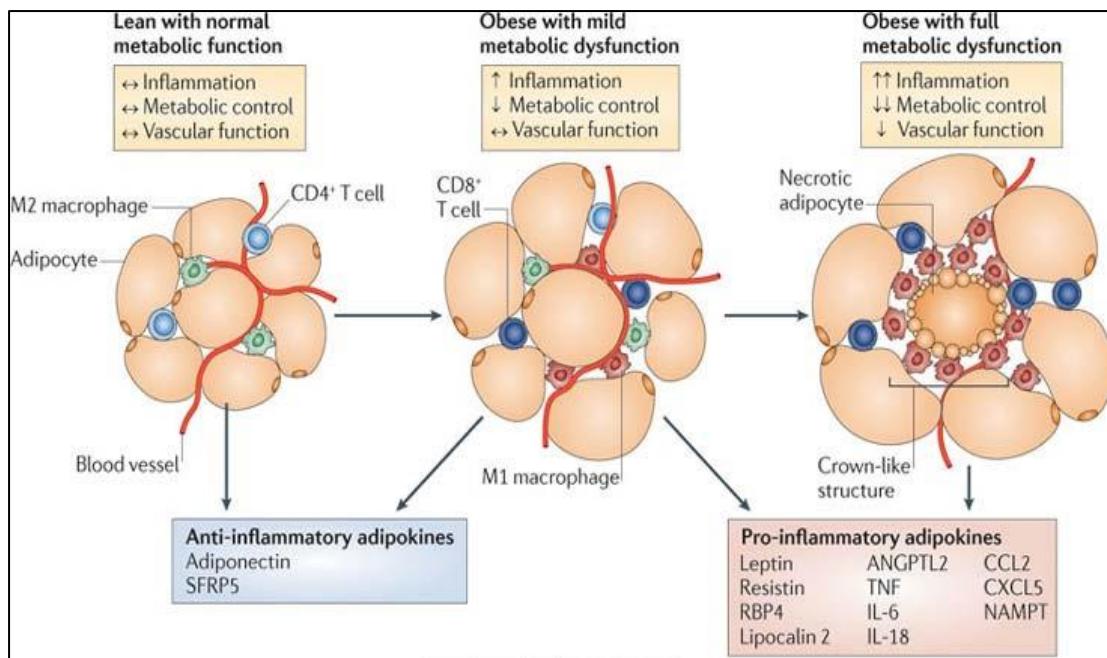


Figure 1.5: Adipokines and obesity (Adapted from – www.nature.com)

1.3.1 Adiponectin

Adiponectin, also known as adipocyte complement-related protein of 30 kDa (Acrp30), adipose most abundant gene transcript 1 (apM1), adipoQ and gelatin binding protein of 28 kDa (GBP28), was identified as a protein expressed and secreted by differentiated 3T3-L1 adipocytes and also found to be in abundant quantity in serum [95]. Adiponectin is expressed exclusively in white adipose tissue and adipocytes are the most important source of adiponectin, but serum adiponectin levels do not increase with obesity. Serum adiponectin levels are low in obese subjects but increase with weight loss [96].

Adiponectin, also known as adipocyte complement-related protein of 30 kDa (Acrp30), adipose most abundant gene transcript 1 (apM1), adipoQ and gelatin binding protein of 28 kDa (GBP28), is the most abundant and studied adipokines and is identified as a protein exclusively released in adipocytes [98]. It is found in plasma at levels of 3-30 mg/ml. Both trimers and oligomers, but not monomers, of adiponectin are present in the circulation [97]. A proteolytic cleavage product containing the globular domain of adiponectin also circulates at physiologically significant levels and has biological activity [99]. Adiponectin increases fatty acid oxidation while decreases glucose production, and also plays a role as anti-inflammatory [100]. Numerous studies reveal the negative correlation of plasma adiponectin and obese subjects depicting a higher concentration of adiponectin in non-obese subjects [101]. Individuals with low concentrations of plasma adiponectin have reduced insulin sensitivity, and tend to develop Type II diabetes [102]. Elevated plasma adiponectin levels were found in

low BMI diabetic and non-diabetic subjects [103]. The physiological role of adiponectin in humans is not yet clearly elucidated [101].

1.3.2 Leptin

Leptin is another adipokine product of the obese (*ob*) gene, which reduces appetite by sending a satiety signal to the central nervous system [104]. It is a 16 kDa hormone and mainly released by adipocytes in order to control body weight [98]. Concentration of leptin circulation in plasma is proportional to total body adiposity and direct nutritional state [105]. It is present in human serum in ranges of 1-15 ng/ml in non-obese individuals, and levels more than 30 ng/ml in individuals with $BMI \geq 30 \text{ kg/m}^2$ [106]. Leptin promotes basal metabolism and β -oxidation. Unlike adiponectin, leptin levels are positively correlated with BMI and degree of adiposity [117, 118]. Despite the higher concentration of leptin in obesity, it is unable to block weight gain effectively. This condition is termed as leptin resistance.

1.3.3 Other Important Adipokines

Interleukin-6 (IL-6) is an inflammatory cytokine that is correlated with hyperglycaemia, insulin resistance, and type 2 diabetes mellitus [107]. Mice chronically exposed to IL-6 develop hepatic insulin resistance [108]. About 25% of circulating IL-6 in humans is released by subcutaneous adipose tissue [109].

Tumor necrosis factor-alpha (TNF- α) is a cytokine whose expression is increased in adipose tissue and highly found in circulation of obese and insulin resistant individuals [110]. TNF- α has an important effect on whole body lipid and glucose metabolism [111], it inhibits tyrosine phosphorylation of IRS-1 which decreases insulin signalling [112]. Moreover, TNF- α is directly involved in the activation of pro-inflammatory subcellular pathways and induces the production of reactive oxygen species (ROS) [113].

Plasminogen activator inhibitor-1 (PAI-1) is secreted in high concentration by adipose tissue. Plasma and adipocyte concentrations of PAI-1 correlate with the levels of visceral fat and triglycerides [114]. It has been suggested that PAI-1 may probably destroy adipocytes causing hypertrophy [115]. Macrophage chemo-attractant protein -1 (MCP-1) is a novel adipokine which has an important role in the development of obesity-associated insulin resistance [116]. The expression of MCP-1 by adipose tissue induces inflammatory reactions and insulin resistance, suggesting their suppression of MCP-1 is important for the management of metabolic syndrome [91].

Chapter 2

Literature Review

Literature Review

2.1 Potential Health Benefits of Bioactive Compounds in the Development of Obesity

To prevent chronic disease, increasing fruit and vegetable consumption has been the longstanding advice. Consistently, fruit and vegetable intake is negatively correlated with the occurrence of obesity and metabolic syndrome [136, 137], and further, increasing fruit and vegetable consumption may decrease risk for heart disease and diabetes [138, 139]. Not only do fruits and vegetables provide a low-calorie, high-fibre option, but researchers within the past several decades have also identified more than thousands of phytochemicals that naturally occur in the produce we eat [140].

Discovery of bioactive compounds has invited researchers to explore the vast array of beneficial effects these compounds possess. Much interest surrounds the use of bioactive compounds, through increased consumption and supplementation, to prevent or alleviate symptoms of metabolic syndrome as drug therapy is not only costly, but often elicits negative side-effects [144, 141]. Currently, it is estimated that humans consume about 1-3g of bioactive compounds daily, depending on their level of fruit and vegetable intake [142]. Nearly all bioactive compounds have been shown to possess health-promoting properties, typically in a dose-dependent manner. The American Dietetic Association (ADA) acknowledges the significance of consumption of bioactive compounds and therefore supports the marketing of functional foods, either conventional or modified, when there is substantial scientific evidence of benefit [143].

Cell culture and animal model studies have indicated that the anti-obesity effects occur through modification of the adipocyte lifecycle [61]. Polyphenols are a class of bioactive phytochemicals that are likely candidates as anti-obesity agents as several studies have suggested they can modulate the adipocyte lifecycle [145, 146]. The strongest evidence for this effect comes from: phenolic acid derivatives such as chlorogenic acid [147, 148]; flavonols e.g. quercetin [146]; and flavones such as luteolin [145]. These classes of polyphenols are widely distributed in plants and therefore are consumed regularly as part of the human diet.

Mechanisms of action of phytochemicals on adiposity

The plausible mechanisms of action of certain vegetable phytochemicals include:

- (a) Reducing adipose tissue mass by inhibiting the proliferation of precursor cells [145, 146];

- (b) Increasing the rate of apoptosis during the adipocyte lifecycle [145, 146];
- (c) The inhibition of dietary triglyceride absorption via reduction in pancreatic lipase formation [149].

Obesity has been associated with a chronic inflammatory status [148] and the strong anti-inflammatory activity may be one of the mechanisms of action for counteracting the negative physiological effect of the obesogenic state [61].

The development and maintenance of obesity involves many complex molecular mechanisms and interconnected cell signalling pathways and to discuss recent advances in this topic is well beyond the scope of this review. Therefore the current review is restricted to a summary of proposed mechanisms of action of the major vegetable phytochemicals [61].

2.1.1 Polyphenols

Polyphenols are a class of phytochemicals widespread in fruits and vegetables that have demonstrated one or more potential anti-obesity effects. Dietary polyphenols may suppress growth of adipose tissue by modulating adipocyte metabolism [150, 151]. Unfortunately to date, the effects of polyphenols on human adipocytes have not been studied systematically; most studies having been conducted on murine cell lines such as 3T3-L1 and in the tissues of laboratory animals [152, 153].

Polyphenols, including their functional derivatives, esters and glycosides, have one or more phenol groups with one hydroxyl substituted aromatic ring [154]. According to their structure and the type and number of structural elements binding to the rings, polyphenols are grouped into different classes. The classes of polyphenols for which there is most evidence of potential anti-obesity properties are:

- The simple phenolic acids such as chlorogenic, coumaric, gallic and caffeic acid.
- The flavonoid sub-classes - flavonols e.g. quercetin, kaempferol, myricetin and isorhamnetin and the flavones e.g. luteolin and apigenin.

2.1.1.1 Phenolic acids: chlorogenic acid and related compounds

Naturally occurring phenolic acids contain two distinguishing constitutive carbon frameworks: hydroxyl-cinnamic and hydroxyl-benzoic structures. Although the basic skeleton remains the same, the numbers and positions of the hydroxyl groups on the aromatic ring create the variety. Common hydroxyl-cinnamic acid derivatives are coumaric, caffeic and ferulic acids which can be frequently found in foods as simple esters with quinic acid or glucose. The most widely occurring of these is chlorogenic acid [61]. Unlike hydroxyl-

cinnamates, hydroxyl-benzoic acid derivatives are mainly present as glycosides. The most common forms in plant foods are p-hydroxybenzoic and vanillic acids [155]. Figure 2.1 depicts some polyphenols with reported anti-obesity properties.

Hsu and Yen (2006) [152] investigated the inhibitory effect of dietary phenolic acids on mouse pre-adipocytes. Chlorogenic and coumaric acids caused significant inhibition of cell growth as well as enhancing apoptosis. Gallic acid while not affecting the adipocyte cell cycle did increase the number of apoptotic cells. A recent study by Son et al. (2010) [156] evaluated the effects of feeding ferulic acid on lipid metabolism of mice. This dietary phenolic acid suppressed the weight gain due to the high fat diet and inhibited fatty acid biosynthesis.

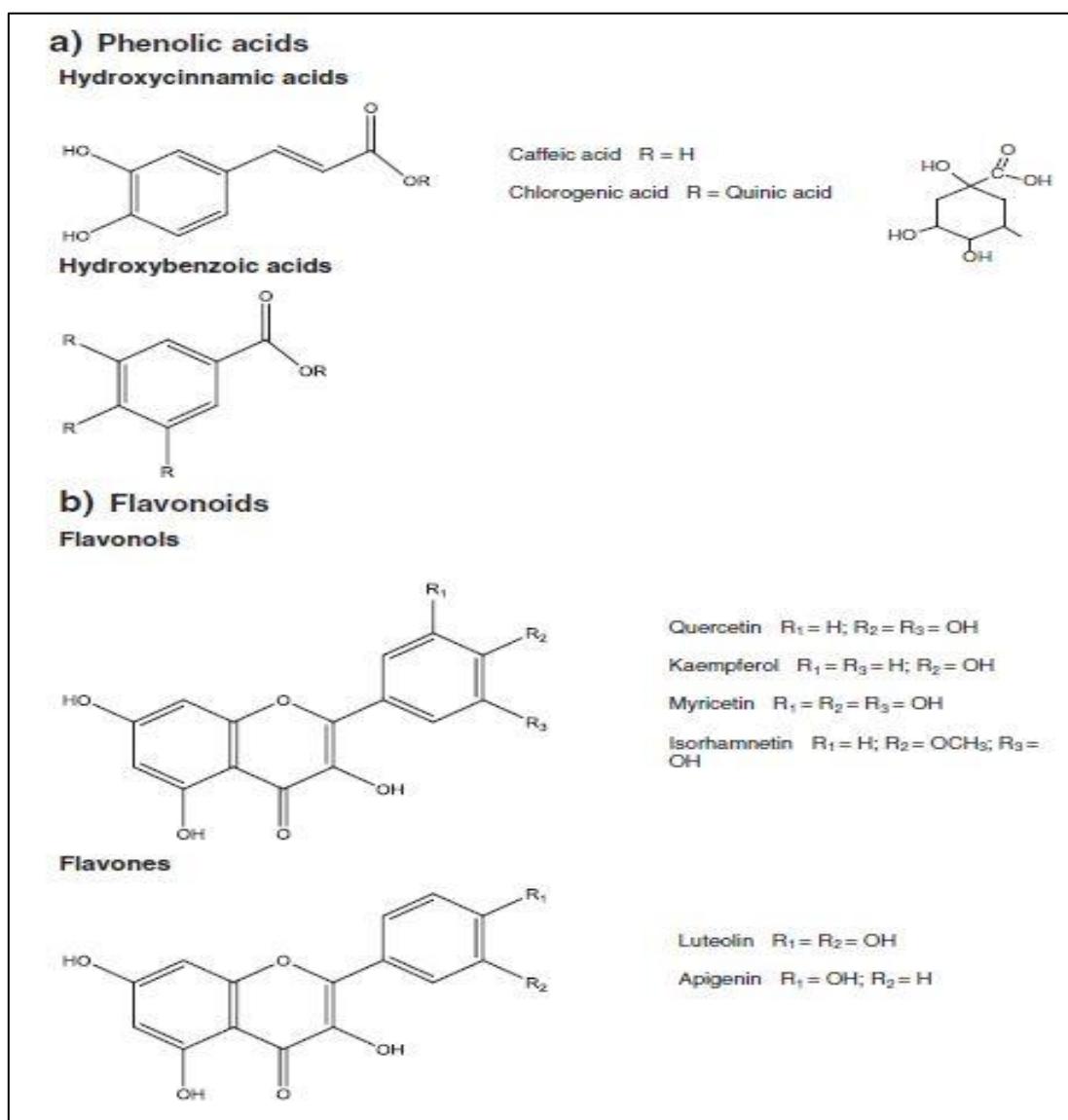


Figure 2.1: Phytochemicals with reported anti-obesity effects (Adapted from - David J. Williams et al., 2013 [61])

2.1.1.2 Flavonoids: flavonols, flavones and anthocyanins

Flavonoids are a class of polyphenols that are widely distributed in vegetables and can be further differentiated into sub-classes according to their structure. They share the common skeleton of diphenylpropanes (C6–C3–C6). The main difference between the two important sub-classes, i.e. the flavonols and the flavones is the presence of a hydroxyl group at C3 in flavonols. They both usually occur in plants as glycosides [61].

- Flavonols: quercetin and related compounds :

Quercetin is a dietary flavonol frequently found in fruits and vegetables, for which there is most evidence for its potential anti-obesity effects. It has been shown to inhibit adipogenesis [157] and to induce apoptosis in mouse pre-adipocytes [152, 158, 159]. Kaempferol has also exhibited these potential anti-obesity properties but to a lesser extent [158]. A recent study [160] provided useful insights into the molecular mechanisms by which quercetin influences the regulation of fat cell differentiation and apoptosis.

Park et al. (2008) [161] exposed human adipocytes to quercetin in combination with the isoflavone, genistein and the stilbene, to human adipocytes. The combined treatments caused enhanced inhibition of lipid accumulation in maturing human adipocytes, far greater than the responses to individual compounds. Several studies have revealed that quercetin provides some protective effects against obesity-related inflammation [162, 163]. Quercetin was demonstrated to attenuate markers of inflammation, macrophages and insulin resistance in human adipocytes and reduce circulating markers of inflammation in animal models [61].

- Flavones: luteolin and apigenin :

Park, Kim, and Kim (2009) [164] demonstrated an anti-adipogenic effect of the flavone, luteolin on murine 3T3-L1 pre-adipocytes mediated through decreased lipid accumulation and inhibition of differentiation. An earlier study [159] had shown that the addition of a similar flavone, apigenin induced lipolysis in rat adipocytes.

- Anthocyanins :

Another flavonoid sub-class with potential for anti-obesity-related effects is the anthocyanins, responsible for the red, blue and purple colours in vegetables [165]. In situ, anthocyanins are stabilised by the formation of complexes with other flavonoids and their degradation is prevented by glycosylation and esterification with various organic acids and phenolic acids. In a 2008 study Tsuda [166] showed that anthocyanins possess significant anti-inflammatory properties in obese adipose tissues. Another possible anti-obesity mechanism associated with anthocyanins was reported by Sasaki et al. (2007) [167]. They observed that cyanidin, the most common anthocyanin in foods, reduced blood glucose levels

as well as down-regulating inflammatory protein cytokines such as monocyte chemoattractant protein-1 (MCP-1) in the adipose tissue of mice. Recent studies have demonstrated that an increase in expression of these inflammatory molecules in adipose tissue contributes to the development of insulin resistance [168, 169].

2.1.2 Carotenoids

Carotenoids, though not polyphenols, but rather a sub-class of terpenoids, have been reported to possess anti-obesity and anti-inflammatory abilities [170]. Carotenoids are classified into hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls). They are responsible for the yellow, orange and red colour of many vegetables. α -Carotene is one of the most abundant carotenoids in the diet and can be converted in the body to an active form of vitamin A. β -Carotene inhibits inflammatory gene expression in lipopolysaccharide-stimulated macrophages. Possible anti-obesity roles for both these carotenes have been postulated based on the finding that the plasma of overweight and obese children had significantly lower levels of α -carotene and β -carotene when compared to healthy weight children [171].

2.2 *Moringa oleifera*

Moringa oleifera is the most widely cultivated species of the genus Moringa of the family Moringaceae. It is a drought-resistant wispy tree that grows in and has traditional usage as a food product in Asian countries [119, 120]. It is a small tree of the sub-Himalayan regions North West India, which is now indigenous many regions in Africa, Arabia, South East Asia, the Pacific and Caribbean islands and South America. *Moringa* is also referred as horse radish tree (referring to the taste of its roots), drumstick tree (describing the shape of its pods) and less frequently referred to as 'The Tree of Life' or 'Miracle Tree' due to

Table – 2.1: Taxonomical classification of *Moringa oleifera* (Adapted from - ITIS Standard Report Page)

Kingdom	Plantae
Subkingdom	Viridiplantae
Super division	Embryophyta
Division	Tracheophyta
Class	Magnoliopsida
Order	Brassicales
Family	Moringaceae
Genus	Moringa
Species	<i>Moringa oleifera</i>

its economic importance and versatility with high nutritive, agricultural, medicinal, domestic, industrial and environmental benefits [121]. Table 2.1 gives the taxonomical classification of *Moringa oleifera*.

2.2.1 Nutritional Content

Moringa oleifera is one of the most nutrient-rich plants yet discovered. *Moringa* provides a rich and rare combination of nutrients, amino acids, antioxidants, anti-aging and anti-inflammatory properties used for nutrition and healing. A large number of reports on the nutritional qualities of *Moringa* now exist in both the scientific and the popular literature. According to literatures, *Moringa* contain large amounts of several important nutrients including vitamin C, vitamin A, Calcium, Potassium, Proteins, and all the essential amino acids in good proportions. A nutrition chart for dried *Moringa* leaves is given in Table 2.2.

2.2.2 Possible health benefits

The plant has been used from ages as a traditional medicine for the treatment of ascites, rheumatism, venomous bites, pneumonia [122, 123], circulatory disorders, metabolic and

endocrine disorders, and general nutritional deficiencies [127]. It has some usage for the prevention of diabetes and glucose disturbance, [124] wound healing, [125] and as an aphrodisiac [126].

Nutrients	Content (Dry weight basis)	References
Protein	20.72 - 25.29 %	[120, 134]
Fatty acids Dry basis	5.37 - 5.75 %	[120]
Carbs	37.98 %	[134]
Dietary fibre	13.71 %	[134]
Ash Content	8.53 - 15.09%	[120, 134]
calcium	870 - 3468 mg/100g	[134, 135]
Phosphorus	228 - 600 mg/100g	[134, 135]
Magnesium	300 - 831 mg/100g	[134, 135]
Sodium	50 mg/100g	[135]
Potassium	300 mg/100g	[135]
Copper	960 - 1170 µg/100g	[134, 135]
Manganese	11.28 mg/100g	[134]
Iron	105 mg/100g	[134]
Zinc	2.04 mg/100g	[134]
Vitamin C	17.3 mg/100g	[230]
Vitamin A	16.3 mg/100g	[230]
phenolic content	181.3 - 200.0 mg catechin equivalent /100g	[120]
Phytate	31.1 mg/g	[135]
Oxalate	4.1 mg/g	[135]

Table 2.2: Nutrition chart for *Moringa oleifera*

The plant has been also used in culinary purposes by wrapping the leaves around food products to preserve their quality and reduce bacterial contamination [128] due to mixed antioxidant, antibacterial, [129] and protease inhibiting properties [130]. Most parts of this plant are said to hold some medicinal properties even including the seeds, fruit pods [131], and flowers [132] although the leaves are thought to be the main medicinal component. The leaves are also known to have the highest antioxidant potential of all plant parts [133].



Figure 2.2: *Moringa oleifera* (Drumsticks)

2.3 *Brassica oleracea*

Brassica oleracea comprises cruciferous family of vegetables varieties having worldwide economic importance, such as kale, broccoli, Brussels sprouts and cauliflower.

Brassica oleracea var. *acephala* or Kale is a descendent of the wild cabbage, a plant thought to have originated in Asia Minor and to have been brought to Europe around 600 BC. It is also known as borecole. Their wild forms have become widely distributed from their place of origin and are found on the coasts of northern Europe and Britain. Apparently, all the principal forms of kale we know today have been known for at least 2,000 years [234]. Kale is hardy cool-season green of the cabbage family. Although kale tolerates summer heat, it grows best in the spring and fall. The highly curled, bluish-green leaves (some varieties have plain leaves) do not form a solid head. Flower-like cultivars of kale are quite colourful [235]. In addition to serving as ornamentals, kale plants are used for greens as a garnish or in salads and may be cooked in place of cabbage [234]. Table 2.3 describes the taxonomical classification of *Brassica oleracea*.

Different varieties of kale:

- Curly Kale (Common - ruffled leaves with dark green or purple color)
- Lacinato Kale (dark blue-green leaves with a slightly wrinkled and firm texture)
- Red Russian Kale (flat, fringed leaves with a red tinge and a reddish-purple tinge to the stems)
- Redbor Kale (dark red to deep purple-ornamental kale)

Table 2.3: Taxonomical classification of *Brassica oleracea* (Adapted from - ITIS Standard Report Page)

Kindom	Plantae
Subkindom	Viridiplantae
Super division	Embryophyta
Division	Tracheophyta
Class	Magnoliopsida
Order	Brassicales
Family	Brassicaceae
Genus	Brassica
Species	<i>Brassica oleracea</i>



Figure 2.3: *Brassica oleracea* (Kale)

2.3.1 Nutritional Content

Kale has gained recent widespread attention because of its health-promoting, sulphur containing phytonutrients. It is one of the most nutritious vegetables from the garden. One cup of this leafy green vegetable contains significant amounts of vitamins A, C, K and B6, and significant amounts of the minerals potassium, calcium, iron and manganese. It is also a very good source of dietary fibre (Refer Table 2.4).

Nutrients	Content (Raw fresh basis)	References
Protein	2.47 - 4.28 g/100g	[231, 232]
Omega-3 fatty acids	0.1 g/100g	[231]
Carbs	7.32 - 8.75 g/100g	[231, 232]
Dietary fibre	2.6 - 3.6 g/100g	[231, 232]
Calcium	93.60 - 150 mg/100g	[231, 232]
Phosphorus	36.4 - 92 mg/100g	[231, 232]
Magnesium	23.4 - 47 mg/100g	[231, 232]
Sodium	29.9 - 38 mg/100g	[231, 232]
Potassium	296.4 - 491 mg/100g	[231, 232]
Copper	200 µg/100g	[232]
Manganese	0.54 - 0.659 mg/100g	[231, 232]
Iron	1.17 - 1.47 mg/100g	[231, 232]
Zinc	0.31 - 0.56 mg/100g	[231, 232]
Vitamin C	53.3 - 120 mg/100g	[231, 232]
Vitamin A	9990 - 17707.3 IU/100g	[231, 232]
Vitamin K	704.8 - 1062.1 µg/100g	[231, 232]
phenolic content	227 - 285 mg catechol equivalent /100g	[233]

Table 2.4: Nutrition chart for *Brassica oleracea*

2.3.2 Potential health benefits

- Kale is loaded with powerful anti-oxidants; this includes beta-carotene, vitamin C, as well as various flavonoids and polyphenols.
- Kale is one of the world's best sources of vitamin K. Vitamin K is an important nutrient that is involved in blood clotting.
- Detoxification of body: presence of isothiocyanates made from Kale's glucosinolates [234].
- Anti-inflammatory: presence of omega-3 fatty acids [234].

- Cancer prevention: sulphur containing compounds (sulforaphane), antioxidants including carotenoids and flavonoids and chlorophyll (inhibit absorption of carcinogenic heterocyclic amines).
- Cholesterol reduction: high amount of dietary fibre.
- Improves bone density: rich in vitamin K and also have good percentage of calcium.

2.4 *Ocimum basilicum*

Sweet Basil is a common name for *Ocimum basilicum*. It is also known as Great basil or Saint Joseph's worth [236]. Basil, originally from India, is a half-hardy annual plant, best known as a culinary herb. *O.basilicum* is a cultivated plant which is widely distributed in the tropics of Africa and Asia. It is cultivated commercially for its green, aromatic leaves, which are used fresh or dried as a flavoring or spice. Depending on the species and cultivar, the leaves may taste somewhat such as anise, with a strong, pungent, and often sweet smell [237]. Sweet basil has been

Table 2.5: Taxonomical classification of *Ocimum basilicum* (Adapted from - ITIS Standard Report Page)

Kindom	Plantae
Subkindom	Viridiplantae
Super division	Embryophyta
Division	Tracheophyta
Class	Magnoliopsida
Order	Lamiales
Family	Lamiaceae
Genus	<i>Ocimum</i>
Species	<i>Ocimum basilicum</i>

used for thousands of years as a traditional medicinal herb. The phyto-chemical evaluation of *O.basilicum* shows that it is rich in alkaloids, tannins, phytates, flavonoids and oligosaccharides. The essential oil and oleoresin extracted from the leaves and flowering tops of basil plant via steam distillation & hydro distillation contains some of the most precious compounds i.e. linalool and eugenol. It is also rich in saponines, tannins, flavonoids and glycosides, enzymes and organic acids [238].



Figure 2.4: *Ocimum basilicum* (Sweet Basil)

Different Species of Basil:

The basil comes in many different varieties, each with its own unique chemical composition and characteristic flavour. The flavour and character of any particular variety of basil is affected to a great extent by many external environmental factors, such as temperature, the type of soil, the geographic location, and even the amount of rainfall received by the individual plant. Some varieties can be summarised as follows:

- *Ocimum basilicum* - Best known cultivars (Sweet basil)
- *Ocimum americanum* - Lime Basil or Hoary Basil
- *Ocimum sanctum* or *tenuiflorum* - Holy Basil or Tulsi
- *Ocimum citriodorum* - Thai Lemon Basil (hybrid of *O. americanum* and *O. basilicum*).
- *Ocimum gratissimum* – Clove Basil
- *Ocimum minimum* – Greek Bush Basil or Greek Spicy Globe Basil
- *Ocimum kilimandscharicum X basilicum* – African Blue Basil or Camphor Basil (hybrid)

2.4.1 Nutritional Content

The seeds of sweet basil produce an aromatic essential oil which is prominent in the production of several drinks, cosmetics, and food of consumption. Basil oil contains various bioactive compounds that include camphene, cis-ocimene, camphor, linalool, methyl chavicol, γ -terpineol, citronellol, geraniol, methyl cinnamate and eugenol, and other terpenes [248]. A nutrition chart for dried and powdered basil leaves is given in Table 2.6.

2.4.2 Potential health benefits

Basil has been used as a folk remedy for an enormous number of ailments, including boredom, cancer, convulsion, deafness, diarrhea, epilepsy, gout, hiccup, impotency, insanity, nausea, sore throat, toothaches, and whooping cough. Basil has been reported in herbal publications as an insect repellent. Recent scientific research has investigated the health benefits associated with basil's essential oils. Studies reveal the anti-viral, anti-microbial, antioxidant, and anti-cancer properties of the oils; further research is underway [242, 243]. Leaves and flowering parts of *O.basilicum* are traditionally used as hypolipidemic, antispasmodic, aromatic, carminative, digestive, galactogogue, stomachic, and tonic agents [244]. They have also been used as a folk remedy to treat various ailments such as; feverish

illnesses, poor digestion, nausea, abdominal cramps, gastro-enteritis, migraine, insomnia, depression, gonorrhea, dysentery, and chronic diarrhea exhaustion. Externally, they have been applied for the treatment of acne, loss of smell, insect stings, snake bites, and skin infections [245]. Fathiazadeal *et al.*, in 2012 [246] argued that cardio protective effects of *O.basilicum* are correlated with its antioxidant compounds. Antioxidants are compounds which restrain oxidative damage through variable mechanisms such as reacting with free radicals, chelating catalytic metals, and acting as oxygen scavengers. There is an increasing interest recently to elucidate the association of different antioxidants with stress-related conditions such as inflammatory disease [247].

Nutrients	Content (Dry weight basis)	References
Protein	15.58 g/100g	[239]
Carbohydrate	66.65 g/100g	[239]
Dietary fibre	10 g/100g	[239]
calcium	321.4 mg/100g	[239]
Phosphorus	774 mg/100g	[239]
Magnesium	266 - 301.4 mg/100g	[239, 240]
Sodium	290 - 314.8 mg/100g	[239, 240]
Potassium	523.8 - mg/100g	[239]
Copper	0.67 mg/100g	[239]
Iron	1.48 mg/100g	[239]
Zinc	55.7 mg/100g	[239]
Vitamin C	354 mg/100g	[239]
Vitamin A	0.29 mg/100g	[239]
Phenolic content	107 mg gallic acid equivalent /100g	[241]
Oxalate	0.41 mg/100g	[239]

Table 2.6: Nutrition chart for *Ocimum basilicum*

2.5 3T3-L1 Cell line model

The two models that have been frequently used to study adipose tissue are adipocytes differentiated either from immortalised pre-adipocyte cell lines in culture or obtained from stromal-vascular cells in adipose tissue [67]. The establishment of these *In vitro* cellular models for adipogenesis has greatly advanced the understanding of the molecular basis of differentiation. The most extensively characterised pre-adipocyte lines are the 3T3-L1 and 3T3-F442A lines, isolated from non-clonal murine Swiss 3T3 cells by Green, Meuth and Kehinde [172, 173]. These cells are morphologically indistinguishable from fibroblasts but are already committed to the adipocyte lineage. When treated with pro-differentiative agents (cyclic adenosine monophosphate (cAMP), insulin and glucocorticoids) they undergo differentiation over a 4-6 day period to form mature fat cells. This is thought to represent a valid model of pre-adipocyte differentiation *in vivo*, as indicated by transplantation studies. Evidence shows that the fully differentiated adipocytes mimic the metabolism of adipocytes isolated from adipose tissue.

3T3-L1 cell line has been used widely in more than 5000 published articles on adipogenesis and the biochemistry of adipocytes for the last 30 years, because of its potential to differentiate from fibroblast to complete adipocytes [175]. Several investigations use 3T3-L1 cells because it helps in identifying key molecular markers including transcription factors and various pathways during pre-adipocyte differentiation [174]. Numerous protocols can be used to induce differentiation from pre-adipocytes to adipocytes, but the most commonly used agents are insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) [176] at concentrations of 1 µg/mL, 0.25 µM, and 0.5 µM, respectively. Pre-adipocytes contain fewer amounts of lipid droplets accumulated, but four days after induction they start to accumulate lipids that grow in size and number over the differentiation time [175].

There are, however, several potential drawbacks to the exclusive use of established pre-adipocyte cell lines in studies of adipogenesis, including their aneuploid status, which may influence their competence to undergo differentiation, and the fact that they exist out of the context of their normal extracellular matrix and supporting structures. Another important limitation is that they do not allow the assessment of depot-specific differences in fat cell behaviour. *In vivo*, white adipose tissue is located in a variety of locations, including omental, retroperitoneal, and subcutaneous depots. Pre-adipocytes isolated from different areas have different adipogenic potential. Additionally, the metabolic behaviour of mature fat cells differs from depot to depot.

Other cells lines commonly used are 10T1/2 mesenchymal stem cells, and various human vascular stem cells isolated from individual patients.

Hasegawa (2001) [177] investigated the anti-obesity effects of garcinia extract isolated from the fruit of the *Garcinia cambogia* on 3T3-L1 cells. Garcinia extract could inhibit accumulation of lipid droplets and reduce the peak droplet area of 3T3-L1 cells cultured with insulin.

Hsu et al (2003) [178] obtained extract of *Toona sinensis* leaves with 50% alcohol solution, and then studied its lipolysis effects in 3T3-L1 differentiated adipocytes. Its lipolytic activity induced by *Toona sinensis* leaf extract was diminished, which may be involved in the protein kinase C pathway and may be down-regulated by cyclic adenosine monophosphate (cAMP).

Ahn et al (2006) [179] indicated that Kochujang, Korean fermented red pepper paste, is a mixture of fermented soybeans, wheat, and red pepper powder, decreased lipid accumulation in 3T3-L1 adipocytes by inhibiting adipogenesis through down-regulation of sterol regulatory element-binding protein 1-c and peroxisome proliferator-activated receptor γ (PPAR γ) and by stimulation of lipolysis due to the increase of hormone-sensitive lipase activity.

Phenolic acids (chlorogenic acid, gallic acid, o-coumaric acid and m-coumaric acid) decreased the cell population growth of 3T3-L1 pre-adipocytes, and the half maximal inhibitory concentration of a substance (IC50) values were respectively 72.3, 43.3, 48.2, and 49.2 mM [152]. Chlorogenic acid, o-coumaric acid, and m-coumaric acid caused cell cycle arrest, while gallic acid did not. But it could increase the number of apoptotic cells in both time- and dose-dependent manners.

According to Jeon and Kim (2006) [180], pine needle extract treatment suppressed both glycerol-3-phosphate dehydrogenase activity and expression of PPAR γ in cultured 3T3-L1 adipocytes. Further animal experiments showed that pine needle extract significantly decreased body weight gain and visceral fat mass in rats fed with high fat diet. It could also reduce plasma parameters, like total cholesterol.

Murosaki et al (2007) [181] reported that a combination of caffeine, arginine, soy isoflavones and L-carnitine could significantly decrease 3T3-L1 differentiation and lipid accumulation, while it also could significantly increase lipolysis in 3T3-L1 adipocytes.

Zizyphus jujuba was investigated about the effects on adipocyte differentiation of 3T3-L1 preadipocytes by Kubota et al. (2009) [182]. The results showed that chloroform fraction

of *Zizyphus jujube* extract had the highest inhibitory effect on lipid accumulation and glycerol-3-phosphate dehydrogenase activity.

Hsu and Yen (2006) [152] reported that naringenin, rutin, hesperidin, resveratrol, naringin and quercetin could inhibit 3T3-L1 pre-adipocytes, and quercetin had the highest inhibitory effects (71.5%). Apoptosis assays and western analyses indicated that quercetin efficiently inhibited cell population growth and apoptosis induction in 3T3-L1 pre-adipocytes. Oleanolic acid, a triterpenoid compound, is one of the major components in vegetables and plants. It was reported that oleanolic acid could reduce the induction of PPAR γ and CCAAT-enhancer-binding proteins α (C/EBP α) [183]. The study mentioned by Furuyashiki et al (2004) [186] showed that 10-30 μ M EGCG decreased lipid accumulation by 25-50 % in differentiating 3T3-L1 adipocytes.

Kaempferol and quercetin, flavonoids extracted from *Euonymus alatus*, significantly improved insulin mediated glucose uptake when added to differentiated 3T3-L1 adipocytes at 5-50 μ M for 3 days (Fang et al. 2008) [158]. The addition of (-)-catechin to differentiated 3T3-L1 adipocytes at 50 μ M for 24 hours also increased insulin-mediated glucose uptake (Cho et al. 2007) [187].

According to Bazuine et al. (2005) [188] genestein, an isoflavone derivative, showed significant inhibition of insulin-mediated glucose uptake when incubated at 50 μ M, 15 min prior to insulin treatment in 3T3-L1 adipocytes. Berberine, an isoquinoline alkaloid, isolated from Chinese medicinal herbs such as *Coptidis rhizoma* and *Cortex phellodendri*, enhanced basal glucose uptake in normal and insulin-resistant 3T3-L1 adipocytes when added at 5 μ M for 6 hours (Kim et al. 2007) [189].

The anti-obesity effect of extract of purple sweet potatoes on 3T3-L1 adipocytes was investigated by Ju et al (2011) [184]. Differentiated 3T3-L1 adipocytes were treated with a purple sweet potato extract at concentrations of 1, 2, and 3 mg/mL for 24 hours. The results showed that the extract could diminish leptin secretion, suppressed the expression of messenger ribonucleic acid (mRNA) of lipogenic and inflammatory factors and promoted lipolytic action.

Sung et al. (2011) [185] demonstrated that water extract of *Lethariella cladonioides* could cause a significant increase in glycerol release and reduce the protein expression of the adipogenic transcription factors, PPAR γ and CCATT/enhancer-binding protein α (C/EBP α).

Recently in 2015 E. Mutai et al. [190] studied about the Antioxidant, Enzyme Inhibitory and Anti-Obesity Potential of Sorrel Calyx Extracts in 3T3-L1 Adipocytes and they

said that *Hibiscus sabdariffa L.* (sorrel) contains bioactive compounds that are important in modulating obesity through anti-oxidant related mechanisms and inhibition of adipogenesis in 3T3-L1 adipocyte cells.

The anti-lipogenic effects of *Taraxacum officinal* (dandelion) extracts was established by Belén García-Carrasco et al in (2015) [191] on adipocytes as well as radical scavenging and reducing activity. Importantly, along with previous results indicating that cell populations cultivated in the presence of the dandelion extracts decrease in 3T3-L1 adipogenesis capacity, these results suggests that these extracts might represent a treatment option for obesity-related diseases by affecting different processes during the adipocyte life cycle.

In 2015, Young et al. [192] investigated the anti-adipogenetic activity of 300 plant extracts using Oil Red O staining assay in a 3T3-L1 cell line. Their results indicate that three plants, including the stem and leaf of *Physalis angulata*, the whole grass of *Solidago virgaurea*, and the root of *Dioscorea nipponica*, produced over 90% inhibition of adipogenesis. Kaempferol-3-O-rutinoside, which demonstrated a 48.2% inhibitory effect on adipogenesis without cytotoxicity, was isolated from the butanol layer of a water extract of *S. virgaurea* guided by the anti-adipogenesis assay in 3T3-L1. PPAR-and C/EBP expression levels were determined using western blot, and they concluded that kaempferol-3-O-rutinoside has a strong anti-adipogenic effect in 3T3-L1 cells through the suppression of increases in PPAR-and C/EBP expression.

Meilinah et al., (2015) [193] evaluated the cytotoxic effects, anti-obesity and anti adipogenesis potential of ethanol extract of *Detam1* soybean seed (EEDS), *Jati Belanda* leaves (EEJB) and their combinations on 3T3-L1 cells, and they concluded that Ethanol extract of Detam1 soybean seed and *Jati Belanda* leaves possess the inhibitory potential on G6PD, triglyceride and cholesterol activities in 3T3-L1 cell line and the most active compound showed by ethanol extract of *Jati Belanda* leaves.

Ju-Hyun et al., in 2011 [194] also showed that *Lethariella cladonioides* (LC) suppressed lipid accumulation and increased the amount of glycerol release into the medium. It also suppressed adipogenic gene expression in 3T3-L1 adipocytes and decreased body weight and modulated the lipid metabolism in obese mice. Although the exact mechanisms by which these effects take place remain to be elucidated, this study's results provide a basis for proposing that LC may have future application in the treatment of obesity.

Through reviewing these previous articles about in vitro 3T3-L1 cell assays, it was found that this can be an ideal method to investigate and study the mechanisms of plant extracts containing anti-obesity dietary components.

2.6 Identification of Polyphenols and other secondary metabolites (UPLC-HRMS/MS)

The analysis of polyphenols in food samples is relatively complex not only due to the great variety of compounds that can be present, which differ in polarity and size (from simple phenolic acids to tannins), but also because many of these compounds in food products are found at very low concentration levels. Liquid chromatography coupled with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) is the most effective technique for the structural characterization and determination of both low and high molecular weight polyphenols in food samples [195, 196, 197].

The determination of polyphenols in complex matrices by LC requires high resolution and long analysis times. In the last years, ultra-high performance LC (UHPLC), either using sub- $2\mu\text{m}$ particle packed columns [198, 199] or porous-shell columns (with sub- $3\mu\text{m}$ superficially porous particles) [200, 201], has opened up new possibilities for improving the analytical methods for complex sample matrices, being able to achieve 5- to 10-fold faster separations than with conventional LC, while maintaining or even increasing resolution. Today, UHPLC coupled to MS (UHPLC-MS (/MS) is one of the most widely employed techniques in food analysis and the number of works focusing on the determination of polyphenols is increasing [197].

It is noteworthy that beyond the qualitative and quantitative studies of bioactive compounds, an emerging trend relies on the analysis of compositional profiles and fingerprints as a source of information to be exploited for classification and authentication purposes [202, 203]. The number of applications involving a chemometric data analysis has increased dramatically in the last years. Both LC-MS and LC-HRMS provide data of exceptional quality to be further analysed by chemometric methods such as principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA). Data to be analysed comprise concentrations of polyphenols of interest (profiling approach) or instrumental signals consisting of intensity counts as a function of m/z and retention time (fingerprinting approach).

2.6.1 Sample treatment procedures

Liquid chromatography (LC) is by far the best analytical technique for qualitative and quantitative analysis of phenolic compounds. Within this context, several sample preparation methods have been developed in recent years to improve the extraction of polyphenols from food samples. The extraction approach obviously depends on the nature of the sample matrix

as well as on the chemical properties of the phenolics, including molecular structure, polarity, concentration, number of aromatic rings and hydroxyl groups [204]. Different extraction solvents such as methanol, ethanol, acetone, water, ethyl acetate, diethyl ether and their combinations have been mentioned in the literature [205], with liquid–liquid extraction (LLE) and solid-phase extraction (SPE) being probably the most used techniques for the fractionation/purification step. The selection of appropriate solvents can improve limits of detection (LOD) and reduce matrix effects in LC-MS analysis. The most effective extractants typically are mixed aqueous-organic solvent systems employing methanol, ethanol, or acetone [206], since phenolic compounds are generally more soluble in polar organic solvents than in pure water.

2.6.2 Mass spectrometry (MS)

Mass spectroscopy (MS) is an analytical technique for the determination of the elemental composition of a molecule and for elucidating the chemical structures of molecules. It is a very sensitive technique and even from micro gram amounts good spectra can be obtained. A mass spectrometer is an analytical instrument used for determining the molecular weight of a compound. Technically, mass spectrometers are divided into three parts: ionization source, analyser, and detector, which should be maintained under high vacuum conditions so as to maintain the ions travel through the instrument without any hindrance from air molecules. The sample is ionized in the ionization source and the rising ions are sorted and separated according to their mass (m) to charge (z) ratio (m/z) in the mass analyser. Both negative and positive charged ions can be observed. Once the separated ions flow into the detector, the signals are transmitted to the data system where the mass spectrum is recorded. The molecular ion (parent ion) has to be identified giving the molecular weight of the compound. From the fragmentation patterns of the compound information about substructures can be attained. Therefore, mass spectrometry is used to determine the molecular weights of pure compounds or compounds in a mixture.

2.6.2.1 Electrospray ionization mass spectrometry (ESI-MS)

In ESI method, a solution of a substance is sprayed through a capillary into a chamber. Charged droplets are produced by an applied potential of a few kV, and in the following are driven by the electric field to move into the pre-analyser region. ESI-MS is a powerful analytical method, because it allows one to analyse the molecular ions of polar and higher molecular compounds in aqueous solution.

2.6.3 Liquid chromatography-mass spectrometry (LC/MS)

HPLC is a useful and powerful method for the separation of complex mixtures, especially when many of the components may have similar polarities. If a mass spectrum of each component can be recorded as it elutes from the LC column, quick characterization of the components is greatly facilitated.

Liquid chromatography-mass spectrometry (LC-MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) are among the most widely used techniques for both quantification and structural characterization of low molecular weight polyphenols but also some oligomer (dimers, trimmers,...) tannins [207].

Peak efficiency and chromatographic resolution provided in UHPLC are higher than in conventional HPLC and, consequently, the coupling of UHPLC with mass spectrometry is typically less affected by possible matrix effects. Another advantage is that UHPLC methods can be considered more cost effective because they typically consume around 80% less organic solvents than conventional HPLC methods. For these reasons, UHPLC-MS (/MS) methods are becoming more popular in the analysis of polyphenols in food [208-211], although many conventional HPLC-MS methods can still be found in the literature.

Reversed-phase mode mainly using C18 stationary phases is the most widely employed chromatographic separation mode for the analysis of phenolic compounds in food samples, although examples using other stationary phases such as C8 [212, 213] or even high strength silica (HSS) T3 [214, 215] can also be found in the literature. Generally, for the separation of polyphenols by reversed-phase chromatography acidified water (with small amounts of formic acid or acetic acid) and methanol or acetonitrile as organic solvents (in some cases also acidified with formic acid or acetic acid) are employed as mobile phases. Formic acid or acetic acid concentration is usually kept as low as possible in order to ensure a satisfactory reversed-phase separation without compromising ionization when acquiring in negative ionization mode, and typically is kept between 0.05-0.5 percent.

Regarding the ionization of polyphenols in LC-MS, electrospray in negative mode is, by far, the most generalized ionization source employed, usually providing the deprotonated molecule $[M-H]^-$, although ESI in positive ionization mode has also been proposed in some specific applications [210, 216]. In the publication by Kim et al. [216], LCMS/MS was used with positive ESI mode in a QTrap MS analyser working in SRM acquisition mode, which yielded the protonated molecule $[M+H]^+$, for the profiling of flavonoids in several citrus varieties native to the Republic of Korea. Electrospray ionization in the positive mode has

also recently been used by Kaliora et al. [217] for the characterization of the phenolic profiles of Greek herbal infusions.

Although less common in the analysis of polyphenols, other atmospheric pressure ionization sources such as atmospheric pressure chemical ionization (APCI) [218, 219, 220] or even atmospheric pressure photoionization (APPI) [221, 222] have also been described. In a recent application, Parets et al. [223] compared the use of UHPLC-ESI-MS/MS and UHPLC-APPI-MS/MS polyphenolic profiles for the characterization and classification of cranberry-based and grape-based natural products and cranberry-based pharmaceutical preparations. APPI(-) using acetone as dopant reagent showed to be more sensitive than ESI(-) for several targeted polyphenols (i.e. gallic acid, vanillic acid, syringic acid, p-coumaric acid, ferulic acid, gylringaldehyde, umbelliferon, and quercetin).

LC-MS/MS or UHPLC-MS/MS methods using triple quadrupole (QqQ) mass analysers are often proposed for the determination of polyphenols in food and plant products because of their high sensitivity in MRM acquisition mode [207]. The use of single quadrupole mass analysers has also been described in the determination of polyphenols, for instance, in wine samples [224] or plant extracts (*Cassicum annum L.* extracts) [211]. Nevertheless, although a general overview of the compounds present in the sample can be obtained with quadrupole MS analysers when full scan MS acquisition is performed, these instruments lack in sensitivity in comparison to QqQ analysers.

Ion-trap mass analysers are typically employed when structural information is required to achieve elucidation of target analytes, because typically full scan MS and product ion scan MS acquisition modes are employed, being able to obtain MS^n spectra which are helpful to establish fragmentation patterns and then to elucidate the structure of a given analyte [207]. For example, Du et al. [225] proposed the use of HPLC-ESI-MS/MS with an ion-trap analyzer for the elucidation of bioactive compounds of five wild *Chaenomeles* fruits.

Recently, the use of QTrap mass analysers, hybrid instruments combining a quadrupole and a liner ion-trap in a similar configuration than a QqQ instrument, is gaining popularity for the analysis of food products. Several applications can be found in the literature dealing with the determination of Polyphenols [207].

2.6.4 High resolution mass spectrometry (HR-MS)

High resolution is achieved by passing the ion beam through an electrostatic analyser before it enters the magnetic sector. In such a double focusing mass spectrometer, ion masses can be measured with an accuracy of about 1 ppm. With measurement of this accuracy, the

exact mass, elemental composition and detailed molecular structure of a given compound can be determined.

Among the multiple advantages of HRMS over classical unit-mass resolution tandem mass spectrometry we can find:

- (i) Differentiation of isobaric compounds (different compounds with the same nominal mass but different elemental composition);
- (ii) Simplification of sample-preparation procedures, thereby leading to faster methodologies requiring less sample manipulation;
- (iii) Information gathered by a single injection that can be used for quantification and screening purposes, including targeted, suspect and non-targeted analysis;
- (iv) Collection of full-scan spectra that can be stored and used in a later stage retrospective analysis, thus permitting the formulation of a posteriori hypotheses involving structural elucidation of unknown or suspected compounds [226, 227].

In the last years, scientists are taking advantage of LC-HRMS methods either employing time-of-flight (TOF) or Orbitrap analysers for the characterization, determination and identification of Polyphenols in foods [194].

TOF and Orbitrap are the most-commonly used analysers for both LC and UHPLC analysis of phenolic compounds in food matrices. In fact, classic HRMS instrumentation (sector or FT-ICR) are too slow, too complex to handle, and probably too expensive to buy and to maintain [228]. On the contrary, recent advances in both TOF and Orbitrap mass analyzers have reduced power requirements, size and instrument costs (especially when compared to FT-ICR) while maintaining high resolving powers of approximately 10.000-40.000 FWHM (full width at half maximum) and 10.000-140.000 FWHM for TOF and Orbitrap, respectively [197].

Finally, after LC separation, detection is mainly performed by negative electrospray ionization [ESI or heated ESI (HESI)], being an excellent tool for identifying phenolic compounds. In fact, although chromatographic separation requires acidic conditions, the response of polyphenols (with the exception for anthocyanins and iso-flavonoids) has been proven to be better in the negative ion mode than in the positive one [197,229].

UPLC-HRMS results in an excellent technique for the tentative identification of unknown components from the interpretation of data such as the exact mass, and MS and MS/MS spectra. Although this kind of studies may be sufficient in some cases, it should be

noted that the final confirmation of the identity of the compounds will require additional assays using standards of the candidates. The full concordance of chromatographic and spectral data may be used as the criterion of positive identification. Finally, regardless of the type of MS analyser, the use of high resolution and accurate mass will surely become routine in food polyphenol analysis as instrument resolving power, accuracy, and sensitivity continue to improve [197].

Chapter 3

Aims and Objectives

Aims and Objectives

Present study aims to investigate and evaluate the anti-adipogenic properties expressed by *Moringa oleifera*, *Brassica oleracea* and *Ocimum basilicum* extracts on 3T3-L1 (pre-) adipocytes and to develop functional food products incorporated with these extracts to combat obesity and related issues.

Research Objectives:

The objectives of the present work are-

- ⊕ Extraction of bioactive compounds from *Moringa oleifera*, *Brassica oleracea* and *Ocimum basilicum* using hydro-alcohol.
- ⊕ Determination of total phenol and flavonoid content and estimate the anti-oxidant activity exhibited by these plant extracts.
- ⊕ Identification of probable bioactive compounds (phenolic acids and flavonoids) present in these extracts using UPLC-HRMS/MS.
- ⊕ Determination of cytotoxicity effects for these extracts on 3T3-L1 cell line model.
- ⊕ Evaluation of plant extracts for their anti-adipogenic properties on 3T3-L1 cell line model.
- ⊕ Formulation of functional food products to tackle obesity incorporated with plant extracts with anti-adipogenic properties. – Anti-obesity soup mix powder.

Chapter 4

Materials and Methods

Material and Methods

All the chemicals and reagents mentioned in the experimental analysis and assays were procured from Sigma-Aldrich, USA unless specified.

4.1 Preparation of Plant extracts

4.1.1 Chemicals and Reagents

Ethanol (absolute); Hydrochloric acid; Milli Q water.

4.1.2 Instruments and Apparatus

Electric hot air oven (Quest International); Grinder (Philips); Hand held pH meter (Hanna Instruments); Orbital Shaker (Orbitek – Scigenics Biotech); Rotary flash evaporator (Heidolph 2); Freeze Drier (Lyolab – LSI Pvt. Ltd.); Deep Freezer (Vestfrost Solutions); Precision balance (Sortorius, Presica 205 ASCS); Whatman filter no. 1.

4.1.3 Method

M. oleifera, *B. Oleracea* and *O. Basilicum* leaves were purchased from Sabala Agro Product Pvt. Ltd., Bangalore in February 2017. As specified by the company, plants were harvested at their peak and were stored hygienically. These leaves were dehydrated in temperature controlled electric oven (46° C for 52 hours) and then grinded to form a nutrient rich fine powder. The same dehydrated powder has been used in the present study.

20 g of dried plant powder was taken in a conical flask and volume was made up to 200 ml with 80% ethanol (1:10 ratio). The pH of the solution was adjusted to 2 with the help of a hand held pH meter using 2 M HCl solution. The mouth of the conical flasks were covered with cotton plug and aluminium foil. These conical flasks were than kept in an orbital shaker for 12 hours at 37° C . Obtained extracts were filtered first through normal filter paper and then through Whatman No.1 filter paper to get a clear solution. Rotary flash evaporator (heidolph) at 50° C was used to remove the ethanol from the liquid extracts. The concentrated samples were then transferred into 3 steel bowls for lyophilisation. The samples were kept in the freeze drier for overnight and the powdered extracts were obtained the next day. These extracts were weighed for calculation of yield and were then dissolved in Milli Q water at a conc. of 100 mg/ml (Stock). These stock samples were then stored at -20° C in a deep freezer, until further use for working preparation. A simplified overall procedure for extraction is explained in Figure 4.1.



Figure 4.1: Procedure for Phytochemical extraction from plant materials

4.2 Phytochemical Investigation

Phyto come from the Greek word "phuton" meaning plant hence the chemical/nutrient found in plant are called phytochemical or phytonutrient. It refers to the natural chemical compounds found in plants that make up its colours.

4.2.1 Chemicals and Reagents

Folin-ciocalteu's Reagent (FCR); Quercetin; Ferulic acid; DPPH(2,2-diphenyl-2-picryl hydrazyl); Sodium carbonate; Aluminium chloride; Sodium acetate; Methanol; Distilled water.

4.2.2 Instruments and Apparatus

UV-Vis Spectrophotometer (Spectro Star Nano – BMG Labtech); Precision weight (Sortorius, Presica 205 ASCS); Pipettes (5ml, 1ml, 200µl, 10µl) (Eppendorf, Tarsons, Thermo fisher); 6,12 and 24 well plates (Corning Costar TC).

4.2.3 Determination of total phenolic content

The phenolic compound in food that is originated from one of the main classes of secondary metabolites in plants derived from phenylalanine and to lesser extent in some plant, also from tyrosine. Chemically phenolic can be defined as substance possessing an aromatic ring bearing one or more hydroxyl group, including their functional derivatives. The total phenolic content of plant extracts was determined with Folin-Ciocalteu method (Van Sumere C.F, 1989) [249].

Principle:

Folin-Ciocalteu reagent is sensitive to reduce the compound Phenolic hydroxyl group reduces the phenolic reagent to form chromogens that can be detected using spectrophotometer by monitoring the absorbance at 756nm .The method is an oxidation-reduction reaction in which the phenolate ion is oxidized while the phosphotungstic-phosphomolybdic compound are reduced to a blue compound in alkaline solution. The non-specificity of the Folin-Ciocalteu reagent makes it a suitable reagent for total phenol analysis [251].

Protocol:

In this method, Ferulic acid (FA) was used to make the standard curve and values were evaluated as the mg equivalent of FA per gram of extract (mg FAE/g). To obtain the standard curve, various concentration of FA solution [5, 10, 20, 30, 40, 50 µg/ml] were prepared and the volume was made up to 250 µl by distilled water. 1. 25 ml of FCR (diluted 1:10, v/v), and 1ml of Na₂CO₃ (7.5%, w/v) were added to the tubes. The tubes were left for incubation for 90 minutes in dark at room temperature. The activity assay was performed in triplicates for each treatment. Similarly for the plant extract (10 mg/ml), different volumes (50 and 100 µl) were taken. Absorbance measurements were carried out at 756nm on a UV-visible spectrophotometer against the reagent blank (without Ferulic acid). Standard calibration curve for weight of FA against the absorbance was plotted.

With the help of standard calibration curve's linear regression equation, the total phenolic content of the extracts were determined.

4.2.4 Determination of total Flavonoids content

Flavonoid constitutes a relatively diverse family of aromatic molecules that are derived from phenylalanine and malonyl-coenzyme A via the fatty acid pathway. They are one of the major classes of phenolic, derived from a combination of the shikimic acid and malonic acid pathway [250]. Flavonoids perform major role in plant such as, protect against ultraviolet radiation, defence against pathogens and pests, pollen fertility, signalling with microorganism, auxin transport regulation and pigmentation [272]. The total phenolic content of plant extracts was determined with aluminium chloride colorimetric method (Akrout A et al., 2011) [252].

Principle:

Flavonoids react with aluminium chloride form an acid stable complex with the keto group and either the hydroxyl group in ring A or ring C of flavonoid. It also form acid unstable complexes with orthodihydroxyl group in ring A or ring B. The complex absorbs light strongly at 415nm and can be detected on UV-VIS spectrophotometer.

Protocol:

In this method, Quercetin was used to make the standard curve and values were evaluated as the mg equivalent of quercetin per gram of extract (mg QE/g). 1mg of quercetin was dissolved in 1ml methanol. To obtain the standard curve, various concentrations of quercetin were taken (25, 50, 75, 100, 150, 200 µg/ml) and the volume was made up to 1000 µl with methanol. 0.5ml of 1.2% aluminium chloride and 0.5ml of 1.2% sodium acetate was added into all test tubes. Similarly for plant extracts (10mg/ml), different volumes (50 and 100µl) were taken. The activity assay was performed in triplicate for each treatment. Measurement was carried out at 415nm on a UV-VIS Spectrophotometer against reagent blank (without quercetin).

With the help of standard calibration curve's linear regression equation (wt. of standard *vs.* absorbance), the total phenolic content of the extracts were determined.

4.2.3 Determination of Anti-oxidant Activity

Antioxidants protect cells against the damaging effects of reactive oxygen species otherwise called, free radicals such as singlet oxygen, super oxide, peroxy radicals, hydroxyl radicals and peroxy nitrite which results in oxidative stress leading to cellular damage [253]. Natural antioxidants play a key role in health maintenance and prevention of the chronic and degenerative diseases, such as atherosclerosis, cardiac and cerebral ischemia, carcinogenesis, neurodegenerative disorders, diabetic pregnancy, rheumatic disorder, DNA damage and ageing [254]. Antioxidants exert their activity by scavenging the 'free-oxygen radicals' thereby giving rise to a fairly 'stable radical'.

Free radical scavenging activity of plant extracts was determined by in vitro assay model - DPPH free radical scavenging activity.

Principle:

DPPH (2, 2-diphenyl-1-picryl hydrazyl) radical is scavenged by anti-oxidant through the donation of proton forming the reduced DPPH. The colour changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength of

517nm. Free radical scavenging activity increased with increasing percentage of the radical inhibition. The degree of discoloration indicates the free radical scavenging potential of the sample/antioxidant by their hydrogen donating ability. The electrons become paired off and solution losses colour stoichiometrically depending on the number of electron taken up [255].

Protocol:

Different volumes (25, 50, 75, 100, 125 and 150 µl) of the diluted extracts (10mg/ml) were made up to 200µl using methanol and then 3mL of a 0.004% methanol solution of DPPH was added to each test tube. After a 30min incubation period at room temperature, the absorbance was read against a blank at 517nm. The percentage of inhibition of DPPH radical was calculated by comparing the result of the control (not treated with the extract) using following equation. The synthetic antioxidant reagents quercetin was used as standard and all tests were carried out in triplicate. The *IC50 of the samples were calculated using linear regression equation derived from the graph of percentage% scavenging activity vs. weight of the sample [256].

$$\text{Percentage of inhibition (\%)} = \frac{(\text{Absorbance for positive control} - \text{Absorbance for sample})}{\text{Absorbance of control}}$$

*IC50 = Concentration of sample to provide 50% inhibitory activity.

4.3 Identification of bioactive compounds (UPLC-HRMS/MS)

4.3.1 Chemicals and Reagents

MS grade Methanol, Acetonitrile, Acetic acid, and H₂O.

4.3.2 Instruments and Apparatus

UPLC-ESI-HRMS/MS (UPLC system: eKspert™ UltraLC110; HRMS system: SCIEX Triple TOF MS 5600) (CIL, CFTRI-Mysore); Precision balance (Sortorius, Presica 205 ASCS); 0.22 µm PES syringe filter (Millex-GP, Merck Millipore); Pipettes (5ml, 1ml, 200µl, 10µl) (Eppendorf, Tarsons, Thermo fisher).

4.3.3 Sample preparation

Lyophilised plant extracts were dissolved in MS grade methanol at a concentration of 10mg/ml (Stock) and were than filtered through 0.22 µm PES syringe filter. 10 µl of these stock samples were dissolved in 990 µl of methanol to get a final concentration of 10µg/ml (10µl was injected in the instrument). These samples were then stored at -20 °C to avoid degradation until analysed.

4.3.4 UPLC Conditions

For the evaluation of metabolites in plant extracts, UPLC coupled to a Triple TOF HRMS mass spectrometer was used to generate accurate mass data (CFTRI, Mysore). The chromatographic separation of the extracts (10µl) was accomplished using a 25 minutes long gradient chromatographic method on a Phenomenex Kinetex C18 100A, reverse phase column (L 30 × ID 2.1 mm, 1.7µm). For chromatographic elution, solvent mixtures were used consisting of 0.1% acetic acid in deionized water (Eluent A) and 0.1% acetic acid in 80% acetonitrile and 20% methanol (Eluent B). The following gradient elution program at a flow rate of 0.7 ml/minute was applied: 0–15 min—99% A; 15–18 min—78% A; 18–19.1 min—5% A; 19.1–26 min—99% A. Chromatographic separation was monitored by the electrospray ionization mass spectrometer (ESI-MS) detector.

4.3.5 HR-MS Conditions

MS detection was performed in a 3200 QTRAP Mass spectrometer (AB Sciex, USA) equipped with an electro spray ionisation source (ESI) and a triple, quadrupole-ion trap mass analyser that was controlled by the Analyst 1.5 software. ESI worked in the negative-ion and positive -ion mode and the optimum values of the source parameters were: capillary temperature 550° C, curtain gas 40 psi, nebulizer gas 60 psi, source voltage - 5500 ISV, mass range – 100 to 2000 *m/z*. Nitrogen was used as curtain and collision gas. For each compound, the optimum conditions of Multiple Reaction Mode (MRM) were determined in the infusion mode. Triplicate injections were made for each standard solution and sample. The analytes were identified by comparing retention time and *m/z* values obtained by MS and MS² with the mass spectra from corresponding standards, tested under the same conditions. The limits of detection (LOD) and quantification (LOQ) for phenolic compounds were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations.

4.4 In-vitro studies (3T3-L1 cell line model)

The establishment of *In vitro* cellular models for adipogenesis has greatly advanced the understanding of the molecular basis of differentiation. 3T3-L1 pre-adipocyte cell line is the most extensively used cell line for adipogenesis related studies.

4.4.1 Chemicals and Reagents

3T3-L1 mouse fibroblasts (ATCC, American Type Culture Collection, Manassas, VA, U.S.A.) ; Dulbecco's modified Eagle's medium (DMEM) powder; Fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, USA); Antibiotic and antimycotic solution (Gibco, Thermo Fisher Scientific, USA); Dexamethasone; Insulin (bovine); Methylisobutylxanthine (IBMX); Sodium bicarbonate; Trypsin solution (Gibco, Thermo Fisher Scientific, USA); Curcumin; Dimethyl sulfoxide (DMSO); Oil red O stain; Hematoxylin dye; MTT dye (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); Isopropanol; Formaldehyde (40%) solution; Sodium carbonate; Sodium hydroxide; Copper sulphate; Sodium potassium tartrate; Bovine serum albumin (BSA); Folin-ciocalteu's Reagent (FCR).

4.4.2 Instruments and Apparatus

Bio-safety Cabinet (MARK Ultraclean solutions); Compound light microscope; Refrigerator (Godrej); -80° C Deep freezer (Ultralow temperature freezer U410 premium – New Brunswick); Refrigerated centrifuge (Sigma 3 – 30K); REMI centrifuge; Micro-pipettes (5ml, 1ml, 200µl, 10µl) (Eppendorf, Tarsons); 0.22 µm PES syringe filter (Millex-GP, Merck Millipore); CO₂ Incubator(NUAir); Precision balance (Sortorius, Presica 205 ASCS), Compound light microscope (Olympus CX30).

4.4.3 Cell line maintenance, Differentiation and Treatment

In this study, 3T3-L1 cell line was used to model the inhibitory effects of *M. oleifera*, *B. Oleracea* and *O. basilicum* against pre-adipocyte differentiation of adipocyte cells. Differentiation of 3T3-L1 pre-adipocytes was performed by following the ATCC protocol. Cells were seeded and maintained with Pre-adipocyte Expansion Medium (PEM) (DMEM supplemented with 10% FBS and 1% antibiotic solution) for 48 h by incubating at 37°C and 5% CO₂, or after reaching 100% confluence. Differentiation was induced two days after 100% confluence of cells using identical volume of Differentiation medium (DM) (DMEM supplemented with 10% FBS, 1% antibiotic solution, 0.5 mM 1-methyl 3-isobutylxanthine (MIX), 0.1 µM dexamethasone, and 10µg/ml insulin) and incubated for 48 h at 37°C in humidified atmosphere containing 5% CO₂. This was considered as day 0. Post incubation, the Differentiation Medium was replaced with Adipocyte Maintenance Medium (AMM)

(DMEM supplemented with 10% FBS, 1% antibiotic solution and 10 μ g/ml insulin); AMM was changed every 72h. Finally, the cells were fully differentiated at day 10 after induction. Pre-adipocyte cells could be fully differentiated between 8 to 12 days after DM application.

To examine the effect of plant extracts on adipocyte differentiation, 3T3-L1 cells were treated with different concentrations of the extracts (1, 2 and 3 mg/ml for *BO* and 0.45, 0.60 and 0.75 mg/ml for *OB* and *MO*). Curcumin was taken as standard drug against obesity (Bradley S. et al., 2016) [3]. 10 mg of curcumin was dissolved in 10ml of DMSO and was filtered through 0.22 μ m syringe filter in a biosafety cabinet before adding to the cells (20mM concentration). 1 day post the cells reached full confluence, extract and standard treatment was given for 24 hours (pre-treatment) and then the differentiation was induced. The treatment was continued with every change in media until day 10. Also, a flask of cells containing non-differentiated cells was maintained until day 10.

This method was followed for every experimental analysis and assays related to 3T3-L1 cell lines.

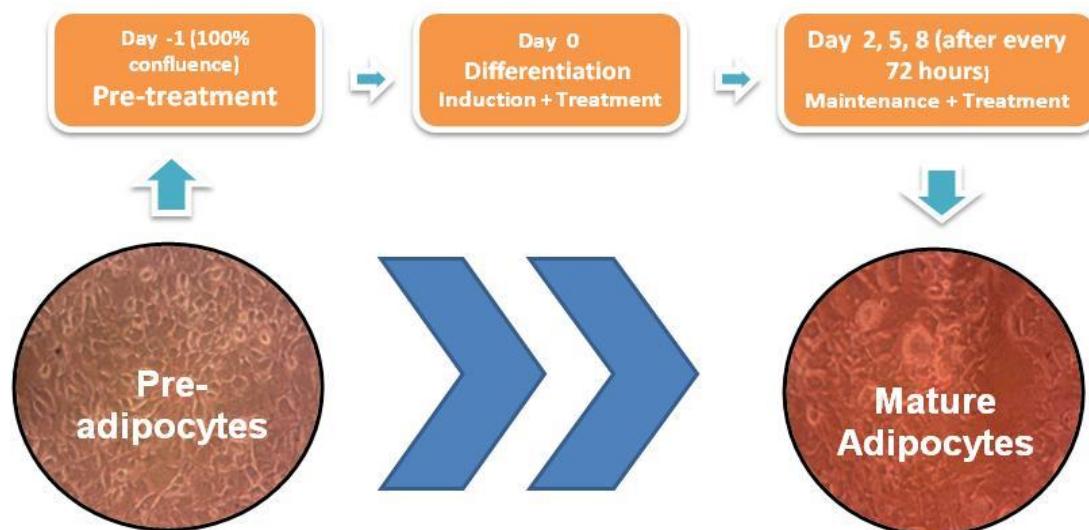


Figure 4.2: (Pre)-adipocyte differentiation timeline

4.4.4 MTT proliferation assay

Principle:

Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption max is dependent on the solvent employed. This reduction takes place only when mitochondrial reductase enzymes are active,

and therefore conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve. [257, 258, 259].

Protocol:

A relationship between 3T3-L1 cell viability and absorbance was established in the present study using MTT proliferation assay. Briefly, a 25 cm² flask (80% confluence) was trypsinized followed by the addition of 7 ml fresh PEM onto the cells. These cells were centrifuged (REMI centrifuge) in a sterile 15 ml centrifuge tube for 5 minutes and the supernatant was discarded. Cell pellet was re-suspended in 1ml fresh PEM and 25µl cells (~ 75 X 10²) from this suspension were seeded into each well of 96 well micro-plate. The final volume was made up to 100 µl using fresh PEM. These cells were then incubated at 37° C in 5% CO₂ for 24 hours.

After 24 hours, media was removed and fresh media with different concentrations of extracts and standard drug were added to the wells, such that the final volume for each well remained 100 µl. Cells were again incubated for 32 hours in 5% CO₂ at 37° C. 20 µl of MTT solution (5 mg/mL PBS) was added to each well after incubation including 1 set of wells with MTT solution but no cells (Blank). The micro-plate was incubated for 3.5 hours at 37° C in incubator. The supernatant (media) was carefully removed without disturbing the bottom of the wells. 150µL of dimethyl sulfoxide (DMSO) was added to each well. The plate was covered with tinfoil and was agitated for 15 minutes to dissolve the formazan crystals. The absorbance was measured at 590 nm by microplate reader (Spectro Star Nano – BMG Labtech) to determine the formazan formed by viable cells with a reference filter of 620 nm. [257, 258] The percentage of viable cells was calculated by defining the cell viability without treatment as 100%.

4.4.5 LDH release assay

Principle:

The lactate dehydrogenase assay is a means of measuring either the number of cells via total cytoplasmic lactate dehydrogenase (LDH) or membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. This method is simple, accurate and yields reproducible results. The assay is based on the reduction of NAD by LDH. The resulting reduced NAD (NADH) is utilized in the stoichiometric conversion of a tetrazolium

dye. The resulting colored compound is measured spectro-photometrically. If the cells are lysed prior to assaying the medium, an increase or decrease in cell numbers results in a concomitant change in the amount of substrate converted. This indicates the degree of inhibition of cell growth (cytotoxicity) caused by the test material. If cell-free aliquots of the medium from cultures given different treatments are assayed, then the amount of LDH activity can be used as an indicator of relative cell viability as well as a function of membrane integrity. [260, 261]

Protocol:

The assay was performed using an optimized Lactate dehydrogenase based *In Vitro* Toxicology Assay Kit (Cat no.TOX7 Sigma-Aldrich, St. Louis, USA) according to the manufacturer's instructions. Briefly, the 3T3-L1 pre-adipocytes were seeded in a 24-well plate and cultured 100% confluence in PEM. The cells were then treated with various concentrations of plant extracts and standard drug for 48 hours in 5% CO₂ at 37° C. As an indicator of cytotoxicity total cytoplasmic enzyme lactate dehydrogenase (LDH) and LDH release were measured for the treated cells. The absorbance was measured at 490 nm with a reference wavelength of 690 nm (background).The percentage of viable cells was calculated by defining the cell viability without treatment as 100%. [262]. Lactate dehydrogenase enzyme is released into the media when there is tissue damage. Increased levels of LDH demonstrated cell damage.

4.4.6 Oil- Red -O lipid staining

Principle:

Oil Red O is a lysochrome (fat-soluble dye) diazo dye used for staining of neutral triglycerides and lipids on frozen sections and some lipoproteins on paraffin sections. Oil Red O Staining is widely used and accepted method suitable for selective staining and detection of neutral lipids in cultured cells. The principle behind the staining is the greater solubility of the dye in the lipid substances than in the usual hydro-alcoholic dye solvents. Oil Red O staining was used for the determination of intracellular lipid accumulation in 3T3 cells.

Protocol:

Oil Red O staining was performed according to the procedure given by Green and Kehinde (1975) [173] with certain modifications. A stock solution of Oil Red O (60 mg in 20ml 100% isopropanol) was prepared and passed through a 0.2 mm filter. To prepare the working solution, 3 parts of stock solution was mixed with 2 parts of distilled water, left for 1 h at room temperature, and filtered through a 0.2 mm filter prior to use. 3T3-L1 cells were

inoculated in 6 well plates and were cultured in PEM supplemented with 1% antibiotics and 10% heat-inactivated fetal bovine serum to reach full confluence for 2 days in a humidified incubator (5% CO₂ at 37° C). Cells were differentiated treated with plant extracts and maintained for 10days. 1 pair of wells was left undifferentiated (negative control).

On day 10 medium was removed from the cells and cells were gently washed with PBS (two times). For fixing the cells, 10% formalin (2ml) was added to the wells and incubated for 30 minutes. Formalin was discarded post incubation and cells were washed twice using water. 60% isopropanol was added to the cells and incubated for 5 minutes. Isopropanol was discarded and cells were covered evenly with Oil Red O working solution (2ml per well). Plate was rotated for even distribution of dye and incubated for 20 minutes. After this the Oil Red O solution was discarded and the cells were washer with water 4 to 5 times until no excess stain is seen. Hematoxylin dye solution was added to the cells and incubated for 1 minute and was than discarded. Cells were again washed with water for 4 to 5 times. Cells were than covered with water and observed under microscope for stained lipid droplets. Lipid droplets appeared red while nucleus appeared blue in colour.

4.4.7 Intracellular Lipid accumulation assay (Oil Red O de-staining method)

Principle:

Intracellular lipid droplets stained with Oil Red O stains can be de-stained with 100% isopropanol and the amount of stain that was present in the cell monolayer can be estimated using spectrophotometer. The amount of Oil Red O stain present in a cell monolayer will be directly proportional its intracellular lipid content.

Protocol:

The method for the assay was a slight modification of a published protocol by Green and Kehinde (1975) [173]. The dye retained by the cells was eluted by incubation with 2ml of 100% isopropanol. The OD was determined at 510nm using a Spectro Star Nano plate reader. Blank wells (without cells) were stained with dye and rinsed in the same manner; these values were subtracted from the experimental (cell) data points to control for stain retention by the walls of the well.

4.4.8 Triglyceride inhibition assay

Principle:

Total triglyceride content present in a cell sample can be estimated using colorimetric, enzymatic method with glycerophosphate oxidase. Triglycerides are enzymatically hydrolyzed by lipase to free acids and glycerol. The glycerol is phosphorylated by adenosine triphosphate (ATP) with glycerol kinase (GK) to produce glycerol-3 phosphate and adenosine diphosphate (ADP). Glycerol-3-phosphate is oxidized to dihydroxy-acetone phosphate (ADP) by glycerol phosphate oxidase producing hydrogen peroxide (H_2O_2).

In a Trinder type colour reaction catalyzed by peroxidase, the H_2O_2 reacts with 4-aminoantipyrine (4AAP) and 4-chlorophenol to produce a red coloured dye (Quinoneimine dye). The absorbance of this dye is proportional to the triglycerides concentrations in the sample when measured at 505nm [263, 264].

Protocol:

3T3-L1 adipocytes were harvested for 10 days after the initiation of differentiation in 6 well plates. On the 10th day cells were washed with PBS and lysed in lysis buffer (1% Triton X-100 in PBS) [265]. Then these cells were scraped, collected in micro-centrifuge tubes and homogenised using a hand-held homogeniser in ice for 2 minutes. After centrifugation (14000 × g for 15 minutes), the supernatant was collected and total triglyceride content in cells was determined using a commercial triglyceride assay kit (Erba Mannheim-Liquixx Triglycerides GPO – PAP Method, End Point kit: Mannheim, Germany) according to company protocol. The results were expressed in TG content (μg) per μg of protein present in the sample. Protein content for the samples was estimated using Lowry's method.

4.4.9 Protein estimation assay (Lowry's method)

Principle:

The phenolic group of tyrosine and tryptophan residues (amino acid) in a protein will produce a blue purple color complex, with maximum absorption in the region of 660 nm wavelength, with Folin- Ciocalteau reagent which consists of sodium tungstate molybdate and phosphate. Thus the intensity of colour depends on the amount of these aromatic amino acids present and will thus vary for different proteins. Most proteins estimation techniques use Bovin Serum Albumin (BSA) universally as a standard protein, because of its low cost, high purity and ready availability. The method is sensitive down to about 10 $\mu g/ml$ and is probably the most widely used protein assay despite being only a relative method, subject to

interference from Tris buffer, EDTA, nonionic and cationic detergents, carbohydrate, lipids and some salts. The incubation time is very critical for a reproducible assay. The reaction is also dependent on pH and a working range of pH 9 to 10.5 is essential.

Protocol:

The protein content for the cell lysates was estimated according to Hartree EF. (1972) [266] with certain modifications. Analytical reagent for estimation was prepared by mixing 100ml of solution A (50 ml of 2% sodium carbonate + 50 ml of 0.1 N NaOH solution) with 2 ml of solution B (10 ml of 1.56% copper sulphate solution + 10 ml of 2.37% sodium potassium tartrate solution). Different concentrations of BSA solution were prepared (0.05 to 1 mg/ ml) and 0.2 ml from each was taken into test tubes. 2ml of analytical reagent was added to each test tube and incubated for 10 minutes (Gentle mixing may be required). Post incubation 0.2 ml of Folin-Ciocalteau reagent (1:1 with distilled water) was added to the test tubes and incubated for 30 minutes. OD was determined at 660nm using Spectro Star Nano plate reader including a blank without BSA. The absorbance value was plotted against protein concentration to get a standard calibration curve. Protein concentration for the unknown samples (cell lysates) was determined using this standard curve. With the help of standard calibration curve's linear regression equation, protein concentration for the unknown samples (cell lysates) was determined using their absorbance values.

4.5 Gene Expression studies

4.5.1 Chemicals and Reagents

Trizma base; Hydrochloric acid; Sodium chloride; Sodium dodecyl sulphate (SDS); RIPA buffer; Protease inhibitor; Phosphatase inhibitor; Bovine serum albumin; Glycerol; Bromophenol blue; β -mercapto ethanol; Acrylamide; bis-acrylamide; Tetramethylethylenediamine – 8.4% (TEMED); Hydrochloric acid; distilled water; Ammonium persulfate (APS); Glycine; Methanol; Tween 20; Luminol; p-Coumaric acid; Hydrogen peroxide; Sodium monobasic phosphate; Sodium dibasic phosphate; Ponceau S staining solution; X-ray film fixer and developer (Kodak); Primary antibody: Adipsin polyclonal goat (Sigma); Secondary antibody: anti-goat horseradish peroxidase (HRP) (Sigma).

4.5.2 Instruments and Apparatus

Bio-Rad Mini-Protean apparatus (Bio-Rad Laboratories, Inc., USA); Refrigerated centrifuge (Sigma 3 – 30K); -80° C Deep freezer (Ultralow temperature freezer U410 premium – New Brunswick); Precision balance (Sortorius, Presica 205 ASCS); Hand-held homogeniser (Sigma-Aldrich); Refrigerator (Godrej); Micro-pipettes (5ml, 1ml, 200 μ l, 10 μ l) (Eppendorf, Tarsons); Cell scraper; Micro-centrifuge tubes; PVDF membranes; X-ray films.

4.5.3 Reagent preparations

- Separating buffer: 9.08g Trizma base + 0.20g SDS + 60ml distilled (d) water; pH adjusted to 8.8 using HCl; Volume make up to 100ml with d. water.
- Stacking buffer: 3.03g Trizma base + 0.02g SDS + 60ml d. water; pH adjusted to 6.8 using HCl; volume make to 100ml with d. water.
- Acrylamide solution (30%): 29.2g acrylamide + 0.8g bis-acrylamide; Volume make up to 100ml with d. water.
- APS solution: 1.25g APS in 10ml d. water.
- Running buffer: 3.03g Trizma base + 14.4g Glycine + 1.0g SDS; volume make up to 1 litre with d. water.
- Transfer buffer: 3.03g Tzima base + 14.4g Glycine + 200ml Methanol (absolute); Volume make up to 1 litre with d. water.
- Separating Polyacrylamide gel (10%): 3.3ml of 30% acrylamide solution + 5ml of separating buffer + 1.6ml d. water + 0.1ml APS solution + 7.5 μ l TEMED.

- Stacking Polyacrylamide gel: 1ml of 30% acrylamide solution + 2ml of separating buffer + 2ml d. water + 0.1ml APS solution + 5 μ l TEMED.
- TBST buffer: 2.42g Trizma base + 9g Sodium chloride + 0.5ml Tween 20; Volume make up to 1 litre with d. water.
- Blocking solution: 100ml TBST buffer + 3g skim milk powder.
- Phosphate buffer Saline (PBS) pH 7.4: 19ml of 0.2M Sodium monobasic phosphate + 81ml of 0.2M Sodium dibasic phosphate + 1.8g Sodium chloride + 100ml d. water.

4.5.4 Protein Isolation

The medium from 75 cm² flasks was carefully discarded, and the adherent cells were washed with ice-cold PBS twice. Cold RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, and 1 mM protease and phosphatase inhibitors) (Sigma, USA) was added (0.8 ml) to the cells, and lysed on ice for 5 min. The plates were swirled occasionally for uniform spreading. The cell scraper was used to gather the lysate to one side, and the lysate was collected and transferred to a precooled micro-centrifuge tube. The cell lysates were then homogenized using a hand-held homogenizer (Sigma-Aldrich) for 2 minutes in ice. These homogenised lysates were centrifuged at 14000 \times g for 15 min at 4 °C to pellet the cell debris. The supernatant was transferred to new precooled micro-centrifuge tubes and stored at -80° C until further analysis.

Protein content was determined for cell lysates using Lowry's method (refer sec. 4.4.9).

4.5.5 Western Blotting

Cell lysates were thawed and 60 μ g of total protein mixed with 20 μ l 6X loading buffer (100 mM Tris-HCL pH 6.8, 20% Glycerol, 4.6% SDS, 0.004% bromophenol blue, 10% β -mercapto ethanol) [267] was boiled for five minutes and separated by SDS-PAGE using a 10% polyacrylamide gel in a Bio-Rad Mini-Protean apparatus (Bio-Rad Laboratories, Inc., USA). Current settings for electrophoretic separation were 20 mA till dye front reaches separating gel and then 25 mA (constant). After the run was completed gel was incubated for 20 minutes in the transfer buffer along with the PVDF membranes that are to be used for transfer (wetted with methanol). The proteins in the gel were transferred to PVDF membrane (PALL) by the method of wet transferring (Power settings: 100 V constant). For detecting whether the protein was transferred to the membrane, the membrane was incubated in Ponceau S staining solution with gentle agitation and then rinsed with distilled water for the

removal of background. After detection, the membrane was continued washing until the stain was completely removed from the protein bands.

After transfer, the PVDF membrane was washed with 15 ml TBST for 5 minutes at room temperature, and blocked for overnight at 4° C in 3% skim milk diluted in TBST (blocking solution). Next day, the membrane was washed three times for 5 minutes each with 15 ml of TBST. The membrane was incubated with primary antibody (1:750 dilutions) in 15 mL TBST with gentle agitation for 3 hours. After washing 4 times for 15 minutes, the membrane was incubated with horseradish peroxidase (HRP)-linked secondary antibody (1:5000 dilutions) in 15 ml TBST with gentle agitation for 2 hours at room temperature, and washed 5 times for 5 min each with 15 ml of TBST.

The signals were detected using chemiluminescence (ECL) solution (50 μ l of 250mM luminol in DMSO, 22 μ l of 90mM p-coumaric acid in DMSO, 3 μ l of 30% (w/v) H₂O₂ solution, and 10ml of 0.1M Tris-HCL pH 8.6) [268]. After sufficient washing of the membrane in TBST, 100 μ l of ECL was spread over the membrane and the membrane was put between two transparent plastic sheets. The X-ray film was exposed to the emitting light from the membrane by placing it over the plastic sheet in dark room for 5 min. The film subjected to developing, fixing, rinsing, and drying.

4.6 Development of Anti- obesity Soup Mix

Medicinal plants reduce obesity by influencing different pathways such as blocking the digestion and absorption of dietary lipids, appetite suppressants, adipocyte differentiation as well as increasing intestinal transit, Beta oxidation, and metabolic rate. Thus, natural plant products provide an exciting opportunity and promise for the new therapeutic approaches to the treatment of obesity and constitute alternative tool to other pharmacological agent.

Soup mix was chosen as the product to be worked upon as an anti-obesity functional food because of its wide acceptance and easy formulations. Also, soup mix powders can be easily reconstituted and thus are perfect choice for the fast moving lifestyle of the recent generation.

4.6.1 Raw material procurement

All the basic raw materials required for soup mix starting from corn starch to regular spices were procured from local market of Mysore. Food grade xanthan gum (gluten free – Bob's Red Mill), maltodextrin (Akshar Chem), whey protein isolate (Optimum Nutrition) were procured online from Amazon.com. Vegetables (carrot, onion and radish) were procured from Reliance Fresh store, Mysore, diced to small cubes and were oven dried (45°C for 48 hours) to get dehydrated vegetables.

4.6.2 Compositional trials

Different commercial and published recipes were analysed and compared to standardise the ingredients and their proportions for the product. After many trials with different formulations viscosity, texture, dilution ration and heating time-temperature combination were standardised. Three formulations (A, B and C) with different ingredient proportions were finalised and were extended for sensory evaluation (Table 4.1).

Dilution ratio – 10g/150ml water; Heating: 5 minutes boiling.

Ingredients	Formulation 'A' (g)	Formulation 'B' (g)	Formulation 'C' (g)
Corn Starch	1	1.2	1.2
Xanthan Gum	0.8	0.7	0.7
Whey Powder	0.5	0.5	0.5
<i>Moringa oleifera</i> Powder	0.2	0.1	0.2
<i>Brassica oleracea</i> Extract	0.4	0.12	0.12
<i>Ocimum basilicum</i> Extract	0.4	0.2	0.2
Turmeric Powder	0.2	0.1	0.2
Black Cumin	0.5	0.3	0.3

Tea Extract	0.1	0.1	0.15
Sunflower Oil	0.4	0.4	0.4
Maltodextrin	0.4	0.12	0.2
Garlic Powder	0.65	1.5	1.5
Onion Powder	0.75	1	1
Salt	1.1	1.2	1.1
Powdered Sugar	0.5	0.5	0.5
Red Chilli Powder	0.1	0.3	0.15
Black Pepper Powder	0.1	0.15	0.15
Chilli Flakes	0.5	0.1	0.05
Dried vegetable mix	1.41	1.41	1.41
Sum (Total, g)	10	10	10

Table 4.1: Different formulation samples for Soup mix powder.

4.6.3 Sensory Analysis

The sensory qualities are essentially to be measured subjectively. With the development of sensory evaluation techniques on scientific lines, the experts were replaced by semi trained panel members. The panel members analyse food products through properly plant experiments and their judgement were quantified appropriately.

Sensory analysis was carried out according to Amerine et al. (1965) [269] with slight modifications in the method. A panel of 10 semi-trained members evaluated the products using a 9 point hedonic scale of 1 to 9 scores with 9 for like extremely and 1 for dislike extremely. Descriptive sensory analysis for all the formulations was conducted to determine: Appearance (App), Taste (T), Texture evaluation (TE), Aroma (A), after taste (AT) and Overall acceptability (OA). Formulation with highest average scores was pursued for further analytical tests.

4.6.4 Proximate Analysis

- **Moisture analysis (Vacuum oven method)**

The moisture content was found out by using Vacuum oven method (Ranganna, 1986) [270]. 10 g of sample was taken in a moisture cup and was kept in vacuum oven at temperature of 70° C. The weight of the sample was taken after every half an hour till a constant weight was obtained. The moisture cups were transferred to desiccator, and allowed to cool and weighed. Moisture content was calculated by the given formula.

Calculation:

$$\text{Moisture content of sample (\% MC)} = \frac{\text{Initial Weight} - \text{Final Weight} \times 100}{\text{Weight of sample}}$$

- **Ash contents (Muffle furnace)**

Ash content of prepared sample was determined by AOAC method, 2000. 10g of sample was weighed in a silica crucible. The weight of empty crucible and crucible with sample was noted. The crucibles with samples were placed on a hot plate burner and the contents were charred until no smoke was coming from the sample. Crucibles were then placed in a muffle furnace at a temperature of 550°C - 600°C for 5 hours (until carbon residue disappeared). Crucibles were then allowed to cool in a desiccator and finally weighed. The % Ash was determined using the given formula.

Calculation:

$$\text{Ash content (g)} = \frac{(\text{Weight of sample (g)} - \text{Weight of crucible and ash (g)}) \times 100}{\text{Weight of empty crucible (g)}}$$

- **Estimation of fat content**

Fat content of the samples was estimated by Soxhlet method as per AOAC, 1990 protocol with continuous refluxing for 14-16 hours. Empty cellulose thimbles were weighed and 10g of moisture free samples were taken into the thimbles. Clean, dry flat bottom standard joint flask (250ml) were weighed, marked and fixed into the Soxhlet extractor. Samples were placed in the extractor and petroleum ether (46° C-60° C) was added in excess. Water condenser was fixed over the extractor (Run the extraction set using iso-mantle with regulator for 14-16 hours). After extraction, petroleum ether was evaporated at 60°C in a flash evaporator and the fat content recorded until a constant weight was reached. The additional weight gained by the flask after complete evaporation gives the fat content of sample. Fat % was calculated using the given formula.

Calculation:

$$\text{Fat (\%)} = \frac{\text{Weight of ether extract} \times 100}{\text{Weight of the sample}}$$

- **Estimation of protein by Micro Kjeldahl method**

Protein content of samples was determined by Kjeldahl method (AOAC, 1984). 0.5g of sample was taken in a 250 ml Kjeldahl flask taking care that it does not stick to the sides of the flask. To this, 1 g of catalyst mixture (2.5g SeO₂, 100g K₂SO₄ and 20g CuSO₄.5H₂O) and 25ml concentrated H₂SO₄ was added and kept for digestion in the digestion chamber. Digestion proceeded until the mixture became clear after which it was cooled. The mixture was made up to 100ml using distilled water. 1ml aliquot was distilled with 20ml of 30% NaOH solution. The liberated ammonia was absorbed by 5ml 2% boric acid solution with mixed indicator (bromocresol blue and methyl red). The mixture was then titrated against

0.01 N HCl until a stable pink colour appears. The nitrogen content of the sample was determined from the liberated ammonia from which the protein content was measured using the conversion factor 6.25.

Calculation:

% Nitrogen value

$$= \frac{\text{Titration value} \times \text{Normality of HCl} \times \text{Molecular weight of N} \times \text{Made up volume} \times 100}{\text{Sample weight} \times \text{Aliquot volume} \times 1000}$$

And

$$\text{Protein content (g)} = \% \text{ Nitrogen value} \times 6.25$$

Factor 6.25 is based on the assumption that plant protein contains 16 % nitrogen.

- **Estimation of crude fibre**

The crude fibre content was determined using AOAC (1976) method. 2gm of sample was digested with 200ml of 1.25% sulphuric acid for 30 min. After filtration through a linen cloth, the residue was washed with boiled distilled water until it was free from acid. The acid free residue was then digested with 200 ml of 1.25% NaOH for 30 minutes. The contents were filtered hot through linen cloth. Cleaned and dried Gooch crucible was filled 3/4th its column with dried asbestos powder and was fixed on to the filtration device. It was washed with distilled water. While adding distilled water suction was applied to facilitate the asbestos to settle tightly at the bottom of Gooch crucible. The packed volume should not be more than 1/8th of the total volume of the Gooch. If required the surface of the asbestos may be levelled by pressing with a glass rod having a flat end. The packed Gooch Crucible was washed under suction, by distilled water until the washings were clear. Dried in a hot air oven at 120°C for one hour and stored in a desiccators until further use. The sample residue was transferred to the prepared gooch crucibles. Finally, the residue was washed with 15 ml of 95% ethyl alcohol. The content of crucible was dried to a constant weight at 100°C. The dried residue was ignited in a muffle furnace at 550 ±15°C for 2 hours; the percentage loss in weight was expressed as crude fibre.

Calculation:

$$\text{Crude fibre (\%)} = \frac{\text{Weight of digested sample and crucible} - \text{weight of Ash and crucible}}{\text{Weight of sample}}$$

- **Total Carbohydrate content**

Total Carbohydrate content of the developed product was calculated by difference method.

4.6.5 HPLC analysis of water soluble vitamins

HPLC conditions: For chromatographic elution, solvent mixtures were used consisting of 0.1% TFA (Tri flouro acetic acid) in acetonitrile (HPLC grade) (Eluent A) and 0.1% TFA in water (Eluent B); Flow rate: 1 ml/minute; λ_{max} : 265nm; Detector: UV-Vis and PDA detector.

Extraction solution: 50 ml acetonitrile + 10ml glacial acetic acid and volume made up to 1000ml with distilled water.

Sample preparation: To 10g of sample 50ml of extraction solution was added. Then it was kept in shaking water bath maintained at 60° C for 45 minutes followed by filtration through 0.22 μm syringe filter. 20 μl from this filtered sample was injected in the instrument (Nollet et al., 2012) [271].

4.6.6 Extraction of bio-active compounds from the food product

Product was extracted in both methanol (100%) and ethanol (80%) for a comparative study. 20g of soup mix powder was taken in a conical flask and volume was made up to 200 ml with a) 80% ethanol b) 100% methanol (1:10 ratio). The pH of the solution was adjusted to 2 by a hand held pH meter using 2 M HCl solution. The mouth of the conical flasks were covered with cotton plug and aluminium foil. These conical flasks were then kept in an orbital shaker for 12 hours at 37° C. Obtained extracts were filtered first through normal filter paper and then through Whatman No.1 filter paper to get a clear solution. Rotary flash evaporator (heidolph) at 50° C was used to remove the ethanol from the liquid extracts. The concentrated samples were then transferred into 3 steel bowls for lyophilisation. The samples were kept in the freeze drier for overnight and the powdered forms extracts were obtained the next day. These powdered extracts were weighed for calculation of yield and were then dissolved in Milli Q water at a conc. of 100 mg/ml. These extracts were then stored at - 20° C in a deep freezer as a stock until further use for working preparation.

4.6.7 Phytochemical Investigation for food product extracts

Total phenolic and flavonoid contents were estimated for ethanol and methanol food extracts by following the previously explained protocols (Refer sec. 4.2.3 & 4.2.4).

4.6.8 Statistical analysis

All data were expressed as mean \pm standard deviation (Std. Dev.) from triplicates for each experiment. Statistical analysis was performed using the Statistical Analysis Software (SAS) (version 9.2). Differences between control and treatment groups were determined by one-way analysis of variance (ANOVA) and followed by Tukey's multiple comparison tests. A P-value of < 0.05 was considered statistically significant.

Chapter 5

Results and Discussions

Results and Discussion

The results for phytochemical analysis, bioactive compound identification and *In-vitro* evaluation of *Moringa oleifera* (*MO*), *Ocimum basilicum* (*OB*) and *Brassica oleracea* (*BO*) extracts for anti-adipogenic effects are presented in this section.

5.1 Extraction yield

Yield % for ethanol plant extracts is given in Table 5.1. Yield of *MO* was observed to be highest followed by *BO* and *OB*. Ethanol was selected as solvent as it is easier to penetrate the cellular membrane to extract the intracellular ingredients from the plant material. It is also the most effective one producing the highest extraction yield compared to non-polar solvents (Aveen, 2015) [273].

Plant species	Yield % (Ethanol extract)
<i>M. oleifera</i> (<i>MO</i>)	24.9 ± 0.89
<i>B. oleracea</i> (<i>BO</i>)	23.1 ± 0.93
<i>O. basilicum</i> (<i>OB</i>)	20.8 ± 0.88

Table 5.1: Ethanol extract yield % for Plant materials

5.2 Phytochemical Investigation

Quantitative phytochemical examinations of *MO*, *BO* and *OB* showed that the ethanol extracts contained phytochemicals such as phenols and flavonoids. It was also observed that these extracts were rich in anti-oxidant properties.

5.2.1 Total phenolic content

Phenolic compounds are secondary metabolites ubiquitously found in plants, mainly acting as UV protectors. Estimation of total phenolic content was carried out according to the standard calibration curve (Figure 5.1) and the results were expressed as mg Ferulic acid equivalent/g of extract using the standard calibration curve equation.

$$\text{The linear equation for the standard} \quad Y = 0.0071X + 0.0123 \quad R^2 = 0.988$$

Where Y denotes the absorbance at 765 nm and X denotes the total phenolic content (TPC) in the plant extract. The total phenolic content in *MO*, *BO* and *OB* was found to be 65.75, 23.86 and 45.69 mg FAE/g of plant extract (Table 5.2) respectively.

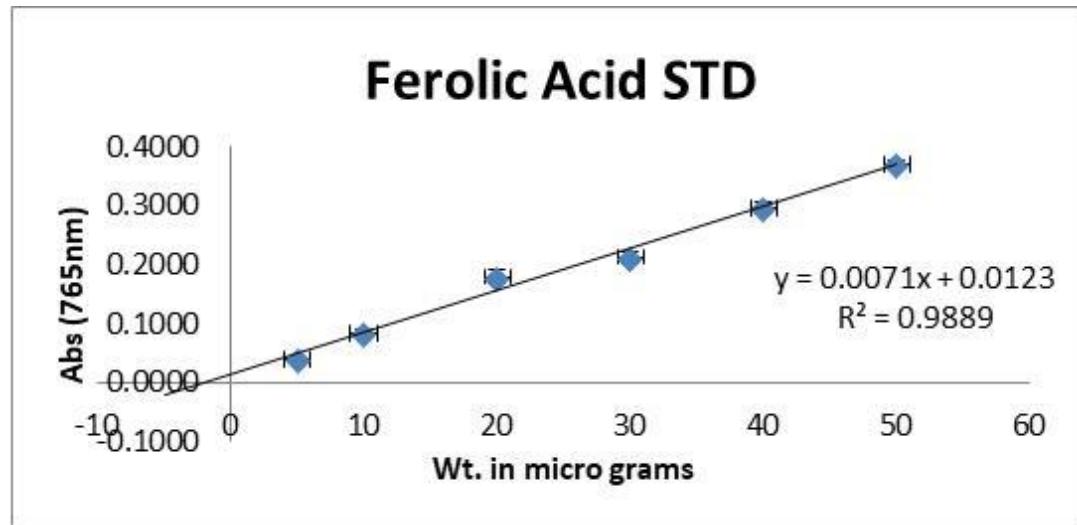


Figure 5.1: Standard calibration curve (Total Phenolic content: Ferulic acid)

5.2.2 Total Flavonoid content

A standard calibration curve was prepared to calculate the percentage of flavonoid content using the following equation:

$$\text{The linear equation for the standard} \quad Y = 0.001X - 0.024 \quad R^2 = 0.985$$

Where Y is the absorbance at 415 nm and X is the total flavonoid content in the plant methanol extract, as presented in Figure 5.2.

From the standard calibration curve, the total flavonoid content in the ethanol extract of *MO*, *BO* and *OB* was found to be 29.64, 18.19 and 29.97 mg QUE/g of plant extract (Table 5.2) respectively, which is considered a relatively high percentage of flavonoid content.

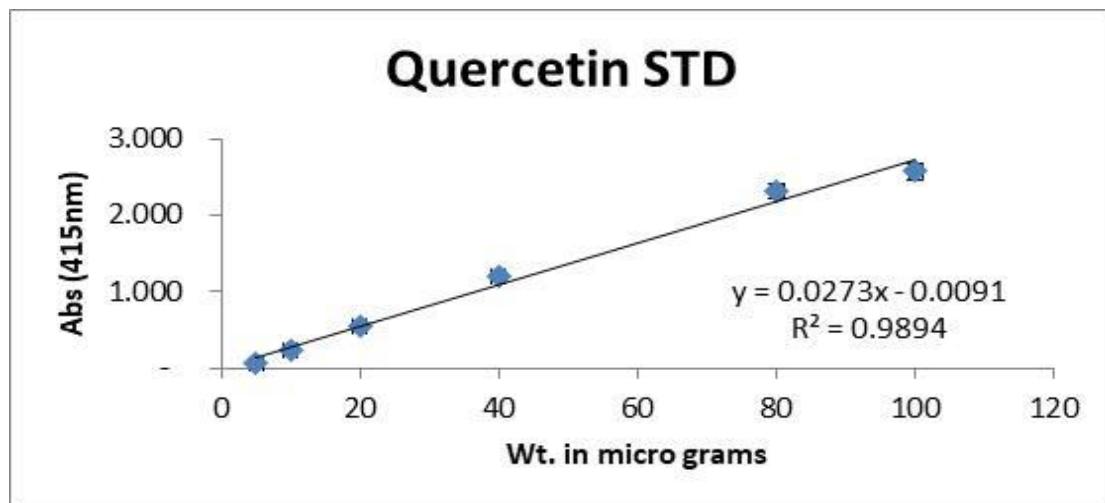


Figure 5.2: Standard calibration curve (Total Flavonoid content: Quercetin)

Ethanol Extracts	Total phenolic content (mg FAE/g plant extract)	Total flavonoid content (mg QE/g plant extract)
<i>M. oleifera (MO)</i>	65.75 ± 5.94	26.94 ± 0.95
<i>B. oleracea (BO)</i>	23.86 ± 0.81	18.19 ± 1.19
<i>O. basilicum (OB)</i>	45.69 ± 2.93	29.97 ± 0.68

Table 5.2: Total Phenolic and Flavonoid content for Plant extracts.

These studies showed that *MO* and *OB* had relatively higher phenolic and flavonoid content than *BO* (Table 5.2).

5.2.3 Anti-oxidant Activity (DPPH assay)

The potential anti-oxidant activity for the ethanol extract of *MO*, *BO* and *OB* showed a high effective free radical scavenging in the DPPH assay. This method is based on the capture of DPPH by antioxidants producing a decrease in absorbance. The increased consumption of DPPH by a sample is proportional to increased antioxidant activity [274]. The results are expressed in terms of IC₅₀ value (Concentration of sample providing 50% inhibitory activity). The IC₅₀ value for *MO*, *BO* and *OB* was found to be 181.25 ± 7.96 , 953.41 ± 24.32 and 198.92 ± 8.04 respectively compared to Ferulic acid (anti-oxidant reference) ($Y = 4.3068X + 15.959$; $IC_{50} = 8.07 \pm 0.26$; $R^2 = 0.937$) (figure 5.3, 5.4, 5.5 and 5.6).

Several previous studies have demonstrated that plant extracts rich in flavonoids and phenolic acids can scavenge free radicals [277]. The efficiency of flavonoids and phenolic acids as antioxidant polyphenols is attributable to their unique molecular structures. The hydroxyl groups of flavonoids, especially at the 3' OH and 4' OH of their three-carbon chain, make them potent electrons donors and terminators of chain reactions [276], hence their antioxidant effect. On the other hand, the antioxidant activity of phenolic acids has been attributed to the ability of the phenolic ring in their structure to stabilize and delocalize unpaired electrons [275].

Clearly, the antioxidant activity of *OB* and *MO* was relatively very high than *BO* which is also evident from the higher content of total phenolic and flavonoid content of these extracts.

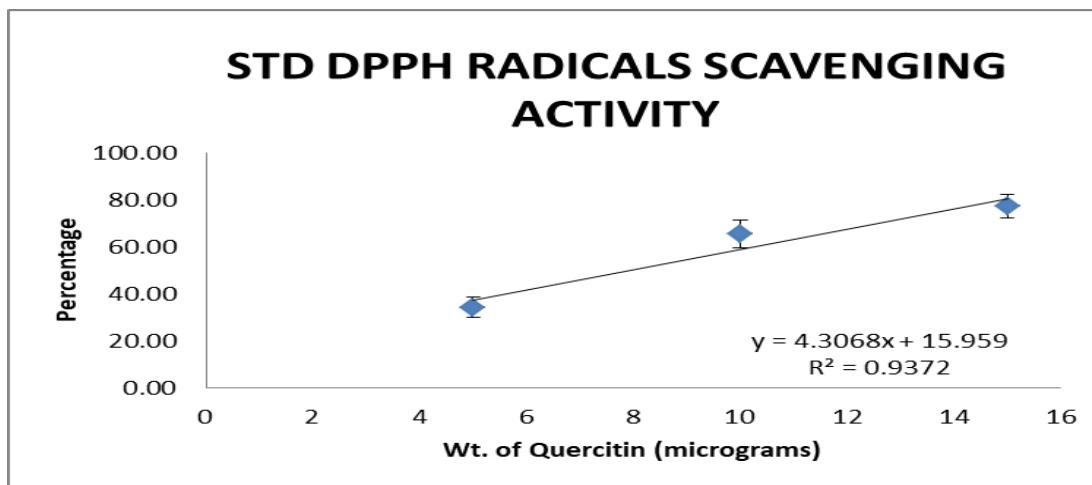


Figure 5.3: Standard calibration curve (DPPH Free radicals scavenging activity: Quercetin)

These levels of anti-oxidant activity are considered high for leaves and can be correlated with the levels of flavonoids and phenolic compounds. These high values, allows us to infer that the ethanol extract of *MO*, *BO* and *OB* may have thermogenic potential or can be used as functional foods. After pharmacological studies, these plant extract may play a key role in preventing cardiovascular disease, cancer and degenerative diseases, due to their high antioxidant potential. It is important to remember that natural antioxidants occur in all parts of plants. These antioxidants include carotenoids, vitamins, phenols, flavonoids, dietary glutathione, and endogenous metabolites. Several studies have revealed that the phenolic content in the plants are associated with their antioxidant activities, probably due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers [278].

Ethanol Extracts	Free-radical scavenging activity (IC_{50} - $\mu\text{g/ml}$) (DPPH assay)
<i>M. oleifera</i> (<i>MO</i>)	181.25 ± 7.96
<i>B. oleracea</i> (<i>BO</i>)	953.41 ± 24.32
<i>O. basilicum</i> (<i>OB</i>)	198.92 ± 8.04

Table 5.3: Anti-oxidant activity for Plant extracts in terms of IC_{50} value ($\mu\text{g/ml}$)

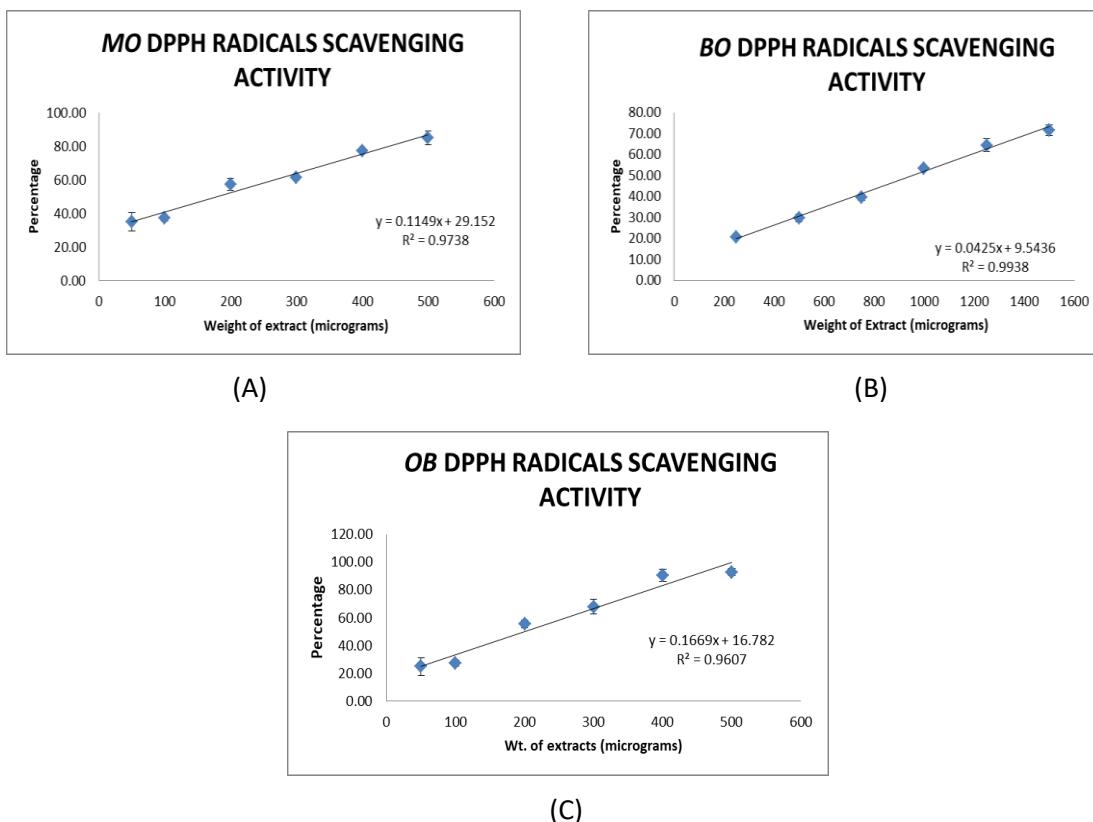


Figure 5.4: Weight of extracts vs. Percentage radical scavenging activity (DPPH assay); A) *Moringa oleifera*; B) *Brassica oleracea*; C) *Ocimum basilicum*.

5.3 Identification of bioactive compounds (UPLC-HRMS/MS)

Metabolites were extracted from dried leaves and subsequent targeted phenol-flavonoid profiling of extracted material was carried out. Profiling was performed under negative ion mode. From the crude UPLC–MS Total ionic chromatogram (TIC) (Intensity vs. Retention time) total of 6 intensity peaks for *O. basilicum*, and 7 intensity peaks for *B. oleracea* and *M. oleifera* were identified (Figure 5.5, 5.6 and 5.7). Table 5.4, 5.5 and 5.6 gives the list of probable compounds identified in each plant extract along with their mass by charge (*m/z*) ratios, retention times (minutes) and major MS¹ (MS/MS) fragment ions. To identify the compounds online mass spectra libraries and previously published research articles were used as a reference. Retention times for the probable compounds were marked in the Total ionic chromatograms.

7 major flavonols were found in *M. oleifera* (Table 5.4). In which 2 were quercetin derivatives and 2 were Kaempferol derivatives. Charlene Makita et al. (2016) [279] also found Quercetin acetyl hexose, Quercetin acetyl di-hexose, Kaempferol hexose and Isorhamnetin acetyl hexose in the UHPLC-qTOF-MS fingerprinting analysis of *Moringa oleifera*.

Probable Compounds	Molecular ions [M-H] ⁻ or m/z value	Retention time (minutes)	MS/MS fragment ions
Quercetin acetyl hexose	505	7.34	301
Kaempferol hexose	447	7.76	285
Kaempferol acetyl hexose	489	7.98	285
Caftaric acid	311	14.34	241,161
Quercetin acetyl di-hexose	667	18.09	624,462
Chicoric acid	473	18.91	311,179
Isorhamnetin acetyl hexose	519	19.51	315

Table 5.4: List of probable bioactive compounds in *M. oleifera*

Probable Compounds	Molecular ions [M-H] ⁻ or m/z value	Retention time (minutes)	MS/MS fragment ions
Caffeic acid derivative	359	7.12	197,179
Kaempferol Hexose	447	11.77	285
Sinapic acid	222.8	14.24	148,121
Caftaric acid	311	14.50	241,161
Caffeic acid	178.7	15.32	135,161
Isorhamnetin 3-O-rutinoside	623	16.9	315
Quercetin acetyl di-hexose	667	18.17	624,462

Table 5.5: List of probable bioactive compounds in *B. oleracea*

Probable Compounds	Molecular ions [M-H] ⁻ or m/z value	Retention time (minutes)	MS/MS fragment ions
Caftaric acid	311	14.50	341,161
Feruloyl tartaric acid	325	15.3	193,149
Quercetin acetyl di-hexose	667	18.17	624,462
Kaempferol derivative	533	18.94	489,285
Chicoric acid	473	18.97	311,179
Quercetin derivative	607	19.41	463,301

Table 5.6: List of probable bioactive compounds in *O. basilicum*

In case of *B. oleracea* 7 major bio-actives compounds were identified (Table 5.6). Sinapic acid, Caffeic acid and Caftaric acid were some of the most important polyphenols that were found in the extract. Previous studies have also confirmed the presence of Sinapic acid, Caffeic acid, Vanillic acid, p-Coumaric acid, Ferulic acid and Salicylic acid [280]. One more compound named Danshensu was identified at t = 0.35 (m/z = 197; MS/MS 161).

Caftaric acid and Chicoric acid were the important components found in *O. basilicum* crude extract (Table 5.7). Quercetin and Kaempferol derivatives were also identified in the crude extract. According to previous ligatures Chicoric acid, Caffeic acid and its derivatives and Cinnamic acid were the major phenolics found in the *O. basilicum* [281]. Rosmarinic acid is also present in the *O. basilicum* in significant quantities [281, 282] but was not identified in the present study. At t = 14.07 caffeic acid was also identified (m/z = 178; MS/MS 135,161).

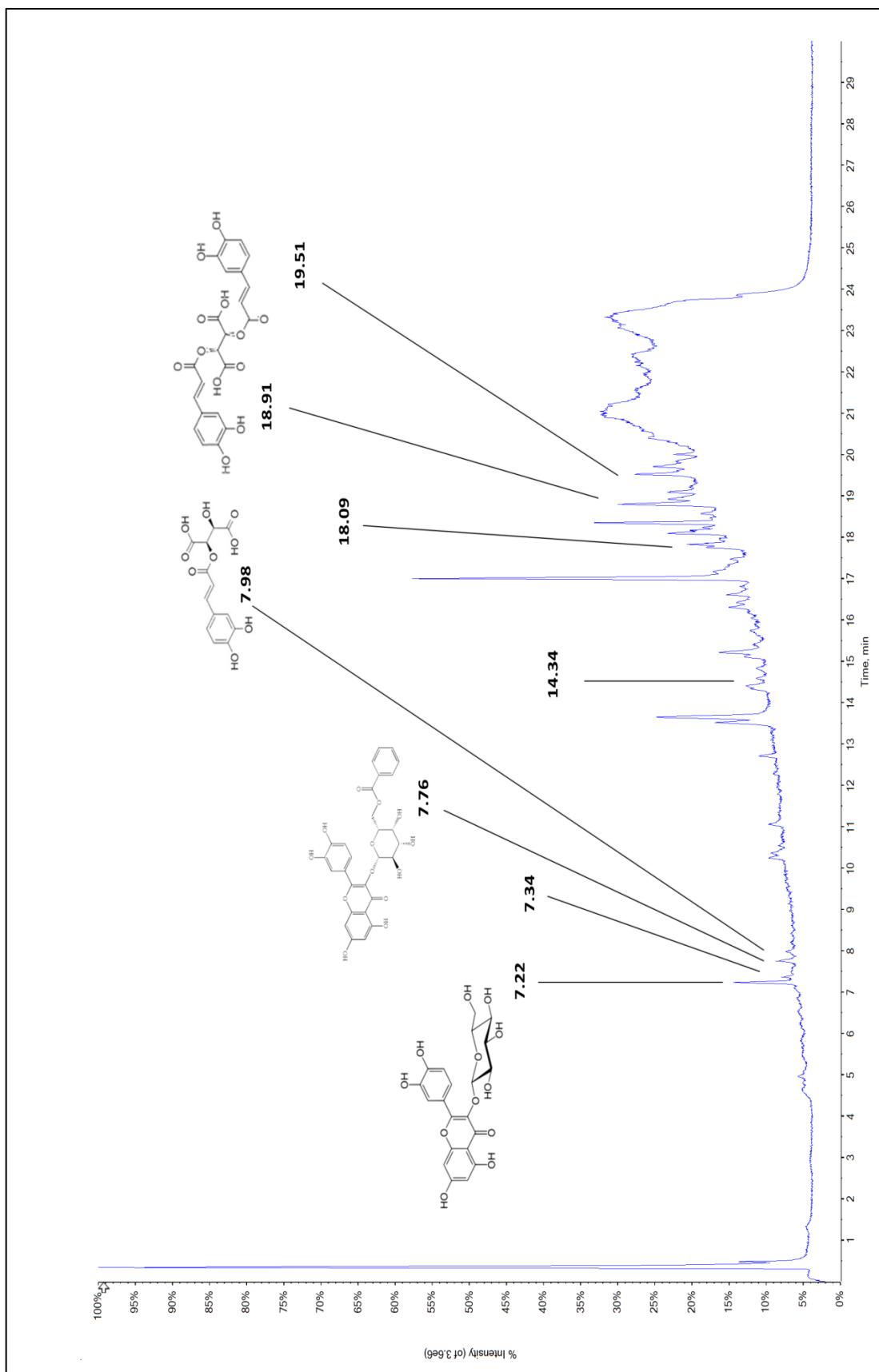


Figure 5.5: Total Ionic Chromatogram (% Intensity vs. Time) *Moringa oleifera*

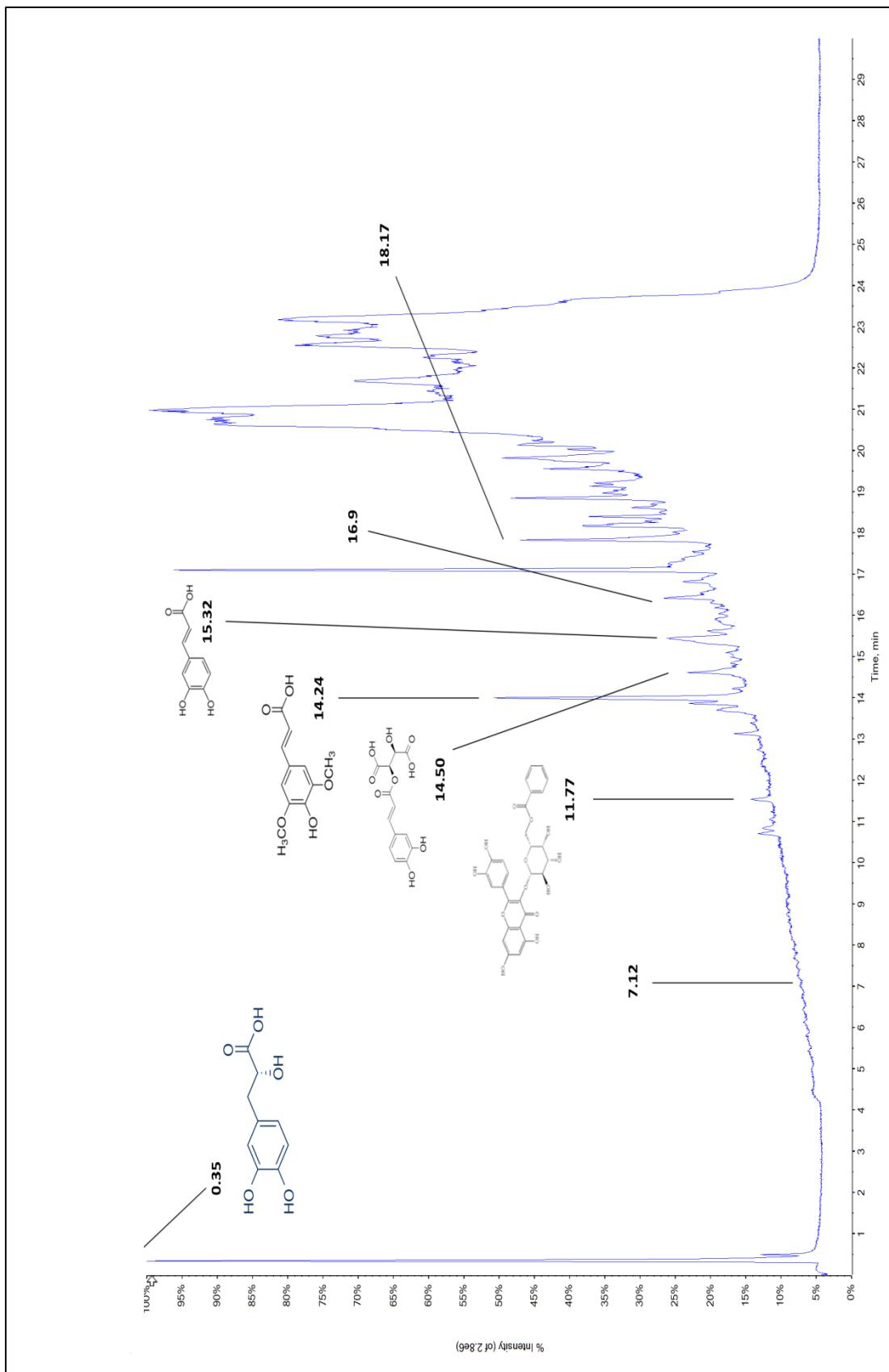


Figure 5.6: Total Ionic Chromatogram (% Intensity vs. Time) *Brassica oleracea*

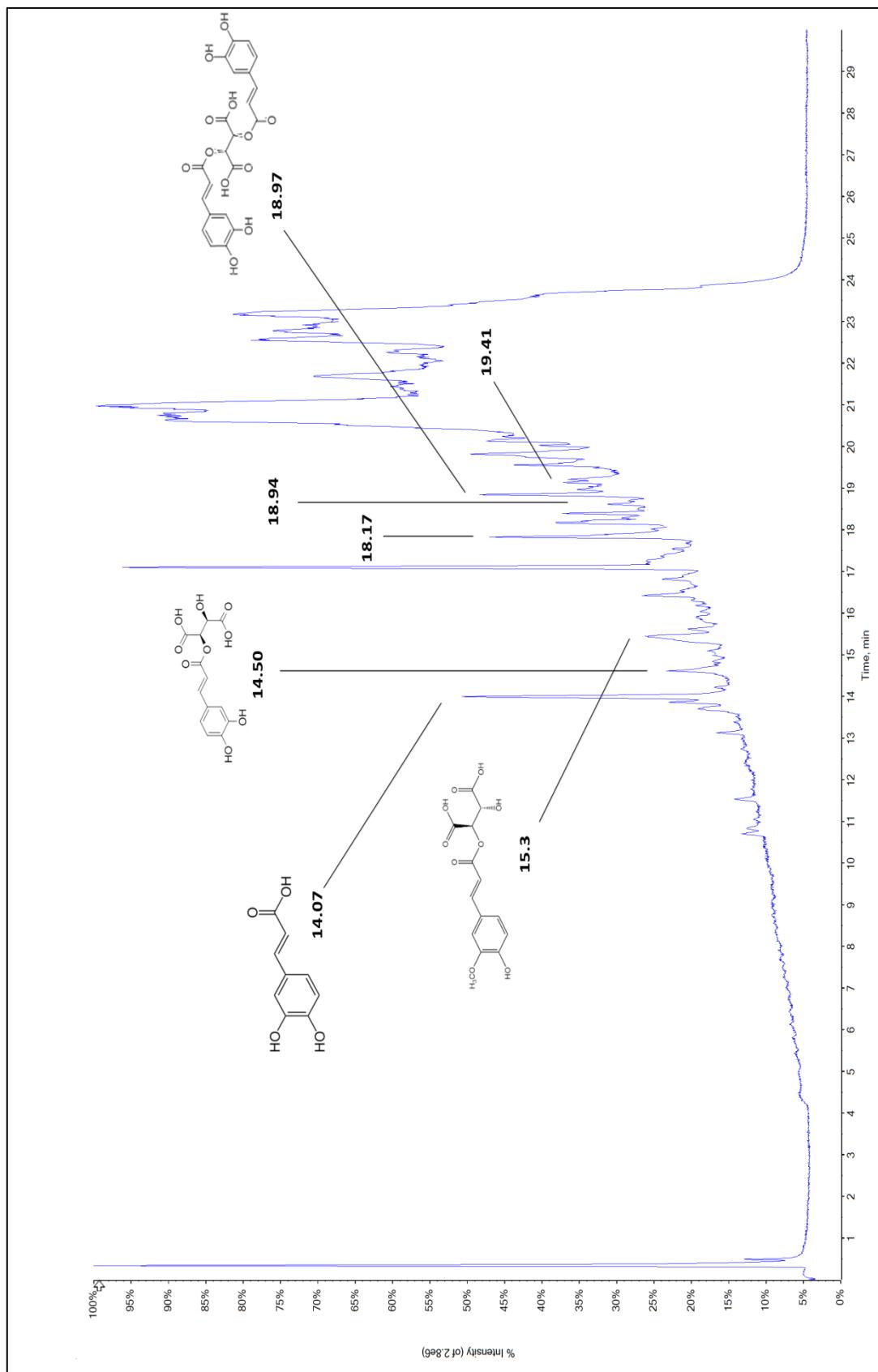


Figure 5.7: Total Ionic Chromatogram (% Intensity vs. Time) *Ocimum basilicum*

5.4 Effect of plant extracts on cell viability

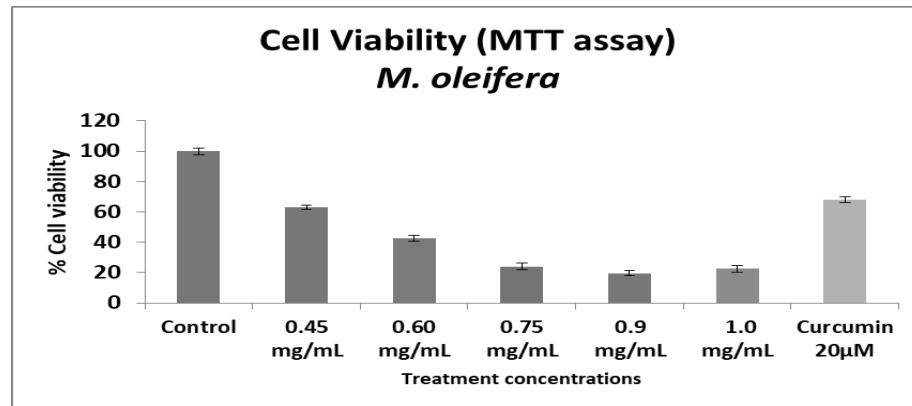
To determine the effects of plant extracts on cell viability during adipocyte differentiation, 3T3-L1 cells were treated with a range of concentrations (0*, 0.45, 0.6, 0.75, 0.9 and 1.0 mg/mL for *OB* & *MO*; and 0*, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/ml for *BO*) of plant extracts for 32 & 48 hours, and the cell viability was determined using the MTT assay and LDH assay, respectively. Concentration for standard drug (curcumin) was taken as 20mM. Also, non-differentiated cells were maintained only on PEM ('-ve control). The cell viability results are expressed as the percentage test cells surviving compared to control cells. Treatments were performed in triplicates.

Viability of 3T3-L1 cell populations treated with *MO* for 32 and 48 hours (MTT & LDH release assay) both was markedly decreased to the extent that the survival rates of cells were only about 60% even at the initial concentration of 0.45 mg/mL (Figure 5.8 (A) and Figure 5.9 (A)). However, *OB* and *BO* did not show any significant effect on 3T3-L1 cell viability at concentrations up to 0.75 mg/mL and 4 mg/mL respectively ($p > 0.5$). (Figure 5.8 (B) & (C) and Figure 5.9 (B) & (C)).

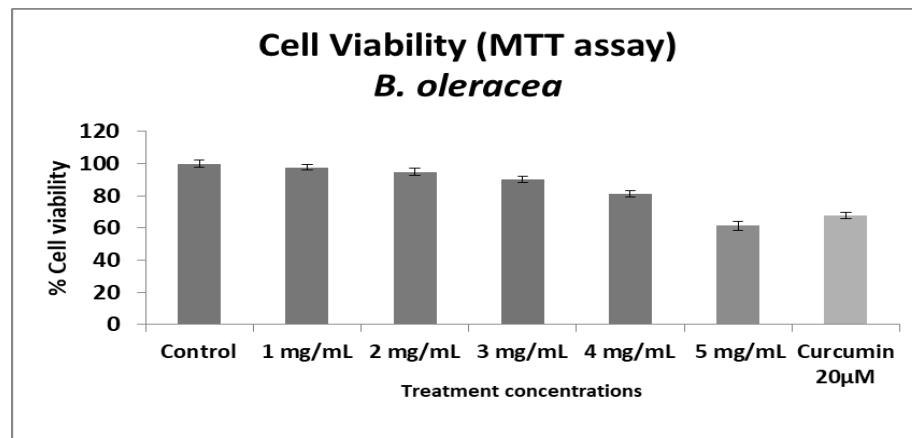
LDH enzyme is released into the media when there is tissue damage. Increased levels of LDH demonstrate cell damage and the amount of LDH released in negatively correlated with cell viability. Minimum damage or minimum LDH release was seen in the positive control. Cell viability in treatments was evaluated with respect to the control (taking control as 100%).

During adipocyte differentiation in the presence of *OB* and *BO*, average cell viability was more than 90%. Based on the results, *OB* and *BO* at the concentrations used were not cytotoxic to 3T3-L1 pre-adipocytes. On the other hand, cytotoxicity levels shown by *MO* were relatively very high and thus treatment concentration must be further reduced to check its effects on lipid accumulation and anti-obesity properties. Because the viability of control, *BO* and *OB* treated, and non-differentiated cells was higher than 90 %; 3T3-L1 cells were maintained for 10 days in case of *OB* and *BO* for further observations in terms of lipid accumulation, morphology and biomarker measurements. To focus specifically on the non-cytotoxic range of *OB* and *BO*, concentrations of 0.45-0.75 mg/mL and 1-3mg/ml respectively, were used in the following experiments.

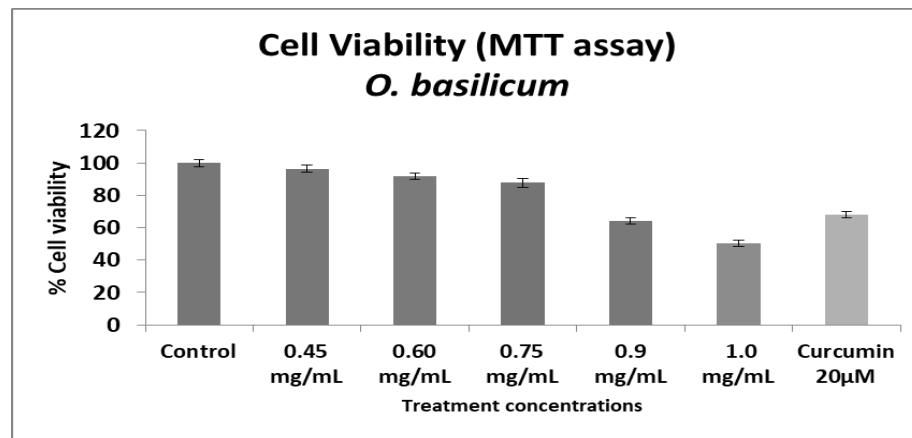
* Positive control (differentiated cells without any treatment)



(A)

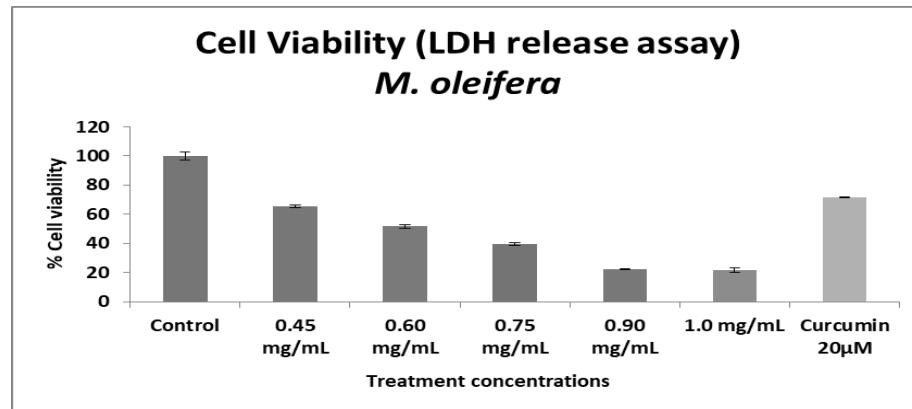


(B)

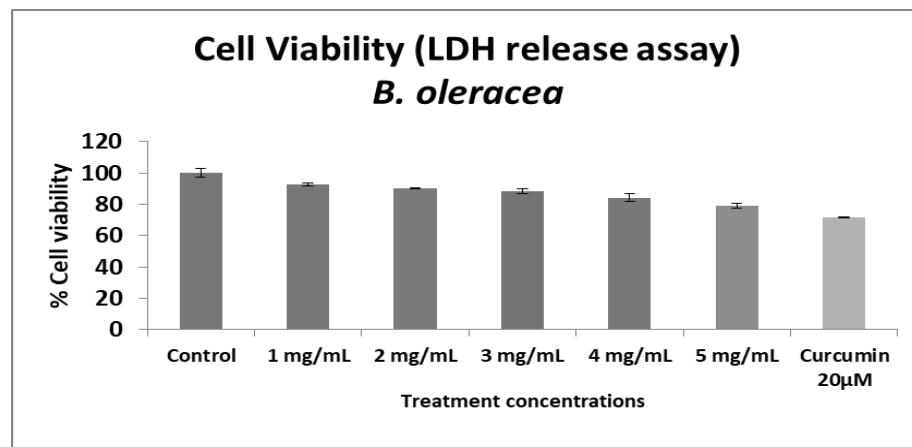


(C)

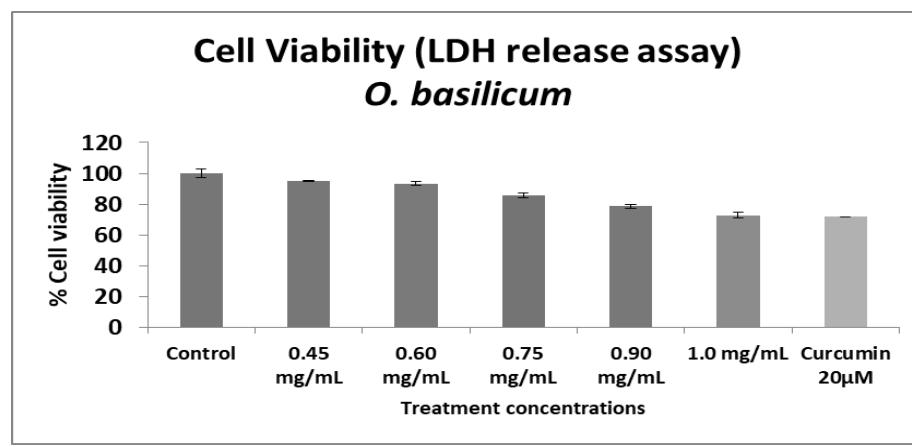
Figure 5.8: % Cell viability vs. Treatment concentrations (MTT cell viability assay – 32 hour incubation); A) *Moringa oleifera*; B) *Brassica oleracea*; C) *Ocimum basilicum*



(A)



(B)



(C)

Figure 5.9: % Cell viability vs. Treatment concentrations (LDH release assay – 48 hour incubation);
A) *Moringa oleifera*; B) *Brassica oleracea*; C) *Ocimum basilicum*

5.5 Effect of plant extracts on Lipid accumulation in Adipocytes

The inhibitory effect of *OB* and *BO* on lipid accumulation was evaluated on day 10 of treatment, after the differentiation was induced by adding the differentiation media containing various concentrations of plant extracts. The retained dye by the intracellular lipids was eluted with isopropanol and measured at 510 nm. Treatments were compared to positive control (differentiated cells without treatment) and negative control (non-differentiated cells). There were significant statistical differences ($P < 0.05$) between non differentiated cells, control and *OB* or *BO* treated cells. The results were expressed as percentage of positive control (100%). Non-differentiated cells showed a lipid accumulation of $27.58 \pm 1.52\%$.

The oil red O staining results for *BO* and *OB* are shown in Figure 5.11 and 5.13. There was significant difference ($P < 0.05$) between non differentiated cells, control, (1 to 3 mg/mL) *BO* treated cells, and (0.45 to 0.75 mg/mL) *OB* treated cells. Cells treated with 3mg/mL *BO* down regulated the lipid droplet accumulation by $44.93 \pm 2.28\%$ compared to control, whereas cells treated with 0.75 mg/mL *OB* down regulated the lipid accumulation by $54.95 \pm 1.82\%$. Clearly, *OB* extract had higher lipid inhibition properties in comparison to *BO* extract, indicating towards its higher potency against obesity. Also, standard drug (curcumin) inhibited the lipid accumulation by $68.31 \pm 0.60\%$ with respect to control.

Differentiation was induced in 3T3-L1 cells for 10 days with or without *BO* or *OB*. Oil red O stained adipocytes treated with *BO* (1-3 mg/mL) and *OB* (0.45-0.75 mg/mL) were photographed at day 10. Microscopic observation was conducted with an OLYMPUS Model CX 30 at 20X magnification. Figure 5.10 and 5.12 shows the pictures obtained for *BO* and *OB* treatments in comparison with negative and positive control. The pictures clearly show the difference between non-differentiated cells and control at day 10. Non-differentiated cells (Figure 5.10 (A) and 5.12 (A)) maintained the initial fibroblast morphology and show no lipid accumulation whereas for control (Figure 5.10 (B) and 5.12 (B)), where the intracellular lipid accumulation is noticeable by oil red O staining. As shown in Figure 5.12 (D-F), the concentration of *BO* (1 to 3 mg/mL) did not highly attenuate the lipid accumulation in differentiated adipocytes while concentrations of *OB* (0.45 to 0.75 mg/mL) did significantly attenuate the lipid accumulation as demonstrated by oil red O staining (Figure 5.10 (D-F)). These observations match with the results obtained in the dye quantification at 510 nm by spectrophotometry.

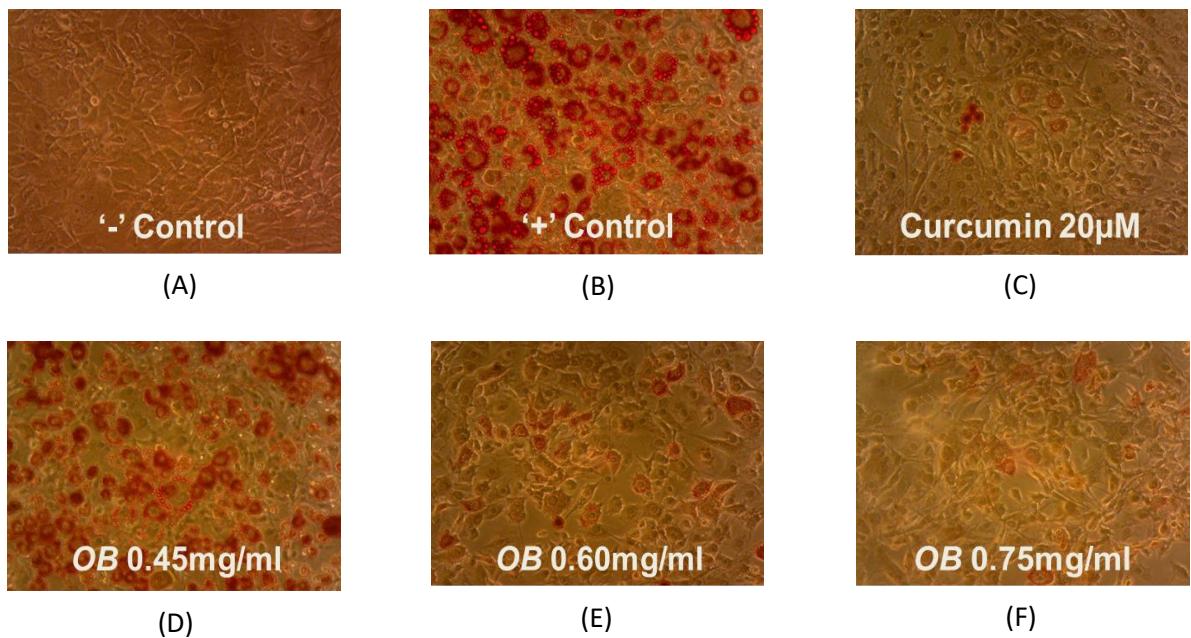


Figure 5.10: Oil Red O stained mature adipocytes under 20X optical zoom – Treatment with *Ocimum basilicum*; A) Undifferentiated Control ('-'); B) Differentiated Control ('+'); C) Standard drug- Curcumin (20 μ M); D) Treatment- OB (0.45mg/mL); E) Treatment- OB (0.60mg/mL); F) Treatment- OB (0.75mg/mL)

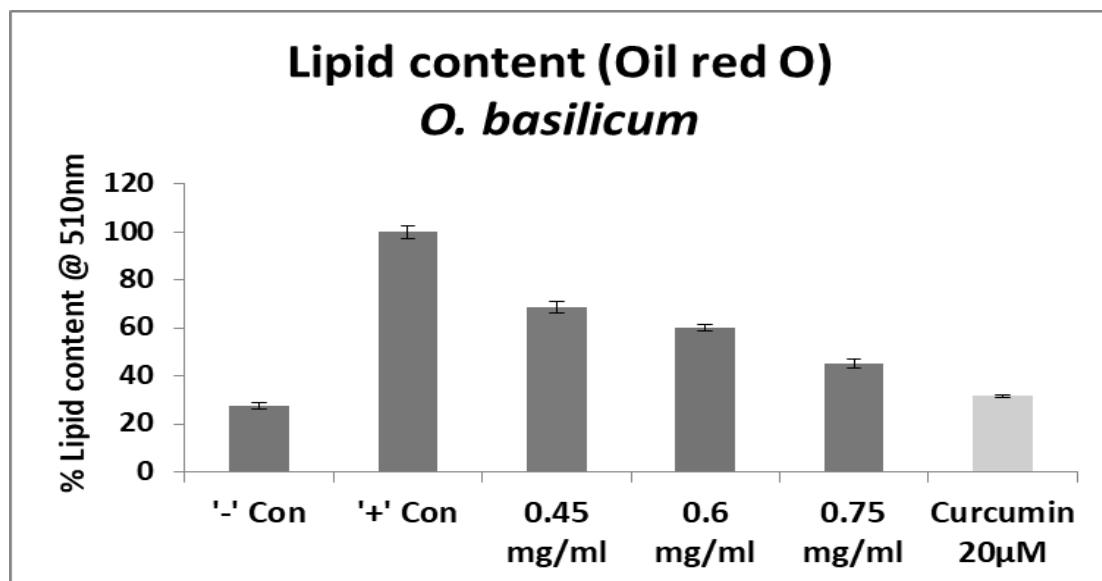


Figure 5.11: Inhibition of Intracellular lipid accumulation in mature adipocytes when treated with different concentrations of *Ocimum basilicum* (OB); ('-' Con: Undifferentiated control; '+' Con: Differentiated untreated control)

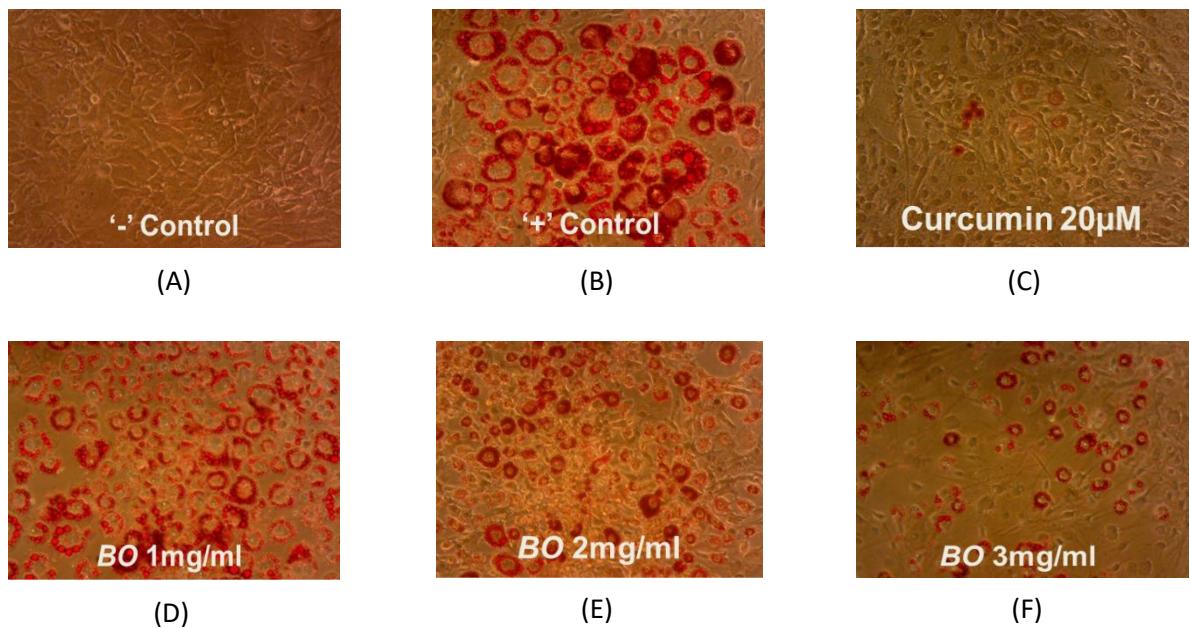


Figure 5.12: Oil Red O stained mature adipocytes under 20X optical zoom – Treatment with *Brassica oleracea*; A) Undifferentiated Control ('-'); B) Differentiated Control ('+'); C) Standard drug- Curcumin ($20\mu M$); D) Treatment- BO (1.0mg/mL); E) Treatment- OB (2.0mg/mL); F) Treatment- OB (3.0mg/mL)

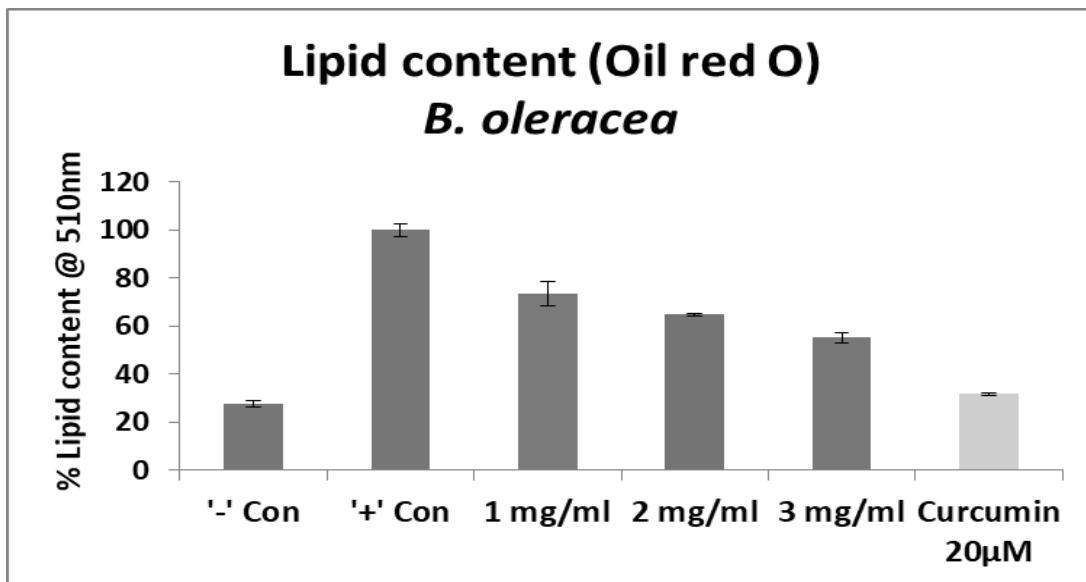


Figure 5.13: Inhibition of Intracellular lipid accumulation in mature adipocytes when treated with different concentrations of *Ocimum basilicum* (OB); ('-' Con: Undifferentiated control; '+' Con: Differentiated untreated control)

5.6 Effect of plant extracts on Total triglyceride content

3T3-L1 (pre)-adipocytes were treated with different concentrations of plant extract samples during its differentiation period for 10 days (refer sec. 4.4.3). In case of *BO* 2mL/mL and 3mg/mL concentrations significantly blocked the triglyceride accumulation during differentiation of 3T3-L1 cell (Figure 5.14). While in case of *OB* 0.60 and 0.75 mg/mL concentrations were enough to significantly inhibit the triglyceride accumulation (Figure 5.15). Moreover, the inhibitory effect of *OB* and *BO* extracts on triglyceride accumulation exhibited in a dose dependent manner. Treatments were compared to positive control (differentiated cells without treatment) and negative control (non-differentiated cells).

There were significant statistical differences ($P < 0.05$) between non differentiated cells, control and *OB* or *BO* treated cells. The triglyceride values were expressed in μg of Triglycerides per μg of protein present in the sample.

For *BO* no significant difference was found between differentiated control and 1mg/mL concentration treatment while for 3mg/mL a significant inhibition of 28% was observed. Also in the case of *OB* 0.45, 0.60 and 0.75 mg/mL concentrations inhibited the TG accumulation by 6, 15 and 47 %, respectively. Cells in undifferentiated control accumulated 32% TG with respect to differentiated control. Also standard drug (Curcumin) inhibited the TG accumulation by 62 % and there was no significant difference found between undifferentiated control and Standard drug treatment.

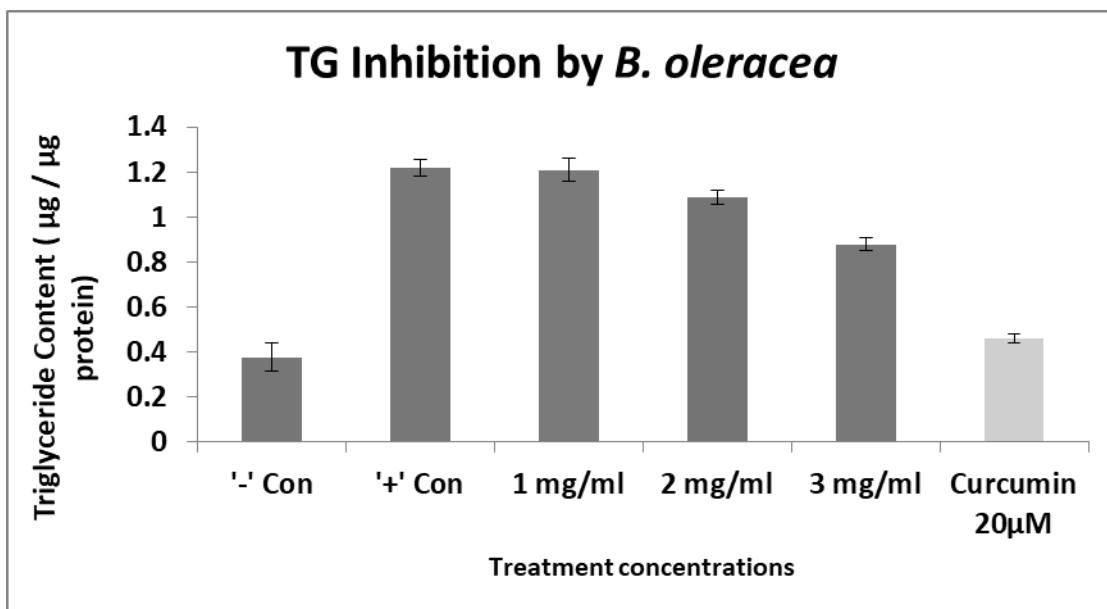


Figure 5.14: Inhibition of Triglyceride accumulation in mature adipocytes when treated with different concentrations of *Brassica oleracea* (*BO*); ('-' Con: Undifferentiated control; '+' Con: Differentiated untreated control)

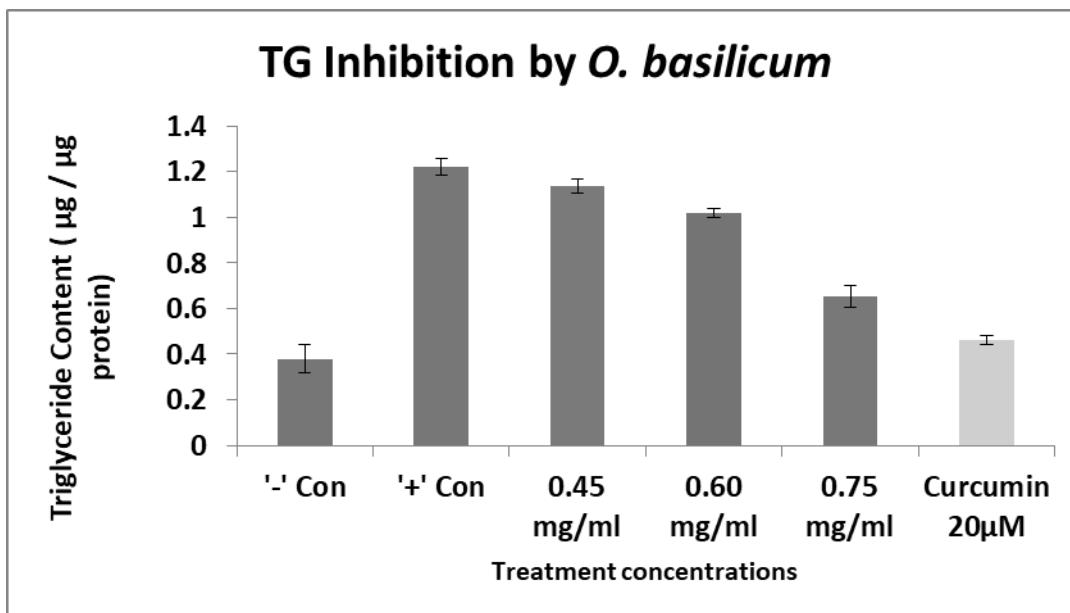


Figure 5.15: Inhibition of Triglyceride accumulation in mature adipocytes when treated with different concentrations of *Ocimum basilicum* (OB); ('-' Con: Undifferentiated control; '+' Con: Differentiated untreated control)

5.7 Effects of *B. oleracea* and *O. basilicum* on expression of Adipsin during cell differentiation.

Western blot analysis was employed to investigate the effect of *B. oleracea* and *O. basilicum* extracts on the expression of adipocyte transcription factor, Adipsin at cellular protein level.

Adipsin, a serine protease homologue, exhibits an apparent molecular weight of 44 and 37 kDa due to glycosylation of a core 28 kDa polypeptide [283, 284]. Adipsin is identified as complement factor D in human subjects since they share 98% amino acid sequence similarity and exhibit the same enzymatic activity [285]. Adipose tissue is the main source of adipsin secretion but it is detectable in the sciatic nerve and bloodstream [286].

3T3-L1 differentiated cells without plant extract treatment were used as '+' control, while undifferentiated cells were utilised as '-' control. The results were expressed as protein expression relative to that of control (qualitative estimation of protein signals on X-ray films).

Figure 5.16 and 5.17 shows the protein expression of adipsin in *B. oleracea* and *O. basilicum*, respectively.

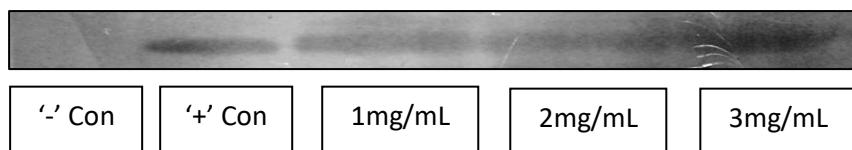


Figure 5.16: Western blot for the protein expression of Adipsin when treated with Ethanol extract of *Brassica oleracea*. ('-' Con: Undifferentiated control; '+' Con: Differentiated untreated control)

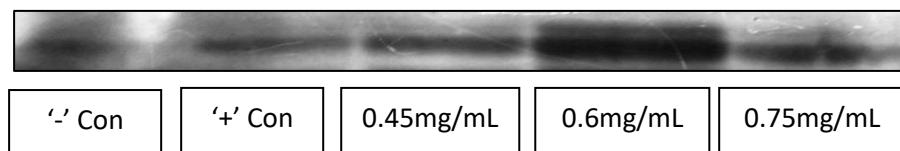


Figure 5.17: Western blot for the protein expression of Adipsin when treated with Ethanol extract of *Ocimum basilicum*. ('-' Con: Undifferentiated control; '+' Con: Differentiated untreated control)

Adipsin is negatively correlated with adipogenesis. Though the expression level in undifferentiated adipocytes is negligible and mature adipocytes express high levels of adipsin, the expression decreases with increased fat cell volume. Adipsin mRNA levels are significantly decreased in genetically obese mice (db/db and ob/ob) and also in a mouse model of chemically-induced obesity [287]. With increased body weight adipsin expression goes down. Thus adipsin expression in adipocytes can be a useful evaluation parameter for anti-obesity drugs.

Adipocytes treated with *B. oleracea* (*BO*) showed significant changes in protein expression of Adpsin in a dose dependent manner (Figure 5.15). Adipsin expression was negligible in undifferentiated control and was highest in cells treated with 3mg/mL concentration of *BO*. The expression level increased with increasing concentration of the treatment. There was no significant difference found between positive control and treatment of 1 mg/mL ($p > 0.5$).

This was the exact case with cells treated with *O. basilicum* (*OB*). With increasing concentrations of treatment the expression level increased. It was found that expression of Adipsin was highest at 0.6 mg/mL treatment concentration of *OB* and also, there were significant differences found between treatments and control and no significant difference between 0.60 and 0.75 mg/ml treatment (*OB*).

From these results it was evident that *BO* and *OB* both plant extracts significantly increased the protein expression of Adipsin in cells. Thus, it can be concluded that both extracts exhibited anti-adipogenic properties (as adipsin expression levels are negatively correlated with Obesity). It was also evident that adipsin levels expressed by *OB* treated cells were higher than *BO* treated cells indicating higher potency of *OB* extract against obesity.

5.8 Product Development – Anti-obesity Instant Soup powder Mix

5.8.1 Sensory Analysis and Final composition

All three formulations for Soup mix (A, B and C) were evaluated on the basis of sensory attributes by a panel of 10 semi-trained members on a 9 point hedonic scale (Refer sec. 4.6.3). The mean scores of sensory panel were analysed for appearance, taste, consistency, aroma, after taste and overall acceptability. The results are depicted in Table 5.7.

Formulations	Appearance	Taste	Consistency	Aroma	After Taste	Overall Acceptability
A	7.0	6.7	6.9	6.8	6.7	6.8
B	7.6	7.7	7.9	7.4	7.2	7.6
C	6.8	6.7	7.2	6.8	6.8	6.8

Table 5.7: Sensory evaluation scores based on 9 point hedonic scale

From the results it can be easily concluded that the overall acceptability of Formulation B was the highest (7.6: between moderately to like very much). Thus, formulation B was finalised as the Product composition and all further assays were done using formulation B as the sample.

- Finalised Heating time and temperature: 5 minutes, Boiling.
- Dilution ratio: 10g in 150 mL water.

5.8.2 Proximate analysis for Soup Mix

Proximate analysis of macro nutrients was performed for the Soup Mix (Refer sec. 4.6.4). The results are depicted in Table 5.8. The results are represented in Mean \pm Std. Dev. The results showed high quantity of Protein (15.4%) and crude fibre (6.3%) content in the sample with respect to the commercial samples. Also the amount of fat was significantly low.

Obesity is reported to be less in populations that consume a diet rich in fibre and prevalent in populations that consume a low-fibre in their diet [288]. High fibre content of the beverage mix suggests that this product can be potential food against obesity.

5.8.3 HPLC analysis for water soluble vitamin estimation

Water soluble vitamins were estimated using HPLC analysis (Refer Sec. 4.6.5). The results were expressed as Mean \pm Std. Dev. in mg per 10 gram of sample (Table 5.9).

Results showed that the soup mix contained high quantities of Vitamin C, B1, B2 and B6. High content of Vitamin C suggests high anti-oxidant activity of the soup mix which is an essential clause for the product to work against obesity. Also, vitamin B complex is essential in the process of carbohydrate to glucose conversion and thus higher amounts of Vitamin B complex can play a major role against obesity.

Proximate analysis (per 10g of powder)	
Energy (kcal)	32.12 ± 1.84
Protein (g)	1.54 ± 0.14
Carbohydrate (g)	5.06 ± 0.27
of which Sucrose (g)	0.5 ± 0.02
Total Fat (g)	0.48 ± 0.02
Fibre (g)	0.63 ± 0.01
Moisture (g)	0.7 ± 0.02
Total Ash (g)	1.56 ± 0.08
Minerals (g)	1.45 ± 0.06

Table 5.8: Proximate analysis of Macronutrients for the soup mix

Vitamins analysis by HPLC (per 10g of powder)	
Vitamin C (mg)	2.67 ± 0.03
Vitamin B1 (mg)	2.00 ± 0.011
Vitamin B2 (mg)	0.53 ± 0.02
Vitamin B5 (mg)	0.34 ± 0.07
Vitamin B6 (mg)	0.35 ± 0.032
Vitamin B9 (mg)	0.29 ± 0.025

Table 5.9: HPLC analysis of Water soluble vitamins

Ingredients	Weight in grams
Corn Starch	1.2
Xanthan Gum	0.7
Whey Powder	0.5
<i>Moringa oleifera</i> Powder	0.1
<i>Brassica oleracea</i> Extract	0.12
<i>Ocimum basilicum</i> Extract	0.2
Turmeric Powder	0.1
Black Cumin	0.3
Tea Extract	0.1
Sunflower Oil	0.4
Maltodextrin	0.12
Garlic Powder	1.5
Onion Powder	1
Salt	1.2
Powdered Sugar	0.5
Red Chilli Powder	0.3
Black Pepper Powder	0.15
Chilli Flakes	0.1
Dried vegetable mix	1.41
Sum (Total, g)	10

Table 5.10: Formulation B: Final Product Composition.



Figure 5.18: Anti-obesity Soup Mix



Figure 5.19: Soup Mix labelling

5.8.4 Yield of Extraction and Phytochemical screening of Soup Mix.

Phytochemicals were extracted from the Soup mix by 100% methanol extraction and 80% Ethanol extraction (Refer sec. 4.6.6). Yield of extraction was calculated and these extracts were investigated for Total phenolic and flavonoid content. Results are shown in Table 5.11.

Ferulic acid was taken as the standard for estimating total phenolic content. A standard calibration curve was plotted with weight of Ferulic acid in micrograms vs. absorbance value (Figure 5.20). For flavonoid determination, Quercetin was used as the standard to plot the calibration curve (Figure 5.21).

Results were expressed in mg FAE/g of extract (total phenolic content) and mg QE/g of extract (Total flavonoid content).

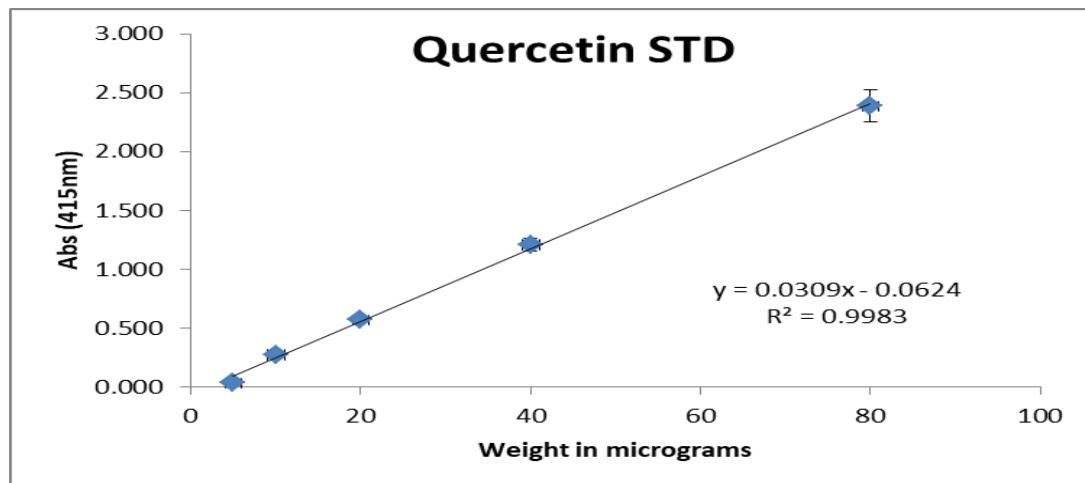


Figure 5.20: Standard calibration curve (Total Flavonoid content: Quercetin)

Analysis	Ethanol Extract	Methanol Extract
Yield (%)	21.81 ± 0.8	24.1 ± 0.52
Total Phenolic content (mg FAE/g)	38.22 ± 4.5	44.48 ± 3
Total Flavonoid Content (mg QE/g)	9.16 ± 1.03	11.25 ± 1.2

Table 5.11: Yield %, total Phenolic and Flavonoid content for Soup mix extract.

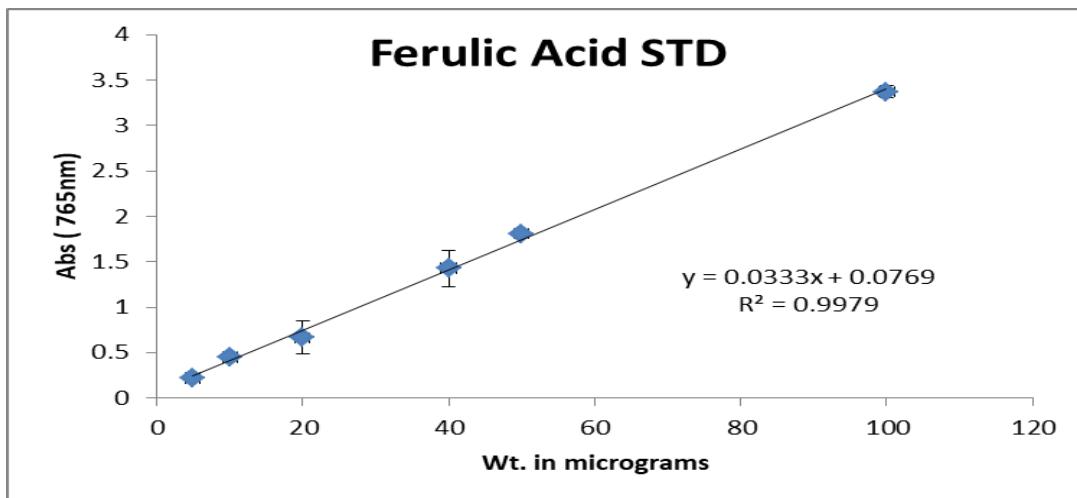


Figure 5.21: Standard calibration curve (Total Phenolic content: Ferulic acid)

Soup mix was found to be a very good source of Polyphenols owing to its high phenolic and flavonoid content. Both flavonoid and phenolic content along with yield % of Methanol extract were relatively higher than Ethanol extracts. These values suggests that Soup mix can be a potential functional food against obesity, but further studies including *In-vivo* studies are necessary to substantiate the clause.

Chapter 6

Conclusion & Future directions

Conclusion

Overweight and obesity have become a major public health issue. It is a serious leading metabolic disease in the world, like high blood pressure, diabetes, strokes and cancer. The data in 2011 showed that each year at least 2.8 million people die due to being overweight or obese. Eating a healthy diet can also help prevent obesity. Consumption of bioactive compounds from diet or dietary supplementation is one of possible ways to control obesity and to prevent or reduce the risks of getting various obesity-related diseases.

The current study was designed to investigate the anti-adipogenic effects of *Moringa oleifera*, *Brassica oleracea* and *Ocimum basilicum* and to identify the major bioactive compounds responsible for their activity against obesity. This study demonstrated that the ethanol extracts of *Moringa oleifera*, *Brassica oleracea* and *Ocimum basilicum* contained significant amount of polyphenols and they also showed high antioxidant activities, suggesting that these plants can be one of the potential sources of safer natural antioxidants. An array of major phenolic antioxidant compounds were identified for each of these plant extracts by the help of UPLC-HRMS/MS analysis, which can counteract the damaging effects of free radicals and can protect the human body against mutagenesis. Thus, replacement of synthetic antioxidants with secondary metabolites exhibiting safe and effective antioxidant activities (because of their manifestations on human health) from abundantly available plant sources such as *M. oleifera*, *B. oleracea* or *O. basilicum* may be advantageous.

The present study indicated that *B. oleracea* and *O. basilicum* extracts are not cytotoxic to 3T3-L1 cells at physiological or supra-physiological concentrations and does not significantly affect the cell viability, while this is not true in the case for *M. oleifera*. *M. oleifera* did significantly affect the cell viability at relatively low concentrations.

Our findings here demonstrate that *B. oleracea* (vegetable) and *O. basilicum* (culinary herb), which are widely consumed around the world, shows promising potential as anti-obesity agents because of their ability to significantly inhibit lipid and triglyceride accumulation in mature adipocyte cells. Compared to control 3T3-L1 cells (Differentiated and undifferentiated), the following results were obtained:

O. basilicum (*OB*) suppressed lipid accumulation more than *B. oleracea* (*BO*) during 3T3-L1 cell differentiation. *OB* down regulated lipid accumulation by 31% and 55% at 0.45 and 0.75 mg/mL, respectively, while *BO* down regulated lipid droplet accumulation only by 26% and 45% at high concentrations of 1 and 3 mg/mL without any significant cell damage. *OB* and *BO*, both also inhibited total triglyceride content of the mature adipocytes by 46% at 0.75 mg/mL and 27% at 3mg/mL, respectively.

Treatment of cells with *OB* up regulated Adipsin expressions to a significant level with respect to differentiated control at all treatment concentrations, while change in expression level was not significant at lower concentration of *BO* (1 and 2 mg/mL). At 3mg/mL, *BO* also significantly enhanced the expression levels of Adipsin. These results suggest that *OB* was able to inhibit adipogenesis in 3T3-L1 cells at relatively lower concentrations than *BO*.

These findings confirmed that both *BO* and *OB* at physiological and supra-physiological concentrations affect the regulation of fat cell volume and number and inhibits lipid droplet accumulation during adipocyte differentiation. Further studies on gene expression and *In-vivo* (animal model) may substantiate the hypothesis and conclude the potential anti-obesity benefits of these plants.

‘Anti-obesity Instant soup mix powder’, a functional food against obesity, incorporated with *B. oleracea* and *O. basilicum* extracts, developed under this project, was widely acceptable with a hedonic scale value of 7.6 in terms of overall acceptability (sensory evaluation). Product was found to be rich in protein and crude fibre content. Vitamin C and Vitamin B complex were also present in significant amount. The phytochemical analysis for product also confirmed its high content of total phenolics and flavonoids. These results suggested that Soup mix can be a potential functional food against obesity, but further studies including *In-vivo* studies are necessary to confirm the clause.

Chapter 7

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