Development of evidence based novel synbiotic components for the prevention and management of lifestyle associated diseases

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By

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CERTIFICATE

This is to certify that the thesis entitled **"Development of evidence based novel synbiotic components for the prevention and management of lifestyle associated diseases"** is an authentic record of research work carried out by **Mr. Arun K B**, under my guidance in partial fulfillment of the requirements for the award of Degree of Doctor of Philosophy in Biochemistry from Cochin University of Science and Technology, and further that no part of this thesis have been submitted elsewhere for any other degree. All the relevant corrections, modifications and recommendations suggested by the audience and the doctoral committee members during the pre-synopsis seminar of **Mr. Arun K B** have been incorporated in the thesis.

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DECLARATION

I hereby declared that the thesis entitled **"Development of evidence based novel synbiotic components for the prevention and management of lifestyle associated diseases"** includes the results of investigations carried out by me at Agro Processing and Natural Products Division, National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram as a full time Research Scholar under the guidance of Dr. P. Nisha and further that no part of this thesis have been presented before for any other degree.

The results and observations whichever included in this thesis is completely known to me and found true.

Thiruvananthapuram
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Arun. K. B.

Dedicated to

My Family & My Teachers…

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Introduction and Review of Literature

1.1 Preface

Diseases date back to the dawn of human race. As humans have evolved, so too have their diseases: some that were once rare have become common, others have disappeared and new varieties have emerged (Blaser, 2006). Many of these changes have taken place in the wake of important transformations in human civilizations and ecology. Poor lifestyle is the overriding health problem that is associated with high risk of many chronic diseases with an array of health consequences. Chronic diseases, often referred to as non-communicable diseases, typically emerge in middle age after long exposure to an unhealthy lifestyle involving tobacco use, a lack of regular exercise, and consumption of typified "fast food" diets which lacks fibre but rich in saturated fats, sugars, and salts. This lifestyle leads to higher levels of risk factors, such as hypertension, diabetes, and cancer that act independently and synergistically. The risk factors are frequently undiagnosed or inadequately managed in health services designed to treat acute conditions. The disease profile of the world is changing rapidly, especially in low and middle income countries.

Chronic non-communicable diseases are currently the main cause of both disability and death worldwide. This heterogeneous group of diseases, including cardiovascular conditions, cancers, chronic respiratory conditions and diabetes, affect people of all ages and social classes (WHO, 2002). Globally, of the 58 million deaths that occurred in 2005, approximately 35 million, or 60%, were due to chronic causes. Most of them were due to cardiovascular disorders and diabetes (32%), cancers (13%), and chronic respiratory diseases (7%) (Abegunde et al., 2007). This burden is predicted to worsen in the coming years. A WHO study projected an increase of global deaths by

a further 17% in the period 2005-2015, meaning that of the 64 million estimated deaths in 2015, 41 million people will die of a chronic disease (WHO, 2005). Chronic life style associated diseases are now the leading cause of death around the world, with developing countries hit hardest, killing 36 million people a year, according to a new report from the World Health Organization (WHO, 2011).

The prevalence of having one or more of the leading lifestyle-related chronic conditions increased steadily in year wise. If these increases continue, particularly among younger adults, managing patients with multiple chronic conditions in the aging population will continue to challenge public health and clinical practice. The current burden of chronic diseases reflects the cumulative effects of unhealthy lifestyles and the resulting risk factors over the life span of people. Some of these influences are present from before a child is born.

1.2 Life style associated diseases

The lifestyle-related diseases are chronic diseases including cancer, diabetes and heart diseases. It is also known as non-communicable diseases (NCDs) against which worldwide actions are being taken. However, as the prevention of such diseases required considerations based on the current state of one's lifestyle and behavior factors such as diet, physical activities, smoking and alcohol consumption as well as the issues related to those, the concept of "lifestyle-related diseases" was introduced (Yeh and Kong, 2013). Together with fast economic development and growing westernization of lifestyle in the past few decades occurrence of life style related diseases has reached terrifying dimensions among Indians in the recent years. Another interesting fact is that, the advancements in medical field decreases the mortality due to chronic diseases,

however the increasing incidence of these chronic diseases even in younger generation has become a problem.

Lifestyles play an important role in determining chronic diseases and lifestyle changes are likely to be responsible for a significant proportion of their increase over time. Smoking alone is estimated to be responsible for 22% of cardiovascular diseases in industrialized countries, and for the vast majority of some cancers and chronic respiratory diseases (WHO, 2002). Alcohol abuse is deemed to be the source of 8%-18% of the total burden of disease in men and 2%-4% in women. The public expenditure on health and long-term care is increasing suggesting an increase from 5.7% of GDP in 2005 to 9.6% in 2050 (Franco and Jeremy, 2008).

The 5 leading causes of death - cancer, diabetes, heart disease, chronic lower respiratory disease and cerebrovascular disease - accounted for more than half of all deaths and represent a high percentage of the world's health care costs (WHO, 2011). Similar disease strategy is following in India's disease profile also (Figure 1.1). Other chronic conditions exert a heavy toll in terms of disease, disability, quality of life, and economic costs. Because the roots of the chronic conditions that are the leading causes of morbidity and mortality can be traced to lifestyle factors - principally smoking, diet, and physical inactivity - it is likely that, despite significant reductions in the prevalence of smoking, the continuing erosion of a low-risk lifestyle profile could result in an increase in the incidence, prevalence, and co-occurrence of lifestyle-related chronic conditions.

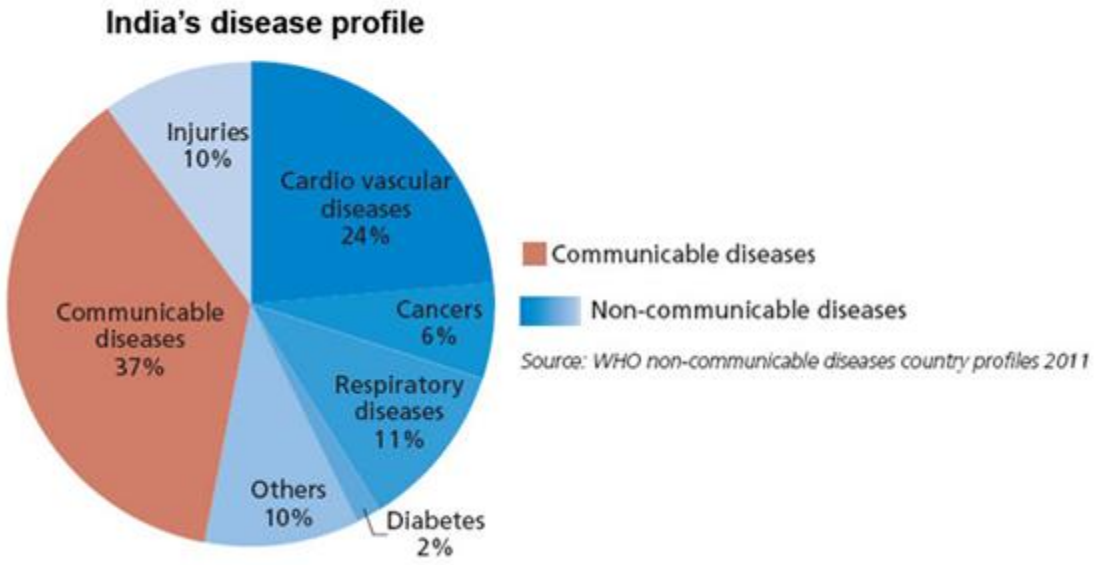

Figure 1.1 The Disease Profile of India (Source: WHO NCD report, 2011)

1.3 Cancer

Today, cancer is a common household word, with each of us closely associated with at least one near and dear one, a family member or a friend, a neighbor or a colleague, diagnosed with cancer. Cancer is a genetic term for a large group of diseases that can affect any part of the body (Conti et al., 2010). One defining feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries and which can invade adjoining parts of the body and spread to other organs. This process is referred to as metastasis. Metastases are the major cause of death from cancer. Thus cancer arises as one normal cell transformed into a tumor cell in a multistage process. These changes are the result of the interaction between a person's genetic factors and 3 categories of external agents – physical carcinogens, chemical carcinogens and biological carcinogens.

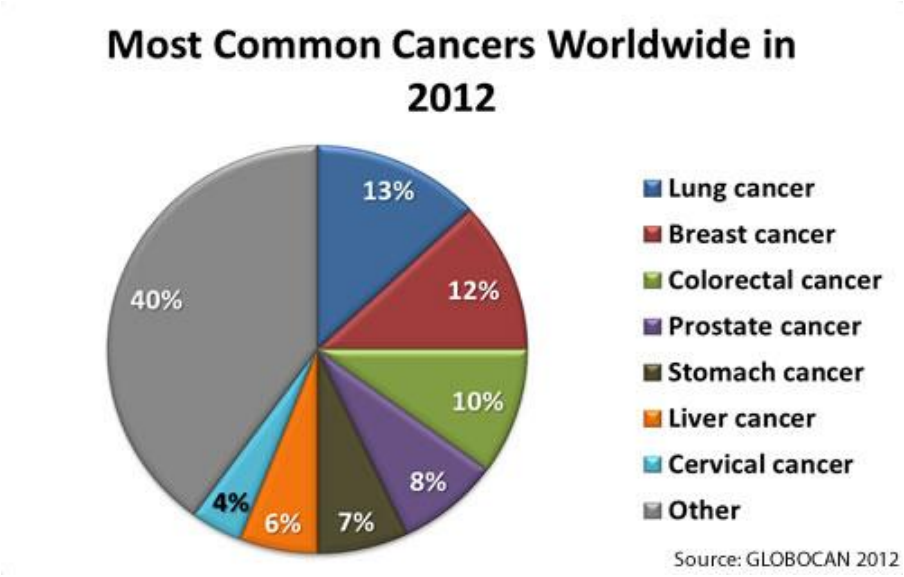

Figure 1.2 Common cancers worldwide in 2012 (Source: GLOBOCAN 2012)

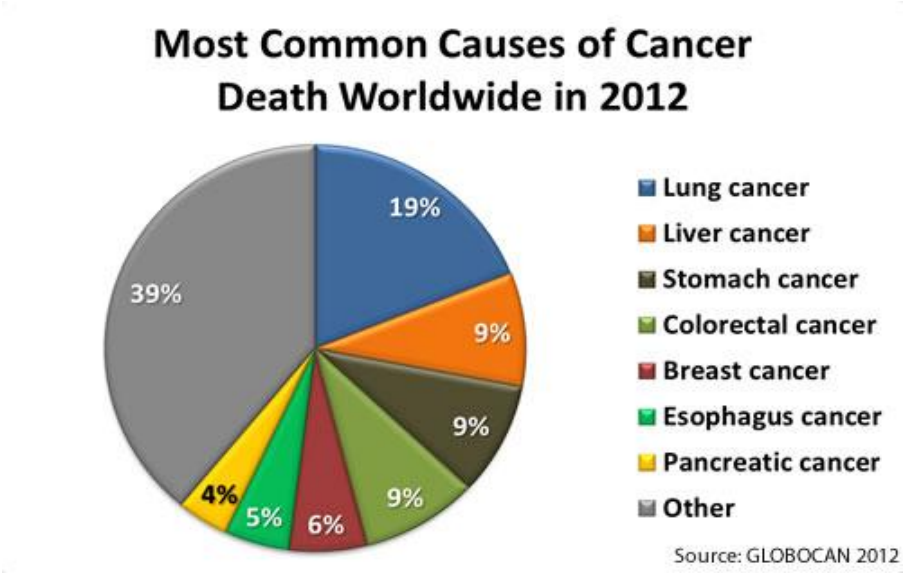

Figure 1.3 Causes of cancer death worldwide in 2012 (Source: GLOBOCAN 2012)

Cancers figure among the leading causes of morbidity and mortality worldwide (Figure 1.2 and 1.3), with approximately 14 million new cases and 8.2 million cancer related deaths in 2012 (World Cancer Report, 2014). The number of new cases is expected to rise by about 70% over the next 2 decades. Among men, the 5 most common sites of cancer diagnosed in 2012 were lung, prostate, colorectum, stomach,

and liver cancer. Among women the 5 most common sites diagnosed were breast, colorectum, lung, cervix, and stomach cancer. Reports have shown that around one third of cancer deaths are due to the behavioral and dietary risks: high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, alcohol use (Anand et al., 2008). More than 30% of cancer deaths could be prevented by modifying or avoiding key risk factors. Tobacco use is the single most important risk factor for cancer causing about 20% of global cancer deaths and around 70% of global lung cancer deaths.

Cancer causing viral infections such as Hepatitis B virus/ Hepatitis C virus and Human Papilloma Virus are responsible for up to 20% of cancer deaths (liver and cervical cancer respectively) in low- and middle-income countries (de Martel et al., 2012). Infection with Human immunodeficiency virus substantially increases the risk of cancer such as cervical cancer and Kaposis sarcoma. More than 60% of world's total new annual cases occur in Africa, Asia and Central and South America. These regions account for 70% of the world's cancer deaths. It is expected that annual cancer cases will rise from 14 million in 2012 to 22 within the next 2 decades.

According to International Agency for Research on Cancer (Ferlay et al., 2013), 1.1 million new cancer cases were estimated in India and contribute 7.8% of global cancer burden and 8.33% of global cancer deaths. The five most common cancers in both sexes in India were cancers of the breast (14.3%), cervix uteri (12.1%), oral (7.6%), lung cancer (6.9%) and colorectum cancer (6.3%) comprising 47.2% of the 28 cancers reported.

1.3.1 Colorectal Cancer

Colorectal cancer is a major cause of morbidity and mortality throughout the world. It accounts for over 9% of all cancer incidences (World Cancer Research Fund, 2007). It affects men and women almost equally with nearly 1.4 million new cases diagnosed in 2012. It is predicted that worldwide the number of cases will rise to 1.36 million for men and 1.08 million for women by 2035 (World Cancer Research Fund International, 2014).

In India, the annual incidence rates (AARs) for colon cancer and rectal cancer in men are 4.4 and 4.1 per 100000, respectively. The AAR for colon cancer in women is 3.9 per 100000. Colon cancer ranks 8th and rectal cancer ranks 9th among men. For women, rectal cancer does not figure in the top 10 cancers, whereas colon cancer ranks 9th (NCRP, 2013). In the 2013 report, the highest AAR in men for CRCs was recorded in Thiruvananthapuram (4.1) followed by Bangalore (3.9) and Mumbai (3.7). The highest AAR in women for CRCs was recorded in Nagaland (5.2) followed by Aizwal (4.5) (NCRP, 2013).

Risk factors for CRC can be broadly divided into genetic and environmental or lifestyle-related factors. Most CRCs are sporadic, although genetic factors increase the risk considerably.

1.3.1.1 Genetic factors

Genetic factors can be classified as those associated with colonic polyposis and those not associated with colonic polyposis. Among the colonic polyposis syndromes, familial adenomatous polyposis (FAP) and its variants (Turcot, Gardner, and attenuated FAP) and MYH-associated polyposis are the most common. Hereditary nonpolyposis colon

cancer (HNPCC) comprises the non-colonic polyposis category.

- FAP is characterized by multiple colonic adenomatous polyps appearing in childhood with subsequent transformation to malignancy at an average age of 45 years and is caused by a germline mutation in the APC gene on chromosome 5 (Burt et al., 1995).
- Turcot syndrome is a variant of FAP in which there exists an association between multiple colorectal adenomas and primary neuroepithelial brain tumours as a result of a germline APC mutation or mutations in mismatch repair genes (MMR).
- Gardner syndrome includes mandibulomaxillary osteomas and multiple epidermoid cysts along with multiple colonic polyps.
- Attenuated FAP is associated with the same genetic mutation as FAP but is characterized by fewer adenomas and a later average age at CRC presentation.
- MYH-associated polyposis is inherited in an autosomal recessive pattern, with mutations in the base excision repair gene mutY homologue (Dolwani et al., 2005).
- Lynch syndrome is an autosomal dominant condition and is caused by a defect in one of the MMR genes. The peculiarity of Lynch syndrome is the early average age of onset of colorectal malignancy and the predominance of right-sided colonic lesions (Van Vliet et al., 2011; Guarinos et al., 2010).

Recent genome-wide analyses of solid tumours in CRC have shown mutations in between 20 and 100 protein-encoding genes (Wood et al., 2007). A number of key genetic and epigenetic alterations which lead to malignant transformation have been

identified in CRC, and these include aberrations in genes involved in the chromosomal instability pathway, the microsatellite instability pathway, the hMYH pathway and the CpG island methylation pathway (Migliore et al., 2011). Recent data have shown that *KRAS* and *BRAF* mutations predict response to anti-epidermal growth factor receptor therapy (Markman et al., 2010).

1.3.1.2 Environmental factors

The environmental factors can be summarized as follows:

- Age and gender: Older men are at a high risk (25% higher in men than in women) (Jernal et al., 2010)
- Diabetes mellitus associated with insulin resistance: This linked to the long-term effects of insulin-like growth factors (Giovannucci, 1995; Yuhara et al., 2011)
- Decreased dietary fibre and fruit intake, Decreased physical activity, Presence of coronary heart disease (Sriamporn et al., 2007)
- Alcohol consumption (Cho et al., 2012); Obesity (Harris et al., 2009); Cigarette smoking (Ji et al., 2002); red meat consumption (English et al., 2004)
- Ulcerative colitis: The extent, duration, and activity of disease are primary determinants (Ekbom et al., 1990)
- Ethnicity: The African American population is at an increased risk.
- Long-term immunosuppression following organ transplantation, especially renal transplantation (Collins et al., 2012)

1.3.1.3 Different stages of CRC

The different stages of colorectal cancer (Figure 1.4) are described below:

- **Stage 0** (Also known as Duke A stage). - The earliest stage. It is still within the

mucosa (inner layer) of the colon or rectum - also called carcinoma *in situ*.

- **Stage I** (Also known as Duke B stage). - It has grown through the inner layer of the colon or rectum, but has not yet spread beyond the wall of the rectum or colon.
- **Stage II** (Also known as Duke C stage). - It has grown through or into the wall of the colon or rectum. However, it has not reached the nearby lymph nodes yet.
- **Stage III** (Also known as Duke D stage). - The nearby lymph nodes have been invaded by the cancer, but it has not yet affected other parts of the body.
- **Stage IV** (Also known as Duke E stage). - It has spread to other parts of the body, including other organs, such as the liver, the membrane lining the abdominal cavity, lung, or ovary.

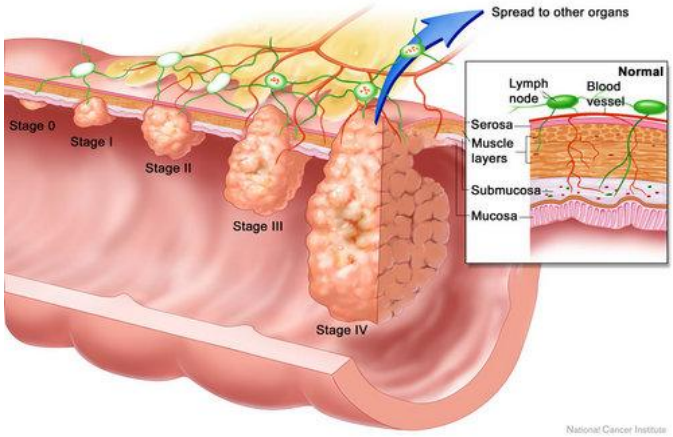

Figure 1.4 Different stages of colorectal cancer (Source: Terese Winslow, National Cancer Institute, 2005)

1.3.1.4 Current treatment strategies

Currently four important approaches are used for the treatment of CRC - surgery, chemotherapy, radiotherapy and targeted therapies. The mainstay of CRC treatment is surgery. In early stage disease (stage 0 or I), surgical excision can be used without need for further treatment options, as the recurrence rate is very low (Kobayashi et al., 2011).

Adjuvant therapy (chemo or radio therapy) can increase survival benefit of stage II and III patients. Patients with stage IV disease require chemotherapy or targeted therapies combined with surgery, where appropriate (Hagan et al., 2013).

Due to better understanding of the molecular pathology of cancer, a number of targeted agents have been developed which have demonstrated improved outcome in CRC patients, with combination chemotherapy. A number of different drugs have significant antitumour activity in CRC, including the systemic drugs 5-fluorouracil (5-FU), irinotecan, oxaliplatin, bevacizumab, cetuximab and panitumumab, and the oral drug capecitabine. Different combinations of these drugs, such as the FOLFOX regimen (leucovorin, 5-FU and oxaliplatin), the FOLFIRI regimen (leucovorin, 5-FU and irinotecan) and the XELOX regimen (oxaliplatin and capecitabine), with or without a monoclonal antibody agent, have been shown to improve outcomes in CRC (Edwards et al., 2012).

Over the past few decades, CRC incidence has been rapidly increasing, especially in developed countries (Center et al., 2009). The considerable geographic variation in incidence of CRC suggests that life style, especially dietary factors, may play vital roles in the development of CRC (Huxley et al,. 2009). The role of diet and lifestyle factors has long been suspected and investigated in CRC development, with specific dietary constituents, in addition to excessive caloric intake, weight gain, physical inactivity, smoking, and heavy alcohol intake all thought to result in elevated risk (Giovannucci et al., 1992). The differences in rates by country, and elevated risk among immigrants from a low- to high-risk country (Flood et al., 2000), support that environmental factors are important in CRC risk. In one study, it was estimated that

dietary factors contributed to nearly 50% of all CRC cases diagnosed, while the attributable risk was only about 10% for family history (Kune et al., 1992). Therefore, a healthy diet and lifestyle are seen as essential in primary prevention of CRC (Shike, 1999).

1.4 Diabetes

Diabetes mellitus (DM) is a metabolic disorder resulting from a defect in insulin secretion, insulin action, or both. Insulin deficiency in turn leads to chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism (Sicree et al., 2006). It is the most common endocrine disorder and International Diabetic Federation (2013) most recent estimates indicate that 8.3% of adults – 382 million people – have diabetes, and the number of people with the disease is set to rise beyond 592 million in 2035. India ranks second in having diabetic patients (Figure 1.5).

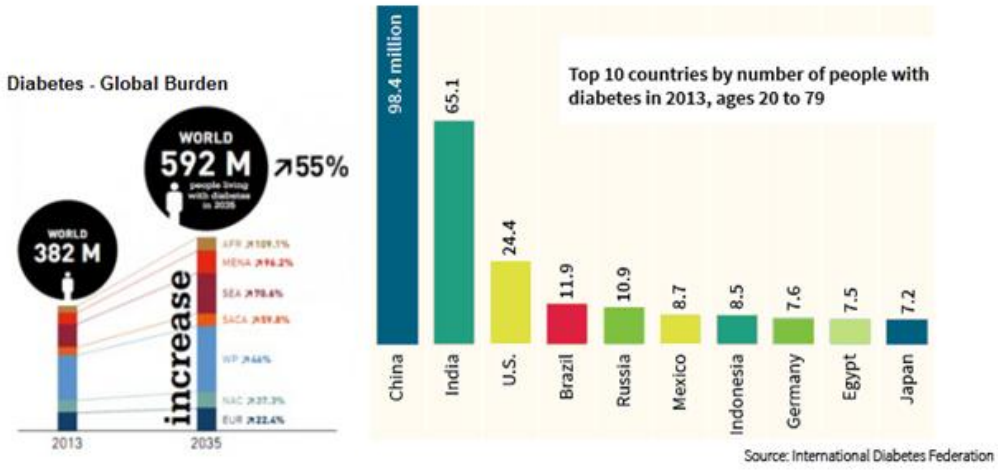

Figure 1.5 Global diabetes burden and list of top 10 countries (Source: IDF Diabetes Atlas, 2013)

1.4.1 Diabetes classification

Diabetes mellitus is classified based on its etiology and clinical presentation. As such, there are four types or classes of diabetes mellitus (Sicree et al., 2006):

- Type 1 diabetes
- Type 2 diabetes
- Gestational diabetes, and
- other specific types

Type 1 diabetes is caused by an autoimmune reaction, where the body's defense system attacks the insulin-producing beta cells in the pancreas. As a result, the body can no longer produce the insulin it needs. Type 1 diabetic patients required daily administration of insulin in order to control the levels of glucose in their blood. The incidence of type 1 diabetes is increasing in both rich and poor countries. The reasons for this are still unclear but may be due to changes in environmental risk factors, early events in the womb, diet early in life, or viral infections.

Type 2 diabetes is the most common type of diabetes and usually occurs in adults. In this case, the body is able to produce insulin but either this is not sufficient or the body is unable to respond to its effects (also known as insulin resistance), leading to a build-up of glucose in the blood. Obesity, poor diet, physical inactivity, advancing age, family history of diabetes, ethnicity and high blood glucose during pregnancy affecting the unborn child are the main risk factors for the development of Type 2 diabetes. The number of people with Type 2 diabetes is growing rapidly worldwide. This rise is associated with economic development, ageing populations, increasing urbanization, dietary changes, reduced physical activity, and changes in other lifestyle patterns (WHO, 1994).

Women who develop a resistance to insulin and subsequent high blood glucose during pregnancy are referred to as gestational diabetes. The condition arises because

the action of insulin is blocked, probably by hormones produced by the placenta. Babies born to mothers with gestational diabetes also have a higher lifetime risk of obesity and developing type 2 diabetes.

In addition to Type 1, Type 2 and gestational diabetes, there are a range of other types – Maturity onset diabetes of the young, neonatal diabetes etc. which are usually rare but run strongly in families. Other types also include genetic defects of the pancreatic ß cell or in insulin action pathways (insulin receptor mutations or post-receptor defects) (Raffel et al., 1997) as well as disease of the exocrine pancreas (e.g., Pancreatitis, pancreatic reaction, or cystic fibrosis), and endocrinopathies producing insulin counter regulatory hormones excess (e.g., Cushing's syndrome, acromegaly) are less common causes of diabetes (American Diabetes Association, 2001).

1.4.2 Diabetes associated complications

People with diabetes are at risk of developing a number of disabling and life-threatening health problems. Consistently high blood glucose levels can lead to serious diseases affecting the heart and blood vessels, eyes, kidneys, and nerves. People with diabetes are also at increased risk of developing infections. In almost all high-income countries, diabetes is a leading cause of cardiovascular disease, blindness, kidney failure, and lower-limb amputation. Maintaining blood glucose levels, blood pressure and cholesterol close to normal can help delay or prevent diabetes complications.

Cardiovascular disease is the most common cause of death and disability among people with diabetes (Go et al., 2013). The cardiovascular diseases that accompany diabetes include angina, myocardial infarction, stroke, peripheral artery disease, and congestive heart failure. About 65 percent of people with diabetes die from

heart disease and stroke. Current diabetes guidelines also emphasize that cardiovascular disease risk reduction should be a focus of therapy (Inzucchi et al., 2012). In people with diabetes, high blood pressure, high cholesterol, high blood glucose and other risk factors contribute to the increased risk of cardiovascular complications. When hyperglycemia develops in type 2 diabetes, it produces toxic effects on the endothelium both directly (Cosentino, 2004) and through the formation of advanced glycation end products (Basta et al., 2004) which can themselves perpetuate an inflammatory response in the endothelium. However, atherosclerosis may occur only as many as ten years after the development of diabetes (Caterina et al., 2006). Evidence now exists that insulin allows the initiation and perpetuation of vascular inflammation, through the increased gene expression of VCAM-1, MCP-1, macrophage colony stimulating factor, CD-40L, and similar molecules (Madonna et al., 2004). Therefore, the increase in insulin production and plasma concentration that accompanies the compensated phase of insulin resistance appears to increase atherogenic risk directly.

Retinopathy (eye diseases), nephropathy (kidney diseases) and neuropathy (nerve damage) are also seen associated with diabetes. Due to damage in nerves and blood vessels diabetic patients are prone to have many foot and oral problems.

1.4.3 Type 2 diabetes mellitus

Type 2 diabetes is the most common form of diabetes. It was first described as a component of metabolic syndrome in 1988 (Patlak, 2002), characterized by hyperglycemia, insulin resistance, and relative insulin deficiency. It results from interaction between genetic, environmental and behavioral risk factors (Olokoba et al., 2012). Type 2 diabetic patients are vulnerable to various forms of both short- and long-

term complications, which often lead to their premature death. Apart from life style changes as described earlier, there is a strong inheritable genetic connection in type 2 diabetes. Several genes – TCF7L2, PPARG, FTO, KCNJ11, NOTCH2, WFS1, CDKAL1, and IGF2BP2 are involved in the development of Type 2 diabetes.

Type 2 diabetes is characterized by insulin insensitivity, reduced insulin production, and eventual pancreatic beta-cell failure (Khan, 1994). This leads to a decrease in glucose transport into the liver, muscle cells, and fat cells. There is an increase in the breakdown of fat with hyperglycemia. The body attempts to arrest hyperglycemia, by drawing water out of the cells and into the bloodstream. The excess sugar is excreted in the urine. This is why diabetics present with constant thirst, drinking large amounts of water, and polyuria as the cells try to get rid of the extra glucose which subsequently leads to glucosuria. As hyperglycemia prolongs, the body cells are devoid of glucose which forces the cells to seek alternative mobilizable energy sources especially fatty acids stored in adipose tissue which in turn increases ketosis and acidosis.

Type 2 diabetes should be regarded as a potentially preventable disease. Life style management is apparently the cornerstone of management of Type 2 diabetes. It is recognized as being an essential part of diabetes and cardiovascular disease prevention. A study done in Australia Aborigines demonstrated marked improvement in carbohydrate and lipid metabolism in patients with type 2 DM who reverted to a traditional lifestyle (O'Dea, 1984). An important large-scale prospective study in China, examined the effects of diet and exercise upon the rate of progression of IGT to diabetes; both the measures, alone or together reduced the progression of the disease by

40% after 6 years (Pan et al., 1997). Studies have shown that there was significant reduction in the incidence of Type 2 diabetes with a combination of maintenance of body mass index of 25 kg/m^2, eating high fibre and unsaturated fat and diet low in saturated and trans-fats and glycemic index, regular exercise, abstinence from smoking and moderate consumption of alcohol (Olokoba et al., 2012). Insulin is also important in Type 2 diabetes when blood glucose levels cannot be controlled by diet, weight loss, exercise and oral medications. Oral hypoglycaemic agents - sulphonylureas, biguanides, alpha glucosidase inhibitors and thiazolidenedione are, also useful in the treatment of Type 2 diabetes (Kumar and Clark, 2002). The main objective of these drugs is to correct the underlying metabolic disorder, such as insulin resistance and inadequate insulin secretion.

1.5 Diet

Dietary practices are the usual decisions taken by an individual or culture what foods to eat. Traditional plant-based diets including foods such as cereals and vegetables are increasingly being replaced by diets that are richer in added sugars and animal fats. This nutritional transition, combined with a general trend towards a more sedentary lifestyle, is an underlying factor in the risk of developing chronic diseases. An unhealthy diet has been implicated as risk factors for the development of chronic non-communicable diseases like colon cancer, diabetes and cardiovascular diseases (Scardina and Messina, 2012). The average food consumption (in terms of calories and fat content but lesser dietary fibre) appears to have increased steadily in countries around the world. As a result of changes in the way we eat and live, these chronic diseases are increasingly affecting both developed and developing countries. Indeed, diet-related chronic diseases

are the most common causes of death in the world. Worldwide, diets low in fruits rank third for deaths attributable to individual risk factors (Ezzati and Riboli, 2013).

1.5.1 Diet and Colorectal cancer

Diet has long been thought to have a role in the etiology of CRC, particularly when a poor diet is combined with excess calorie intake and weight gain, physical inactivity, and unhealthy practices, such as smoking and consuming a great deal of alcohol. It has been estimated that 45 percent of all colorectal cancer cases can be prevented in high-risk populations through modifications of diet, physical activity habits, and weight control (WCRF and AICR, 2007). According to the recent report from the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR), there is convincing evidence that dietary fiber protects against colorectal cancer and that red and processed meat and alcohol (particularly in men) increase the risk of the disease (WCRF and AICR, 2011).

Consumption of red meat might be related directly to the incidence of CRC or indirectly because a diet high in meat tends to be low in vegetables, fruit, and fibre. Bidoli et al. (1992) found that high intake of refined starches, eggs, cheese, and red meat increased risk of CRC. Risk of colorectal cancer was about twice as great among those who consumed these foods more frequently. On the other hand, more frequent consumption of tomatoes was associated with a 50% and 60% reduction in risk of colon and rectal cancer, respectively. The carcinogenic effect of heterocyclic amines, produced during cooking of red meat, has been suggested as the link between red meat and CRC (Ryan-Harshman and Aldoori, 2007). High consumption of fruit was associated with a 32% reduction in risk of CRC, while high intake of cereal fibre did

not lower risk of CRC (Terry et al., 2001) among Swedish women. Some researchers have suggested that a diet high in fat and meat and low in dietary fibre might affect the integrity of colonic cells (Rieger et al., 1999). Others have suggested that certain plant cell-wall constituents, suberin and lignin, adsorb heterocyclic amines and thus protect against CRC (Harris et al., 1996). Potato skins contain suberin. Wheat bran contains lignin. Folic acid supplementation was found to exert a substantial decrease in colon cancer among ulcerative colitis patients (Lashner et al., 1989). Calcium and vitamin D are thought to reduce risk of CRC through mechanisms that decrease cell proliferation or promote cell differentiation (Peters et al., 2001). All these evidences suggest that diet has an effect on the incidence of CRC and might be affected by the multifactorial nature of CRC. Making appropriate choices across food groups, particularly with respect to fruit, vegetables, and fibre, is the key to healthy eating.

1.5.2 Diet and Diabetes

Type 2 diabetes is a global public health crisis that threatens the economies of all nations, particularly developing countries. Fueled by rapid urbanization, nutrition transition, and increasingly sedentary lifestyles, the epidemic has grown in parallel with the worldwide rise in obesity (Hu, 2011). An unhealthy diet has been regarded as a major contributor to the development of type 2 diabetes. Excessive caloric intake is a major driving force behind escalating obesity and type 2 diabetes epidemics worldwide, but diet quality also has independent effects. In particular, higher dietary glycemic load and trans fat are associated with increased diabetes risk, whereas greater consumption of cereal fiber and polyunsaturated fat is associated with decreased risk (Hu et al., 2001). Evidence also indicates that higher consumption of sugar-sweetened beverages

increases the risk of type 2 diabetes (Malik et al., 2010). Both vegetable and animal ghee, which are used for cooking in India and other South Asian countries, have an extremely high trans fatty acid (Popkins, 2001) content plays a role in the development of insulin resistance (Lopez-Garcia et al., 2005). Studies showed that higher consumption of white rice is associated with increased risk of diabetes, whereas consumption of brown rice, a whole grain, protects against the disease (Sun et al., 2010).

In the past couple of decades, evidence from prospective observational studies and clinical trials has converged to support the importance of individual nutrients, foods, and dietary patterns in the prevention and management of type 2 diabetes. The quality of dietary fats and carbohydrates consumed is more crucial than is the quantity of these macronutrients. Diets rich in whole grains, fruits, vegetables, legumes, and nuts; moderate in alcohol consumption; and lower in refined grains, red or processed meats, and sugar-sweetened beverages have been shown to reduce the risk of diabetes and improve glycaemic control and blood lipids in patients with diabetes (Ley et al., 2014). Fiber has been studied in the treatment of diabetes for many years because increased fiber content decreases the glycemic index of foods (Anderson et al., 1979; Jenkins et al., 2002; Marangoni and Poli, 2008) which would lead to smaller increases in blood glucose, and thus reduced blood glucose.

1.5.3 Diet and Cardiovascular diseases

Cardiovascular disease is likely the most studied chronic human disease, yet it remains the most common throughout the world and is the leading cause of premature morbidity and mortality in industrialized countries (Caterina et al., 2006). Research on the reason

behind the development of coronary heart disease has been ongoing for approximately a century (Connor, 1999). The role of diet is crucial in the development and prevention of cardiovascular diseases. Initially, cholesterol - an important component in diet, was found to play an important role in development of atherosclerotic plaque and myocardial infarction thereafter. Later the dietary fatty acids were found as the determinants of serum cholesterol level (Menotti, 1999). The study of lipoprotein metabolism showed that the cholesterol-rich LDL fraction, not total cholesterol, was most strongly related to the development of atherosclerosis (Gofman et al., 1950). The LDL cholesterol fractions are usually increased by saturated and trans fatty acids present in diet (Clarke et al., 1997). Complex interactions between diet, lifestyle, and lipoprotein metabolism determine the development of atherosclerosis and its complications.

Obesity is a recognized risk factor for cardiovascular disease. The mechanisms responsible for this increased risk appear to be related to obesity itself and to insulin resistance. Several studies have linked insulin resistance to systemic inflammation (Grimble, 2002), possibly as the result of increased concentrations of circulating free fatty acids (Boden and Shulman, 2002). The low mortality from cardiovascular disease among traditionally living Eskimos and Alaskan Natives has been attributed to less atherosclerosis in the coronary arteries and elsewhere than in non-natives because of a high dietary intake of n−3 poly unsaturated fatty acids from fish or fish-derived products (Hu et al., 2003). Diet can affect the vast majority of modifiable risk factors for cardiovascular disease, which are now identified as explaining a very large part of

the variability in the occurrence of a first acute myocardial infarction. Proper selection of diet offers incredible opportunities for prevention of cardiovascular disease.

Diet interventions and natural bioactive supplements have now been extensively studied to reduce the risks of non-communicable diseases, as a cause of prevention instead of cure. Consuming predominantly plant-based diets reduces the risk of developing obesity, diabetes, cardiovascular diseases, and some forms of cancer. Plant-based diets are high in vegetables and fruits, whole grains, pulses, nuts and seeds, and have only modest amounts of meat and dairy. These diets help to achieve and maintain a healthy weight, reduce blood pressure, and are also rich in sources of dietary fibre (which protects against colorectal cancer).

1.6 Dietary fibre

The term "dietary fibre" was coined by Hipsley (1953), but the health benefits of high fibre foods have been long appreciated. In 430 BC, Hippocrates described the laxative effects of coarse wheat in comparison with refined wheat (Slavin, 1987). In the 1920s, J.H. Kellogg published extensively on the attributes of bran (Slavin, 1987), claiming it increased stool weight, promoted laxation, and prevented disease. Burkitt (1971) is usually credited with re-popularizing the idea that dietary fibre protects against development of Western diseases, including diabetes, cardiovascular disease, colon cancer, and obesity. He observed that rural Ugandans consuming a diet rich in dietary fiber had a low rate of colorectal cancer.

According to AACC report (2001) dietary fibre is defined as the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine.

Dietary fibre includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibres promote beneficial physiological effects, including laxation, and blood cholesterol and glucose attenuation. The beneficial effects of DF for human health have been widely reported (Champ & Guillon, 2000; Reddy, 1982; Schneeman, 1987). Dietary guidelines recommend an increase in consumption of DF rich products, such as cereals, vegetables and fruits.

Tungland and Meyer (2002) suggested several different classification systems to classify the components of dietary fibre: based on their role in the plant, based on the type of polysaccharide, based on their simulated gastrointestinal solubility, based on site of digestion and based on products of digestion and physiological classification. The most widely accepted classification for dietary fibre has been to differentiate dietary components on their solubility in a buffer at a defined pH, and/or their fermentability in an *in vitro* system using an aqueous enzyme solution representative of human alimentary enzymes. Thus most appropriately dietary fibre is classified into two categories such as water- insoluble/less fermented fibres: cellulose, hemicellulose, lignin and the water- soluble/well fermented fibres: pectin, gums and mucilages (Anita and Abraham, 1997). The classification of dietary fibre on the basis of water solubility and fermentability is shown in Table 1.1.

Dietary fibre is naturally present in cereals, vegetables, fruits and nuts. The amount and composition of fibres differ from food to food (Desmedt and Jacobs, 2001). A fibre-rich diet is lower in energy density, often has a lower fat content, is larger in volume and is richer in micronutrients. This larger mass of food takes longer to eat and its presence in the stomach may bring a feeling of satiety sooner, although this feeling

of fullness is short term (Rolls et al., 1999). It is suggested that healthy adults should eat between 20 and 35 g of dietary fibre each day.

Table 1.1 Classification of dietary fibre components based on water solubility/fermentability

Characteristic	Fibre component	Description	Main food sources
Water insoluble/Less fermented	Cellulose	Main structural component of plant cell wall. Insoluble in concentrated alkali, soluble in concentrated acid.	Plants (vegetables, sugar beet, various brans)
	Hemicellulose	Cell wall polysaccharides, which contain backbone of β glucosidic linkages. Soluble in dilute alkali.	Cereal grains
	Lignin	Non-carbohydrate cell wall component. Complex cross-linked phenyl propane polymer. Resists bacterial degradation.	Woody plants
Water soluble/Well fermented	Pectin	Components of primary cell wall with D-galacturonic acid as principal components. Generally water soluble and gel forming	Fruits, vegetables, legumes, sugar beet, potato
	Gums	Secreted at site of plant injury by specialized secretary cells. Food and pharmaceutical use.	Leguminous seed plants (guar, locust bean), seaweed extracts (carrageenan, alginates), microbial gums (xanthan, gellan)
	Mucilages	Synthesized by plant, prevent desiccation of seed endosperm. Food industry use, hydrophilic, stabilizer.	Linseed, Plant ovate husk mucilage, Ocimum etc.
	Exudates	Exudates are considered complex mixtures of plant origin with solid or semi solid consistency such as resins, oleoresins, balsams and gums	Gum acacia, Gum karaya, Gum tragacanth

Source: Dhingra et al., 2012 (modified).

Several non-starch food provide up to 20–35 g of fibre/100 g dry weight and other those containing starch provide about 10 g/100 g of dry weight and the content of fibre of fruits and vegetables is 1.5–2.5 g/100 g of dry weight (Selvendran and Robertson, 1994). Lambo et al. (2005) reported, cereals to be one of the main sources of dietary fibre, contributing to about 50% of the fibre intake in western countries, 30–40% dietary fibre may come from vegetables, about 16% from fruits and the remaining 3% from other minor sources. Dietary fibre content of various food sources are shown in Table 1.2.

Table 1.2 Dietary fibre content of various food sources

Source	Dietary fibre (g/100 g edible portion)		
	Total	**Insoluble**	**Soluble**
Grains			
Barley	17.3	–	–
Corn	13.4	–	–
Oats	10.3	6.5	3.8
Rice (dry)	1.3	1.0	0.3
Rice (cooked)	0.7	0.7	0.0
Wheat (whole grain)	12.6	10.2	2.3
Wheat germ	14.0	12.9	1.1
Legumes & pulses			
Green beans	1.90	1.40	0.50
Soy	15.0	–	–
Peas, green frozen	3.5	3.2	0.3
Kidney beans, canned	6.3	4.7	1.6
Lentils, raw	11.4	10.3	1.1
Lima beans, canned	4.2	3.8	0.4
White beans, raw	17.7	13.4	4.3
Vegetables			
Potato, no skin	1.30	1.0	0.30
Bitter gourd	16.6	13.5	3.1
Beetroot	7.8	5.4	2.4
Fenugreek leaves	4.9	4.2	0.7
Ladyfinger	4.3	3.0	1.3
Spinach, raw	2.6	2.1	0.5
Turnips	2.0	1.5	0.5
Tomato, raw	1.2	0.8	0.4
Green onions, raw	2.2	2.2	0.0
Eggplant	6.6	5.3	1.3

Cucumbers, peeled	0.6	0.5	0.1
Cauliflower, raw	1.8	1.1	0.7
Celery, raw	1.5	1.0	0.5
Carrot, raw	2.5	2.30	0.20
Broccoli, raw	3.29	3.00	0.29
Fruits			
Apple, unpeeled	2.0	1.8	0.2
Kiwi	3.39	2.61	0.80
Mango	1.80	1.06	0.74
Pineapple	1.20	1.10	0.10
Pomegranate	0.60	0.49	0.11
Watermelon	0.50	0.30	0.20
Grapes	1.2	0.7	0.5
Oranges	1.8	0.7	1.1
Plums	1.6	0.7	0.9
Strawberry	2.2	1.3	0.9
Bananas	1.7	1.2	0.5
Peach	1.9	1.0	0.9
Pear	3.0	2.0	1.0
Nuts and seeds			
Almonds	11.20	10.10	1.10
Coconut, raw	9.0	8.5	0.5
Peanut, dry roasted	8.0	7.5	0.5
Cashew, oil roasted	6.0	–	–
Seasame seed	7.79	5.89	1.90
Flaxseed	22.33	10.15	12.18

Source: Farhath Khanum et al., 2000; Schakel et al., 2001

The diets with a high content of fibre, such as those rich in cereals, fruits and vegetables have a positive effect on health since their consumption has been shown to decrease incidence of several types of diseases as due to its beneficial effects like increasing the volume of fecal bulk, decreasing the time of intestinal transit, cholesterol and glycaemic levels, trapping substances that can be dangerous for the human organism (mutagenic and carcinogenic agents), stimulating the proliferation of the intestinal flora etc. (Heredia et al., 2002; Beecher, 1999). Some functions and benefits of dietary fibre on human health are summarized in Table1.3.

Table 1.3. Functions and benefits of dietary fibre on human health

	Functions	Benefits
1.	Adds bulk to the diet, making feel full faster	May reduce appetite
2.	Attracts water and turns to gel during digestion, trapping carbohydrates and slowing absorption of glucose	Lowers variance in blood sugar levels
3.	Lowers total and LDL cholesterol	Reduces risk of heart disease
4.	Regulates blood pressure	May reduce onset risk or symptoms of metabolic syndrome and diabetes
5.	Speeds the passage of foods through the digestive system	Facilitates regularity
6.	Adds bulk to stool	Alleviates constipation
7.	Balances intestinal pH and stimulates intestinal fermentation production of short-chain fatty acids	May reduce risk of colorectal cancers

Source: Dhingra et al., 2012.

On the other hand, there is a wide developing market for natural food antioxidants based on two aspects: well documented protective effect against cancer and cardiovascular diseases of these natural antioxidants (Garewall, 1997), and a general rejection of synthetic antioxidants by the consumers. Fruits, vegetables, and beverages are important sources of natural antioxidants, which are extracted using different solvents and expensive processes such as supercritical fluids (Starmans and Nijhuis, 1996). Flavonoids and other plant phenolics have been reported to have several biological effects such as antioxidant activity, inhibition of platelet aggregation, and antimicrobial and antiinflammatory action (Ho et al., 1992). Also, dietary flavonoids have been associated with a reduced risk of cardiovascular diseases and cancer (Ohigashi et al., 1997). These plant foods generally possess dietary fibre with high SDF/IDF ratio. The dietary fibre acts as a mesh entrapping antioxidants like vitamins C and E, bioactive compounds (flavonoids and carotenoids) and polyphenolic compounds (Saura Calixto and Larrauri, 1996). Hence the concept of antioxidant dietary fibre is

gaining more importance now a day. Dietary fibre and antioxidants provide properties associated with slow glucose absorption, high colonic fermentability, lower serum cholesterol levels, enhancement of immune functions and protection against oxidative damage (Saura-Calixto, 2010). Antioxidants and dietary fibre are known fact that a significant proportion of the antioxidants contained in fruit and vegetables are linked to dietary fibre. These compounds are not bio accessible in the human small intestine, but they may exert significant health effects when they reach the colon. They are the dietary fibre with exceptional amount of associated polyphenolic antioxidants, which combines the effect of both dietary fibre and natural antioxidants in a single material (Saura-Calixto, 2010).

In other words antioxidant dietary fibre (ADF) is defined as a natural product that combines the beneficial effects of dietary fibre and natural antioxidants, such as polyphenol compounds. ADF can be used as a dietary supplement to improve gastrointestinal health and to prevent cardiovascular diseases, and on the other as an ingredient in seafood and meat products to prevent lipid oxidation. The function of natural antioxidants and dietary fibre in foods and biological systems has received much attention. Fruits and vegetables play a significant role in the human diet providing protection against cellular damage caused by exposure to high levels of free radicals, while also aiding digestion. This is attributed to the fact that these foods provide an optimal mix of antioxidants such as vitamin C and E, polyphenols, carotenoids, and complex carbohydrates.

Therefore fruits and vegetables account for a small part of our daily calorie

intake; however their benefits to health surpass their calorie contribution. The contributory factors are due to the presence of vitamins and provitamins, such as ascorbic acid, tocopherols and carotenoids and, in addition to that, they are also rich in a wide variety of phenolic substances. Phenolic substances are a category of phytonutrients that exert strong antioxidant properties. The vegetables such as kale, beets, pepper, broccoli, spinach, carrot, cabbage have high antioxidant activities besides antioxidant nutrients such as ascorbic acid, tocopherols, and carotenoids, fruits and vegetables are also a good source of polyphenol compounds.

Dietary fibre has been reported to have stool bulking property, reduces transit time and thereby decreases the amount of carcinogen that comes in contact with the gut wall (Hill, 1974). Improvements in diabetic control and reduction in insulin and sulfonylurea requirements have been reported in both mild (Kiehm et al., 1976; Kay et al., 1981) and moderate (Albrink et al., 1979; Rivellese et al., 1980) diabetics on taking high fibre diets. Pectin (Kay and Truswell, 1977), guar gum and gum arabic also show a hypolipidic effect in humans, lowering both serum cholesterol and triglycerides (Takahashi et al., 1993). A variety of fibre rich foods such as wheat straw, oats, soy bran, rice bran, apples, legumes, mucilaginous fibre (Heller et al., 1980) were shown to reduce the atherogenicity of semi-synthetic diets.

Fibre in foods can change their consistency, texture, rheological behaviour and sensory characteristic of the end products, the emergence of novel sources of fibres, have been offering new opportunities in their use in food industry (Guillon and Champ, 2000). Fibre can even be produced from sources that might otherwise be considered waste products. For example, wheat straw, soy hulls, oat hulls, peanut and

almond skins, corn stalks and cobs, spent brewer's grain and waste portions of fruits and vegetables processed in large quantities can be converted into fibre ingredients, which may be highly functional in certain food applications (Katz, 1996). Dietary fibre holds all the characteristics required to be considered as an important ingredient in the formulation of functional foods, due to its beneficial health effects.

The importance of food fibres has led to the development of a large and potential market for fibre-rich products and ingredients and in recent years, there is a trend to find new sources of dietary fibre that can be used in the food industry (Chau and Huang, 2003). Supplementation has been used to enhance fibre content of foods. Supplementation has been focused on cookies, crackers and other cereal-based products, enhancement of fibre content in snack foods, beverages, spices, imitation cheeses, sauces, frozen foods, canned meats, meat analogues and other foods also has been investigated (Hesser, 1994).

1.7 Prebiotics

The concept of prebiotics is relatively new and was first defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health (Gibson and Roberfroid, 1995). This definition was later refined to (Gibson et al., 2004): a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits. *Lactobacilli* and *bifidobacteria* are the usual target genera for prebiotics. All prebiotics are dietary fibre and posses some properties (Slavin, 2013):

- Resists gastric acidity, hydrolysis by mammalian enzymes, and absorption in the upper gastrointestinal tract;
- Is fermented by the intestinal microflora;
- Selectively stimulates the growth and/or activity of intestinal bacteria potentially associated with health and well-being.

Foods high in prebiotics have been consumed since prehistoric times. Prebiotics occur naturally in foods such as leeks, asparagus, chicory, Jerusalem artichokes, garlic, onions, wheat, oats, and soybeans (Leach and Sobolik, 2010). Some known prebiotics (inulin) are low digestible carbohydrates and are associated with impaired gastrointestinal tolerance, especially when consumed in large quantities (Grabitske and Slavin, 2009) while other prebiotic fibers (e.g., wheat dextrin, polydextrose) exhibit high gastrointestinal tolerability (Pasman et al., 2006).

An important mechanism of action for dietary fiber and prebiotics is fermentation in the colon and changes in gut microflora. The human large intestine is one of the most diversely colonized and metabolically active organs in the human body (Gibson et al., 2010). The colonic environment is favourable for bacterial growth due to its slow transit time, readily available nutrients, and favourable pH. Together with the gut immune system, colonic microflora contributes significantly prevents the invasion of pathogenic bacteria to gastrointestinal (GI) tract. The intestinal flora obtains energy through fermentation of carbohydrates not digested in the upper gut. The main substrates are dietary carbohydrates that escape digestion in the upper GI tract. Colonic bacteria use a range of carbohydrate hydrolyzing enzymes to produce hydrogen, methane, carbon dioxide, short chain fatty acids (SCFAs mainly acetate, propionate and

butyrate), and lactate. Certain colonic bacteria generate energy from these fermentation products. Dietary components that stimulate fermentation lead to an increase in bacterial mass and consequently fecal mass and, thus have a stool bulking effect. It is estimated that about 30 g of bacteria are produced for every 100 g of carbohydrate that is fermented (Slavin, 2003). Overall, a number of factors influence the composition of the microflora. These include changes in physiological conditions of the host (e.g., age stress, health status), composition of the diet, and environmental circumstances (e.g., antibiotic therapy, hygiene with antiseptics, *etc.*) (De Filippo et al., 2010).

Aberrant gut microbiota profiles have been associated with obesity, type 1 and type 2 diabetes and non-alcoholic fatty liver disease. Prebitotics are reported to improve glucose homeostasis in prediabetes and type 2 diabetes (Barengolts, 2013). Dietary fructans are nutritionally interesting oligosaccharides that strictly conform to the definition of prebiotics and (in view of experimental studies in animals and of less numerous studies in humans) exhibit interesting serum or hepatic lipid lowering properties. Other nondigestible/fermentable nutrients, which also modulate intestinal flora activity, exhibit cholesterol or triglyceride lowering effects (Delzenne and Williams, 2002). Thus prebiotic fibre, a class of dietary fibre that may act to beneficially alter the colonic microflora and other health benefits, has generated intense scientific, and consumer attention.

1.8 Probiotics

Probiotic bacteria have become increasingly popular during the last two decades as a result of the continuously expanding scientific evidence pointing to their beneficial effects on human health. Etymologically the term probiotic is derived from the Greek

language meaning “for life” but the definition of probiotics has evolved over time simultaneously with the increasing interest in the use of viable bacterial supplements and in relation to the progress made in understanding their mechanisms of action. The term was originally used to describe substances produced by one microorganism that stimulated the growth of others and was later used to describe tissue extracts that stimulated microbial growth and animal feed supplements exerting a beneficial effect on animals by contributing to their intestinal flora balance (Fuller, 1999). The most widely used definition was - probiotics are live microbial feed supplements which beneficially affect the host animal by improving microbial balance (Fuller, 1989). The present definition as conferred by WHO and FAO was - live microorganisms which when administered in adequate amounts confer a health benefit on the host (2001).

Taking into consideration their definition the number of microbial species which may exert probiotic properties is impressive. Some of the most important representatives are listed in Table 1.4. As far as nutrition is concerned only the strains classified as lactic acid bacteria are of significance and among them the ones with the most important properties in an applied context are those belonging to the genera *Lactococcus* and *Bifidobacterium* (Holzapfel et al., 2001).

In order for a potential probiotic strain to be able to exert its beneficial effects, it is expected to exhibit certain desirable properties (Kechagia et al., 2013). The ones currently determined by in vitro tests are

- acid and bile tolerance which seems to be crucial for oral administration,
- adhesion to mucosal and epithelial surfaces, an important property for successful immune modulation, competitive exclusion of pathogens, as

well as prevention of pathogen adhesion and colonization

- antimicrobial activity against pathogenic bacteria
- bile salt hydrolase activity.

Table 1.4. Microorganisms considered as probiotics (Source: Holzapfel et al., 2001)

***Lactobacillus* species**	***Bifidobacterium* species**
L. acidophilus	*B. adolescentis*
L. casei	*B. animalis*
L. crispatus	*B. bifidum*
L. gallinarum	*B. breve*
L. gasseri	*B. infantis*
L. johnsonii	*B. lactis*
L. paracasei	*B. longum*
L. plantarum	
L. reuteri	
L. rhamnosus	
Other lactic acid bacteria	***Non lactic acid bacteria***
Enterococcus faecalis	*Bacillus cereus var. toyoi*
E. faecium	*Escherichia coli strain nissle*
Lactococcus lactis	*Propionibacterium freudenreichii*
Leuconostoc mesenteroides	*Saccharomyces cerevisiae*
Pediococcus acidilaccii	*S. boulardii*
Sporolactobacillus inulinus	

As there are no specific parameters essential to all probiotic applications, the best approach to establish a strain's properties is target population and target physiologic function specific studies (Mercenier et al., 2008).

Milk products fermented by various strains of the genera *Lactobacillus*, *Streptococcus*, *Lactococcus* and *Bifidobacterium* have shown different antimutagenic activities (Abdelali et al., 1995). Oral application of the carcinogens with lactic acxid bacteria and yogurt was found to reduce DNA induced by carcinogens in rats (Pool-Zobel et al., 1996). Probiotics are found to bind the hetrocyclic amines (carcinogens) which are formed during cooking of meat (Orrhage et al., 1994). The carcinogenic

effect of endogenous toxic and genotoxic compounds is probably influenced by the activity of the bacterial enzymes NAD(P)H dehydrogenase, nitroreductase, β glucuronidase, β-glucosidase, and 7-α-dehydroxylase (Rowland, 1991). *Bifidobacteria* and *Lactobacilli* have lower activities of these xenobiotic-metabolizing enzymes than do *Bacteroides*, *Clostridia*, and *Enterobacteriaceae*. SCFAs produced during fermentation of dietary fibre by probiotics have very important role in maintenance of gut health. At the end, it results in a decrease in pH and thereby preventing overgrowth of pathogenic bacteria. These short chain fatty acids usually act as a source of carbon for colonocytes and they carry out important metabolic activities like modulation of bioactive food components, vitamin synthesis by intestinal microbiota. Its function shapes the host intestinal anatomy and also gut mucosal immune system (Wollowski et al., 2001; Moreaum and Gaboriau-Routhiau, 2000). Among these short-chain fatty acids, butyrate is found to play a defensive role in DNA oxidative damage induced by H_2O_2. It may also decrease the altered cell proliferation and induce programmed cell death process in altered cells (Rosignoli et al., 2001). *Lactobacillus salivarius, Pediococcus pentosaceus* and *Enterococcus faecium* exhibited anti proliferative properties. The proposed mechanism was given to the synergic induction by directly adhering to colon cancer cells and triggering bio production of butyric and propionic short chain fatty acids (Thirabunyanon and Hongwittayakorn, 2013). Recent studies explored that *L. casei* and *L. rhamnosus* GG cellfree supernatants (CFS) are able to inhibit colon cancer cell invasion by influencing levels of the tight junction protein zona occludens-1 (ZO-1) and matrix metalloproteinase-9 (MMP-9) activity in cultured metastatic human colorectal carcinoma cells (Urbanska et al., 2009). Azoxymethane

induced colon cancer in rodents was found to reduced due to stimulated growth of *Bifidobacterium* species in the colon. The growth of *Bifidobacteria* leads to lowering of pH, which is attributed to further inhibit aberrant crypt foci, crypt multiplicity and growth of *E. coli* and *Clostridia* in rats (Lijinsky et al., 1985; Reddy et al., 1997). Dietary intake of *Bifidobacterium longum* showed that there is a significant inhibition in tumor multiplicity as well as a decrease in the size of tumor volume. It also alters the intermediate biomarkers of colon cancer, thereby providing strong anti-tumor activity and in another study, it was noted that expression levels of ras-p21 and cell proliferation in colonic mucosa cells was decreased upon addition of *B. longum* to the rat diet and thereby providing anti-tumor activity (Singh et al., 1997; Reddy, 1998).

It has been suggested that the intestinal microbiota composition is associated with conditions such as allergies, intestinal inflammatory diseases, cancer, diabetes, cardiovascular diseases and dyslipidaemia (Holmes et al., 2011; Larsen et al., 2010). It has also been suggested that altered intestinal microbiota leads to increased intestinal permeability and mucosal immune response, contributing to the development of diabetes. Increased intestinal permeability is a result of reduced expression of tight junction proteins, eventually favoring the translocation of bacterial lipopolysaccharide (LPS), which may result in metabolic endotoxemia and insulin resistance (Secondulfo et al., 2004; Cani et al., 2008). Probiotics may be involved in the maintenance of a healthier gut microbiota, and have also been identified as effective adjuvants in insulin resistance therapies (Moroti, 2012; Ejtahed et al., 2012; Andreasen et al., 2010).

Probiotics help reduce blood cholesterol in three different ways (Saini et al., 2010):

- Probiotics create acids that counter cholesterol production: As probiotic bacteria absorb fiber from the intestines, they generate acids. One of the specific acids, i.e. proprionic acid, reduces production of cholesterol by the liver.
- Probiotics break down liver bile acids: Probiotics break down bile acids and, therefore, the liver has to make additional bile acids, using up more cholesterol in the progression.
- Probiotics actually eat cholesterol: Probiotic bacteria have been shown to break down cholesterol and use it for nourishment.

Probiotic products should have a minimum concentration of 10^6 CFU/mL or gram; furthermore the strains must be able to grow under manufacture and commercial conditions and should retain viability under normal storage conditions (Sanders 2008).

1.9 Synbiotics

Synbiotics are defined as the combination of appropriate probiotics and prebiotics, where the latter form the substrate for the growth and development of selective indigenous or introduce beneficial bacteria in the colon (Raman et al., 2016). The prebiotics commonly in use are inulin, fructo oligosaccharide, galacto oligosaccharide, lactose, etc., and the probiotics investigated include *Bifidobacterium* and *Lactobacillus* spp. Prebiotics form the food for the growth of probiotics. The mechanism of action of synbiotics is a collective effort of pro- and prebiotics. An ideal synbiotic supplement should contain an appropriate combination of prebiotics with probiotics where the former selectively favours the later, should exhibit synergistic relationship between viable beneficial bacteria and their selective substrate and should produce additive or synergistic effect (Patel and Patel, 2010).

Administration of synbiotics could prevent the initiation or early stage of cancer and also treat the existing tumors. Synbiotic interventions bring about significant alterations in the composition of the colonic microbiome leading to the altered metabolic activity of the organ. It reduces the exposure of cytotoxic agents, including mutagens and carcinogens, to the intestinal lining; decreases cell proliferation in the colonic tissue; and develops mucosal structure.

The alarming increase in inappropriate use of antibiotics and development of bacterial resistance makes pro-, pre- and synbiotics a very interesting field for research. At present, these agents have shown several beneficial effects in a variety of gastrointestinal and non-gastrointestinal disorders including colon cancer. All three of them offer dietary means to support the balance of the intestinal flora. As altered balance of the intestinal flora is an important cause of several gastrointestinal diseases, they may be used to correct such disorders like local immunological dysfunction, destabilize intestinal function, prevention of infections caused by pathogenic microorganisms and disturbed intestinal metabolism. Thus, these three agents hold immense potential for delivering novel therapies in different diseases in future (Chakraborti, 2011).

1.10 Objectives

From the literature review, it was evident that the unhealthy diet serves as an important risk factor for majority of the non-communicable diseases. Diet is one of the major modifiable risk factors for NCDs. Colon cancer, diabetes and cardiovascular diseases can be managed and prevented by maintaining a healthy diet. Food environments are increasingly characterized by higher prices of fruits and vegetables, low prices of

processed industrialized foods, access and availability of fast food shops, increasing trend of eating outside the home and larger portion sizes. However, consumers are now aware of the harmful effects of unhealthy diet. This has resulted in growing demand of food products rich in dietary fibre, probiotics, prebiotics and synbiotics. Researchers are in continuous search of new dietary fibre sources with better health beneficiary effects that can incorporate for the development of new synbiotic components. Indian subcontinent is a treasure house of various cereals, legumes, tubers, spices and medicinal plants. Apart from this, there are so many agro based industries in India which dispose tons of agro residues as waste with no use. These agro industrial residues can be utilized as a new source of dietary fibre.

With this background, the present work was proposed to develop evidence based novel synbiotic components for the prevention and management of lifestyle associated diseases.

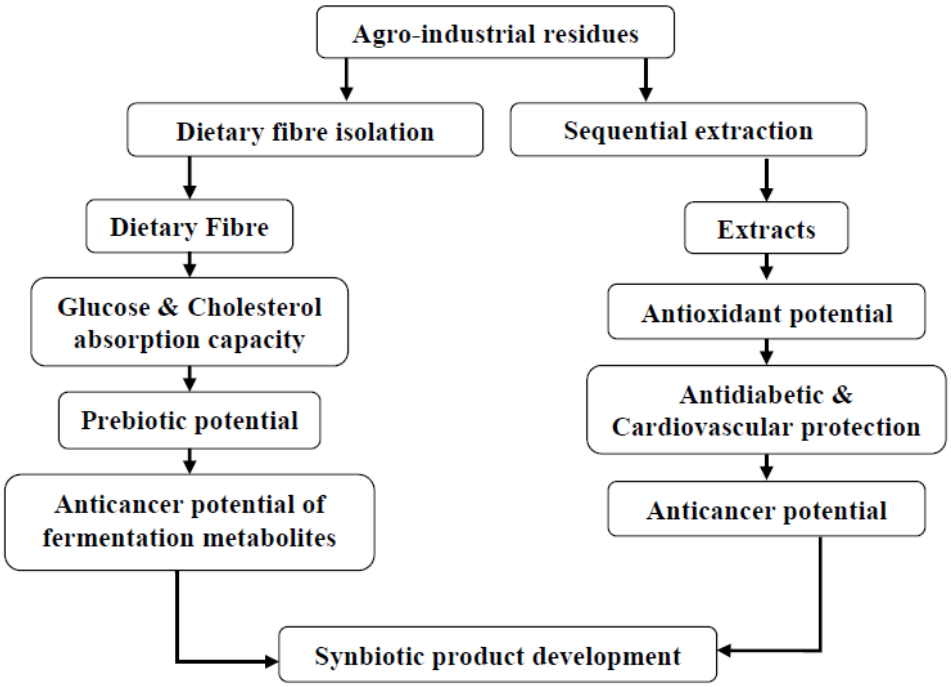

Figure 1.6 Outline of the work

The outline of the work is depicted in Figure 1.6 and the main objectives are summarized below:

1. Screening of agro-industrial residues for antioxidant dietary fibre.
2. Antidiabetic and cardiovascular protective effect assessment.
3. Prebiotic potential of soluble dietary fibre from the selected agro-industrial residue.
4. Assessment of anticancer potential of secondary metabolites obtained from fermentation of soluble dietary fibre.
5. Analyzing the anticancer potential of crude extracts of selected agro-industrial residue.
6. Development of synbiotic components and viability study.

The thesis is organized into six chapters. An attempt has been made to describe the major lifestyle diseases (diabetes, cardiovascular diseases and colorectal cancer), probiotics, prebiotics and synbiotics in Chapter 1. Screening of different agro-industrial waste for antioxidant dietary fibre, based on soluble dietary fibre content and antioxidant potential, is discussed in Chapter 2. Based on the preliminary experiments, plantain (*Musa paradisiaca*) inflorescence was chosen for further antidiabetic, cardiovascular protection, prebiotic potential and anticancer studies. Effect of dietary fibre and various extracts of plantain inflorescence in modulating Type 2 diabetes and associated cardiovascular problems are described in Chapter 3. The antidiabetic effect was analysed by inhibition of α-amylase, α-glucosidase, and glycation process; glucose uptake assay and glucose absorption capacity of soluble dietary fibre. The cardio vascular protection was assessed in terms inhibition of LDL oxidation and angiotensin

converting enzyme and cholesterol lowering effect of soluble dietary fibre. The prebiotic and anticancer potential of plantain inflorescence is discussed in Chapter 4. Prebiotic potential was assessed using two probiotic species – *Lactobacillus casei* and *Bifidobacterium bifidum*. Anticancer effect of extracts and dietary fibre fermentation byproducts- short chain fatty acids, were analysed in HT 29 colon cancer cell lines in terms of DNA damage, apoptosis, cell cycle arrest, nitochondrial membrane potential and expression studies of different proteins involved in apoptosis. Synbiotic product development and its stability are discussed in Chapter 5. The synbiotic product was developed by lyophilisation and spray drying techniques. Chapter 6 summarizes the work with main conclusions and future scope and directions of work in this area.

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Chapter 2

Screening of agro-industrial residues for the isolation of antioxidant dietary fibre

2.1 Introduction

For decades, agriculture has been associated with production of essential food crops. At present, agriculture above and beyond farming includes forestry, dairy, fruit cultivation, poultry, bee keeping, mushroom, etc. Today, processing, marketing and distribution of crops and livestock products etc. are all acknowledged as part of current agriculture. The agro-industry mainly comprises of the post-harvest activities of processing and preserving agricultural products for intermediate or final consumption.

The agro-industry is regarded as an extended arm of agriculture. It could cover a variety of industrial, manufacturing and processing activities based on agricultural raw materials; and also activities and services that go as inputs to agriculture (Bhattacharya, 1980). It helps in processing agricultural products such as field crops, tree crops, livestock and fisheries and converting them to edible and other usable forms. India is the second largest producer of food in the world (Chetan Ghate, 2012). In India, the agro industry has a huge potential in economy, as it provided a direct employment to about 14 million people and indirect employment to about 35 million people (Anoop, 2014).

Agro-industrial waste, which includes both natural (organic) and non-natural wastes, is a general term used to describe waste produced on a farmland and industries related to agricultural sector. Agricultural and food industry wastes constitute a significant proportion - over 30%, of worldwide agricultural productivity, represents valuable biomass and potential solution to problems of animal nutrition and worldwide supply of protein and calories if appropriate technologies can be developed (Ugwuanyi, 2009). The non utilization of these vast resources constitutes significant loss of value.

Most of the agro-industrial wastes are highly perishable and seasonal, and are a problem to the processing industries and pollution monitoring agencies. This problem can be resolved by utilizing its high value compounds, including the dietary fibre fraction that has a great potential in functional foods preparation. Agro-industrial waste is of potentially of good enough quality to be recycled as raw materials for other applications or may be reprocessed to higher value products. Various studies have showed that these agro-industrial residues are potent sources of dietary fibre and antioxidants (Vijayalaxmi et al., 2015; Arab et al., 2011; Sowbhagya et al., 2007). The increasing interest to find new sources of dietary fibres with specific bioactive constituents, may add new beneficial properties to the traditionally commercialized products.

In this scenario, we have selected some locally available agro-industrial residues for the isolation of dietary fibre and antioxidants. Literature review on selected sources are summarized on Chapter 2 along with the screening of selected agro-industrial residues based on the antioxidant potential of their solvent extracts as well as dietary fibre content.

2.1.1 Potato peel

Potato (*Solanum tuberosum* L.) is the world's fourth important food crop because of its high yield and great nutritive value. It is an economically important staple crop in both developed and developing countries. Potato is an excellent source of carbohydrate, protein, vitamins and minerals and is also one of the richest sources of antioxidants (Buono et al., 2009).

The potato processing industry generates high amount of peel as by-product, which is a good source of several functional ingredients including antioxidants. Of over

30 metric ton of annual production, tenth goes to industry to produce variable products including french fries and potato chips while the remaining part mainly consumed freshly (FAOSTAT, 2009). By-products from potato processing, like any other food processing industry, are principally organic materials thus management and disposal are crucial toward a clean industry. Although potato peel does not pose any serious disposal and environmental problems, meaningful utilization of this nutrient-rich waste has recently drawn much attention among the scientific community (Prasad and Pushpa, 2007; Mabrouk and El Ahwany, 2008; Sabeena Farvin et al., 2012).

Potato peel was reported to be an excellent substrate for the production of thermostable α-amylase, a starch hydrolyzing enzyme extensively used in different food industries, under solid-state controlled growth conditions (Fadel, 1999). Potato peel also has acquired attention as a natural antioxidant in food system due to its high content of polyphenols, which was reported to be 10 times higher than their levels in the flesh (Malmberg and Theander, 1984) accounting for approximately 50% of all polyphenols in potato tuber (Friedman, 1997). Potato peel is reported to be a good source of dietary fibre and polyphenols which demonstrates antihyperglycemic effect in experimental rats (Singh and Rajini, 2004; Singh et al., 2005). Investigators reported that the diabetic rats fed with freeze dried powder of potato peel caused a significant decrease in blood glucose level and effectively attenuated diabetic alterations in rats. The nutritionally and pharmacologically important components such as phenolic compounds, glycoalkaloids and cell wall polysaccharides in potato peel may be used as natural antioxidants, precursors of steroid hormones and dietary fibre (Schieber and Saldana, 2009). In another study by Mohdaly et al. (2010), the methanolic extract of potato peel was found

to have potential antioxidant capacity. Apart from the matured potatoes, the new or young potatoes, harvested before complete maturity, are being used widely in many cooking recipes (Navarre et al., 2010). They have thin skin that is considered to be a good source of fibre, whereas mature potatoes have thick skin and their flesh is drier than young ones. Till now there are not any reports which compare the antioxidant and dietary fibre status of peels from young and mature potatoes.

In India, Potato is grown in 14 states and projections for 2020 suggest that potato production reach approximately up to 50 million metric ton (Pandey et al., 2009). Fresh potatoes can be processed into several value added fried and non-fried products having longer shelf life. The market for such processed potato products is currently booming in India. The demand for processed potato products like chips, french fries, flakes etc., is increasing continuously in the present liberalized economy. There are many potato processing industries in India which comprises four main segments: potato chips or crisps, french fries, potato flakes/powder and other processed products. These industries produce considerable amount of potato peel as waste which is discarded as such. On this background and as mentioned earlier that and young potatoes are widely used in India along with mature potatoes, we were curious to know the antioxidant and dietary fibre status of the peel obtained from both young and mature potatoes.

2.1.2 Rice bran and Wheat bran

Cereals are the important staple food both in developed and developing countries. Cereals are rich sources of dietary fibre and antioxidants (Roa and Muralikrishna, 2006) and evidence suggests that cereal consumption prevents the risk of developing many chronic diseases, especially those related to metabolic syndrome i.e., type 2 diabetes

and cardiovascular diseases (Jacobs et al., 1998; Willet et al., 2006). The scientific and technological boom in agriculture sector has made India self-reliant in production of major food staples- Rice and Wheat. Bran is the outer layer of any kernel of grain, where most of the relevant antioxidants, B vitamins and fibres are located. In order to protect the endosperm and grain, bran is mainly comprised of water insoluble fibre. Due to high fibre content, possible hull contamination and rapid development of rancidity, bran has limited food application.

Rice (*Oryza sativa* L.) is a member of *Poaceae* family formerly known as *gramineae*. More than 650 million metric tons of rice is produced, annually world over, constituting greater than 75% of all cereal grains. Rice is the most important cereal product in Asia and is an overwhelming staple food in most populations of this region (Wadsworth, 1992). Milling of paddy yields 70% of rice (endosperm) as the major product and by - products consisting of 20% rice husk, 8% rice bran and 2% rice germ (Van Hoed et al., 2006). The brownish portion of rice which is taken out in fine grain form during de-husking and milling of paddy is the rice bran. The bran is the hard outer layer of rice consisting of aleurone and pericarp. Rice bran contains an array of micronutrients like oryzanols, tocopherols, tocotrienols, phytosterols, 20% oil and 15% protein, 50% carbohydrate [majorly starch, dietary fibres like beta-glucan, pectin, and gum] (Hernandez et al., 2000).

Rice bran, which was earlier used primarily as animal feed, is now finding major application in the form of rice bran oil (Van Hoed et al., 2006). Rice bran is also a potential source of both soluble and insoluble dietary fibre with contribution 20-51% (Saunders, 1990), the level twice to that of oat bran. Tomlin and Read (1988) and

Burton et al. (2000) suggested the uses of rice bran for the enrichment of nutraceutical foods. γ-oryzanol, tocotrienols and tocopherols present in rice bran are reported to have antioxidant properties (Ju and Vali, 2005).

Wheat (*Triticum aestivum* L.) is an extensively cultivated food crop and forms an important staple food in many countries. The wheat kernel or caryopsis can be roughly divided into 3 parts: the endosperm, the germ, and multiple histological outer layers (that is, outer and inner pericarp, seed coat, and nucellar epidermis), commonly denominated as botanical bran (Delcour and Hoseney 2010). The bran, together with the aleurone layer and remnants of starchy endosperm and germ, end up in a range of milling by-products, which are recovered at different stages in the mill. In contrast to refined flour, these bran-rich products are typically used for animal feed. However, wheat bran has a rich nutritional profile and shows beneficial physiological effects, making consumption of bran-rich food products more interesting from a health perspective than products based on refined flour. Because consumers become more aware of its benefits, wheat bran is increasingly added to mostly cereal-based food products. Wheat bran contains high levels of starch (6% to 30%), protein (14% to 26%), lipids (3% to 4%), lignin (3% to 10%), minerals (5% to 7%), phytic acid (4.5% to 5.5%), phenolic acids (0.4% to 0.8%), high levels of dietary fibre and other minor constituents (Hemdane et al., 2016).

Bran obtained during milling of rice and wheat is gaining importance worldwide due to its beneficial nutritive and biological effects. Various reports are there to show the antioxidant properties of crude extracts from rice and wheat bran (Arab et al., 2011; Zhou and Zhou, 2004; Adom et al., 2005). Nowadays, the advancements in technology

have lead to the usage of a small percentage of bran in various food products. Conversely there are fewer studies related to the nutraceutical benefits and the possible mechanism of action underlying the biological activity of crude extracts from bran.

India is the second largest producer of rice and third largest producer of wheat in the world. Bran is the most valuable by-product of milling industry which is a rich source of protein, vitamins, minerals and many biologically active chemicals. Rice bran is a potential source of vegetable oil. However, the majority of bran is right away used either as an ingredient in animal feed or as fuel in boilers (Fabian and Ju, 2011). Therefore the present study focused to utilize bran obtained from processing of rice and wheat as one of the sources of antioxidant dietary fibre.

2.1.3 Spent cumin

Cumin (*Cumin cyminum*), an herbaceous annual plant that belongs to the *Umbelliferae* family, is one of the most globalized spices in the world and considered as the second most popular spice after black pepper (Neda et al. 2013). It is native to middle-east Asian region, and today, grown all over the world for its flavorful seeds. Cumin seeds are extensively admired for their characteristic aroma and are used as an essential flavoring ingredient in many cuisines. Cumin also have medicinal properties, including antimicrobial, antioxidant (Gachkar et al., 2007), antitumor, anti-inflammatory (Soleymani et al., 2011) and hypoglycaemic effect (Dhandapani et al., 2002). Essential oil and oleoresin from seeds are used in the perfume and pharmaceutical industries (Lewis, 1984).

Cumin is an important component of many of the Ayurvedic decoctions. Cumin is specifically used in Ayurveda for the conditions like bloating, vomiting, diarrhoea,

dysentery, malabsorption syndrome, fever and skin diseases. In Ayurveda the active principles from the raw materials are extracted with water, oil, ghee, milk, etc. where the extraction of active principles into the extracted phase, will not be complete. Hence, most of the residues from the ayurvedic industries are very rich sources of valuable bioactive phytochemicals. After hot aqueous extraction of cumin seed for the drug preparation in ayurveda, which retains major part of the essential oil, aglycons and other active terpenoids, are discarded as waste, except in veterinary feeds to a small percentage. Cumin seeds used after the preparation of these decoctions are usually discarded as waste which is misused for adulterating cumin. Spice processing industries also generate a large quantity of spent cumin (SC) after extraction of essential oil and oleoresin. Currently, around 400 tons of SC residues are disposed of as waste every year (Sowbhagya et al., 2007).

The waste from these industries constitutes a large proportion of urban solid wastes; represent persistent waste stream and present disposal problems. The by-products of agro-industries are remarked as a good source of phenolic compounds. Sowbhagya et al. (2007) reported that SC is a potential source of dietary fibre. Thus, the value addition of such industrial waste is a matter of great interest for research (Balasundram et al. 2006). The SC seeds generated from the ayurvedic industry are chosen for the current study based on the commercial potential as it undergoes minimum thermal processing during preparation and thereby ascertaining the retention of bio-actives after processing. Value addition to SC will help the related industries to generate additional income. Raw cumin (RC) is reported to have antioxidant, antitumor and antidiabetic activity (Bettaieb et al., 2010; Ekta and Dwijendra, 2014; Jagtap and

Patil, 2010). However no such studies have so far done with spent cumin and hence we included spent cumin for the screening of antioxidant dietary fibre in our study.

2.1.4 Plantain

Plantain/Banana (*Musa paradisiaca*) is an herbaceous plant cultivating mainly in southern parts of Asia for their fruits. *Nendran* is one of the widely cultivated varieties of *Musa paradisiaca* species in southern parts of India. Different parts of plantain (*Musa paradisiaca-Nendran* variety) - peel, stem and inflorescence - were selected for screening purpose.

2.1.4.1 Plantain peel

Currently, peels of a variety of fruits get focused as natural source of antioxidants and dietary fibre. With these grounds, banana peel has attracted attention as recent reports suggest it as a very good source of dietary fibre and antioxidants. Banana/plantain is one of the major fruit crops in India which is the largest producer of bananas in the world. Plantain occupies the fourth world rank of the most significant foodstuffs after rice, corn and milk (INIBAP 2002). Plantain is similar to unripe bananas in outward appearance, larger and their flesh is starchy rather than sweet, used mostly unripe, and require cooking. 'Peel' is the main by-product of the banana processing industry which represents approximately 30% of the fruit. This by-product creates an environmental problem because it contains large quantities of nitrogen and phosphorus and its high water content makes it susceptible to modification by microorganisms (Tchobanoglous et al. 1993). The banana fruits are consumed at different stages of maturity and the amount of peels is expected to increase with the development of processing industries that utilize the green and ripe banana. Banana peel flour potentially offer new products

with standardized compositions for various industrial and domestic uses (Emaga et al. 2007).

Various studies have been conducted to investigate possible value addition to banana peel including the production of banana peel flour (Ranzani et al. 1996), the effects of ripeness stage on the dietary fibre components and pectin of banana peels (Emaga et al. 2008). Peel constitutes 30% of the *Nendran* (Kachru et al. 1995) which is an important issue of environmental pollution as waste disposal is a major problem in the region.

2.1.4.2 Plantain stem

Plantain stem is an actively growing aerial part closely packed leaf sheaths. It functions as a vascular bridge for the flow of water and nutrients from roots to leaves and finally to the fruit bunch. It is often used as a vegetable for culinary purposes in India. Juice from plantain stem is a well-known remedy for stomach and urinary disorders (Mustaffa and Sathiamoorthy, 2002). It helps in the treatment for removal of stones in the kidney, gall bladder, and prostate and is also used as an antidote for snake bite. The K, Ca, Mg, Si, and P contents of ashes of banana pseudo stems were 33.4, 7.5, 4.34, 2.7, and 2.2%, respectively (Cordeiro et al., 2004). Plantain stem was reported to be a potential source of polyphenols or natural antioxidants and dietary fibre (Bhaskar et al., 2011), which can be used in the food, nutraceutical, and pharmaceutical industries (Saravanan and Aradhya, 2011). Currently, only <2% of plantain stem production is used for human consumption and for production of fibre (Uma et al., 2005). Fibres from banana stem are being used as natural sorbent, bioremediation agent for bacteria in natural water purifier, mushroom production, handicrafts and textiles (Mohapatra et al., 2010).

2.1.4.3 Plantain inflorescence

The inflorescence of plantain is used as a vegetable in southern part of Asia, especially in southern India, Malaysia, Taiwan, Srilnaka and also in some of the African countries. It is used traditionally for the treatment of dysentery, menorrhagia and diabetes (Imam and Akter, 2011). Various reports have been published describing the biological potential of plantain inflorescence. Gomathy et al. (1989) reported that the juice of inflorescence of plantain resulted in significant lowering of cholesterol and triglycerides in the serum and tissues in rats and the cholesterol lowering effect of plantain inflorescence is due to the presence of pectin. Oral administration of chloroform extract of the plantain flowers in rats resulted in significant reduction in blood glucose (Pari and Umamaheswari, 2000). Jayamurthy et al (2011) reported that the plantain inflorescence is a rich source of phenolic compounds with potential radical scavenging activities. Inflorescence from *Musa* species have been reported to have hypoglycemic and antiglycation effect (Bhaskar et al., 2011).

The production of plantain has considerable economic importance. After harvesting, however, a large amount of pseudo-stem and inflorescence residue is left behind in plantation soil to be used as organic material. It has been estimated that a few tons per hectare of pseudo-stem are produced annually (Cordeiro et al., 2004). Various reports have identified the residues obtained from plantain as a source of biologically active compounds. In present study we have selected *Nendran* variety of plantain as it is one of the important plantain varieties grown in Kerala. If we are able to validate scientifically that the plantain parts – stem, inflorescence and peel can be utilized as a source of antioxidant dietary fibre, there is a great potential to develop an array of value

added health products from plantain.

2.1.5 Objectives

The main objectives of Chapter 2 can be summarized as follows:

- Collection of locally available agro-industrial residues
- Screening of agro-industrial residues based on soluble dietary fibre content and antioxidant potential
- Determination of proximate composition, chemical constituents and physico-chemical properties of selected agro-industrial residue

2.2 Materials and Methods

2.2.1 Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), Griess reagent, α amylase, α glucosidase, protease, lipase, gluco-amylase, pepsin, catechin and polyphenol standards were purchased from Sigma Aldrich Chemicals Pvt. Ltd., St Louis, USA. Quercetin, ascorbic acid, gallic acid, mannitol and Folin–Ciocalteau reagent, aluminium chloride hexahydrate, potassium acetate, sodium nitroprusside (SNP), ferric chloride, Ethylenediaminetetraacetic acid (EDTA), thiobarbituric acid (TBA), trichloro acetic acid (TCA), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH), boric acid, bromocresol green and sodium hydroxide were purchased from Sisco Research laboratories Pvt. Ltd., Mumbai, India. Copper (II) chloride and neocuproine were purchased from Alfa Aesar, Heysham, England. All reagents were of analytical grade.

2.2.2 Collection of agro-industrial residues

Based on literature survey and availability we have selected five different sources of agro-industrial residues for screening purpose. Screening was done based on antioxidant potential and the dietary fibre content in the selected agro-industrial residues.

Potatoes at two different stages of maturity – mature and young, collected from a farm at Nilgiris, Tamilnadu, India. The young and matured potatoes were harvested after 14 and 20 weeks from the planting, respectively. The raw materials were hand rinsed thoroughly under a stream of tap water to remove the dirt and were blotted with cotton cloth. Potatoes were peeled manually. The peel of young potato (YP) was easy to remove and softer as compared to the peel of matured potato (MP). The peels were then freeze dried using lyophilizer (VirTis genesis, USA). The lyophilized peels were powdered, sieved (40 mm mesh) and stored under refrigeration until further use.

Rice bran (RB) and wheat bran (WB) were obtained from a mill in Ernakulam district of Kerala, India. The bran was sieved through 40 mm mesh size, oven dried at 55°C and stored in air tight container until use.

Spent cumin (SC) seeds for the screening purpose were obtained from Kottakkal Arya Vaidya Sala, Kottakkal, Kerala, which is one of the leading manufacturers of Ayurvedic formulations in India. Cumin seeds were then freeze-dried using lyophilizer (VirTis genesis, USA), powdered, then sieved (40 mm mesh) and stored under refrigeration until further use.

Plantain peel (PP), from the variety *Nendran* was collected from one of the local banana chips making industry, located at Thiruvananthapuram district of Kerala, India. The peels were washed, pre treated with cold water containing 0.5% citric acid for 10

min, drained and sliced into small pieces. It was then dried in an oven at 50°C for 16–24 h. Plantain inflorescence (PI) and stem (PS), from *Nendran* variety, identified as *Musa paradaisica*, was collected from one of the local banana farm, located at Thiruvananthapuram district of Kerala, India. The inflorescence and stem were washed, drained and sliced into small pieces. It was then freeze dried using lyophilizer (VirTis genesis, USA). The dried plantain samples were ground using a blender (Ultra centrifugal mill ZM200, Retsch, Germany) and sieved using 20 mm mesh (Vibro Sifter-PVS30, Prism Pharma Machinery, India) to obtain fine powder and stored in a cool dark place under until further use.

The outline of the work has been described in Figure 2.1.

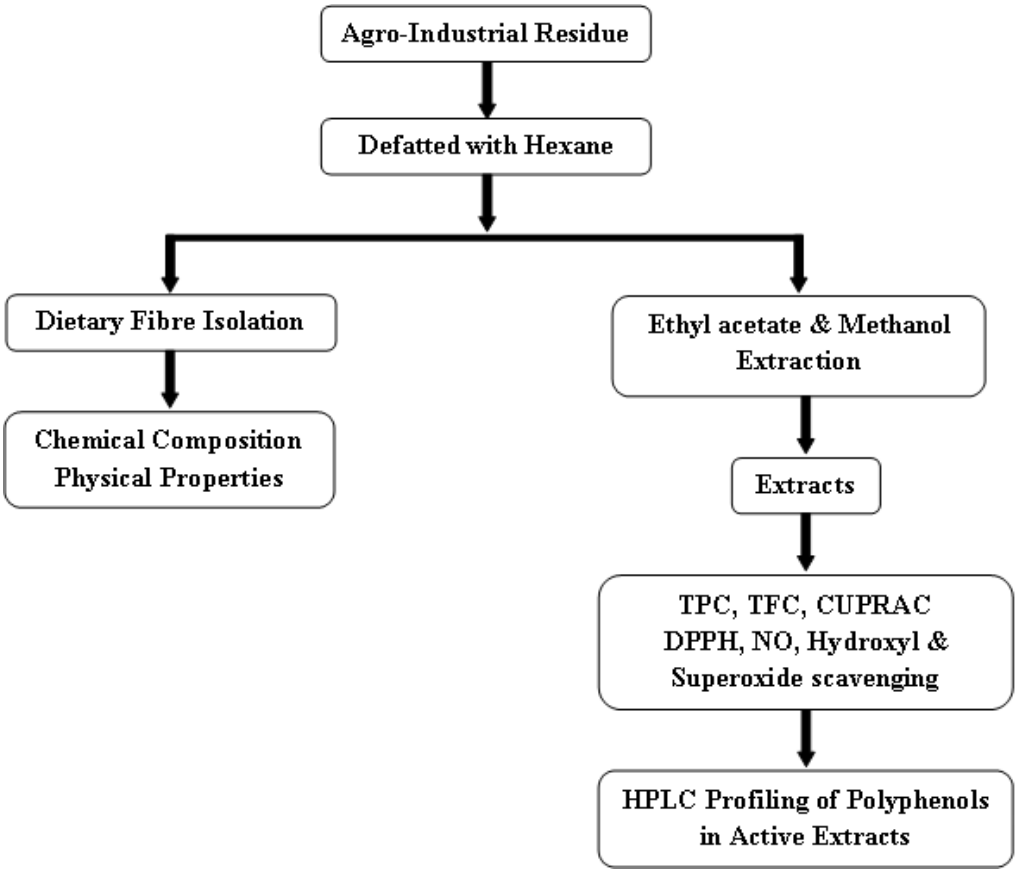

Figure 2.1. Outline of Chapter 2

2.2.3 Screening of agro-industrial residues based on soluble dietary fibre content and antioxidant potential

2.2.3.1 Sample preparation

Powdered samples were defatted with hexane to remove the fat content. For defatting, 500 mL of hexane was added to 100 g of moisture free sample and kept for overnight with mild stirring. After that, the hexane solvent is decanted and the sample was dried properly.

2.2.3.2 Isolation of dietary fibre

The total dietary fibre from selected samples was isolated according to Bureau of Indian Standard Method (IS: 11062, 1984) with slight modifications. Briefly, 3 g of defatted, moisture free sample was mixed with 50 mL water and autoclaved at 120°C for 20 min. It was then cooled and the pH was adjusted to 1.5 with 5 M HCl followed by the addition of 50 mg pepsin and 200 mL of chloroform. It was incubated at 37°C for 20 h with mild stirring. After incubation the pH was adjusted to 6 with 3 N NaOH and 25 mL phosphate buffer, 100 mg pancreatin, 20 mg glucoamylase and few crystals of thymol were added. This mixture was incubated for 18 h at 37°C with mild stirring. After incubation the contents were centrifuged at 3,000×g (Remi Centrifuge-C-30BL, India) for 30 min, the residue was collected and washed with acetone and diethyl ether and lyophilized to constant weight to obtain the insoluble dietary fibre. To the supernatant ethanol was added in 1:4 (v/v) and again centrifuged for 30 min at 3,000×g (Remi Centrifuge-C-30BL, India). The residue was collected and washed with alcohol, acetone and diethyl ether and lyophilized (VirTis genesis, USA) to constant weight to obtain the insoluble dietary fibre.

2.2.3.3 Extraction of antioxidants

The defatted samples were sequentially extracted using ethyl acetate and methanol at 1:10 (w/v) at ambient temperatures (30 ± 2°C). The extraction was started with ethyl acetate with mild stirring (200 rpm, Remi Motors Ltd., India). In between the process extracts were collected and fresh ethyl acetate was added; and continued until the solvent remain colourless. The ethyl acetate extracts were pooled together and was filtered through Whatman No. 1 filter paper using rotary vacuum pump (Vacuum Techniques-VT-201, India). The residue obtained after ethyl acetate extraction was dried and extraction of the residue was continued with methanol and proceeded as same as that of ethyl acetate. Both the extracts were concentrated under reduced pressure in rotavapor (BUCHI R215, Switzerland) made up to 100 mL with respective solvents in standard flask and yield of the extracts were calculated. The ethyl acetate and methanol extracts were stored at 4°C until further biochemical analysis.

2.2.3.4 Yield of the extracts

1 mL of the extract was pipetted out to a pre-weighed petridish and kept in an oven for 2 h at 100°C. After that the weight of the petridish was measured. The petridish was kept in oven till the weight become constant. The difference in final weight and initial weight of the petridish gives the yield of extract in 1 mL and converted to dry weight basis (mg/g of dry source).

2.2.3.5 Total Phenolic Content (TPC)

The total phenolic content of the extracts was determined using Folin-Ciocalteau reagent as described by Singleton and Rossi (1965). Phenolic content from the sample reduce the metal and change the colour from yellow to prussian blue. The assay relies

on the transfer of reducing equivalents (electrons), in the alkaline medium, from phenolic compounds to phosphomolybdic/ phosphotungstic acid complexes, manifested in the formation of blue colour complexes that are determined spectrophotometerically at 725 nm. The intensity of the colour is directly proportional to the phenolic content and the phenolic content is expressed as milligram gallic acid equivalence per gram dry weight of extract (mg GAE/g dry weight). Different volumes of samples and standard (gallic acid) were diluted in methanol and made up to 720 µL. Further, 80 µL of Folin's reagent and 200 µL of 7.5% Na_2CO_3 was added and incubated for 90 min at room temperature. The absorbance was then read at 725 nm using spectrophotometer using a multiplate reader (Synergy 4 Biotek, USA). A control and blank was also performed simultaneously. All tests were performed three times.

2.2.3.6 Total Flavonoid Content (TFC)

The total flavonoid content was determined according as the aluminium chloride colorimetric method described by Chang et al (2002). All tests were performed in triplicates. The aliquots was mixed with 1.5 mL of 95% alcohol, 0.1 mL of 10% aluminium chloride hexahydrate ($AlCl_3.6H_2O$), 0.1 mL of 1 M potassium acetate (CH_3COOK), and 2.8 mL of distilled water. After incubation at room temperature for 40 min, absorbance of the reaction mixture was measured at 415 nm. Quercetin was chosen as a standard. The data were expressed as milligram quercetin equivalents per gram dry weight of extract (mg QE/g dry weight).

2.2.3.7 Cupric Ion Reducing Antioxidant Capacity (CUPRAC) assay

CUPRAC assay was performed according to the method of Apak et al. (2004) with some modifications. The test mixture contained 1 mL of 10 mM of $CuCl_2$, 7.5 mM

neocuproine, and 1 M ammonium acetate buffer (pH 7.0). Briefly, 1mL of sample in the concentration range of 100 –1000 µg/mL was added to the test mixture to achieve final volume of 4 mL. The test mixtures were incubated for 30 min at room temperature and then absorbance at 450 nm was recorded against a blank. Here trolox (TR) was used as the standard. As the molar absorptive of TR in the CUPRAC method is $\varepsilon = 1.67\times 10^4$ L/mol/cm, and the calibration curve for pure TR is a line passing through the origin, the TR equivalent molar concentration of the extract sample in final solution may be found by dividing the observed absorbance to the ε for TR (Guclu et al., 2006). The TR equivalent antioxidant capacity may be traced back to the original extract considering all dilutions, and proportionate to the initial mass of sample taken to find a capacity in the units of micromoles TR per gram dry matter.

2.2.3.8 Free radical scavenging potential

2.2.3.8.1 DPPH scavenging activity

The DPPH scavenging activity of different extracts was evaluated according to the method of Brand Williams (Brand-Williams et al., 1995). 1 mL of 0.1 mM DPPH solution in methanol was mixed with 1 mL of extract solution of varying concentrations. Corresponding blank sample were prepared and gallic acid was used as reference standard. Mixture of 1 mL methanol and 1 mL DPPH solution was used as control. The mixture was shaken well and incubated for 30 min in dark. The reaction was carried out in triplicate and the decrease in absorbance was measured at 517 nm after incubation using a multiplate reader (Synergy 4 Biotek.USA). The scavenging activity was expressed as IC_{50} (µg/mL). The percentage inhibition was calculated using the formula

DPPH radical scavenging activity (%)

$$= \left(\frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}}\right) \times 100$$

2.2.3.8.2 Nitirc oxide (NO) scavenging activity

Nitric oxide scavenging activity was estimated according to the method of Marcocci et al. (1994) with slight modification. The reaction mixture contained 1 mL of 10 mM SNP, phosphate buffered saline (pH 7.4) and various concentration of extracts in a final volume of 1.1 mL. After incubation for 150 min at room temperature, pipetted out 100 µL into wells plate and 100 µL of Griess reagent was added. The mixture was incubated for 10 min at 25°C. The pink chromophore generated was measured spectrophotometerically at 540 nm against a blank sample. All tests were performed in triplicates. Ascorbic acid was used as the standard. The percentage inhibition of nitric oxide radical generation was calculated using the following formula:

NO radical scavenging activity (%)

$$= \left(\frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}}\right) \times 100$$

2.2.3.8.3 Hydroxyl (OH) radical scavenging activity

Hydroxyl radical scavenging activity of different extracts was determined as described by Elizabeth and Rao (1990) with a slight modification. Hydroxyl radical was generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (the Fenton reaction). The reaction mixture contained, in a final volume of 1 mL 2-deoxy-2-ribose (2.8 mM), KH_2PO_4-KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 µM); EDTA (100 µM); H_2O_2 (1 mM); ascorbic acid (100 µM) and various concentrations of the extracts. After incubation for 1 h at 37°C, 0.5 mL

of the reaction mixture was added to 1 mL 2.8% TCA, then 1 mL 1% aqueous TBA was added and the mixture was incubated at 90°C for 15 min to develop the colour. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. Catechin was used as a positive control and the percentage radical scavenging capacity was determined using the formula:

OH radical scavenging activity (%)

$$= \left(\frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}}\right) \times 100$$

2.2.3.8.4 Superoxide radical scavenging activity

Superoxide radical scavenging activity of different extracts was measured by the reduction of NBT according to a previously reported method (Fontana et al, 2001). The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals, which reduce nitro blue tetrazolium (NBT) to a purple formazan. The 1 mL reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 μM), NBT (50 μM), PMS (15 μM) and various concentrations of sample solution. After incubation for 5 min at room temperature, the absorbance at 562 nm was measured against an appropriate blank to determine the quantity of formazan generated. Quercetin was used as the standard and the percentage radical scavenging capacity was determined using the formula,

Superoxide radical scavenging activity (%)

$$= \left(\frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}}\right) \times 100$$

2.2.4 Determination of proximate composition, physical properties and chemical constituents of selected agro-industrial residues

2.2.4.1 Proximate analysis of selected agro-industrial residue

Moisture, ash, fat, protein, carbohydrate and crude fibre were estimated by the standard procedure of the AOAC, (1990) as described below.

2.2.4.1.1 Determination of moisture

3 g of fresh sample was accurately weighed in a pre-weighed petridish and dried in a hot air oven for 2 h at 100 ± 2ºC. The dish with sample was cooled in desiccators and weighed. This exercise was repeated till the difference in weight between two successive weighing becomes constant. From the weight loss during drying, amount of moisture was calculated using the following formula and the moisture can be represented in percentage.

$$Moisture\ (\%) = \frac{(W1 - W2)}{W} \times 100$$

W1 = Weight of sample with petridish before drying

W2= Weight of sample with petridish after drying

W = Weight of sample

2.2.4.1.2 Determination of ash content

Ash content represents the inorganic residue remaining after destruction of organic matter. It may not necessarily be exactly equivalent to mineral matter as some losses may occur due to volatilization. 1 g of dried sample was accurately weighed into pre-weighed, clean crucible. The crucible was heated to the point of charring of the sample on a hot plate. The crucible with the carbon residue obtained as a result of ignition, was placed in muffle furnace at temperature of 650°C until the carbon residue disappears.

The sample is allowed to cool and then weighed. From the difference in weight obtained the ash content was calculated using the formula:

$$\text{Total ash content (\%)} = \frac{\text{Weight of crucible with ash (g)}}{\text{Weight of cruicble with sample (g)}} \times 100$$

2.2.4.1.3 Crude fat estimation

10 g of dried sample was taken in a thimble and plug the top of the thimble with a wad of fat-free cotton. Extract the sample with hexane using Soxhlet apparatus for 6-8 h on a heating mantle at 40°C. At the end of the extraction period, remove the thimble from the apparatus and concentrate the extract at rotavapor at 40°C. Dry at 100ºC for 1 h, cool and weigh. The difference in weights gives the ether soluble material present in the sample.

$$\text{Crude Fat (\%)} = \frac{\text{Weight of hexane soluble material}}{\text{Weight of sample}} \times 100$$

2.2.4.1.4 Determination of total protein

Nitrogen content in the plants mainly appears as proteins and amino acids and thus the total amount of nitrogen indicates the amount of total proteins and amino acids. Nitrogen content was estimated by the *Kjeldahl* method which was based on the determination of the amount of reduced nitrogen (NH_2& NH) present in the sample. The various nitrogenous compounds were converted into ammonium sulphate by boiling with concentrated H_2S0_4. The ammonium sulphate formed was decomposed with an alkali (NaOH), and ammonia liberated was absorbed in excess of neutral boric acid solution and then titrated against standard acid.

Nitrogenous organic compound + $H_2S0_4 \rightarrow (NH_4)_2SO_4$

$(NH_4)_2SO_4 + 2NaOH \rightarrow Na_2SO_4 + 2NH_3 + 2H_2O$

$NH_3 + H_3B0_3 \rightarrow NH_4 + H_2BO_3$

$H_2BO_3 + H \rightarrow H_3BO_3$

0.5 g each of sample and digestion mixture (copper sulphate + potassium sulphate) was weighed into a Kjeldahl flask and 10 mL of concentrated H_2S0_4 was added. The Kjeldahl flask was then heated on a mantle (in slanting position) until colour of solution changes to pale blue green. This clear solution was made up to 25 mL under cold conditions. The Kjeldahl apparatus was set up for protein estimation. 20 mL of 4% boric acid and 1 mL of mixed indicator (bromocresol green) was taken in conical flask and placed under condenser. 5 mL of sample with 20 mL of 40% NaOH and 10 mL water were added to distillation tube through funnel. When water starts boiling inside the round bottom flask, steam produced then passes into distillation tube. NH_3 evolved in distillation tube is trapped in boric acid. Upon ammonia evolution, the colour of boric acid changes to blue. For maximum ammonia evolution, the process is continued for 20 min. The solution was then titrated with standard HCl (0.01 N) till blue colour of the solution disappears.

Amount of nitrogen in the samples was calculated by the following equation

$$\% \text{ of Nitrogen} = \frac{14 \times \text{Normality of HCl} \times 100}{\text{Weight of sample} \times 1000}$$

$$\% \text{ Protein} = \% \text{ of Nitrogen} \times 6.24$$

2.2.4.1.5 Determination of carbohydrate

Carbohydrate is found by difference method and expressed as percentage of carbohydrate.

$$\text{Carbohydrate (\%)} = 100 - [\text{Moisture} + \text{Ash} + \text{Fat} + \text{Protein}]$$

2.2.4.2 Chemical composition of dietary fibre

2.2.4.2.1 Determination of hemicelluloses, cellulose and lignin content

Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were both measured using Van Soest method (Van Soest and Wine, 1967; Van Soest, 1987). Reflux extraction was conducted on the samples in a neutral detergent and acid detergent solution, separately for 90 min. The filtration residue was washed with hot distilled water and ethanol, then oven-dried until constant weight. The difference of weight between the starting material and the oven-dried sample was used to calculate NDF and ADF content. 72% sulphuric acid was added to the samples and kept under magnetic stirring at 27°C for 3 h. After being washed with hot distilled water and filtered, the residues were dried in order to weigh the amount of lignin. Finally, cellulose and hemicellulose were quantified from the contents of NDF, ADF and lignin, as follows:

$$\text{Hemicellulose} = \text{NDF} - \text{ADF}$$

$$\text{Cellulose} = \text{ADF} - \text{Lignin}$$

2.2.4.2.2 Determination of pectin

The pectin was determined according to Koubala et al. (2008) with slight modifications. 5 g sample treated with 1 M H_2SO_4 (pH 1.5) was stirred continuously for 1 h at 85°C. The residues were removed by filtering and pectins were precipitated with 96% ethanol. The precipitated pectins were centrifuged, collected and washed with 96 % ethanol and oven dried at 50°C.

2.2.4.3 Physical properties of dietary fibre

2.2.4.3.1 Water holding capacity (WHC)

Water holding capacity was determined according to the method of Ruperez (2001).

200 mg (residue dry weight) of the powdered sample in a centrifuge tube was soaked in 20 mL water and shaken at 37°C for 24 h. It was then centrifuged at 14,000 rpm (Kubota 7780, Japan) for 10 min, supernatant was discarded and residual hydrated weight was noted. The water holding capacity was calculated from the difference in weight of material before and after centrifugation**.**

$$\text{WHC (g/g)} = \frac{\text{Residue hydrated weight} - \text{Residue dry weight}}{\text{Residue dry weight}}$$

2.2.4.3.2 Water retention capacity (WRC)

Water retention capacity, defined as the quantity of water that remains bound to the hydrated fibre following the application of an external force (pressure of centrifugation) was determined according to the method described by Robertson et al. (2000). 1 g dried sample was weighed into a graduated centrifuge tube, adding 30 mL of water and it was hydrated for 18 h, centrifuged (3,000×g, 20 min; Kubota 7780, Japan) and the supernatant solution was removed by passing through a sintered glass crucible (G4) under applied vacuum. The hydrated residue weight was recorded and then sample was dried at 105°C for 2 h to obtain its residual dry weight.

$$\text{WRC (g/g)} = \frac{\text{Residue hydrated weight (after centrifugation)} - \text{Residue dryweight}}{\text{Residue dry weight}}$$

2.2.4.3.3 Swelling capacity

Swelling capacity of the dietary fibre was determined as described by Robertson et al. (2000). It is defined as the ratio of the volume occupied when the sample is immersed in excess of water after equilibration to the actual weight. Accurately weighed dry sample (0.2 g) was placed in a graduated test tube, 10 mL of water was added and it was hydrated for 18 h, and the final volume attained by the sample was measured.

$$\text{Swelling capacity (mL/g)} = \frac{\text{Volume occupied by Sample}}{\text{Sample weight}}$$

2.2.4.3.4 Oil holding capacity

The oil holding capacity (OHC) was determined according to Ruperez et al. (2001). 200 mg (residue dry weight) of the powdered sample in a centrifuge tube was soaked in 20 mL olive oil and shaken at 37°C for 24 h. It was then centrifuged at 14,000 rpm (Kubota 7780, Japan) for 10 min, supernatant was discarded and the weight (residue weight after centrifugation) was noted. The oil holding capacity was calculated using the formula:

$$\text{OHC (g/g)} = \frac{\text{Residue weight after centrifugation} - \text{Residue dry weight}}{\text{Residue dry weight}}$$

2.2.4.4 HPLC profiling and quantification of polyphenols in extracts of selected agro-industrial residue

The extracts and the reference compounds (1 mg/mL) solutions were prepared in methanol and filtered through 0.45 µm PTFE filter; 20 µL was injected into the HPLC system. The analysis was performed on a Prominence UFLC system (Shimadzu, Japan) containing LC-20AD system controller, Phenomenex Gemini C18 column (250 × 4.6 mm, 5 µm), a column oven (CTO-20A), a Rheodyne injector (USA) with a loop of 20 µL volume and a diode array detector (SPD-M20A).

The HPLC analysis was performed by the method of Rodriguez-Delgado et al. (2001) with some modifications. The mobile phase used was – solvent A: methanol – acetic acid – water (10:2:88, v/v) and solvent B: methanol – acetic acid – water (90:2:8, v/v) with the gradient program 0-15 min 15% B, 16-20 min 50% B, 21-35 min 70% B, 36-50 min 100% B and finally the column was regenerated in 10 min. The flow rate

was 1 mL/min, the injection volume was 20 µL and column was at room temperature. The fractions were monitored at 280 nm. Sample peaks were identified by comparing with retention times of standard peaks. LC LabSolutions software was used for data acquisition and analysis.

2.3 Results and Discussion

2.3.1 Screening of agro-industrial residues

2.3.1.1 Dietary fibre isolation

Dietary fibre was defined as the portions of plant foods that were resistant to digestion by human digestive enzyme. The plant portions include polysaccharides like lignin and oligosaccharides such as inulin and resistant starches (Anderson et al., 2009). Dietary fibre is commonly classified into two based on their solubility in water – Soluble Dietary Fibre (SDF) and Insoluble Dietary Fibre (IDF). Dietary fibre intake provides many health benefits. A generous intake of dietary fibre reduces risk for developing coronary heart disease, stroke, hypertension, diabetes, obesity, and colorectal cancer (Anderson et al., 2009; Lattimer and Haub, 2010). An appreciable proportion of dietary polyphenols are linked to cell walls and/or indigestible compounds of plant food providing to dietary fibres antioxidant, antimutagenic and other biological properties (Saura-Calixto, 1998; Ajila et al., 2010a). Hence there is a continuous search for new sources of antioxidant dietary fibre. The dietary fibre shows that among the different selected sources, plantain inflorescence possess better fibre content in terms of SDF. The SDF content in PI is 12.45 ± 1.14 % dry weight. The results are shown in Figure 2.2. The IDF was higher in plantain stem.

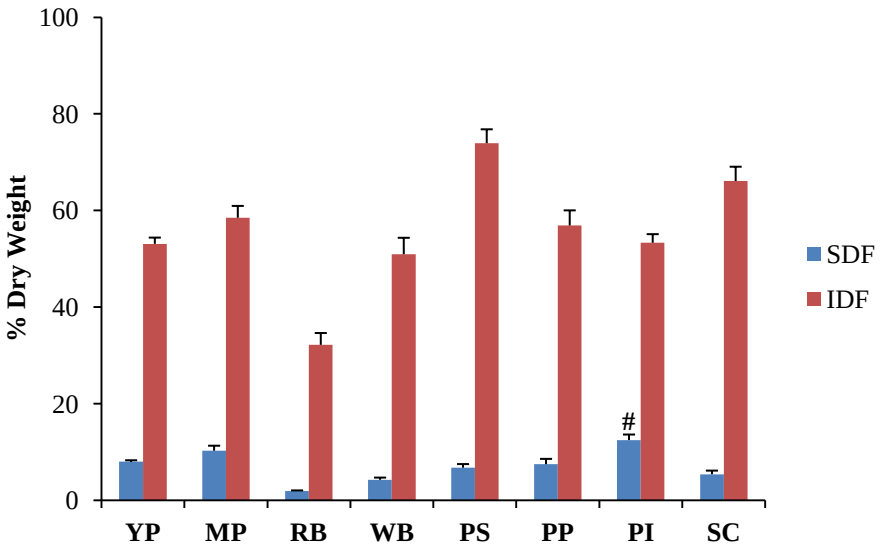

Figure 2.2. Dietary fibre content in selected agro-industrial residues. YP - Young potato peel, MP - Mature potato peel, RB - rice bran, WB - Wheat bran, PS - Plantain stem, PP - Plantain peel, PI - Plantain Inflorescence. SDF - Soluble dietary fibre and IDF - Insoluble dietary fibre. Each value represents mean ± SD (standard deviation) from triplicate measurements. #SDF value of PI significantly different from others ($p \leq 0.05$).

2.3.1.2 Yield of extracts of selected agro-industrial residues

Plants and plant products are rich in biologically and economically important compounds. Solvents extraction is an important way to extract these compounds. Various literature reports showed that extraction efficiency of solvents strongly depend on polarity of the solvent and the nature of the plant matrix (Tomsone et al., 2012). Hexane was used to remove fat from the selected agro-industrial residues. Ethyl acetate and methanol were used sequentially for the extraction purpose. The yield of different extracts was shown in Figure 2.3. The results showed that maximum yield was obtained for methanol extract of plantain inflorescence (84.13 ± 2.14 mg/g PI).

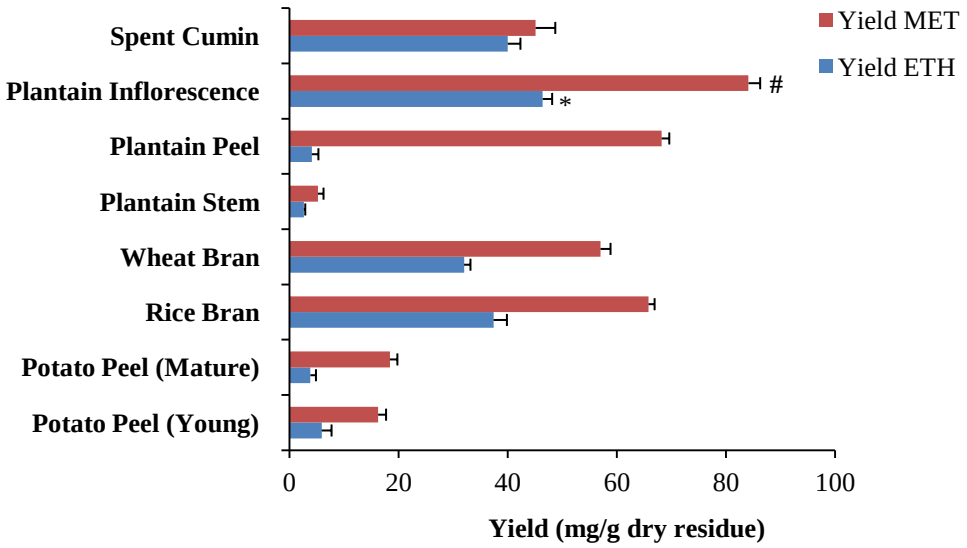

Figure 2.3 Yield of ethyl acetate (ETH) and methanol (MET) extracts of different selected agro-industrial residues. Each value represents mean ± SD (standard deviation) from triplicate measurements. #Yield of methanol extract of PI significantly different from others. *Yield of ethyl acetate extract of PI significantly different from others ($p \leq 0.05$).

2.3.1.3 Total phenolic content (TPC)

Phenolic compounds are considered secondary metabolites and are originated from phenylalanine and tyrosine, the chemical process which occur ubiquitously in plants and are diversified (Naczk and Shahidi, 2004). Total phenolic content of plants is an important parameter for their antioxidant properties. The Folin-Ciocalteu procedure of Singleton and Rossi (1965) has been used as a measure of total phenolics in natural products for many years.

The TPC analysis of various extracts of agro-industrial showed that methanol extract of plantain inflorescence (PI MET) has better phenolic content. TPC of PI MET was found to be 28.5 ± 1.72 mg GAE/g dry weight. The results are shown in Figure 2.4. Plantain stem possess least TPC. It was interesting to find out that the cumin after the

preparation of ayurvedic decoctions still retains 15.89 ± 1.34 mg GAE/g dry weight of TPC. Thus this study proves that agro-industrial residues can be exploited as a source of phenolic compounds.

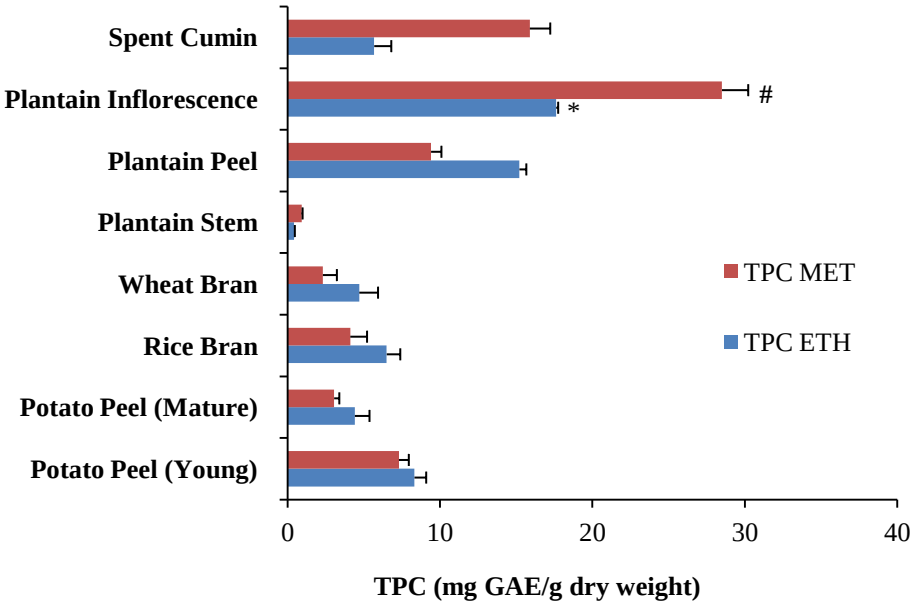

Figure 2.4. Total phenolic content of ethyl acetate and methanol extracts of different agro-industrial residues. Each value represents mean ± SD (standard deviation) from triplicate measurements. #TPC of methanol extract of PI significantly different from others. *TPC of ethyl acetate extract of PI significantly different from others ($p \leq 0.05$).

2.3.1.4 Total flavonoid content (TFC)

Flavonoids, a class of phenolic compounds possessing two phenol subunits, formed from the aromatic amino acids phenylalanine, tyrosine, and malonate are ubiquitous in plants. Determination of TFC is important as these compounds are known to have antioxidant activity and other biological activities. The spectrophotometric assay based on aluminium complex formation is one of the most commonly used procedures for the determination of total flavonoid content. Aluminium chloride forms acid stable and acid labile complexes with hydroxyl and dihydroxyl of flavonoid ring which is measured

photometrically at 415 nm. TFC analysis showed that PI MET possesses highest flavonoid content (14.56 ± 1.82 mg QE/g dry weight). Methanol extract of spent cumin has a flavonoid content of 7.88 ± 1.27 mg QE/g dry weight). The results are shown in Figure 2.5.

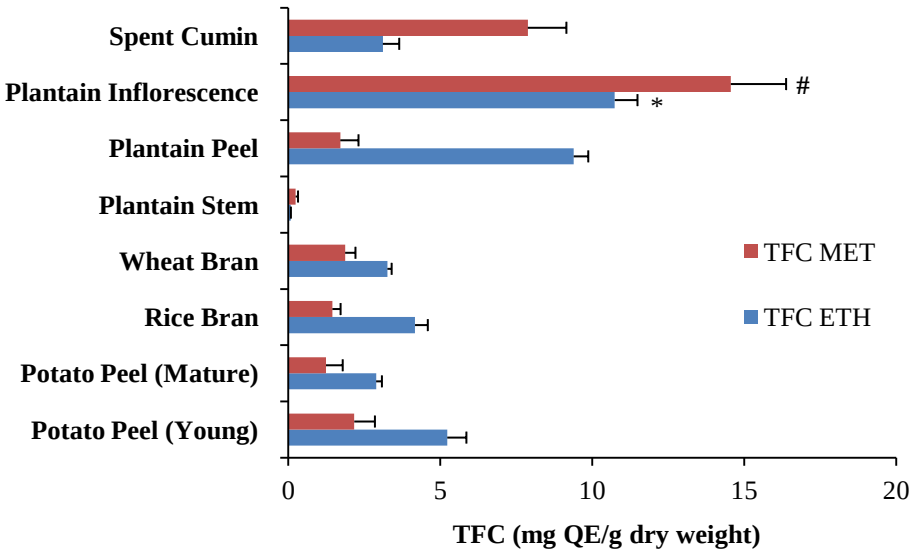

Figure 2.5. Total flavonoid content of ethyl acetate and methanol extracts of different agro-industrial residues. Each value represents mean ± SD (standard deviation) from triplicate measurements. #TFC of methanol extract of PI significantly different from others. *TFC of ethyl acetate extract of PI significantly different from others ($p \leq 0.05$).

2.3.1.5 Cupric ion reducing antioxidant activity

CUPRAC assay is low cost versatile antioxidant assay for dietary polyphenols which utilizes Copper (II)-neocuproine reagent as the chromogenic oxidizing agent. The Cu (I) complex formed as a result of redox reaction with reducing polyphenols was measured at 450 nm and compared with trolox activity. The cupric reducing ability measured for a biological sample may indirectly but efficiently reflect the total antioxidant power of the sample even though no radical species are involved. The cupric ion reducing activity of ethyl acetate and methanol extracts of different agro-industrial residues showed that PI

MET exhibits better reducing potential when compared to others. The reducing potential of PI MET was found to be 4.75 ± 0.08 µM TR/g dry weight. The extracts of plantain stem do not exhibit any reduction potential. The results are depicted in Figure 2.6.

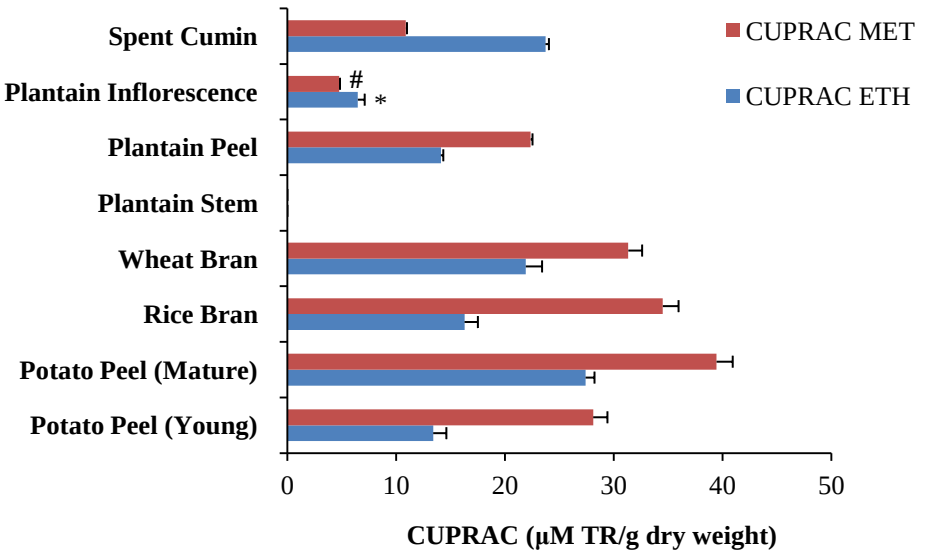

Figure 2.6. Cupric ion reducing antioxidant activity of ethyl acetate and methanol extracts of different agro-industrial residues. Each value represents mean ± SD (standard deviation) from triplicate measurements. #CUPRAC of PI MET significantly different from methanol extract of other sources. *CUPRAC of PI ETH significantly different from ethyl acetate extract of other sources ($p \leq 0.05$).

2.3.1.6 Free radical scavenging activity

Living cells generate free radicals and other reactive oxygen species as a result of physiological and biochemical processes and there is an effective antioxidant system in our body which quenches the excess free radicals. However in some chronic conditions free radical production increases beyond the limits of antioxidant system. Excess free radicals can cause oxidative damage to lipids, proteins and DNA, eventually leading to many chronic diseases, such as cancer, diabetes and other degenerative diseases in

humans (Aiyegoro and Okoh, 2010). Hence there is a need to take antioxidants to scavenge these excess free radicals formed in our body. Polyphenols are gaining a lot of importance as natural antioxidants that can scavenge free radicals and inactivate other pro-oxidants, and can also interact with number of biologically relevant molecules. Evidence suggests that additive and synergistic interactions of natural antioxidants significantly strengthen the protective effects against oxidative damage in the body (Liu, 2003). Hence we analysed the free radical scavenging potential of ethyl acetate and methanol extracts of different agro-industrial residues based on its efficacy to scavenge DPPH, NO, OH and Superoxide free radicals.

2.3.1.6.1 DPPH radical scavenging activity

DPPH radical has been widely used to evaluate the antioxidant properties of natural products. DPPH• (1,1-Diphenyl-2-picrylhydrazyl radical) is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. The radical scavenging potential of the sample was determined by measuring the decrease in absorbance due to DPPH• at 517 nm, representing the formation of its reduced form, 1, 1-Diphenyl-1-2- picrylhydrazine (DPPH), which was yellow in color. Because of the odd electron, the purple coloured methanolic solution shows a strong absorption band at 517 nm.

DPPH radical scavenging activity of ethyl acetate and methanol extract of different agro-industrial residues showed that PI MET exhibits better DPPH scavenging activity. The IC_{50} value PI MET was found to be 46.8 ± 1.72 µg/mL. However the activity of PI MET is less when compared to the standard gallic acid used for the assay (3.15 ± 0.77 µg/mL). Both extracts of PS and ethyl acetate extract of SC does not

exhibit any DPPH scavenging activity. The IC_{50} values for DPPH scavenging activity of ethyl acetate and methanol extracts of agro-industrial residues were shown in Figure 2.7[A].

2.3.1.6.2 Nitric oxide scavenging activity

Nitric oxide is classified as a free radical because of its unpaired electron and displays important reactivity with certain types of proteins and other free radicals such as superoxide. Nitric oxide being a potent pleiotropic mediator in physiological processes and a diffusible free radical in the pathological conditions, reacts with superoxide anion and form a potentially cytotoxic molecule, the peroxynitrite. Peroxynitrite causes nitration or hydroxylation of aromatic compounds especially tyrosine and also triggers adduct formation with dissolved carbon dioxide in body fluids and damages various proteins (Szabo et al, 2007).

At physiological pH, NO is released from sodium nitroprusside (SNP) which reacts with oxygen to produce nitrite ions. NO scavenger competes with oxygen in reacting with NO released from SNP solution in PBS. The nitrite ions react with Griess reagent to form pink chromospheres whose absorbance was measured at 540 nm. The results from the assay depicted that the methanol extract of plantain have better NO scavenging potential (IC_{50}-121.38 ± 1.48 µg/mL). The standard ascorbic acid has an IC_{50} value of 103.59 ± 2.3 µg/mL which is comparable to that of PI MET. Both extracts of PS and ethyl acetate extract of SC does not exhibit any NO scavenging activity. The IC_{50} values for NO scavenging activity of ethyl acetate and methanol extracts of agro-industrial residues were shown in Figure 2.7[B].

2.3.1.6.3 Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was quantified by measuring the inhibition of the degradation of deoxyribose by the free radicals generated by the Fenton reaction. Hydroxyl radical is the most deleterious and reactive among the ROS and it bears the shortest half-life compared with other free radicals. The oxygen derived hydroxyl radicals along with the added transition metal ion (Fe^{2+}) causes the degradation of deoxyribose into malondialdehyde which produces a pink chromogen with thiobarbituric acid (Halliwell et al., 1987). Fe^{3+}-EDTA, H_2O_2 and ascorbic acid reaction system generates hydroxyl radical, which can be detected by their ability to degrade the sugar deoxyribose into fragments. The resulting complex mixture of products is heated under acidic conditions; malonaldehyde is formed that can be detected by its ability to react with thiobarbituric acid to form a pink chromogen. In this method, the assay measures the antioxidant efficacy of the phytochemicals based on their efficacy to compete with deoxyribose for hydroxyl radicals that were produced free in solution from a Fe^{2+}-EDTA chelate.

The results from hydroxyl radical assay showed that ethyl acetate extract of PP exhibits better activity than other extracts. The ethyl acetate extract of PP showed an IC_{50} value of 3.43 ± 1.39 µg/mL even better than the standard catechin (IC_{50}- 8.1 ± 1.19 µg/mL). In case of PI also ethyl acetate extract showed better activity (174.86 ± 2.9 µg/mL). The IC_{50} values for hydroxyl radical scavenging activity of ethyl acetate and methanol extracts of agro-industrial residues were shown in Figure 2.7[C].

2.3.1.6.4 Superoxide radical scavenging activity

Superoxide is biologically quite toxic and is deployed by the immune system to

kill invading microorganisms. The biological toxicity of superoxide is due to its capacity to inactivate iron–sulfur cluster containing enzymes, generate the highly reactive hydroxyl radical, reduce certain iron complex such as cytochrome c and initiates lipid peroxidation (Gulcin et al., 2010). The superoxide anion derived from dissolved oxygen by phenazine methosulphate/NADH coupling reaction reduces nitro blue tetrazolium.

The decrease in the absorbance at 560 nm with the extract thus indicates the consumption of superoxide anion in the reaction mixture. The results from the assay showed that methanol PI exhibits better superoxide radical scavenging activity with an IC_{50} value 173.45 ± 2.19 µg/mL (Figure 2.7[D]). Surprisingly methanol extract of PS also exhibit a little bit activity (IC_{50} - 912.5 ± 1.53 µg/mL). The standard, quercetin exhibited an IC_{50} value 75.64 ± 2.14 µg/mL.

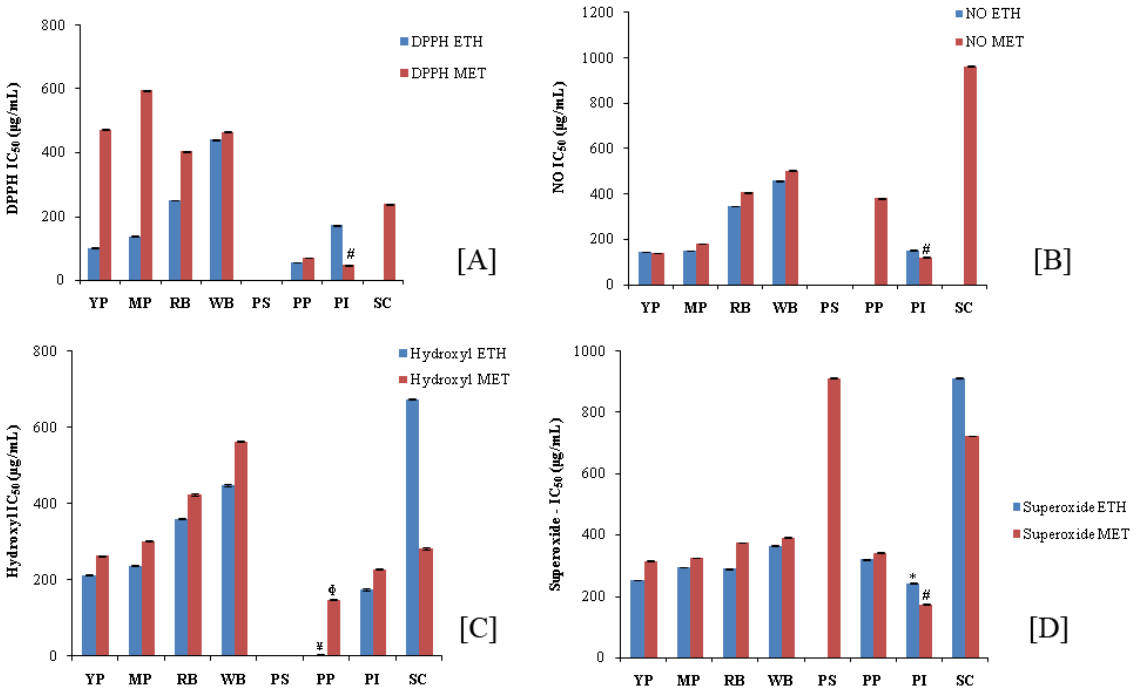

Figure 2.7. Free radical scavenging activity of ethyl acetate and methanol extract of different agro-industrial residues. [A] DPPH radical scavenging activity, [B] NO radical

scavenging activity, [C] Hydroxyl radical scavenging activity and [D] Superoxide radical scavenging activity. YP - Young potato peel, MP - Mature potato peel, RB - rice bran, WB - Wheat bran, PS - Plantain stem, PP - Plantain peel, PI - Plantain Inflorescence, SC-Spent cumin. Each value represents mean ± SD (standard deviation) from triplicate measurements. $^{\#}IC_{50}$ of PP MET significantly different from others. *IC_{50} of PP ETH significantly different from others ($p \leq 0.05$). $^{\Phi}$Hydroxyl IC_{50} of PP MET significantly different from others. $^{¥}$Hydroxyl IC_{50} of PP ETH significantly different from others ($p \leq 0.05$).

Interestingly, the results from different antioxidant assays performed showed that the methanol extract of all the selected agro-industrial residues have better activity than the ethyl acetate extract. This can be explained by the fact that the agro-industrial residues contains polar compounds (usually behind the biological activities), which are more extractable as the polarity of the solvent (used for extraction) increases. Hence the methanol extract of all the selected agro-industrial residues have better activity than the corresponding ethyl acetate extract part.

2.3.2 Proximate composition, physical properties and chemical constituents of selected agro-industrial residues

Based on the investigation of soluble dietary fibre content, total phenolic content, flavonoid content and antioxidant activities (except hydroxyl radical scavenging activity) of selected agro-industrial residues, Plantain Inflorescence was selected for further studies. The dietary fibre from PI was analyzed for its chemical composition in terms of cellulose, hemicelluloses, lignin and pectin content. The dietary fibre was also analyzed for its technological properties in terms of water holding, water retention, oil holding and swelling capacities. The ethyl acetate and methanol extracts of PI were analyzed for the presence of polyphenols by HPLC and the same were quantified. The

results are explained below.

2.3.2.1 Proximate analysis of plantain inflorescence

The proximate composition of PI was analyzed in terms of moisture, ash, fat, protein and carbohydrate content. The results are shown in Table 2.1.

Table 2.1 Proximate analysis of plantain inflorescence

Composition	Proximate content (%)
Moisture	90.8 ± 2.21
Ash	1.68 ± 0.20
Fat	0.42 ± 0.053
Protein	1.71 ± 0.123
Carbohydrate	5.39 ± 2.58

Each value represents mean ± SD (standard deviation) from triplicate measurements.

2.3.2.2 Chemical composition and technological properties of dietary fibre from plantain inflorescence

Plant cell wall polysaccharides, such as pectins, celluloses, hemicelluloses are an extremely diverse set of biopolymers, which play a very important role as structural elements. Dietary fibre mainly consists of soluble (pectins, gums etc.) and insoluble (cellulose, lignin, some hemicelluloses, etc.) fibre fractions (Thebaudin and Lefebvre, 1997).

2.3.2.2.1 Determination of pectin

Pectin is an important component of soluble dietary fibre. It has got wide applications in food and pharmaceutical industries. Pectin is also reported to induce satiety hormones in animal model (Adam et al., 2015). The results of pectin determination showed that PI contains 10.3% of pectin (Figure 2.10).

2.3.2.2.2 Determination of cellulose, hemicellulose and lignin

Cellulose, hemicelluloses and lignin are components of insoluble dietary fibre. Cellulose, hemicelluloses and lignin fractions formed a complex substrate named lignocellulosic biomass which could be used to produce bioethanol (Emaga et al., 2008). Other biological functions of insoluble dietary fibre are explained in Chapter 1. The results for insoluble dietary fibre components are shown in Figure 2.11. The cellulose content of PI was found to be 13.08 ± 0.41 g/100 g of PI. The result points out to the scope of using PI as a source of lignocellulosic biomass, for which further studies are required.

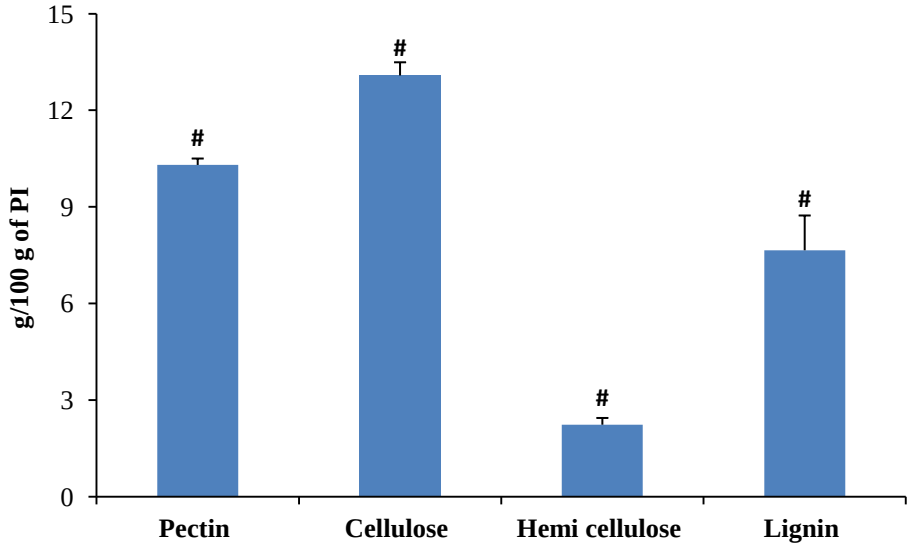

Figure 2.11. Chemical composition of dietary fibre from PI. Each value represents mean ± SD (standard deviation) from triplicate measurements. #Values significantly different from each other ($p \leq 0.05$).

2.3.2.2.3 Determination of technological properties of dietary fibre

The water associated with fibre is an important consideration when investigating the effects of fibre in the diet. Such water will influence the metabolic activity of fibre along the gut. The water holding capacity (WHC) of fibre is a measure of the ability of

a fibre source to immobilize water within its matrix. The fibre which retains more water is easily fermented by microbes of the gut (Robertson and Eastwood, 1981). Technological studies revealed that SDF from PI exhibits more water holding capacity. However the water retention and swelling capacity is more for the insoluble dietary fibre of PI. Oil holding capacity (OHC) is one of the reasons for cholesterol lowering property of fibre. Ingredients with high OHC are useful as emulsifiers for high fat food products. The insoluble dietary fibre from PI shows better oil holding property. The results are shown in Figure 2.12.

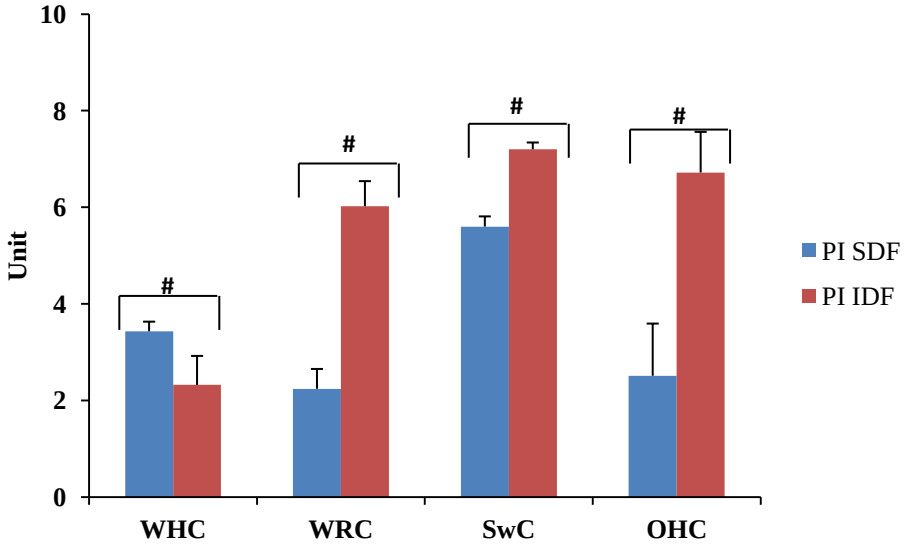

Figure 2.12. Technological properties of dietary fibre from PI. WHC- water holding capacity (g of water/g of fibre), WRC-water retention capacity (g of water/g of fibre), SwC-Swelling capactiy (mL of water/g of fibre), OHC-oil holding capacity (g of oil/g of fibre). Each value represents mean ± SD (standard deviation) from triplicate measurements. #SDF and IDF in each group are significantly different ($p \leq 0.05$).

2.3.2.3 HPLC profiling and quantification of polyphenols in extracts of PI

The polyphenol content in the ethyl acetate and methanol extracts of PI were estimated by high performance liquid chromatography. Thirteen standard polyphenolic

compounds at 1mg/mL concentration were initially analyzed-(1) gallic acid, (2) catechol, (3) chlorogenic acid, (4) caffeic acid, (5) syringic acid, (6) p-coumaric acid, (7) ferulic acid, (8) ellagic acid, (9) myricetin, (10) cinnamic acid, (11) quercetin, (12) kaempferol and (13) apigenin. The retention times are found to be 7.724, 12.773, 23.807, 25.349, 26.298, 27.567, 28.629, 31.155, 31.814, 33.329, 34.531, 37.586 and 38.322 min respectively. Apart from this, polyphenols in the extracts were confirmed by spiking also. For quantification single standard were injected at different concentrations to obtain a straight line equation from which the concentration of that standard compound in the sample was calculated. The chromatogram for ethyl acetate and methanol extracts of PI were shown in Figure 2.13.

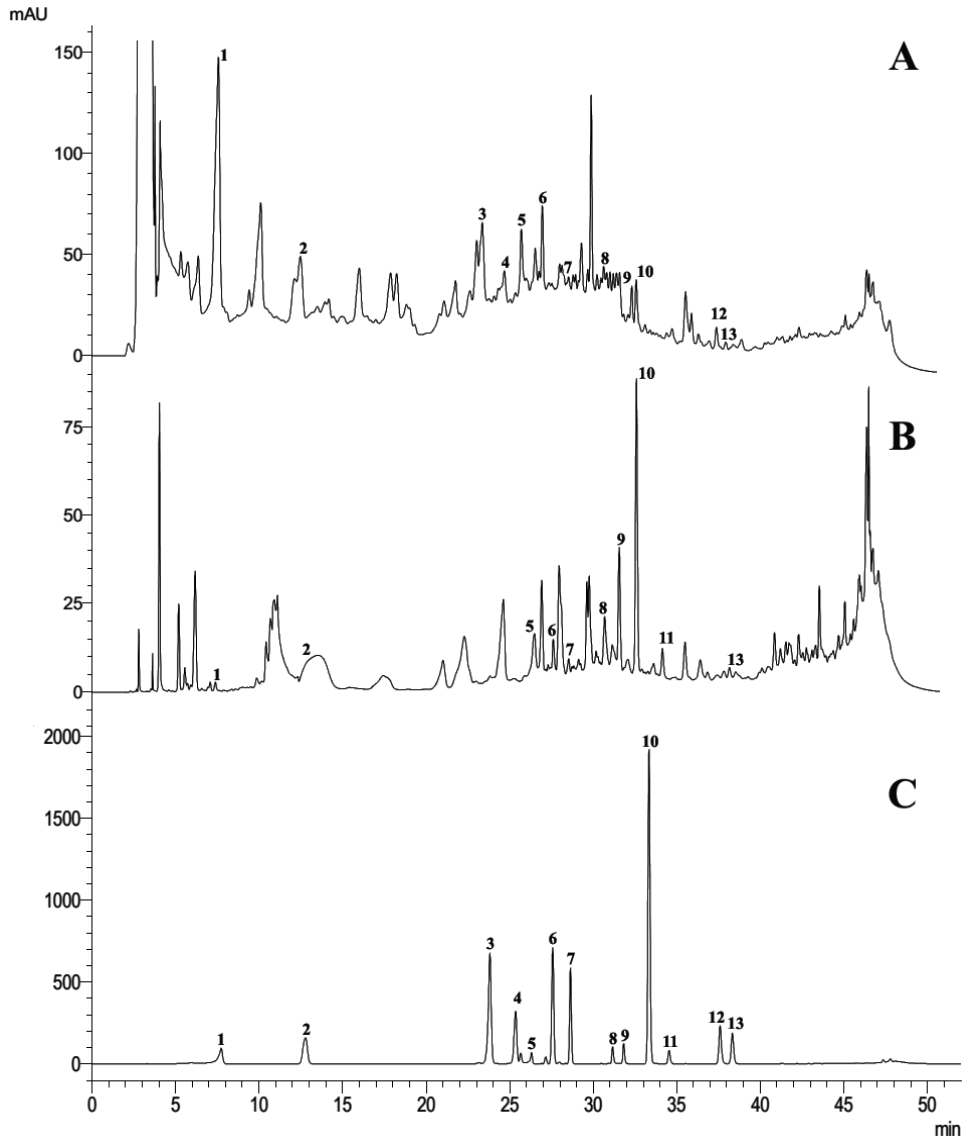

Figure 2.13. HPLC Chromatogram of ethyl acetate and methanol extract of Plantain Inflorescence. (A) Methanol extract, (B) Ethyl acetate extract and (C) Authentic standards. The numbers represents the standard polyphenols which are described in text with retention time.

This is the first study estimating the polyphenols in PI. Ten polyphenols were identified in the PI ETH (1, 2, 5, 6, 7, 8, 9, 10, 11 and 13) and twelve polyphenols were identified PI MET (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13). The quantification results suggest that PI is rich source of polyphenols (Table 2.2). Gallic acid and cinnamic acid were the abundant components in methanolic and ethyl acetate extract of PI (1.29 and 0.359

mg/mL of extract respectively). The polyphenolic content is high in methanol extract of PI and it specifically contained chlorogenic acid, caffeic acid and kaempferol. Quercetin was present only in PI ETH.

Table 2.2. Estimation and quantification of polyphenols in ethyl acetate and methanol extract of PI

Sl.No.	Polyphenols	PI ETH	PI MET
1.	**Gallic Acid**	0.001 ± 0.0002^{a}	1.290 ± 0.120^{b}
2.	**Catechol**	0.098 ± 0.012^{a}	0.323 ± 0.086^{b}
3.	**Chlorogenic acid**	-	0.115 ± 0.048
4.	**Caffeic acid**	-	0.068 ± 0.007
5.	**Syringic acid**	0.047 ± 0.024^{a}	0.233 ± 0.088^{b}
6.	**p-Coumaric acid**	0.009 ± 0.0004^{a}	0.108 ± 0.02^{b}
7.	**Ferulic acid**	0.007 ± 0.0001^{a}	0.019 ± 0.001^{b}
8.	**Ellagic acid**	0.054 ± 0.016^{a}	0.028 ± 0.008^{b}
9.	**Myercetin**	0.093 ± 0.014^{a}	0.072 ± 0.004^{b}
10.	**Cinnamic acid**	0.359 ± 0.084^{a}	0.086 ± 0.001^{b}
11.	**Quercetin**	0.011 ± 0.002	-
12.	**Kaempferol**	-	0.056 ± 0.009
13.	**Apigenin**	0.005 ± 0.0006^{a}	0.025 ± 0.001^{b}

PI-Plantain Inflorescence, PI ETH-ethyl acetate extract of PI, PI MET-methanol extract of PI. The results are expressed in mg/mL of extract. Each value represents mean ± SD (standard deviation) from triplicate measurements. $^{a-b}$ Values in a row with different superscript are significantly different ($p \leq 0.05$).

This result can be correlated with the biological activities of PI, as the health beneficial effects of plant-derived products have been largely attributed to polyphenolic compounds (Anhe et al., 2013). Gallic acid is a well known antioxidant compound and

is reported to have anticancer effect against many cancer cell lines (Paolini et al., 2015; Wang et al., 2014; Sourani et al., 2015). The identified polyphenols have beneficial effect on cancer, diabetes and cardiovascular diseases (Araujo et al., 2011; Xiao and Hogger, 2015; Quinones et al., 2013). Earlier we have stated that the methanol extract of agro-industrial residues is more active than the ethyl acetate extract as methanol extract contains more polar compounds. The HPLC data of methanol and ethyl acetate extract of plantain inflorescence is agreeable with this statement.

2.4 Conclusion

Based on literature survey and local availability, five different sources of agro-industrial residues were selected for screening of antioxidant dietary fibre. Screening was done based on dietary fibre content and antioxidant activities. Among the selected sources plantain inflorescence possessed higher content of soluble dietary fibre content. Soluble dietary fibre is known to have biological effect more than the insoluble fibre. The total phenolic and flavonoid content indicates that methanol extract of PI may have good antioxidant activity. The activity in terms of CUPRAC assay, DPPH and superoxide radical scavenging assays confirms the antioxidant potential of PI. Except in case of the hydroxyl radical scavenging activity, better activity was shown by the extracts of plantain peel. However, we have selected plantain inflorescence for our further studies. The phenolic compounds of PI extracts were identified and quantified using HPLC. The results showed that PI is rich source of polyphenols. Hence we decided further to elucidate the biological properties of dietary fibre and extracts of plantain inflorescence which has been explained in following chapters.

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Chapter 3

Effect of Plantain Inflorescence in modulating type 2 diabetes and associated cardiovascular problems

3.1 Introduction

Food rich in antioxidants and dietary fibre play an important role in everyday life owing to their associated health benefits in the prevention and management of chronic and degenerative diseases. It is well known that the imbalance in the antioxidant defense system in the body leads to oxidative stress which is one of the reasons for the onset of chronic diseases (Fang et al., 2002) and the complications associated with it. Evidences from epidemiological studies suggest a strong correlation between consumption of diets rich in antioxidants and dietary fibre and reduction of chronic diseases (Slavin and Lloyd, 2012). In this respect, there is an increased interest in finding new dietary sources of antioxidants and fibre and validating their role in reducing the risk of the chronic diseases (Pandey and Rizvi, 2009; Pal et al., 2012).

Diabetes is a chronic disease condition which develops due to the failure in regulating the blood glucose level. Recent studies indicate that oxidative stress contributes to the pathogenesis of type 2 diabetes by increasing insulin resistance or impairing insulin secretion (Robertson, 2006; Tangvarasittichai, 2015). Under oxidative stress the free radicals generated inside the body oxidize LDL, which leads to many harmful biological effects including atherosclerotic plaques formation. Prolonged insulin resistance lead to hypertension (Ferrannini et al., 1987) and therefore, type 2 diabetes patients also have significantly higher risk of developing cardiovascular disease (Martin-Timon et al., 2014). The metabolic changes associated with diabetes can be controlled by means of inhibiting enzymes (α-amylase and α-glucosidase) associated with carbohydrate digestion leading to reduction of hyperglycemia, along with reduction of oxidative stress (Matsui et al., 2006). Many studies reported that

natural antioxidants (Dhanya et al, 2015) and dietary fibre (Weickert and Pfeiffer, 2008) possess potential protective effect in managing diabetes and associated complications.

The *Nendran* variety of *Musa paradisiaca* is an herbaceous plant cultivating mainly in southern parts of Asia for their fruits. The inflorescence of plantain species are used as vegetable in different parts of the world. Traditional medical practioners used the inflorescence for the tretament of various diseases (Imam and Akter, 2011). Plantain is reported to be one of the potent antidiabetic plants (Alarcon et al., 1998; Eleazu and Okafor, 2015). Various scientists have reported the blood glucose and cholesterol lowering effect of plantain inflorescence in rats (Pari and Umamaheswari, 2000; Gomathy et al., 1989; Gomathy et al., 1990). It is also reported that plantain inflorescence is a rich source of phenolic compounds with potential radical scavenging activities (Jayamurthy et al., 2011). Apart from this, preliminary study (as described in chapter 2) suggests that the plantain inflorescence is a very good source of dietary fibre.

These reports suggest that plantain inflorescence could be a potential source of phytochemicals with promising antidiabetic potential. Since diabetes is multifactorial in origin, the present study was designed to evaluate the antidiabetic potential of *Musa paradisiaca* (Plantain) inflorescence by exploring the underlying mechanisms in terms of glucose absorption capacity of dietary fibre and antioxidant activity, carbohydrate digesting enzyme inhibition (α-glucosidase and α-amylase) potential, antiglycation and glucose uptake capacity of extracts prepared from the inflorescence. The study also focuses on the cardiovascular protecting efficacy of plantain inflorescence as it is one of the major complications associated with diabetes mellitus.

Various plant based agro-industrial residues were screened for antioxidant activity and dietary fibre content as described in chapter 2, and the results indicated that plantain (*Musa paradisiaca*) inflorescence exhibits better antioxidant and dietary fibre content. The polyphenolic profiling by HPLC indicated the presence of biologically active polyphenols. Our findings from the screening of antidiabetic and cardiovascular potential of plantain inflorescence extracts comprise the subject matter of Chapter 3.

3.1.1 Objectives

The main objectives of this chapter can be summarized as follows:

- To analyze the PI extracts for antioxidant potential in terms of ABTS free radical scavenging and inhibition of *in vitro* ROS production in L6 muscle cells
- To analyze the PI extracts for the antidiabetic effect by inhibition of α-amylase and α-glucosidase assays; antiglycation assay; and its ability to enhance glucose uptake property in differentiated L6 cells
- To investigate the inhibition of LDL oxidation and angiotensin converting enzyme activity of the extracts to examine the cardiovascular protective effect
- To assess the glucose and cholesterol adsorption capacity of the dietary fibre isolated from PI

3.2 Materials and Methods

3.2.1 Chemicals

Dulbecco's modified Eagle's media (DMEM), fetal bovine serum (FBS), horse serum, antibiotic-antimycotic solution (pencillin-streptomycin–amphotericin B mix), rosiglitazone, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), dichloro-dihydro-fluorescein diacetate (DCFH-DA), α-amylase, α-glucosidase,

acarbose, trolox, bovine serum albumin, low density lipoprotein from human plasma (LDL), angiotensin converting enzyme (ACE), N-[3-(2-Furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) and nonylamine were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). 2-NBDG (2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose) was procured from Molecular Probe (Invitrogen Life Technologies, Carlsbad, CA, USA. Glucose and cholesterol were obtained from Sisco Research Laboratories, Mumbai. India. All other reagents used were of analytical grade.

The outline of work included in this chapter is depicted in Figure 3.1.

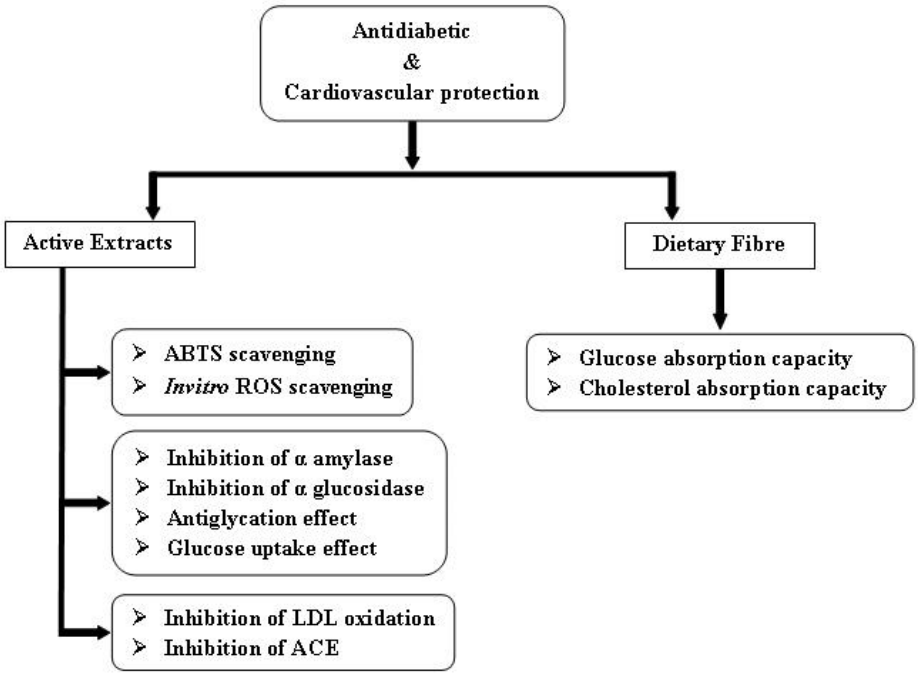

Figure 3.1. Outline of Chapter 3

3.2.2 Sample preparation

Plantain inflorescence, from *Nendran* variety, identified as *Musa paradisiaca*, was collected from one of the local banana farms, located at Thiruvananthapuram district of Kerala, India. A voucher specimen of *Musa paradisiaca* (TBGT 81481) has been deposited in the herbarium of (TBGT) of Jawaharlal Nehru Tropical Botanic Garden

and Research Institute, Palode, Thiruvananthapuram, Kerala, India. The inflorescence washed, drained and sliced into small pieces was then freeze dried using lyophilizer (VirTis genesis, USA). The freeze dried sample was ground using a blender (Ultra centrifugal mill ZM200, Retsch, Germany) and sieved using 20 mm mesh (Vibro Sifter-PVS30, Prism Pharma Machinery, India) to obtain fine PI powder and stored in a cool dark under until further use.

3.2.3 Extraction of fibre

The total dietary fibre from PI was isolated according to Bureau of Indian Standard Method (IS: 11062, 1984). The defatted sample was enzymatically hydrolyzed to obtain soluble and insoluble dietary fibre (SDF and IDF) which was explained in detail in chapter 2 (Section 2.2.3.2).

3.2.4 Extraction of antioxidants

The PI powder was defatted with hexane and sequentially extracted with ethyl acetate (PI ETH) and methanol (PI MET) at room temperature. The extraction method was explained under section 2.2.3.3 in chapter 2. The extracts were stored at 4°C until further biochemical analysis.

3.2.5 Antioxidant assays

3.2.5.1 ABTS scavenging activity

ABTS scavenging potential of extracts were analyzed by the method of Arnao et al. (2001) with some modifications. The working solution was prepared by mixing the stock solutions - 7 mM ABTS and 2.45 mM potassium persulfate solution in equal quantities, and allowing them to react for 14 h at room temperature in the dark. The solution was then diluted with ethanol to make the absorbance in the range 0.70 ± 0.01

units at 734 nm. Different concentrations of extracts were allowed to react with 1 mL of the ABTS solution for 7 min and the absorbance was taken at 734 nm. The ABTS scavenging capacity of the extract was compared with that of ascorbic acid and percentage inhibition was calculated as

$$\text{ABTS radical scavenging activity (\%)} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

3.2.5.2 Determination of intracellular reactive oxygen species (ROS) production

The excess production of ROS is reported to contribute to the development of diabetes mellitus (DM) and diabetic complications, and cardiovascular diseases (CVD) (Alfadda and Sallam, 2012). The efficacy of extracts against intracellular reactive oxygen production was determined in L6 myoblasts.

3.2.5.2.1 Cell culture, treatment conditions and cell viability assay

L6 myoblast cells were obtained from NCCS, Pune, India and were cultured in DMEM supplemented with 10% fetal bovine serum and 0.5% antibiotic-antimycotic solution at 37°C in a humidified atmosphere containing 5% CO_2. Cells at 70–80% confluence were used for all the experiments.

Cytotoxicity of the extracts were standardized based on the concentration using MTT assay (Mosmann, 1983). Cultures were maintained at 37°C in 5% CO_2 incubator. The cells were trypsinized and seeded on 24 well plates (1×10^4 cells per well). Cells after attaining 80% of confluency were treated with different concentrations of the extracts for 24 hours. After the incubation, cells were treated with MTT reagent (0.5 g/L), dissolved in PBS, for the estimation of mitochondrial dehydrogenase activity and inclubated for 4 hours. Mitochondrial dehydrogenase enzyme, which is active only in

live cells, reduces the yellow dye, MTT to purple formazan crystals. The formazan crystals were dissolved in DMSO and the absorbance was read at 570 nm using multimode reader (Synergy 4 Biotek multiplate reader, USA). Each assay was carried out three times, and the results were expressed as the mean ± SD. The percentage viability was calculated as

$$\% \text{ Cell viability} = \frac{\text{The absorbance of test}}{\text{The absorbance of control}} \times 100$$

3.2.5.2.2 Intracellular reactive oxygen species (ROS) levels

The effect of extracts on intracellular ROS levels was measured using DCFH-DA as described by Cathcart et al. (1983). The cells were pre incubated with different sub toxic concentrations of extracts for 24 h followed by H_2O_2 (100 µM) for 15 min. Cells were then washed and incubated with DCFH-DA (20 µM) for 30 minutes and imaged with fluorescent microscope (Pathway 855, BD Bioscience, USA) equipped with filters in the range, excitation, 490 nm; and emission, 525 nm. Quantification of ROS production by cells was determined by measuring the intracellular DCF fluorescent intensity by flow cytometry (BD FACS Aria II, BD Bioscience, USA). The mean fluorescence intensity from 10000 single cell events was collected and analyzed by BD FACS Diva software.

3.2.6 Antidiabetic assays

3.2.6.1 α-amylase inhibition

The method as described by Xiao et al. (2006), based on the starch-iodine test, was adopted for analyzing α-amylase inhibition. Different concentrations of extracts were added to the reaction mixture containing 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM NaCl) and 0.02 U of porcine pancreatic α-amylase were incubated at

37°C for 10 min. followed by incubation at 37°C for 15 min after the addition of 1% w/v of soluble starch. The reaction was terminated by adding 1 M HCl, followed by the addition of iodine reagent (5 mM I_2 and 5 mM KI). The absorbance was read at 620 nm on a microplate reader (Synergy 4 Biotek multiplate reader, USA). A control (representing 100% enzyme activity) was kept without any extract. Appropriate sample controls were included to eliminate the absorbance produced by PI extract. Acarbose was used as the standard. The enzyme activity was measured as follows

$$\% \text{ Relative activity} = \left(\frac{\text{Absorbance of test}}{\text{Absorbance of control}}\right) \times 100$$

3.2.6.2 α-glucosidase inhibition

The α-glucosidase inhibition activity of the extracts was determined by the method of Adisakwattana et al. (2004). In brief, the assay system containing 0.1 M phosphate buffer (pH 7.0), 0.5 mM 4-nitrophenyl α-D-glucopyranoside, extract at different concentrations and 0.1 U/mL α-glucosidase enzyme was incubated at 37°C for 30 min. The reaction was terminated by adding 0.2 M Na_2CO_3 solution. The absorbance of p-nitrophenol released by the enzyme activity was measured at 410 nm. Acarbose was used as the standard. The α-glucosidase inhibition was calculated as

$$\% \text{ Inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100$$

3.2.6.3 Antiglycation property

Antiglycation property of extracts was analyzed as described by Arom (2005). In short, 1 mg/mL of bovine serum albumin was incubated with 200 mM of D-glucose and extracts (different concentrations) in 0.2 M potassium phosphate buffered saline (PBS-pH 7.4) at 60°C for 24 h. The reaction was discontinued by adding 100% trichloro acetic acid and kept at 4°C for 10 min followed by centrifugation at 10,000×g (Kubota-

7780, Japan). The precipitate was redissolved in alkaline PBS. The fluorescence of the glycated end products were measured using excitation at 370 nm and emission at 440 nm (Synergy 4 Biotek multiplate reader, USA).

3.2.6.4 Glucose uptake assay

Glucose uptake assay was performed in differentiated L6 cell lines as described by Chen et al. (2010). Cell culture and treatment conditions are as described earlier under ROS production. Glucose uptake is done in differentiated cells and DMEM medium supplemented with 5% horse serum was used for differentiating L6 cells. Differentiated L6 cells were pretreated with extracts at different concentrations (below IC_{50} concentration, as obtained from MTT assay) for 24 h. Medium was removed and the cells were washed twice with pre cooled phosphate buffered saline (PBS). The cells were then treated with the fluorescent analogue of glucose; 2-NBDG (100 µM), and incubated for 30 min. The uptake of 2-NBDG by the cells was stopped by removing the incubation medium and washing the cells twice with pre-cold PBS. Cells were trypsinised and subsequently resuspended in 1 mL buffer. For each measurement, data from 10000 single cell events were collected using Fluorescence Activated Cell Sorting (BD FACS Aria II, BD Bioscience, USA) and BD FACS Diva software. Rosiglitazone (100 nM) was used as positive control.

3.2.6.5 Glucose adsorption capacity of dietary fibre

Glucose adsorption capacity of fibre was estimated by the method of Ou et al. (2001) with slight modification. Briefly, fibre (50-250 mg) was added to 10 mL of 100 mM glucose solution, mixed well and incubated at 37°C for 6 h. Then it was centrifuged at 4,000 rpm (Kubota-7780, Japan) for 10 min to obtain supernatant. The glucose content

in the supernatant was determined by dinitrosalicylic acid assay (Miller, 1959). 300 µL of supernatant was mixed with 300 µL of DNS reagent (100 mg dinitrosalicylic acid, 20 mg phenol, 0.5 mg sodium sulphite and 100 mg sodium hydroxide dissolved in 100 mL distilled water) and placed in boiling water bath for 15 min followed by addition of 100 µL of potassium sodium tartrate (40%). The assay system was cooled to room temperature and absorbance was recorded at 575 nm. The difference in concentration of control and supernatant gives the concentration of glucose adsorbed by the fibre.

3.2.7 Cardio vascular protection assays

3.2.7.1 Inhibition of LDL oxidation

Malondialdehyde production due to the oxidation of LDL was measured by reaction with TBA (Orrego et al., 2009). Briefly, LDL (50 µg/mL) was incubated with extract (different concentrations). The LDL oxidation was initiated by adding 50 µL $CuSO_4$ (2 mM). The reaction mixture was made up to 1.5 mL with phosphate buffer (pH 7.4) and incubated for 2 h at 37°C. After incubation, 250 µL of TBA (1% in 50 mM of NaOH) and TCA (0.28%) was mixed with 500 µL of reaction mixture and kept for incubation at 95°C for 45 min. The fluorescence of the supernatant obtained after centrifugation (2000 rpm for 10 min) was taken at 515 nm excitation and 553 nm emission. The result was expressed as % of inhibition of LDL oxidation.

$$\%\ Inhibition = \left(\frac{Fluorescence\ of\ control - Fluorescence\ of\ sample}{Fluorescence\ of\ control}\right) \times 100$$

3.2.7.2 Determination of ACE inhibition

The inhibition of angiotensin converting enzyme (ACE) by the extract was determined according using HPLC as per the methods of Anzenbacherova et al. (2001) with slight modifications. Extracts at different concentration were premixed with 15 mU ACE for one min and followed by 200 µL of 0.5 mM FAPGG (N-[3-(2-Furyl)acryloyl]-L-phenylalanyl-glycyl-glycine). It was allowed to react at 37°C for 45 min and the reaction was terminated by keeping the mixture in boiling water bath for 3 min. Suitable blank (FAPGG alone) and control (ACE reacted with FAPGG without inhibitors) was performed under same conditions. FAPGG and FAP (N-[3-(2-Furyl)acryloyl]-L-phenylalanine) formed during the enzymatic reaction were analyzed on Prominence UFLC system (Shimadzu, Japan) containing LC-20AD system controller, Phenomenex Gemini C18 column (250 × 4.6 mm, 5 µm), a column oven (CTO-20A), a Rheodyne injector (USA) with a loop of 20 µL volume and a diode array detector (SPD-M20A). The compounds were separated isocratically with a mobile phase consisting of a mixture of 0.02 M nonylamine (adjusted to pH 2.4 with phosphoric acid): acetonitrile, 67.5:32.5 (v/v). The flow rate was 1 mL/min, the injection volume was 20 µL and column was at 37°C. The eluted fractions were monitored at 305 nm. LC LabSolutions software (Shimadzu, Japan) was used for data acquisition and analysis.

3.2.7.3 Cholesterol adsorption capacity of dietary fibre

Cholesterol adsorption capacity of fibre was estimated by the method described by Abell et al. (1952) with slight modification. Briefly, fibre (50-250 mg) was added to 10 mL of 100 mM cholesterol in ethanol, mixed well and incubated at 37°C for 6 h and centrifuged at 4,000 rpm (Kubota-7780, Japan) for 10 min. 200 µL of supernatant was

mixed with 2 mL chloroform, 2 mL acetic anhydride sulphuric acid mixture, mixed well and placed in dark at room temperature for 30 min. The absorbance was recorded at 680 nm. The difference in concentration of control and supernatant gives the concentration of cholesterol adsorbed by the fibre.

3.2.8. Statistical analysis

The experimental results were expressed as mean ± SD (standard deviation) of triplicate measurements. The data were subjected to one way analysis of variance (ANOVA) and the significance of differences between means were calculated by Duncan's multiple range test using SPSS for windows, standard version 7.5.1, SPSS (SPSS Inc., USA) and the significance accepted at $p \leq 0.05$.

3.3 Results and Discussion

3.3.1 Isolation of dietary fibre

Intake of dietary fibre has been reported to prevent nutritional disorders like gut related problems, cardiovascular diseases, type 2 diabetes, certain types of cancer and obesity (Verma and Banerjee, 2010; Eswaran et al., 2013; Slavin, 2008; Hopping et al., 2010; Nomura et al., 2007; Tucker and Thomas, 2009). Soluble and insoluble fibre (SDF and IDF) from PI was extracted enzymatically. Plantain inflorescence was found to be a rich source of SDF and IDF. The SDF and IDF content of PI were found to be 12.45 ± 1.14% and 53.31 ± 1.78% dry weight of PI, respectively. The results are explained in chapter 2 (Figure 2.2).

The ratio of SDF to IDF plays an important role in defining the nutritional significance of dietary fibre. As per the American Dietetic Association, fibre intakes of 25 to 30 g per day or 10 to 13 g per 1000 Kcal with an IDF/SDF ratio of 3:1 for adults is

recommended (Borderias et al., 2005; Reyes-Caudillo et al., 2008) which refers to a SDF/IDF ratio of 0.33. The SDF/IDF value for various legumes is reported to range between 0.07 – 0.68 (Aldwairji et al, 2014). The SDF/IDF ratio of banana peel was reported to be in a range of 0.18 – 0.25 (Wachirasiri et al., 2009). The SDF/IDF value for PI in the present study is 0.23 and is close to the recommended value, in order to obtain the physiological effect associated with both the soluble and insoluble fractions. As can be seen, the SDF/IDF was much better than that is reported for various legumes.

3.3.2 Extraction of antioxidants

It is well known that major part of phytochemicals is linked with dietary fibre (Spiller, 1986). There is increasing interest to find new sources of dietary fibres with specific bioactive constituents like polyphenols that could be used for the development of food products with added health benefits. An earlier study by the authors (Jayamurthy et al., 2011) reported that PI is a rich source of bioactive compounds. However, phytochemical characterization of bioactive compounds from PI has never been attempted earlier. Antioxidants from PI were extracted sequentially with ethyl acetate and methanol and its phenolic compounds were profiled. The yield of PI ETH and PI MET were 46.4 ± 1.74 and 84.13 ± 2.14 mg/g of PI respectively (Chapter 2, Figure 2.3).

3.3.3 Antioxidant assays

Dietary antioxidants play a major role in the prevention and management of life style associated diseases. Diabetic complications are reported to be arbitrated by increased free radical generation followed by oxidative stress (Suvarna and Ajay Kumar, 2010). It plays key role in developing insulin resistance and diabetes associated cardiovascular diseases. Hyperglycemia induced oxidative stress, impairment in insulin action and

insulin secretion. Apart from this, antioxidant mechanisms are weakened in diabetic patients, which may further enhance oxidative stress (Rains and Jain, 2011; Maritim et al., 2003). Dietary antioxidants can play a major role in the prevention and management of diabetes and associated complications by scavenging the free radicals and thus reducing the oxidative stress. It was found that PI is a good source of many important phenolic antioxidants. Further to confirm its antioxidant potential of PI, the extracts were evaluated for ABTS radical scavenging assay and inhibition of intracellular ROS production in L6 myoblasts induced by H_2O_2.

3.3.3.1 ABTS scavenging potential

ABTS assay measures the relative antioxidant ability to scavenge the radical $ABTS^{+\cdot}$ as compared with a standard amount of Trolox. Interaction with the extract or standard Trolox suppresses the absorbance of ABTS radical, and the results were expressed as TEAC value (Figure 3.2).

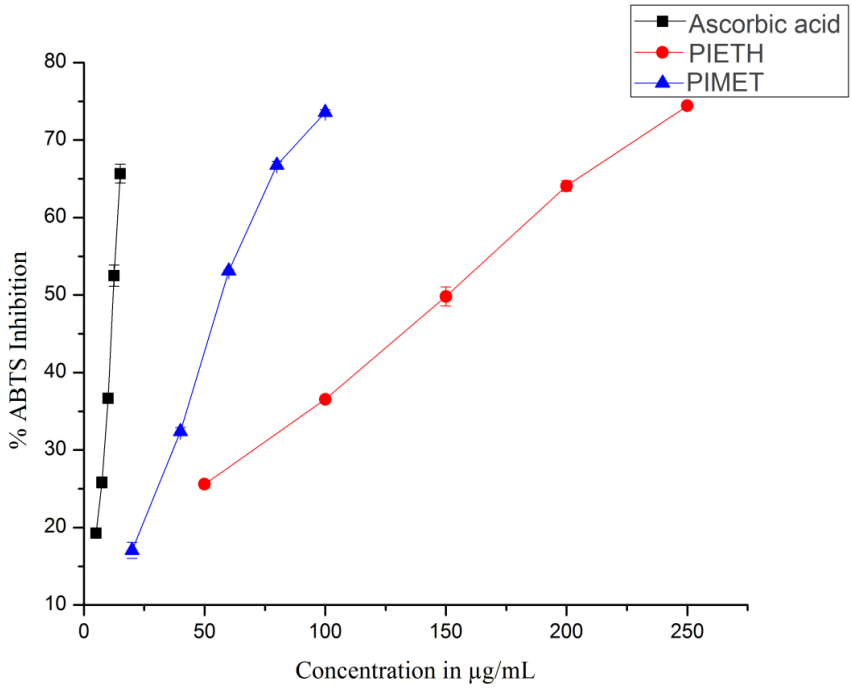

Figure 3.2. ABTS inhibition assays performed with ethyl acetate and methanol extracts

of PI. PI ETH- ethyl acetate extract of plantain inflorescence, PI MET- methanol extract of plantain inflorescence. Graph was plotted by X axis with different concentrations of extracts and Y axis with %inhibition values. Each value represents mean ± SD (standard deviation) from triplicate measurements.*PI MET significantly different from PI ETH values ($p \leq 0.05$).

The data indicated that both PI ETH and PI MET effectively scavenge ABTS radicals and the scavenging potential increased in a dose dependent manner. The standard ascorbic acid exhibited an IC_{50} – 12.05 ± 1.20 μg/mL. Among the extracts PI MET exhibited better scavenging ability (IC_{50} – 57.2 ± 1.15 μg/mL) when compared to PI ETH (IC_{50} – 150.17 ± 1.67 μg/mL). It is reported earlier that the methanolic extracts of plantain flower exhibited promising metal chelating activity, DPPH radical scavenging activity, reducing power and superoxide scavenging activity (Jayamurthy et al., 2011). The better activity of PI MET may be related to the high phenolic content as discussed in Chapter 2.

3.3.3.2 Inhibition of ROS production by methanol extract of plantain inflorescence

It is reported that oxidative stress induced by ROS plays a major role in cellular damage which leads to secondary injury to the cells (Kang, 2012). The production of ROS in the cells leads to depletion in the antioxidant mechanism in the body. Dietary supplimentation of antioxidants can improve the antioxidant status of the body (Alvarado et al., 2006). Since, PI MET possesses significantly higher concentration of phenolic compounds and free radical scavenging capacity; it was selected for studying the effect on *in vitro* ROS production. The potential of PI MET against intracellular ROS was evaluated using H_2DCFDA by fluroscent imaging and flow cytometric analysis.

3.3.3.2.1 Effect of plantain inflorescence methanol extract in viability of L6 cells

Prior to *in vitro* assessment of ROS production, the cytotoxicity of PI MET was examined in L6 myoblasts by MTT assay. In order to find out the working concentrations of PI MET, cells were treated with different concentrations of PI MET (50, 100, 150, 200 and 250 µg/mL) and cell viability was determined. A concentration of 151.02 µg/mL (Figure 3.3) of PI MET caused cell viability to decrease by about 50%.

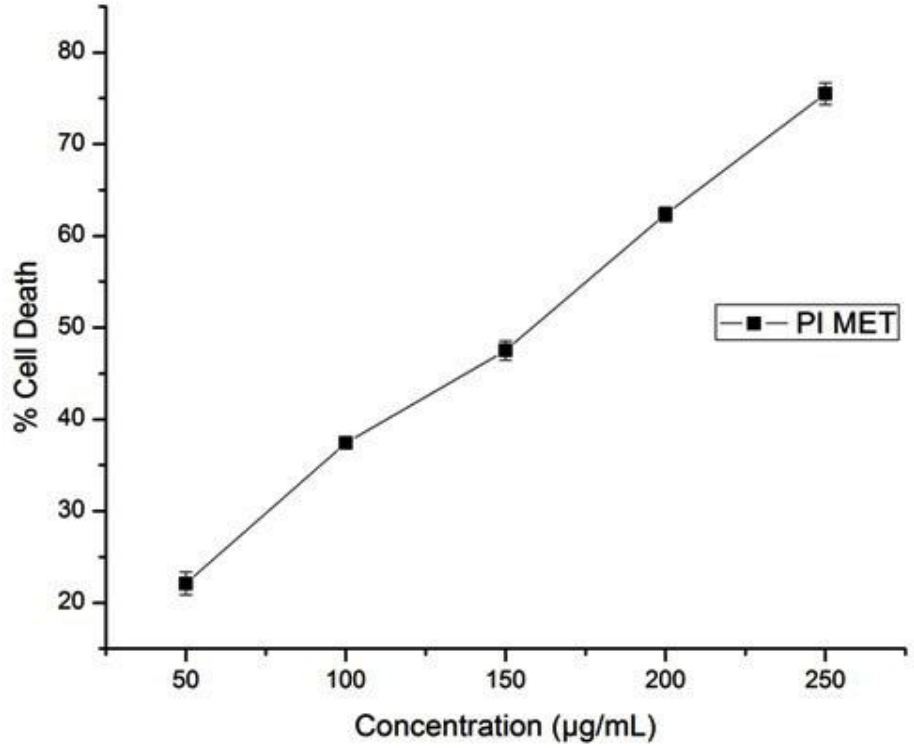

Figure 3.3. Cytotoxic effect of methanol extract of plantain inflorescence (PI MET). Graph was plotted by X axis with different concentrations of plantain inflorescence extracts and Y axis with %cell death values. Each value represents mean ± SD (standard deviation) from triplicate measurements.

For inducing oxidative stress, H_2O_2 has often been reported as a model in different cell types (Small et al., 2013; Sun et al., 2013). In order to determine the subtoxic concentrations of H_2O_2, L6 cells were exposed to H_2O_2 at concentrations

ranging between10 to 500 µM, for 20 min and viability was assayed by MTT. It was found that different concentrations of H_2O_2 significantly reduced cell viability after treatment and 100 µM H_2O_2 caused cell viability decrease by about 30%. Therefore the cells were exposed to a concentration of 100 µM H_2O_2 for inducing oxidative stress for further assays.

3.3.3.2.2 Inhibition of in vitro ROS production in L6 cells

In order to find out the protective effect of PI MET against H_2O_2 induced toxicity, the cells (L6) were initially treated with subtoxic levels of PI MET (50 and 100 g/mL) for 24 h followed by H_2O_2 at a concentration of 100 µM for 20 min. The ROS production was determined by measuring the intracellular the ROS levels, which were visualized by detecting dichlorofluorescein (DCF), derived from the oxidation of H2DCF. The images obtained from confocal microscopy have been illustrated in Figure 3.4[I]. The results indicated that on treatment with 100 µM H_2O_2 a significant increase in ROS level was noticed as compared to the control and the pre-treatment with PI MET efficiently protected cells from H_2O_2 induced toxicity. The fluorescence intensity of the L6 control cells was significantly lower than that of cells with H_2O_2 induced oxidative stress. The ROS level was considerably reduced when cells were pre-treated with PI MET, before treating with 100 µM H_2O_2, as indicated by the reduction in the fluorescence.

These results were further confirmed by quantifying the fluorescence intensity using flow cytometric analysis. The intensity of untreated control (without any treatment), H_2O_2 treated cells (positive control) were 0.31 ± 0.04% and 27.53 ± 2.81% respectively (Fig 3.4[II]). The fluorescence intensity of cells treated with 50 and 100

µg/mL of PI MET extract prior to H_2O_2 treatment was 15.33 ± 1.15% and 7.5 ± 0.56% which was significantly lower than H_2O_2 treated cells. This result suggests that H_2O_2 could induce ROS accumulation in L6 and this ROS production could be effectively inhibited upon treatment with PI MET extract. This activity of PI MET may be correlated to the presence of various antioxidant molecules present in the extracts which which can reduce hydrogen peroxide induced oxidative stress.

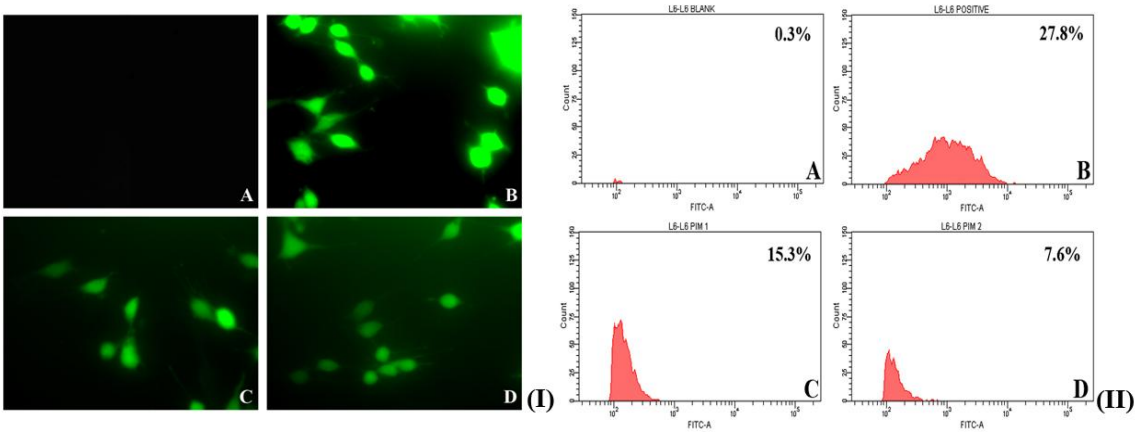

Figure 3.4. *In vitro* scavenging of ROS in L6 cell lines by methanol extract of plantain inflorescence determined by confocal microscope [I] and flow cytometre [II]. (A) Untreated Control, (B) H_2O_2 treated, (C) PI MET – 50 µg/mL, (D) PI MET – 100 µg/mL. Cells were observed and photographed under a confocal microscope at 40X magnification. In flowcytometry histogram, the X axis showed the log scale of fluorescent intensity and Y axis showed the cell count. Images shown are representative of three independent experiments.

3.3.4 Antidiabetic assays

3.3.4.1 Inhibition of α-amylase and α-glucosidase

The control of postprandial plasma glucose levels is significant in the treatment of diabetes and reducing severe vascular complications associated with diabetes (Kwon et al., 2008). The hyperglycemic condition arises due to the hydrolysis of starch by α-amylase and α-glucosidase (Kavitha et al., 2012). Hence one of the therapeutic

approaches in diabetes treatment is the reduction of hyperglycemic condition is by inhibiting α-glucosidases and α-amylase. The inhibitory activity of extracts of PI on α-amylase and α-glucosidase was investigated. The results indicated that PI extracts exhibited good anti α-amylase and α-glucosidase activity (Figure 3.5[A] and 3.5[B]).

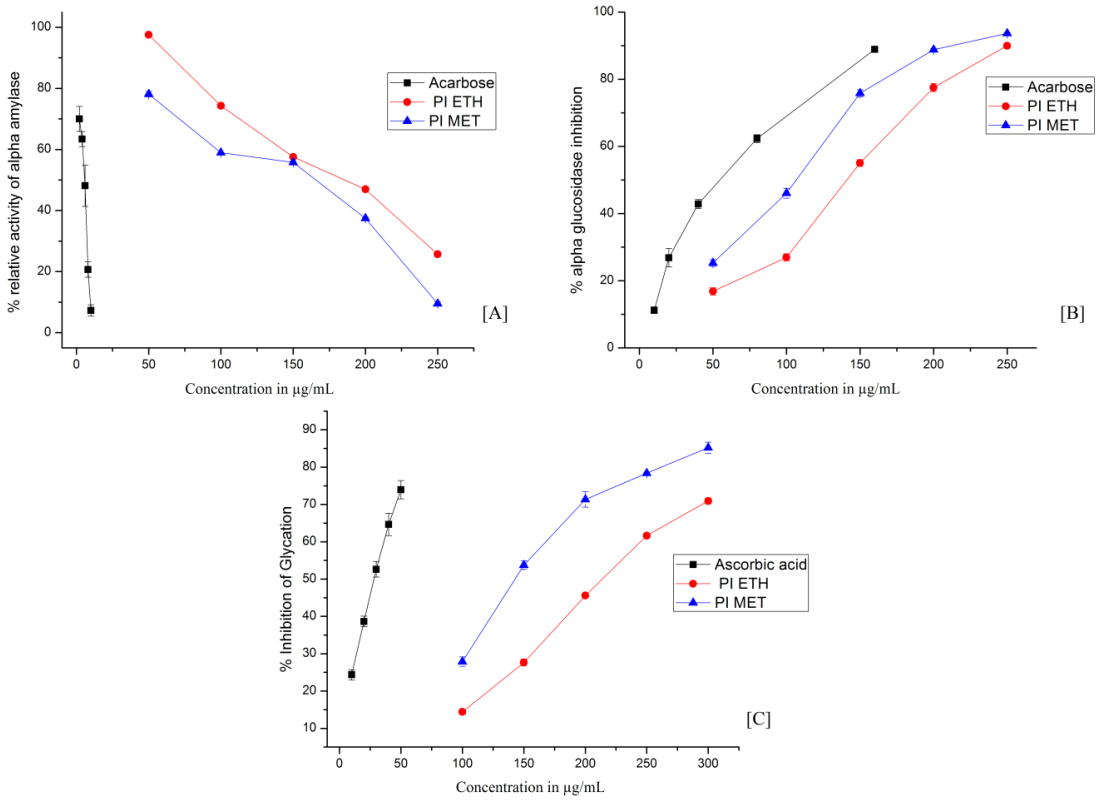

Figure 3.5. Inhibition assays performed with ethyl acetate and methanol extracts of PI. [A] inhibition of α amylase, [B] inhibition of α glucosidase and [C] inhibition of glycation process. PI ETH- ethyl acetate extract of plantain inflorescence, PI MET- methanol extract of plantain inflorescence. Graph was plotted by X axis with different concentrations of extracts and Y axis with %inhibition values. Each value represents mean ± SD (standard deviation) from triplicate measurements. *PI MET significantly different from PI ETH values ($p \leq 0.05$).

There was a dose dependent increase in the percentage inhibitory activity against α-amylase and α-glucosidase enzymes. The PI MET showed an IC_{50} value of 166.14 ± 1.18 and 106.37 ± 1.25 µg/mL; whereas PI ETH showed an IC_{50} value of

183.96 ± 2.42 and 140.03 ± 1.48 µg/mL respectively in the α-amylase and α-glucosidase inhibition assay (Table 3.1). From the results it was clear that the methanolic extract of PI exhibited better activity in inhibiting the enzymes. The IC_{50} values obtained for standard acarbose used in these assays were 5.69 ± 1.13 and 50.2 ± 1.88 µg/mL respectively for α-amylase and α-glucosidase inhibition.

3.3.4.2 Antiglycation property of PI extracts

Hyperglycemic condition causes excess glucose to form adducts with the proteins to form advanced glycated end products (AGEs). AGE is one of the crucial factors in development of diabetic complications such as cardiomyopathy, rheumatoid arthritis, retinopathy, nephropathy and neuropathy (Singh et al., 2014). Experimental studies have showed that interfering AGEs production have beneficial effect in various diabetic complications (Ihm et al., 2010). In this aspect we have evaluated the effect of PI extracts on inhibiting the glycation process. It was found that PI extracts inhibited *in vitro* glycation in a concentration dependent manner (Figure 3.5[C]). Among the extracts PI MET effectively inhibited glycation process with an IC_{50} value of 142.27 ± 1.09 µg/mL (Table 3.1). The IC_{50} value obtained for standard ascorbic acid used in this assays was 27.88 ± 1.46 µg/mL.

Table 3.1. IC_{50} values of PI extracts for various antidiabetic assays

	IC_{50} values (µg/mL)		
Assays	**PI ETH**	**PI MET**	**Standard**
α-amylase	183.96 ± 2.42[a]	166.14 ± 1.18[b]	5.69 ± 1.13[c] (Acarbose)
α-glucosidase	140.03 ± 1.48[a]	106.37 ± 1.25[b]	50.2 ± 1.88[c] (Acarbose)
Antiglycation	213.58 ± 2.11[a]	142.27 ± 1.09[b]	27.88 ± 1.46[c] (Ascorbic acid)

The results are expressed in µg/mL of extract. Each value represents mean ± SD (standard deviation) from triplicate measurements.

[a-b-c] Values in a row with different superscript are significantly different from each other ($p \leq 0.05$).

3.3.4.3 Glucose uptake assay

Insulin plays a key role in regulating the disposal and storage of dietary glucose by enhancing the uptake of glucose into cells. About 75% of the insulin stimulated glucose uptake takes place in skeletal muscle cells (Bhattacharyaa et al., 2014). In type 2 diabetes cells become resistant to insulin and results in impaired glucose transport (DeFronzo and Tripathy, 2009). This creates an imbalance in glucose homeostasis. It is reported that dietary antioxidants enhances the uptake of glucose by cells (Martineau et al., 2006; Dhanya et al., 2015).

From the preliminary antioxidant and antidiabetic assays it was found that methanol extract of PI is exhibiting better biological potential. Therefore, PI MET was further investigated for its role in inducing glucose uptake by the cells. Cellular glucose uptake was monitored by using 2-NBDG which revealed an induction of uptake on pretreatment with PI MET (Figure 3.6). Pretreatment of PI MET (50 and 100 μg/mL) enhanced 2-NBDG uptake to in L6 myotubes 14.23 ± 0.95% and 21.41 ± 0.62% compared to untreated control (0.38 ± 0.08%). 100 nM Rosiglitazone (25.56 ± 1.26%) was used as a positive control. The study confirms that PI MET extracts improved the glucose uptake efficacy of L6 cells in a dose dependent manner.

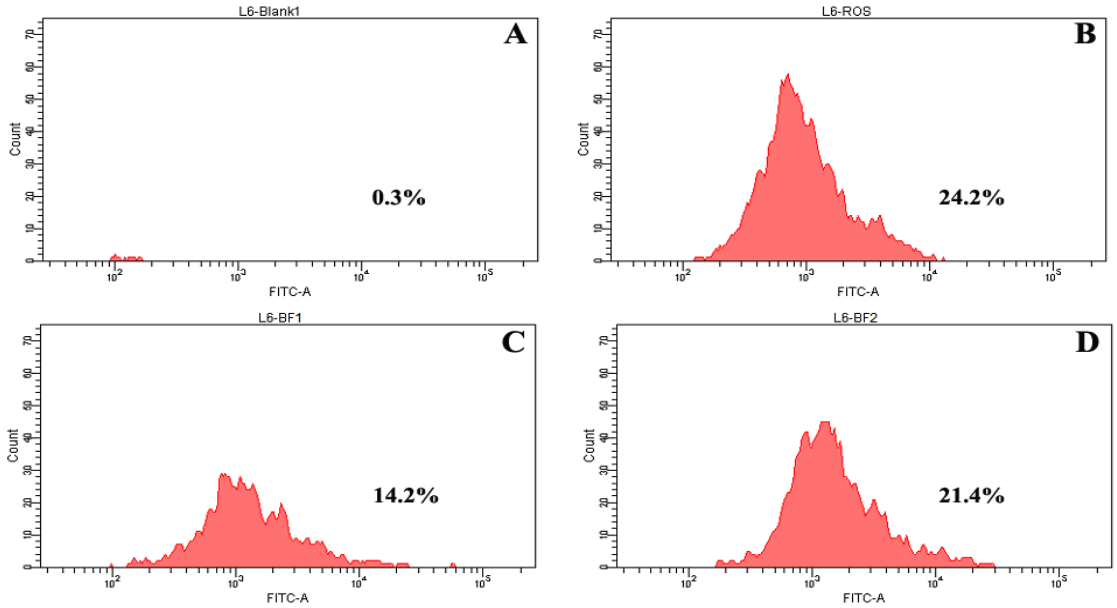

Figure 3.6. Glucose uptake effect of methanol extract of plantain inflorescence. (A) Untreated Control, (B) Rosiglitazone treated, (C) PI MET – 50 µg/mL, (D) PI MET – 100 µg/mL. The X axis showed the log scale of fluorescent intensity and Y axis showed the cell count.

3.3.4.4 Adsorption of glucose by dietary fibre

Dietary fibre consumption has been reported to reduce postprandial glucose responses (Jenkins et al., 2000). The effect is mainly due to the viscous and gel forming properties of fibre which slows down gastric emptying and macronutrient absorption from the gut (Wood et al., 1994, Ahmed et al., 2011). The influence of soluble and insoluble fibre from PI on the postprandial glucose was investigated by its ability to adsorb glucose by *in vitro* glucose adsorption assay.

The study showed that dietary fibre from PI adsorbs glucose effectively (Figure 3.7) and thus can play a potential role in reducing postprandial glucose. The glucose adsorption capacity of fibre was found to increase in a concentration dependent manner. 250 mg of PI soluble dietary fibre was found to absorb 2.116 ± 0.025 mM of glucose

whereas the same concentration of insoluble dietary fibre adsorbs only 1.506 ± 0.085 mM of glucose.

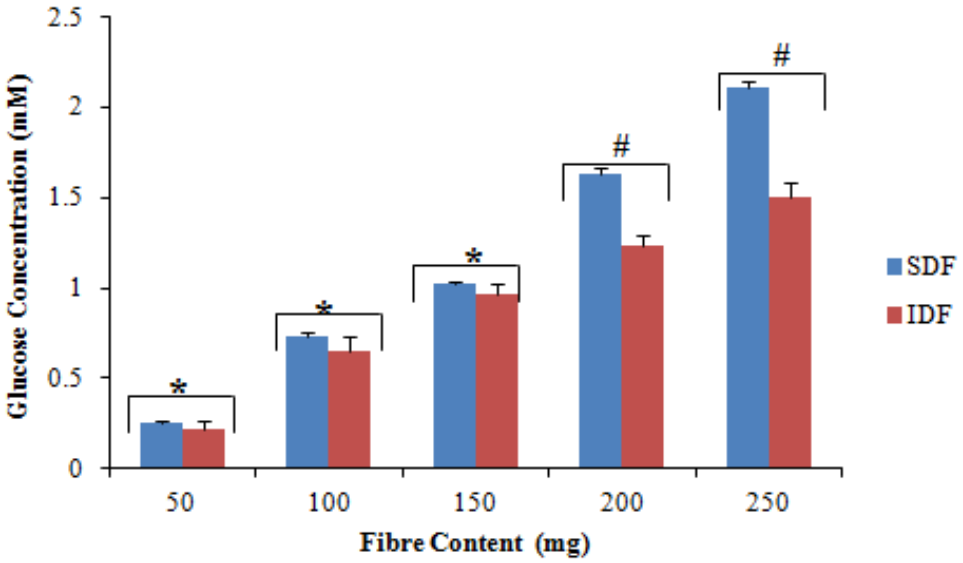

Figure 3.7. Glucose adsorption capacity of dietary fibre from plantain inflorescence. SDF – soluble dietary fibre and IDF – insoluble dietary fibre (IDF). Each value represents mean ± SD (standard deviation) from triplicate measurements. *SDF and IDF not significantly different; # SDF and IDF significantly different from each other ($p \leq .05$).

Cohort studies indicated that consumption of SDF reduces postprandial glucose responses after carbohydrate-rich meals (Schulze et al., 2007, Jenkins et al., 2000). It is reported that diets rich in dietary fibre improve the glucose homeostasis and insulin sensitivity (Fuji et al., 2013). Post meal satiety or decreased subsequent hunger after dietary fibre rich meal and inverse relation with weight gain are the potential mechanisms by which SDF alters diabetes risk (Howarth et al., 2001). The hypoglycemic effect of fibre can be related to viscous nature of soluble fibre, glucose adsorption capacity of fibres, delay gastric emptying and slowing carbohydrate uptake (Ahmed et al., 2011). The results from the present study reveals that PI is a potential

source of bioactive phenolic compounds and dietary fibre and hence its consumption could be helpful in modulating the risk of developing diabetes.

3.3.5 Effect on cardiovascular protection

3.3.5.1 Inhibition of LDL oxidation

Oxidation of low-density lipoprotein (LDL) is known to contribute to atherosclerosis and cardiovascular disease (Heinecke, 2006). Oxidized LDL enhances the expression of pro-inflammatory genes which leads to monocyte recruitment into the vessel wall, dysfunction of vascular endothelial cells and also damage endothelial cells via generation of free radicals (Li and Mehta, 2005). Hence inhibition of LDL oxidation is very important in the prevention and management of atherosclerosis and cardiovascular disease. PI ETH and PI MET were analyzed for its efficacy in inhibiting the oxidation of LDL using *in vitro* protocols. The PI extracts inhibited LDL oxidation in a dose dependant manner (Figure 3.8). From the results it can be seen that PI MET (IC_{50}-169.52 ± 1.77 µg/mL) exhibited better activity as compared to PI ETH (IC_{50}-217.45 ± 2.18 µg/mL). The standard ascorbic acid used in assay exhibited an IC_{50}- 32.3 ± 1.55 µg/mL). Oxidized LDL causes endothelial damage by accumulating in the arterial wall (Witztum, 1994). LDL receptors fail to recognize the oxidized LDL molecules and are taken by macrophages to form foam cells which is an important component of the fatty streak lesions of atherosclerosis (Steinbrecher, 1999). Hence, nutritional strategies to avert oxidation of LDL are expected to result in favorable outcome and thereby manage associated cardiovascular problems in type 2 diabetes.

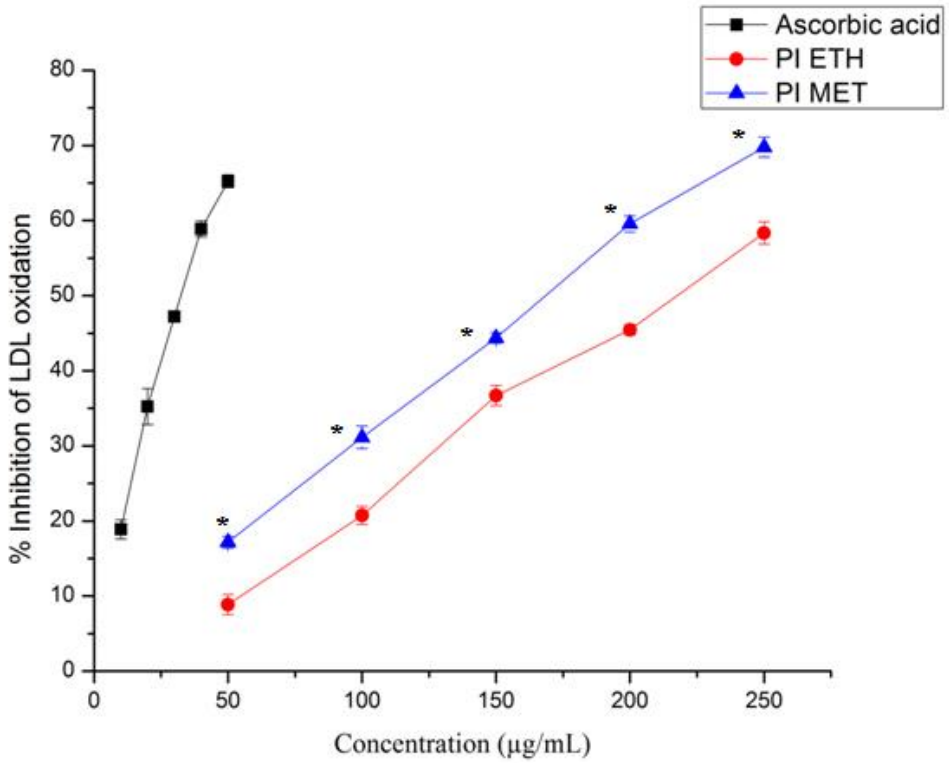

Figure 3.8. Inhibition of LDL oxidation by plantain inflorescence extracts. PI ETH- ethyl acetate extract of plantain inflorescence, PI MET- methanol extract of plantain inflorescence. Graph was plotted by X axis with different concentrations of extracts and Y axis with %inhibition values. Each value represents mean ± SD (standard deviation) from triplicate measurements. *PI MET significantly different from PI ETH values ($p \leq 0.05$).

3.3.5.2 Inhibition of ACE

The advanced stage of type 2 diabetes is characterized by prominent serum ACE activity which leads to arterial abnormalities. Activity of ACE is considered to be a key factor since ACE converts angiotensin I to angiotensin II which is a potent vasoconstrictor and plays a vital role in the regulation of blood pressure (Lastra et al., 2013). Clinical and pharmacological studies have demonstrated that angiotensin II is a critical promoter of insulin resistance and diabetes mellitus. The role of angiotensin II has been implicated in the progression of diabetic cardiovascular complications and

ACE inhibitors have shown clinical benefits (Patel et al., 2014). Hence the inhibition of ACE is a key therapeutic strategy in the treatment of hypertension in type 2 diabetes.

The HPLC analysis showed that PI extracts effectively inhibits ACE activity (Figure 3.9[I]).

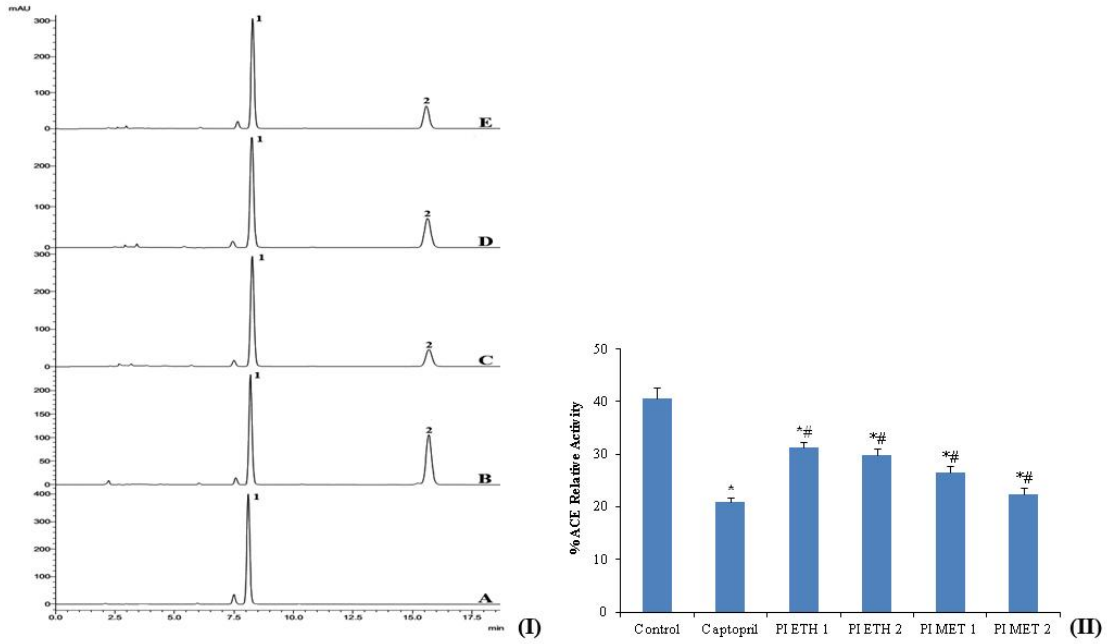

Figure 3.9. Inhibition of ACE enzyme by plantain inflorescence extracts [I] HPLC chromatogram for determining ACE activity. (A) Blank without enzyme, (B) Control without inhibitor, (C) Captopril-standard ACE inhibitor (0.25 µM), (D) 200 µg/mL ethyl acetate extract of plantain inflorescence and (E) 200 µg/mL methanol extract of plantain inflorescence. (1) FAPGG and (2) FAP. [II] Inhibition of ACE enzyme by PI extracts expressed in relative activity of ACE in terms of FAPGG area under the pea. Two concentrations of ethyl acetate (PI ETH1-50 µg/mL and PI ETH2-100 µg/mL) and methanol extracts of PI (PI MET1-50 µg/mL and PI MET2-100 µg/mL) were used. Each value represents mean ± SD (standard deviation) from triplicate measurements.*Significantly different from Control; # significantly different from positive control captopril ($p \leq 0.05$).

The relative activity of ACE was calculated based on the area of FAPGG peak. The relative activity of ACE decreases as the concentration of PI extracts increases.

ACE relative activity was 31.25 ± 1.14% and 29.91 ± 1.21% when treated with 100 and 200 µg/mL of PI ETH. However when treated with PI MET at the same concentration as PI ETH, the activity decreases as 26.49 ± 1.2% and 22.41 ± 1.13% respectively. The control sample without any inhibitor exhibited a relative activity of 40.57 ± 2.17% whereas the same for the standard captopril (0.25 µM) was 20.89 ± 0.88%. As can be seen, both PI ETH and PI MET demonstrated significant inhibition of ACE activity which was comparable to the standard captopril used for the assay. As can be seen that PI MET exhibited better activity than PI ETH in inhibiting ACE activity (Figure 3.9[II]).

3.3.5.3 Adsorption of cholesterol by dietary fibre

Source of serum cholesterol in the body is either from intestinal absorption or by synthesis in liver. Dietary fibre has been reported to lower the levels of circulating cholesterol. Dietary fibres are known to bind the bile acid and its components, and thereby reduce serum cholesterol (Wolever, 1995). In order to understand the cholesterol adsorbing capacity of SDF and IDF from PI, the same was kept in 100 mM cholesterol solution under experimental conditions for 6 h, centrifuged and the supernatant was assayed for residual cholesterol. The results showed that the cholesterol adsorbing capacity of both SDF and IDF followed a dose dependant manner. The SDF exhibited better cholesterol binding ability and was significantly higher than the IDF Figure 3.9. It was interesting to note that 250 mg of SDF adsorbed 2.48 mM of cholesterol. The mechanisms of cholesterol lowering effect of dietary fibre is reported to associate with the disruption of the normal recycling of bile by sequestering bile salts and fatty acids by the fibre (Mackie et al., 2016). Dietary fibre also reported to

decrease the rate of absorption of bile salts and fatty acids significantly, as a result of entanglement with intestinal mucus; and also lowers total and LDL cholesterol levels (Jenkins et al., 2000; Behall et al., 2004). It is evident from the present study that the dietary fibre from PI can reduce the cholesterol level.

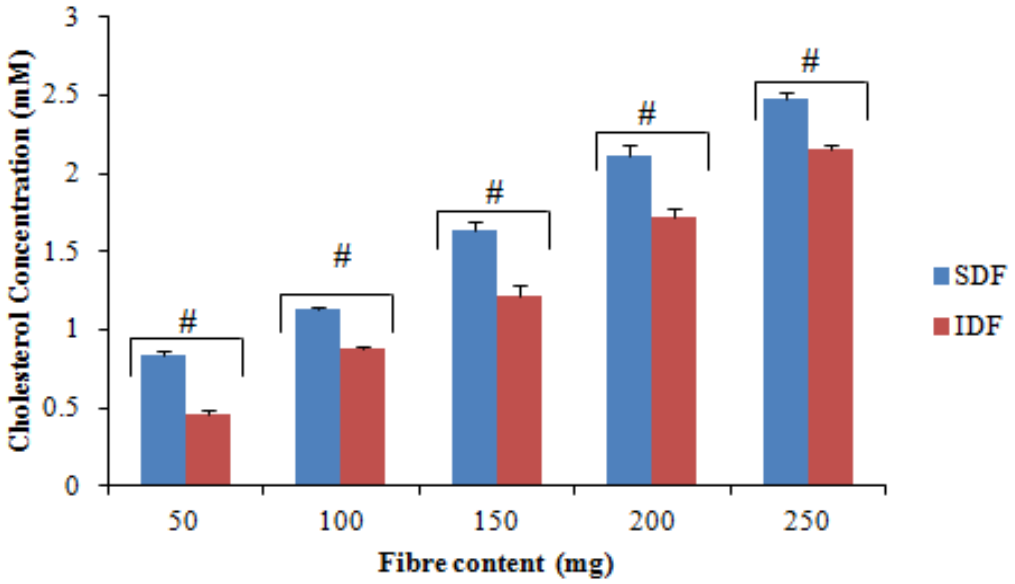

Figure 3.10. Cholesterol adsorption capacity of dietary fibre from plantain inflorescence. SDF –soluble dietary fibre and IDF – insoluble dietary fibre (IDF). Each value represents mean ± SD (standard deviation) from triplicate measurements. * SDF and IDF not significantly different; # SDF and IDF significantly different from each other ($p \leq .05$).

3.4 Conclusion

In conclusion, plantain inflorescence contains bioactive phytochemicals and dietary fibre that can modulate hyperglycemia and the development of diabetic associated cardiac problems. The bioactive compounds demonstrated reduction of the postprandial hyperglycemia by inhibiting the digestive enzymes and enhancing the uptake of glucose by the cells. Over production of reactive oxygen species (ROS) play an important role in the onset of diabetes and the methanol extract of plantain inflorescence was found to

reduce the oxidative stress in L6 myoblasts by scavenging the H_2O_2 induced ROS. The antiglycation properties shown by the extracts can play major role in delaying the onset of some of the diabetic related complications like cardiomyopathy. Further to this, the bioactives from PI also exhibited excellent cardio vascular protection by inhibiting LDL oxidation and ACE activity, which is very important in modulating the cardiovascular complications associated with diabetes.

PI is a very good source of dietary fibre with SDF/IDF ratio better than many reported sources of dietary fibre. Dietary fibre from PI demonstrated potential glucose and cholesterol binding properties. Dietary modulation in the form of functional foods and nutraceuticals is one of the promising approaches in the management of diabetes and its associated complications. The study provide significant evidences for plantain inflorescence to be considered as a dietary supplement for the management of diabetes and its associated complications.

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Chapter 4

Prebiotic and anticancer potential of Plantain Inflorescence

4.1 Introduction

Colon cancer is a world-wide health problem and the second-most dangerous type of cancer, affecting both men and women. Diet is clearly linked to the development of colorectal cancer. Approximately 70% of the causation of colorectal cancer is associated with some dietary factors and it has been found that an optimal dietary approach might prevent much of this disease (World Cancer Research Fund, 1997).

Burkitt (1971) has proposed that the high-fiber diet of Africans was protective against colorectal cancer. Since then, dietary fibre has been postulated to prevent colorectal cancer by various mechanisms like diluting or adsorbing fecal carcinogens, reducing colonic transit time, altering bile acid metabolism, reducing colonic pH, or increasing the production of short-chain fatty acids (Kritchevsky, 1995). A statistically significant reduction in risk of colorectal cancer with higher dietary fiber intake has been observed in most case-control studies (World Cancer Research Fund, 1997). Prebiotics were first described as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health (Gibson and Roberfroid, 1995). This definition was later refined to include other areas that may benefit from selective targeting of particular microorganisms (Gibson et al., 2004): a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, which confers benefits. *Lactobacilli* and *bifidobacteria* are the usual target genera for prebiotics. The fermentation of prebiotics in the colon often generates short chain fatty acids (SCFA). Butyrate, one of the major SCFA, has been found to induce differentiation of phenotype including colorectal tumor cells (Reddy,

1997). The mechanism by which prebiotics promote gut health and there by prevent the risk of developing colorectal cancer is well explained in Chapter 1.

The most commonly used cancer chemotherapy drugs include alkylating agents, antimetabolites, antitumor antibiotics, platinum analogues and natural anticancer agents (Timothy, 2001). 5-fluorouracil (FU) remains a widely used chemotherapeutic drug in the treatment of colorectal carcinoma; however, its anticancer efficacy is partly attributed to its ability to induce p53-dependent cell growth arrest and apoptosis; consequently, mutations or deletions of p53 can cause cells to become resistant to 5-FU (Sui et al., 2014). Due to increased death rate associated with cancer and unfavorable side effects of cancer chemotherapy and radiation therapy, research has been focused on discovery of new anticancer agents derived from nature, especially from plants. Natural products, particularly dietary substances, have played an important role in creating new chemopreventive agents.

Among the selected agro-industrial residues plantain inflorescence (PI) have better antioxidant activity and dietary fibre content. Plantain is one of the most important crops of the tropical plants. Plantain contains a high fibre content, and thus helps to relieve constipation and hence prevention of colon cancer (Okareh, et al., 2015). Plantain bract has medicinal applications in bronchitis, dysentery and on ulcers (Sampath Kumar, et al., 2012); cooked plantain bracts are given to diabetics (Morton, 1987) and culinary uses (edible) in many Asian countries. Antioxidant, antimicrobial and antidiabetic activities of various plantain inflorescences have been reported already (Padam et al., 2012; Roobha et al., 2011; Lakshmi et al., 2014).

However to the best of our knowledge there is no report stating the anticancer and prebiotic potential of Plantain inflorescence (*Musa paradisiaca, Nendran* variety). With this background and from the positive results got for PI in terms of dietary fibre content and antioxidant activity we aim to investigate the prebiotic nature of dietary fibre from PI and the utilization of PI as a source of anticancer agents.

4.1.1 Objectives

The main objectives of this chapter are summarized as follows.

The chapter has been divided into two parts – Part A and Part B.

- Part A
    1. Evaluation of prebiotic potential of soluble dietary fibre isolated from plantain inflorescence in promoting the growth of probiotic species – *Lactobacillus casei* and *Bifidobacterium bifidum*
    2. Evaluating the anticancer potential of supernatant obtained from fermenting soluble dietary fibre using probiotic bacteria against HT29 colon cancer cells
- Part B
    1. Evaluation of anticancer potential of extracts of plantain inflorescence against HT29 colon cancer cells

The outline of the Chapter 4 has been depicted in Figure 4.1.

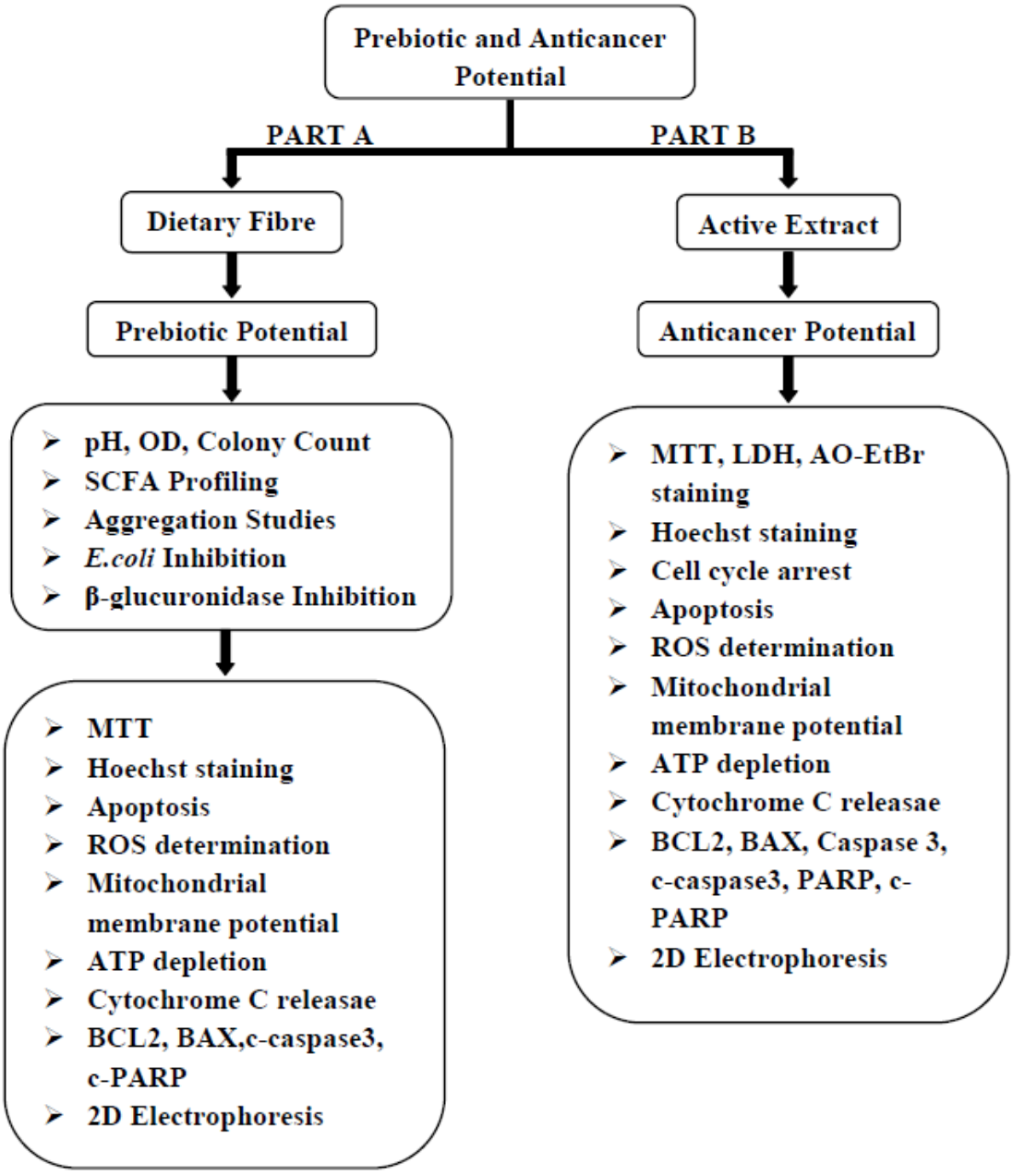


Figure 4.1. Outline of Chapter 4.

PART A

Evaluation of prebiotic potential of soluble dietary fibre and anticancer efficacy of fermentation supernatant

4.2 Materials and Methods

4.2.1. Chemicals

DMEM medium, fetal bovine serum, trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rhodamine 123, bile salts, pepsin, camptothecin, 5-flurouracil and sodium alginate were purchased from Sigma Aldrich Chemicals, St Louis, USA. Antibodies were purchased from Santa Cruz Biotechnology, Inc., USA. Nonlinear immobilized pH gradient strips (IPG strips, pH 3–10 NL, 17 cm) and other materials for 2D electrophoresis and western blotting were purchased from Bio-Rad Laboratories, Germany. Protease inhibitor cocktail was procured from Amresco, USA. Sucrose, calcium chloride, and starch were obtained from Sisco Research Laboratories, India. All microbiological media were obtained from Himedia Laboratories Pvt. Ltd, India and L-cysteine was obtained from Merck chemicals, India. Inulin (Orfati®Inulin) was supplied by Beneo Asia Pacific Pte. Ltd., Singapore. All reagents used were of analytical grade.

4.2.2 Sample

PI, from *Nendran* variety, identified as *Musa paradaisica*, was used for the present study. The sample preparation has been discussed in chapter 3 under section 3.2.2.

4.2.3 Prebiotic potential of soluble dietary fibre from PI

4.2.3.1 Microorganisms

The freeze dried cultures of *Lactobacillus casei* (NCDC17) and *Bifidobacterium bifidium* (NCDC255) were supplied by National Dairy Research Institute, Karnal,

Haryana, India. *Lactobacillus casei* (NCDC17) cultured in MRS broth, *Bifidobacterium bifidium* (NCDC255) cultured in MRS broth and L-cysteine media. Cultures were stored at -80°C in MRS broth/MRS broth with L-cysteine supplemented with 50% glycerol. Bacterial strains were each grown independently in MRS broth for 24 h then sub-cultured in fresh media broth for 24 h at 37°C before use. *E. coli* (MTCC 2622) was obtained from Microbial Type Culture Collection and Gene Bank, Chandigarh, India and cultured in Luria-Bertani medium.

4.2.3.2 Preparation of soluble dietary fibre and fermentation supernatant

The dietary fibre was isolated from PI as described in chapter 2 under section 2.2.3.2. Soluble dietary fibre from plantain inflorescence (PIF-1%) was incorporated in respective media. Inulin (1%) was used as positive control. The culture was diluted in sterile distilled water to obtain 80% transmittance at 600 nm and 1 mL of the diluted culture was inoculated to the media containing SDF/Inulin. A group without SDF/Inulin was used as control. All groups were kept at incubator at 37°C for 48 h. The supernatant was collected by centrifuging the media at 10,000 rpm (Kubota-7780, Japan) for 10 min and kept at -80°C (Thermo Scientific Forma 993,-86C ULT FREEZER, USA) until further use. The supernatant obtained from fermentation of PIF using *Lactobacillus casei* and *Bifidobacterium bifidum* (respectively named as LS and BS) were analyzed for anticancer activity.

4.2.3.3 Evaluation of prebiotic potential of soluble dietary fibre from plantain inflorescence

In this study, *Lactobacillus casei* and *Bifidobacterium bifidum* were used as probiotic strains. *Lactobacillus casei* was grown in MRS broth and *Bifidobacterium bifidum* in

MRS broth containing L-cysteine. For evaluating the prebiotic efficacy 1% of soluble dietary fibre from the PI was added to the MRS broth. Inulin (1%) was used as the positive control. After inoculating with organism the broth was maintained at 37°C up to 24 h. 1 mL of this broth was diluted with sterile distilled water to obtain a transmittance of 80% and mixed well for uniform dilution of organisms. From this 0.5 mL each was inoculated to fresh broth (control, positive control and samples); mixed well and incubated for 72 h at 37°C. At each time interval (0 h, 24 h, 48 h and 72 h), 5 mL of the broth was collected and transferred to pre-weighed centrifuge tubes for various analysis.

4.2.3.3.1 pH measurement

The pH of the broth at each time intervals was measured using pH meter (Eutech pH meter, USA).

4.2.3.3.2 Optical density measurement

The optical density of the broth at each time interval was measured at 600 nm using UV spectrophotometer (Shimadzu UV-2600, USA).

4.2.3.3.3 Dry weight measurement

After taking pH and OD the contents in the tubes were centrifuged at 10,000 rpm (Kubota-7780, Japan) for 10 min and supernatant was taken out. The residue obtained was freeze dried and dry weight of organisms was calculated. The supernatant was kept at -80°C for SCFA analysis.

4.2.3.3.4 Colony count method

At each time interval 1 mL of broth from each group was serially diluted to obtain concentration from 10^{-1} to 10^{-12}. From this 1mL was taken for colony count

measurement using pour plate method (Parisi et al., 1973). After incubation at 37°C for 72 h, the colonies of were counted and expressed as log colony formation units (Log CFU/mL).

4.2.3.3.5 Assessment of short chain fatty acid production

Short chain fatty acid production at different time intervals was analyzed and quantified by HPLC following the method of Guerrant et al. (1982) with some modifications. The supernatant and the standard SCFA solutions were filtered through 0.45 µm PTFE filter; 20 µL was injected into the HPLC system. The analysis was performed on a Prominence UFLC system (Shimadzu, Japan) containing LC-20AD system controller, Phenomenex Gemini C18 column (250 × 4.6 mm, 5µm), a column oven (CTO-20A), a Rheodyne injector (USA) with a loop of 20 µL volume and a diode array detector (SPD-M20A). The mobile phase used was 10% acetonitrile with pH 2 (pH adjusted using ortho phosphoric acid). The flow rate was 0.5 mL/min; the injection volume was 20 µL and column was kept at 37°C. The fractions were monitored at 200 nm. Sample peaks were identified by comparing with retention times of standard peaks and also by spiking the sample with standard SCFA. LC LabSolutions software was used for data acquisition and analysis.

4.2.3.3.6 Aggregation studies

Auto-aggregation and co-aggregation studies were performed according to Pan et al. (2008) with some modifications. After 72 h incubation, cells from 1 mL of broth from each group (control, inulin, PIF) were harvested by centrifugation at 5,000×g (Kubota-7780, Japan) for 15 min and suspended in 4 mL sterile distilled water. The cell suspension was mixed well by vortexing for 15 s and auto-aggregation was determined

during 1 h and 5 h of incubation at 37°C. After 1 h and 5 h, 0.1 mL of the upper suspension was transferred to another tube with 3.9 mL of distilled water and the absorbance (A) was measured at 600 nm. The auto aggregation percentage was expressed as

$$\%\ Auto\ Aggregation\ = \left(1 - \frac{At}{A0}\right) \times 100$$

where At represented the absorbance at time t = 1 or 5 h and A0 the absorbance at t = 0.

The method of co-aggregation experiments was the same as auto-aggregation assay. *E. coli* was used as representative pathogenic strain. Equal volumes (2 mL) of each cell suspension of the three experimental groups and E. coli were mixed together in pairs by vortexing for 15 s. Tubes containing 4 mL of each bacterial suspension on its own was used as control. The absorbance at 600 nm of the suspensions was measured after mixing and after 1 h/5 h of incubation at 37°C. Samples were taken in the same way as those in the auto-aggregation assay. The percentage of co-aggregation was calculated using the equation

$$\%\ Co - aggregation = [1 - \frac{(2Amix)}{(Aexp. + Ae.coli)}] \times 100$$

where Amix= Absorbance of mixed cell suspension, Aexp= Absorbance of experimental group and Ae.coli= Absorbance of *E. coli*.

4.2.3.3.7 Inhibition of E. coli by fermentation supernatant

The ability of fermentation supernatant to inhibit *E. coli* was assessed by disc diffusion method (Bhalodia and Shukla, 2011). Three different volumes (10, 25 and 50 µL) of fermented supernatant from control, Inulin and PIF groups loaded in sterile paper discs

were aseptically placed on the *E. coli* inoculated nutrient agar plates. Then, the plates were incubated at 37°C for 24 h. After the incubation, the zone of inhibition diameter was measured.

4.2.3.3.8 Inhibition of β-glucuronidase enzyme

The ability of fermented supernatant to inhibit β-glucuronidase enzyme produced by E. coli was assessed according to Sekikawa et al. (2002) with some modifications. Briefly, three different volumes (10, 25 and 50 µL) of fermented supernatant from control, Inulin and PIF groups were added to 25 µL of β-glucuronidase enzyme (30 U), 50 µL of phenolphthalein glucuronide substrate (3 mM) and volume made up to 300 µL with 75 mM potassium phosphate buffer (75 mM, pH 6.8). The mixture was incubated for 30 min at 37°C. The reaction was terminated by adding 50 µL ice cold 0.2 M Na_2CO_3 buffer. The absorbance was measured at 405 nm and percentage inhibition was calculated.

4.2.4 Anticancer potential of fermentation supernatant

4.2.4.1 Cell line and culture medium

HT29 colon cancer cell line obtained from National Centre for Cell Science, Pune, India. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 0.5% antibiotic-antimycotic solution at 37°C in a humidified atmosphere containing 5% CO_2. Cells at 70–80% confluence were used for all the experiments.

4.2.4.2 MTT assay for cytotoxic activity

Cytotoxicity of the PIF fermentation supernatant in HT29 cell lines was determined by the MTT assay as described by Mosmann (1983). 1×10^4 cells were plated in each well

of 24-well plates and were placed in the humidified 5% CO_2 incubator at 37°C. The cells grown to 80% confluence were exposed to different volumes of PIF fermentation supernatant from *L. casei* (LS) and *B. bifidum* (BS). The control and the treated cells were incubated for 24 h at 37°C in 5% CO_2 incubator. After incubation, the cell viability was evaluated using the MTT assay. After 24 h, the old media was subsequently replaced with fresh media (1 mL) containing MTT (5 mg/mL in PBS). The cells were incubated at 37 °C for 4 h and the media was carefully removed. The formazan crystals were then dissolved in DMSO. The absorbance of the resulting solution was measured at 570 nm. The cell toxicity was expressed as percentage of control cells treated with 0.1% (v/v) DMSO.

4.2.4.3 Hoechst 33342 staining

Apoptotic activity of PIF fermentation supernatant on HT29 cells were determined by Hoechst 33342 staining as described by Harada et al. (2005) with slight modification. 1×10^4 cells were treated with different volumes of supernatant and incubated for 24 h. Control group cells treated with media containing 0.1% DMSO and H_2O_2 (250 μM) was used as positive control. Cells were washed with PBS and fixed with 70% ethanol for 5 min. After fixation, cells were incubated with Hoechst 33258 stain in PBS (5 μg/mL) for 30 min at 37°C in the dark. Cells were thoroughly washed with PBS and examined under confocal microscopy (BD Pathway 855, BD Bioscience, USA) with an excitation of 350 nm and emission of 460 nm. Apoptotic cells were identified by nuclear condensation and apoptotic bodies.

4.2.4.4 Apoptosis assay by flow cytometry

Apoptosis in HT29 cells was detected using Annexin V-fluorescein isothiocyanate

(FITC)/propidium iodide (PI) apoptosis detection kit (Cayman Chemical Company, USA). Briefly, 1×10^4 cells were treated with different concentrations of PIF fermentation supernatant and incubated for 24 h. Control group cells treated with media containing 0.1% DMSO and camptothecin (50 μM) was used as positive control. Following 24 h incubation the cells were harvested, washed with cold PBS and resuspended in binding buffer. The cells were treated with Annexin V-FITC conjugate and incubated for 15 min at room temperature in the dark condition. The cells were then stained with PI (5 μg/mL) and analyzed by Fluorescence Activated Cell Sorting (BD FACS Aria II, USA) within 1 h following the staining. The data acquisition and analysis were performed using BD FACSDiva^{TM} Software v6.1.2 and a minimum of 10000 cells were analyzed in each group.

4.2.4.5 Determination of intracellular reactive oxygen production

Intracellular reactive oxygen species (ROS) was estimated by using the fluorescent probe, DCFH-DA as described earlier by Ramful et al. (2010). Briefly, the HT29 cells after preincubation with different concentrations of the PIF fermentation supernatant (1, 10 and 50 mg/mL) for 24 h. Positive control cells were treated with H_2O_2 (250 μM) for 20 min. After washing with phosphate buffer saline (PBS, pH-7.4) cells were treated with DCFH-DA (20 μM) for 30min. After washing with PBS, the cells were imaged using confocal microscopy (BD Pathway 855, BD Bioscience, USA) equipped with filters in the FITC range (i.e. excitation, 490 nm; and emission, 525 nm).

4.2.4.6 Detection of mitochondrial membrane potential in HT29 cells

Rhodamine 123 (Rh123) is a fluorescent cationic dye that binds to polarized mitochondrial membrane and accumulates as aggregates in the mitochondria of normal

cells. For analysis of changes in mitochondrial trans-membrane potential, 1×10^4 HT29 cells/well were treated with the different concentrations of PIF fermentation supernatant for 24 h. Then cells were analyzed by flow cytometry after staining for 20 min at 37°C with 0.625 mg/mL Rh123 (Sigma). Data analysis was performed using Fluorescence Activated Cell Sorting (BD FACS Aria II, USA) within 1 h following the staining. The data acquisition and analysis were performed using BD FACSDiva^{TM} Software v6.1.2 and a minimum of 10000 cells were analyzed in each group.

4.2.4.7 Adenisone triphosphate (ATP) production by HPLC analysis

The ATP levels in HT29 cells were determined using HPLC method (Hahn-Windgassen et al., 2005). After treatment, the cells were trypsinized and centrifuged at 800×*g* for 3 min and the pellets were suspended in 4% perchloric acid on ice for 30 min. The pH of the lysates was adjusted between 6 and 8 with 2 M KOH. Precipitated salt was separated from the liquid phase by centrifugation at 13,000×g (Kubota-7780, Japan) for 10 min at 4°C. ATP was quantified on a Prominence HPLC system (Shimadzu, Japan) containing LC-20 AD system controller, Phenomenex Gemini C18 column (250 × 4.6 mm, 5 µm), a column oven (CTO-20A), a Rheodyne injector (USA) with a loop of 20 µL volume and a diode array detector (SPD-M20A). A buffer 20 mM KH_2PO_4 and 3.5 mM K_2HPO_4 $3H_2O$ (pH 6.1) was used as the mobile phase. The flow rate was 1 mL/min, the injection volume was 20 µL and column was at 37°C. The fractions were monitored at 259 nm. Sample peaks were identified by comparing with retention times of standard peaks. LC Lab Solutions software was used for data acquisition and analysis.

4.2.4.8 Detection of Cytochrome C release

Cytochrome C release due to mitochondrial damage was assessed according to Radhakrishnan et al. (2007) with slight modifications. After the treatment of HT29 cells with PIF fermentation supernatant, cells were trypsinized and suspended in 50% acetonitrile solution containing 0.1% TFA and kept at room temperature for 10 min with mild intermediate mixing. Then the mixture was centrifuged at 5,000 rpm (Kubota-7780, Japan) for 10 min to precipitate high molecular weight plasma proteins. Cytochrome C content in the supernatant was quantified on a Prominence HPLC system (Shimadzu, Japan) containing LC-20 AD system controller, Phenomenex Gemini C18 column (250 × 4.6 mm, 5 µm), a column oven (CTO-20A), a Rheodyne injector (USA) with a loop of 20 µL volume and a diode array detector (SPD-M20A). A gradient system consisting of solvent A- 0.1% TFA in water: 0.1% TFA in acetonitrile (70:30) and solvent B - 0.1% TFA in water: 0.1% TFA in acetonitrile (64:36) was used as mobile phase achieving gradient range 0-100% B in 30 min. The flow rate was 1 mL/min, the injection volume was 20 µL and column was at 37°C. The fractions were monitored at 393 nm. Sample peaks were identified by comparing with retention time of standard peak. LC Lab Solutions software was used for data acquisition and analysis.

4.2.4.9 Western blot analysis

Cells (1×10^5) were seeded in a 24-well culture plate for 1 day before treatment with the PIF fermentation supernatant. After 24 h incubation, the cells were collected and lysed for western blot analysis. Antibodies specific against - β-actin, PARP, cleaved PARP, caspase 3, cleaved caspase 3, BCL2, and BAX, for western blot analysis were purchased from Santa Cruz Biotechnology, USA. Protein concentration was determined

with the Bradford (1976) method (Protein Assay Dye Reagent, Bio-Rad Laboratories, Germany). After cell lysis, equal amounts of proteins (40 µg) were separated on a 10% SDS polyacrylamide gel according to the size of the proteins and transferred to a PVDF transfer membrane (Immobilon P™, Millipore®, USA) using Trans-Blot turbo Transfer system (Bio-Rad Laboratories, India). To confirm the transfer of proteins to the membrane, it was stained with Ponceau S solution (Sigma Aldrich, St Louis, USA) for 5 min. Then blots were washed with distilled water to remove ponceau stain. For immunodetection the membrane was washed in wash buffer (TBST - TBS buffer containing 0.1% Tween 20) and blocked using a blocking solution (3% BSA in PBS) for 3h at 37°C. The blots were then washed and incubated with and appropriately diluted primary antibody solution and incubated for overnight on a rotary shaker at 4°C. To remove the unbound primary antibody, blots were washed using the wash buffer. For detection of the blots, the secondary antibody solution horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology, USA) was added and incubated for 2 h at room temperature. The blots were later washed with wash buffer and were incubated with DAB-Peroxidase substrate solution (0.05% DAB, 0.015% H_2O_2, 0.01M PBS, pH 7.2). The stained membrane was then washed with distilled water, and proper gel transfer was verified and imaged using gel doc facility (BioRad, Germany). Protein ladders were used to rule out non-specific binding of antibodies. The pixel density of specific protein bands (ImageJ software) were compared to that of housekeeping gene - β actin and plotted to obtain graphs.

4.2.4.10 Two dimensional electrophoresis

4.2.4.10.1 Protein isolation

Experiments were performed in duplicate where untreated HT29 cells and HT29 cells treated with BS fermentation supernatant/PIMET extract were compared. In order to prepare the cell lysates, 5×10^5 HT29 human colon cancer cells per well were plated on 6-well plates. After 24 h incubation at 37°C, the cells were treated for 24 h with the PIF fermentation supernatant. Cells treated with cell culture medium for the same period of time were used as a control. After incubation, the cells were centrifuged for 5 min at 1000 rpm, washed with phosphate buffered saline, and then a volume of 300 µL lysis buffer was added to each well. Samples were incubated for 30 min on ice and then the isolated protein mixtures were centrifuged at 16,000×g (Kubota-7780, Japan) for 20 min at 4°C to collect the supernatant.

Ice cold solution of 10% TCA in acetone was added in to the supernatant (4:1 ratio) and this mixture was incubated overnight at -20°C and the precipitated proteins were collected by centrifugation at 22,000×g (Kubota-7780, Japan), washed three times with ice cold acetone, air dried and was solubilized in the solubilization buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 50 mM DTT, 0.2% 100X biolyte 3/10 ampholyte, and 0.002% (w/v) bromophenol blue. The total soluble protein concentration was determined according to Bradford's (1976) method. The solubilized proteins were stored at -20°C until further analysis by two-dimensional gel electrophoresis.

4.2.4.10.2 **2D electrophoresis**

The first dimension based on the isoelectric point of proteins was run using a strip based

Isoelectric Focusing System (Protean IEF®, Bio-Rad, USA) at 20°C with a current of 50 μA per strip. Protein sample (130 μg BS/160 μg PIMET treated protein) was loaded onto IPG Dry Strip™ gels of pH 3-10 (Bio-Rad, USA) by in-gel rehydration. IEF was performed with the following conditions: 500 V × 20 min, 4000 V × 2.5 h, and 8000 V for 30000 V-h. Prior to the second dimension (SDSPAGE), the IPG strips were equilibrated for 15 min in 6 M urea, 30% glycerol (v/v), 2% SDS (w/v), 0.375M Tris-HCl (pH 8.8), containing 2% (w/v) DTT, and then for 15 min in the same buffer additionally containing 2.5% (w/v) iodoacetamide. Equilibrated strips were transferred for the second dimension onto SDS-polyacrylamide gels. The proteins were separated on 12% gel. Subsequently, gels were stained using Coomassie Blue R-250. The gels were scanned with a GS-800 calibrated imaging densitometer (Bio-Rad Laboratories GmbH, Munich, Germany) using the QuantityOne software. For each sample, three replicate 2D-gels were comparatively analyzed using the PDQuest 8.0 software (Bio-Rad Laboratories GmbH, Munich, Germany), which allowed automatic detection with manual corrections and quantification of protein spots (based on pixel density). The significance of differences between protein spots was evaluated by Student's t-test and a p value lower than 0.05 was considered as significant. Additional selection criterion was a fold change value (based on ratio of band intensity of sample protein to that of corresponding untreated control protein) of protein spot, which maintains a consistency in repeated experiments.

4.2.4.10.3 Mass spectrometry and protein identification

Gel pieces of selected protein spots (based on differential expression and reproducibility) were cut out and incubated in a buffer containing 200 mM ammonium

hydrogen carbonate and 40% (v/v) acetonitrile at 37°C for 10 min. This was repeated until the gel was completely de-stained gel and stored in 1% acetic acid. The protein samples were submitted to Rajiv Gandhi Centre for Biotechnology Proteomics facility for MALDI-TOF/MS/MS analysis. In-gel digestion was carried out on UltrafleXtreme model (Bruker Daltonik, Bremen, Germany) equipped with smart beam solid state laser (337 nm) in reflectron positive ion mode using 19 kV acceleration voltage. The in-gel digestion was carried out using trypsin and eluted peptides were co-crystallized with α-cyano-4-hydroxycinnamic acid matrix on the target plate (384-well ground steel plate, Bruker Daltonics, Germany) and external peptide mass calibration was applied (Peptide mixture 1) as per the manufacturer's instructions. The PMF data was acquired m/z ratio. The MS/MS fragmentation was carried out for the selected peptide in the LIFT mode of the instrument. In order to confirm the identification, all MS/MS data from LIFT TOF/TOF spectra were combined with the corresponding MS peptide mass and Mascot search was done with following parameters. The mass spectra were imported into the database search engine (BioTools v2.2 connected to Mascot, version 2.2.04; Matrix Science). Mascot searches were done using the NCBI non-redundant database (Human Genome) with the following settings: number of miss cleavages permitted was 1; fixed modifications such as carbamidomethyl on cysteine, variable modification of oxidation on methionine residue; peptide tolerance of 0.7 Da and fragmentation ion tolerance 1.2 Da, enzyme used as trypsin and a peptide charge setting as +1.

4.2.5 Statistical analysis

The experimental results were expressed as mean ± SD (standard deviation) of triplicate measurements. The data were subjected to one-way analysis of variance (one-way

ANOVA) and the significance of differences between means were calculated by Duncan's multiple range test using SPSS for windows, standard version 7.5.1, SPSS (SPSS Inc., USA) and the significance accepted at $p \leq 0.05$.

4.3 Results and Discussion

4.3.1 Prebiotic potential of SDF from PI

4.3.1.1 Change in pH

During fermentation of PIF by probiotics short chain fatty acids are produced as secondary metabolite which decreases the pH of the growth media. Therefore the decrease in pH value can be correlated to the prebiotic activity of SDF. Hence we analyzed the pH change of PIF incorporated medium at different time intervals and compared with that of inulin and control groups. The pH measurement results were shown in Figure 4.2.

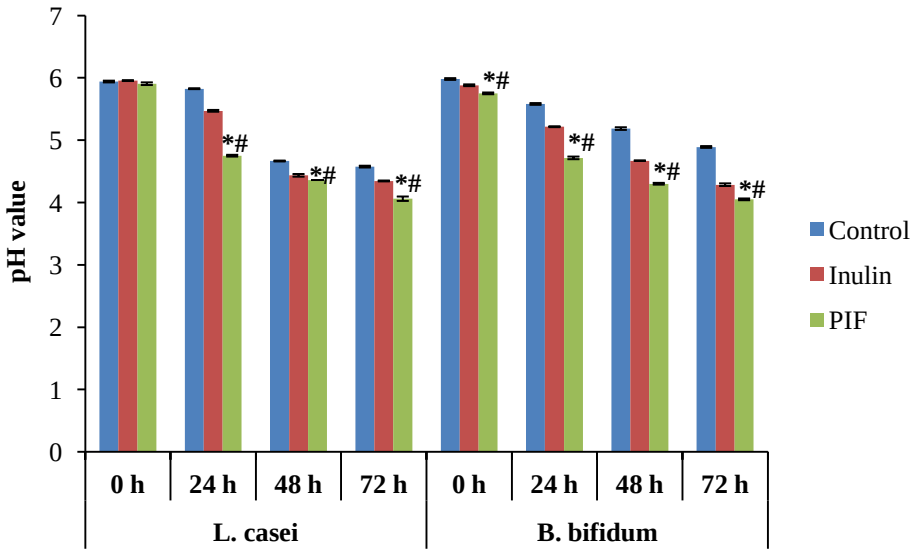


Figure 4.2. Prebiotic potential of PIF determined by pH change for *Lactobacillus casei* and *Bifidobacterium bifidum*. Each value represents mean ± SD from triplicate measurements. PIF – soluble dietary fibre from plantain inflorescence. $p \leq 0.05$

considered significantly different. *Significantly different from control group. #Significantly different from inulin group.

In case of fibre inoculated with *L. casei* the pH was decreased from 5.905 ± 0.021 to 4.06 ± 0.035 as the incubation time increases from 0 to 72 h. The results were better than inulin and control group. Similar results were obtained with *B. bifidum*, with a decrease in pH from 5.75 ± 0.014 to 4.05 ± 0.014. These results indicate that the SDF from PI promotes the growth of probiotic bacteria.

4.3.1.2 Change in optical density

The optical density at 600 nm gives turbidity of the growth medium which can be related to growth of microorganisms indirectly. If PIF has prebitotic potential growth of organisms will be more then the optical density (OD) value will be higher. The OD measurements for *L. casei* and *B. bifidum* are shown in Figure 4.3.

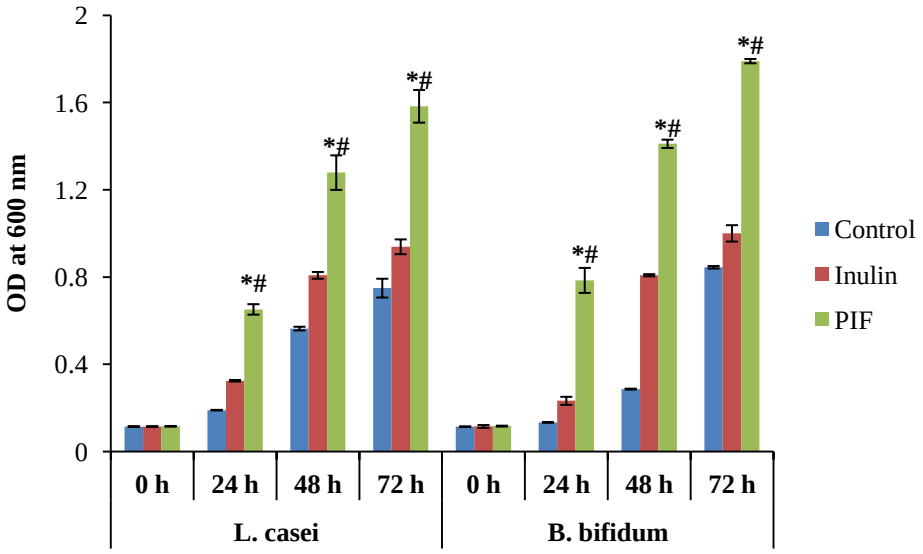


Figure 4.3. Prebiotic potential of PIF determined by change in optical density for *Lactobacillus casei* and *Bifidobacterium bifidum*. Each value represents mean ± SD from triplicate measurements. PIF – soluble dietary fibre from plantain inflorescence.

p≤0.05 considered significantly different. *Significantly different from control group. #Significantly different from inulin group.

The results depicted that the OD value for *L. casei* in PIF incorporated media increases from 0.116 ± 0.001 to 1.583 ± 0.074. In case of *B. bifidum* the OD changes from 0.117 ± 0.002 to 1.79 ± 0.009 as the incubation time increases from 0 to 72 h. The results clearly showed that PIF increases the turbidity that means it promotes the growth of probiotic strains selected for the study. The results obtained for fibre is better than the standard inulin used in the study.

4.3.1.3 Change in dry mass

Dry weight of the selected probiotic strains were calculated after centrifugation. The result from the dry mass determination directly corresponds to the bacterial mass in media. The results are shown in Figure 4.4.

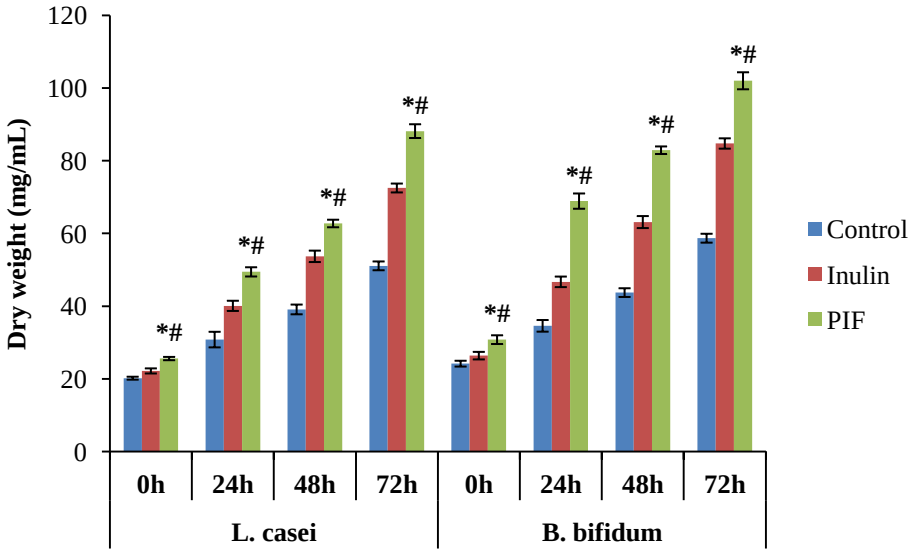


Figure 4.4. Prebiotic potential of PIF determined by change in dry mass for *Lactobacillus casei* and *Bifidobacterium bifidum*. Each value represents mean ± SD from triplicate measurements. PIF – soluble dietary fibre from plantain inflorescence.

$p \leq 0.05$ considered significantly different. *Significantly different from control group. #Significantly different from inulin group.

The dry mass increases from 25.6 ± 0.45 to 88.15 ± 1.89 mg/mL and 30.8 ± 1.2 to 102 ± 2.34 mg/mL respectively for *L. casei* and *B. bifidum* inoculated with PIF as the incubation time increases from 0 to 72 h. Whereas the dry mass change with inulin was found to be 22.2 to 72.51 mg/mL and 26.4 to 84.75 mg/mL respectively for *L. casei* and *B. bifidum*.

4.3.1.4 Determination of colony count

Colony count method is an ideal method for enumerating microorganisms. The number of colony formation unit determined by pour plate showed that PIF promotes the growth of selected probiotic strains significantly. The number of colony formation units increased from 4.2 to 12.45 and 4.2 to 12.46 log CFU/mL for *L. casei* and *B. bifidum* respectively after 72 h incubation. The PIF has better prebiotic activity when compared to inulin. The results of colony count expressed in log values are shown in Figure 4.5.

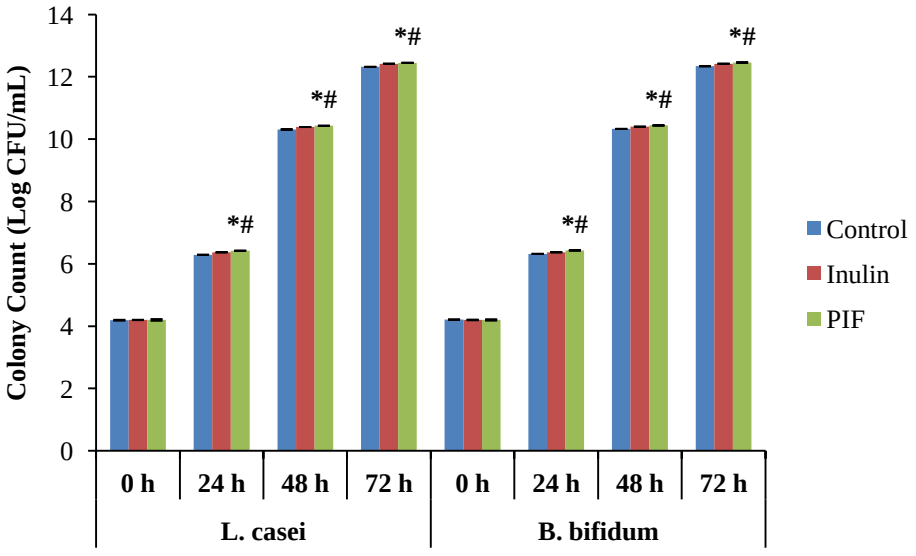


Figure 4.5. Prebiotic potential of PIF determined by colony count (pour plate method) for *Lactobacillus casei* and *Bifidobacterium bifidum*. Each value represents mean ± SD

from triplicate measurements. PIF – soluble dietary fibre from plantain inflorescence. $p \leq 0.05$ considered significantly different. *Significantly different from control group. #Significantly different from inulin group.

These results confirm that the incorporation of PIF in the media for the probiotic species, promotes its growth which is evident from the number of colonies. As the growth of the organisms increased there was a corresponding increase in the dry mass and turbidity of the media. The corresponding decrease in pH may be attributed to production of metabolites, especially short chain fatty acids (SCFA), which was confirmed by HPLC.

4.3.1.5 Assessment of short chain fatty acid production

Probiotic strains salvage energy through the metabolism of undigested carbohydrates which results in the production of secondary metabolites – short chain fatty acids primarily acetate, propionate and butyrate (Topping, 1996) which exert positive effects on human health by various mechanisms. In this study the SCFA production by selected probiotic strains were analyzed by HPLC. The HPLC chromatogram for standard SCFAs were shown in Figure 4.6.

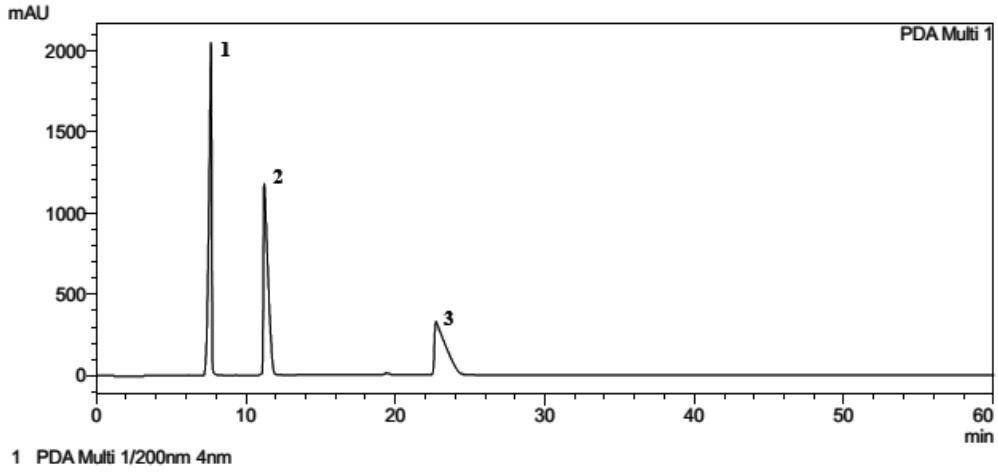


Figure 4.6. HPLC chromatogram of standard SCFAs at 200 nm. (1). Acetic acid, (2) Propionic acid and (3) Butyric acid.

The retention was found to be 7.664, 11.231 and 22.714 respectively for acetate, propionate and butyrate. The SCFAs in experimental groups at different time intervals were identified and quantified. The results are shown in Table 4.1.

Table 4.1. Quantification of SCFA

	Lactobacillus casei			***Bifidobacterium bifidum***		
	Control	**Inulin**	**PIF**	**Control**	**Inulin**	**PIF**
AA 24	25.77 ± 1.52^{a}	42.37 ± 2.16^{b}	54.53 ± 1.48^{c}	30.41 ± 2.11^{A}	45.10 ± 1.49^{B}	61.85 ± 2.32^{C}
AA 48	32.24 ± 2.72^{a}	54.23 ± 2.55^{b}	75.45 ± 1.16^{c}	33.83 ± 1.36^{A}	61.42 ± 2.54^{B}	77.96 ± 1.16^{C}
AA 72	39.88 ± 1.42^{a}	68.92 ± 2.85^{b}	84.07 ± 1.63^{c}	47.10 ± 1.24^{A}	74.28 ± 1.44^{B}	86.07 ± 2.13^{C}
PA 24	7.88 ± 1.77^{a}	16.11 ± 1.46^{b}	31.30 ± 2.40^{c}	16.33 ± 0.91^{A}	22.35 ± 1.16^{B}	39.57 ± 2.32^{C}
PA 48	22.21 ± 1.05^{a}	27.07 ± 1.89^{b}	54.05 ± 1.11^{c}	25.34 ± 2.47^{A}	31.07 ± 2.04^{B}	58.75 ± 1.27^{C}
PA 72	25.60 ± 2.37^{a}	42.85 ± 1.41^{b}	63.85 ± 2.56^{c}	37.33 ± 1.55^{A}	52.95 ± 2.31^{B}	76.84 ± 1.74^{C}
BA 24	4.11 ± 1.20^{a}	11.03 ± 1.09^{b}	21.32 ± 2.14^{c}	11.99 ± 1.04^{A}	17.07 ± 2.18^{B}	30.31 ± 2.63^{C}
BA 48	9.47 ± 1.34^{a}	20.28 ± 1.24^{b}	34.17 ± 2.33^{c}	19.14 ± 1.57^{A}	25.28 ± 2.14^{B}	36.07 ± 1.24^{C}
BA 72	12.84 ± 1.12^{a}	29.28 ± 1.75^{b}	37.60 ± 2.12^{c}	23.89 ± 1.40^{A}	37.28 ± 1.47^{B}	45.60 ± 2.15^{C}

Quantification done at three intervals of incubation time (24 h, 48 h and 72 h). AA- Acetic acid, PA- Propionic acid, BA- Butyric acid. The results are expressed in μg/mL. Each value represents mean ± SD from triplicate measurements $p \leq 0.05$ considered significantly different. ^{a,b,c} Values with different alphabets in same row are significantly different (*Lactobacillus casei*). ^{A,B,C} Values with different alphabets in same row are significantly different (*Bifidobacterium bifidum*).

The results showed that SCFA production increased when PIF was added to growth media of both *L. casei* and *B. bifidobacterium*. In case of *Lactobacillus casei* 29.28 µg/mL and 37.60 µg/mL of butyric acid was produced in presence of inulin and SDF from PI. But butyric acid production was more for *Bifidobacterium bifidum* which was found to be 37.28 µg/mL and 45.60 µg/mL in presence of inulin and PIF. These results agree that the PIF possess prebiotic activity especially with bifidogenic property. The decrease in pH can be correlated to the production of SCFA production.

4.3.1.6 Aggregation studies

Bacterial auto-aggregation and co-aggregation properties play a key role in colonization of the digestive ecosystem and the ability of probiotic strains to exclude pathogens. Aggregation is the reversible assembly of bacterial cells belonging to the same bacterial strain (auto-aggregation) or two different bacterial strains (co-aggregation) (Jankovic et al., 2012). Bacterial adhesion to mucosal surfaces and epithelial cells is one of the important features for the selection of probiotics and auto-aggregation of probiotic strains appears to be necessary for adhesion to intestinal epithelial cells (Del Re et al., 2000). Co-aggregation abilities may form a barrier that prevents colonization by pathogenic microorganisms (Polak-Berecka et al., 2014). In our study we perform this experiment in order to prove that in presence of PIF probiotics will multiply fast and hence they have better aggregation properties when compared to other experimental groups.

The auto-aggregation results are shown in Figure 4.7[A]. The results validated that the auto-aggregation properties of *L. casei* and *B. bifidum* increases in presence of PIF. After 5 h incubation the percentage auto-aggregation increases from 56 to 93.6 and

47.05 to 93.66 respectively for *L. casei* and *B. bifidum* incorporated with SDF from PI which is significantly higher than the inulin incorporated media.

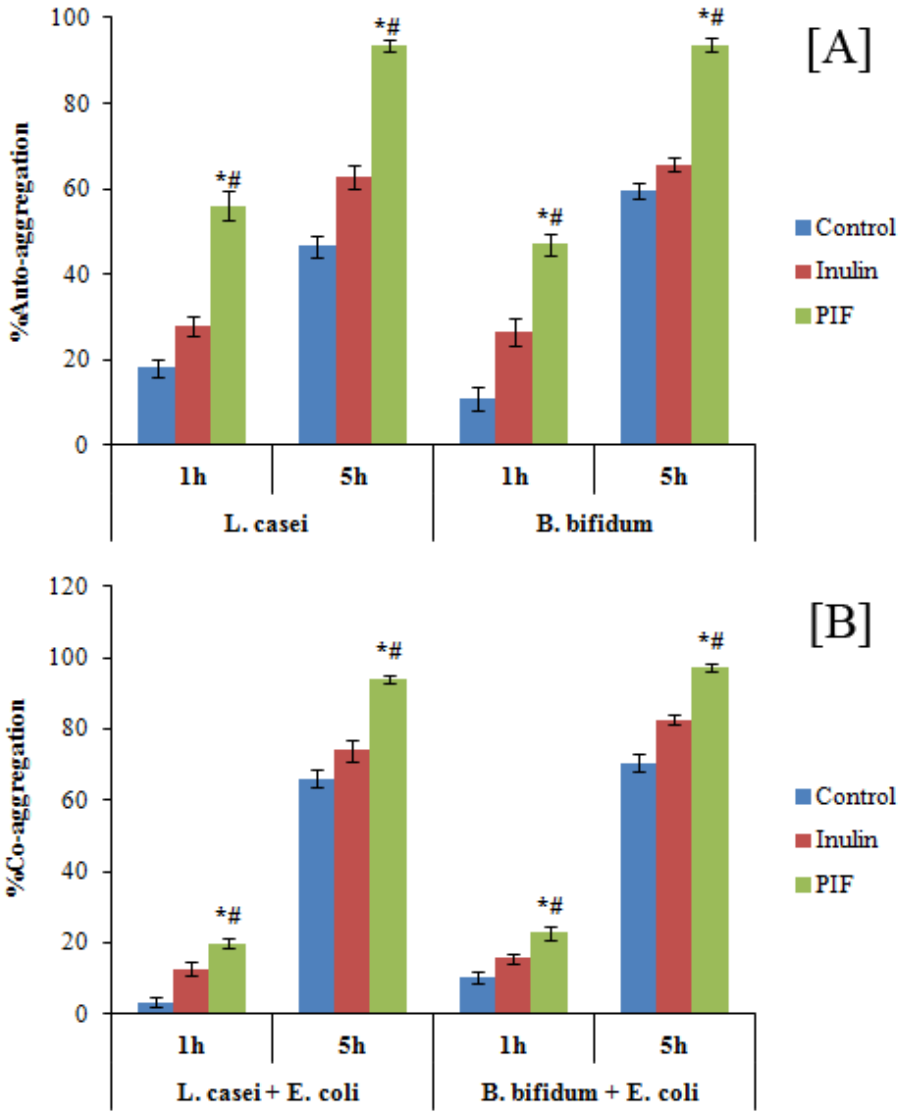


Figure 4.7. Aggregation properties of selected probiotic species in presence of PIF. [A] %Auto-aggregation for *Lactobacillus casei* and *Bifidobacterium bifidum*. [B] %Co-aggregation for *Lactobacillus casei* and *Bifidobacterium bifidum*. Each value represents mean ± SD from triplicate measurements. $p \leq 0.05$ considered as significantly different. *Significantly different from control group. #Significantly different from Inulin group. PIF – SDF from Plantain Inflorescence.

Auto-aggregation allows the formation of biofilm in the gastrointestinal tract which contributes, as a physico-chemical barrier, to the protection of the intestinal wall, along with the stimulation of the immune system (Voltan et al., 2007; Turpin et al., 2010), and to protection against colonization by pathogenic bacteria (Collado et al., 2007, 2008). Furthermore, bacteria with a high auto-aggregation potential will more easily colonize the intestinal and urogenital tracts (Cesena et al., 2001; Collado et al., 2005, 2007, 2008). This will inhibit the adhesion of pathogens and hence prevent pathogen translocation and subsequent infection (Tareb et al., 2013).

The co-aggregation property of selected probiotic species with representative pathogen, E. coli was studied. The co-aggregation results clearly showed that selected probiotic strains grown with PIF, effectively co-aggregate with *E. coli* (Figure 4.7[B]). The co-aggregation properties of *L. casei* and *B. bifidum* increases in presence of PIF significantly ($p \leq 0.05$). After 5 h incubation the percentage co-aggregation increases from 19.85 to 94.2 and 22.75 to 97.1, respectively for *L. casei* and *B. bifidum* incorporated with SDF from PI which is much higher than Inulin incorporated media. Co-aggregation phenomenon reduces the cell–cell distances between probiotics and pathogens, increasing the efficiency of antimicrobial metabolites produced by viable probiotics (Tareb et al., 2013).

Even though the aggregation properties are highly strain dependent (Vlkova et al., 2008), the presence of prebiotics multiplies the probiotic number which indirectly enhances the aggregation properties.

4.3.1.7 Inhibition of E. coli by fermentation supernatant

An alliance between bacteria and colon cancer has been recognized long back (McCoy

and Mason, 1951). The normal microbiota helps in functioning of gut immune system, integrity of the mucosal barrier, ensuring nutrient digestion, absorption, and angiogenesis (Azcarate-Peril et al., 2011). Due to change in dietary pattern, use of antibitotics or any other reasons the normal microbiota will shift towards pathobionts which leads to the induction of an inflammatory state that becomes chronic and significantly increases the risk for colorectal cancer (Akın and Tozun, 2014). One of the important mechanisms by which probiotics promote gastro-intestinal health is by preventing the growth of pathogenic strains. Probiotics achieve this property by the antimicrobial action of secondary metabolites (Corr et al., 2009) produced by fermenting undigested carbohydrates. Anti-microbial metabolites such as organic acids, short chain fatty acids, hydrogen peroxide, reuterin, diacetyl, bacteriocins and bacteriocin-like inhibitory substances are some of the metabolic products of the probiotic bacteria which are suggested to have potential antimicrobial effects (Tharmaraj and Shah, 2009). Hence the antimicrobial effect of fermentation supernatant (LS and BS) against *E. coli*, which is one of the representative pathogen found in gut, were evaluated in the present study.

The antimicrobial effect was determined by measuring the zone of inhibition. The representative photographic images showing zone of inhibition is given Figure 4.8 [I]. From the results it is obvious that supernatant obtained from fibre fermentation inhibits *E. coli*, which is significantly better than that of inulin and control supernatant ($p \leq 0.05$). For 50 µL supernatant zone of inhibition was found to be 1.9 ± 0.14 and 2.3 ± 0.14 cm for *L. casei* and *B. bifidum* from PIF treated group respectively. Whereas the zone of inhibition for inulin group (50 µL) was found to be 1.45 ± 0.07 and 1.4 ± 0.14

cm only (Figure 4.8[II]). The increase in zone of inhibition as the volume of supernatant increases can be related to the amount of short chain fatty acid present in the supernatant.

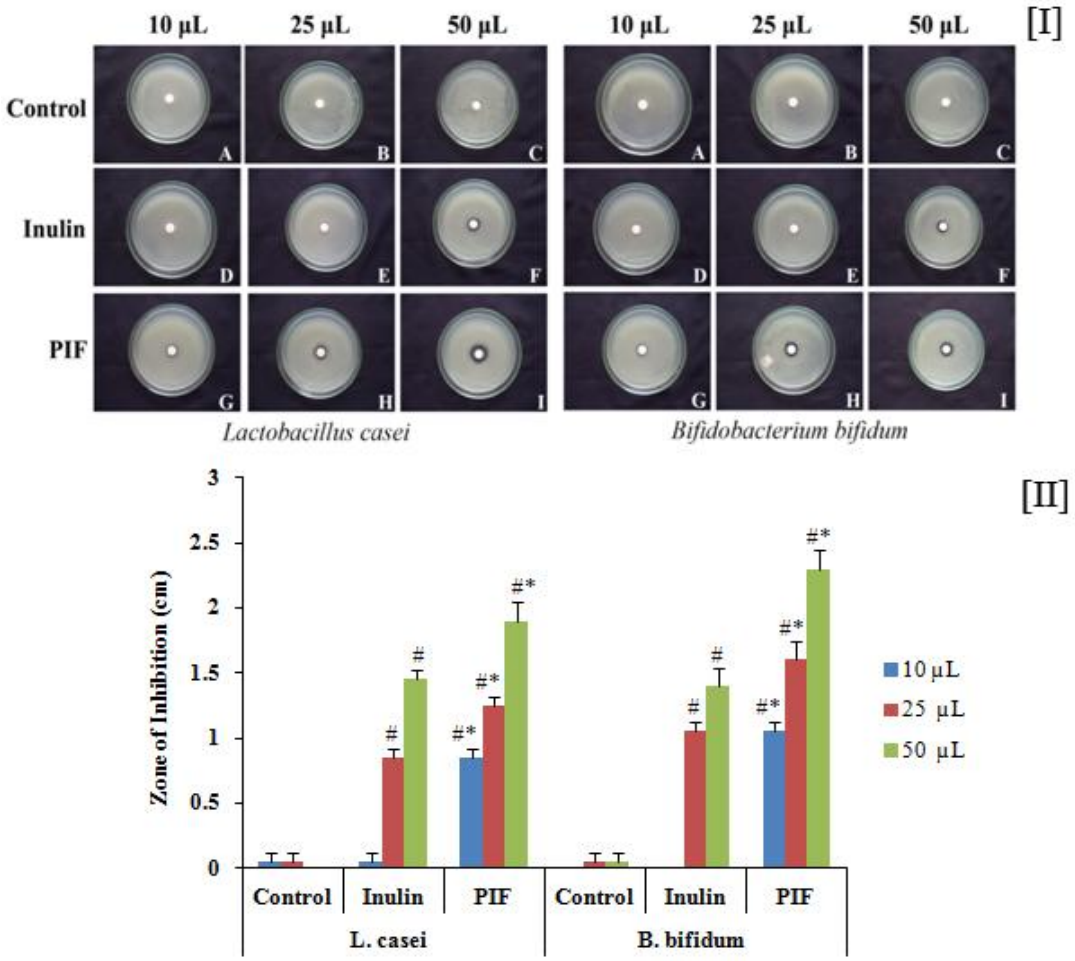


Figure 4.8. Effect of PIF fermentation supernatant on the growth of *E. coli* as determined by disc diffusion assay. [I] Representative images showing zone of inhibition. Three different volumes (10, 25 and 50 µL) of fermentation supernatant from Control (A, B, C); Inulin (D, E, F) and PIF (G, H, I) groups were placed on *E. coli* plates. [II] Graphical representation of antibacterial activity of fermentation supernatant from different experimental groups against *E. coli*. The results represented with zone of inhibition in cm. Each value represents mean ± SD from triplicate measurements. $p \leq 0.05$ considered as significantly different. $^{\#}$Significantly different from control group. *Significantly different from Inulin group. PIF – SDF from plantain inflorescence.

4.3.1.8 Inhibition of β-glucuronidase enzyme

Research correlates elevated levels of β-glucuronidase with increased colon cancer risk (Reddy and Wynder, 1973). In fact, excessive β-glucuronidase activity is one of the primary factors in the etiology of colon cancer. High-fiber diet, probiotics, low meat diet and lower colonic pH are found to reduce β-glucuronidase activity (Gorbach and Goldin, 1990; Lampe et al., 2002; Ling et al., 1994; Kulkarni and Reddy, 1994, Goldin et al., 1982; Kim et al., 1992). Hence the effect of PIF fermentation supernatant in inhibiting β-glucuronidase produced by *E. coli* was investigated.

The β-glucuronidase inhibition activity of fermentation supernatant (10, 25 and 50 µL) of different experimental groups was performed. The results are shown in Figure 4.9. For 50 µL supernatant from PIF and inulin, the percentage inhibition of β-glucuronidase was found to be 42.36 ± 0.58% (PIF) and 21.55 ± 1.13% (inulin) for *L. casei*; and 53.78 ± 0.82% (PIF) and 28.55 ± 3.13% (inulin) for *B. bifidum* respectively.

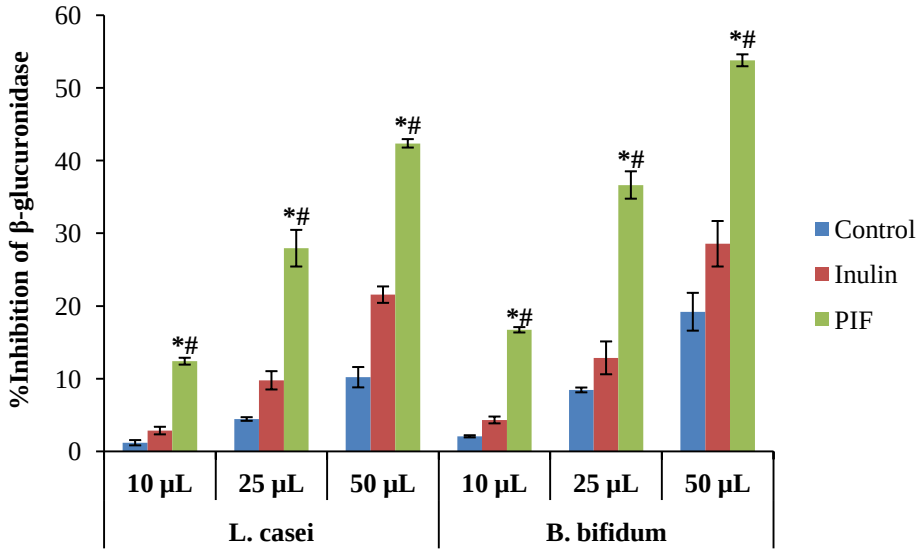


Figure 4.9. β-glucuronidase inhibition by PIF fermentation supernatant of *Lactobacillus casei* and *Bifidobacterium bifidum* (LS and BS). Each value represents mean ± SD from triplicate measurements. p≤0.05 considered as significantly different. *Significantly

different from control group. #Significantly different from inulin group. PIF – SDF from plantain inflorescence.

Thus prebiotic potential studies showed that the soluble dietary fibre from Plantain Inflorescence effectively promotes the growth of selected probiotic strains *Lactobacillus casei* and *Bifidobacterium bifidum*. Also the selected probiotic strains grown in presence of fibre effectively inhibit the growth of *E. coli*, a representative pathogen and also inhibit β glucuronidase which is one of the primary factors in the etiology of colon cancer. Further anticancer potential of the fermented supernatant of the media with SDF from PI and inulin, on the prevention and management of colorectal cancer, was investigated using HT29 colon cancer cells.

4.3.2 Anticancer potential of PIF fermentation supernatant

4.3.2.1 Cytotoxic effect of PIF fermentation supernatant

MTT assay is a rapid and high accuracy colorimetric approach that widely used to determine cell growth and cell cytotoxicity, particularly in the development of new drug. It measures cell membrane integrity by determining mitochondrial activity through enzymatic reaction on the reduction of MTT to formazan.

Cytotoxicity activity of fermentation supernatant were carried out against HT29 cell line at different concentrations to determine the IC_{50} (50% growth inhibition) by MTT assay. Results are graphically represented in Figure 4.10[A]. MTT assay confirms that the supernatants obtained from fermentation of PIF have cytotoxic effect against HT29 colon cancer cells. It was found that the percentage of cell death increased with an increase in the volume of the PIF fermentation supernatant. The IC_{50} value for PIF incorporated with *L. casei* supernatant (LS) was found to be 1510.88 µL. Whereas 905.75 µL of PIF incorporated with *B. bifidum* (BS) was able to inhibit 50% HT29

cells. BS exhibited better cytotoxic effect and this can be correlated with higher content of SCFAs in BS. There are various reports stating the cytotoxic effect of SCFAs on colon cancer cells (Scheppach et al., 1995; Heerdt et al., 1997). LS (750 µL) and BS (450 µL) were used for further analysis

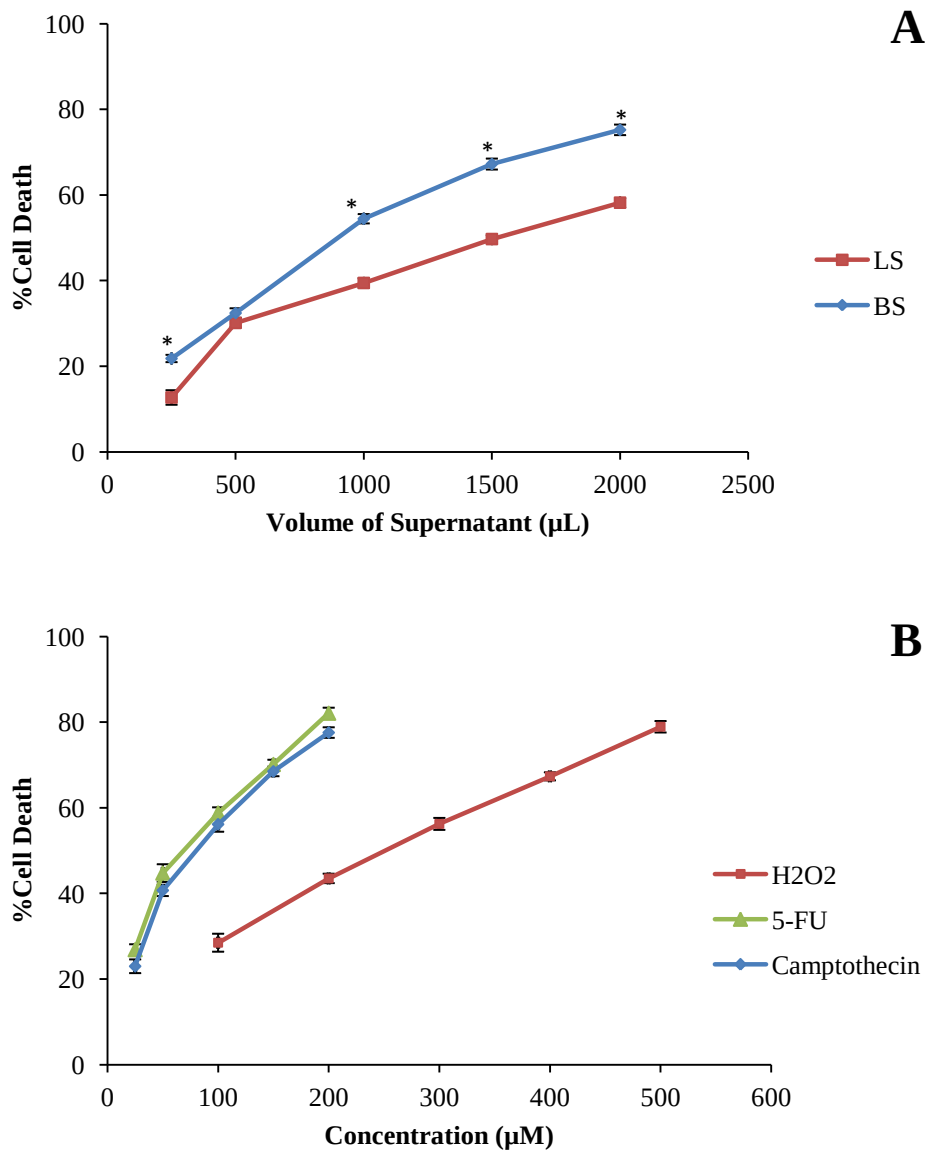


Figure 4.10. Cytotoxic effect of fermentation supernatant and positive controls used for various anticancer assays against HT29 cells. [A] Cytotoxic effect of PIF fermentation supernatant from *Lactobacillus casei* and *Bifidobacterium bifidum* (LS and BS) against

HT29 cells. Each value represents mean ± SD from triplicate measurements. *B. bifidum* percentage cell death values with *symbol are significantly different from that of the corresponding values of *L. casei*. P≤.05 considered as significantly different. [B] Cytotoxic effect of H_2O_2, 5-flurouracil and camptothecin against HT29 cells.

The cytotoxic effect of H_2O_2, 5-flurouracil (5-FU) and camptothecin, which were used as positive control for various assays, were also studied against HT29 cells. The results are shown in Figure 4.10[B]. The incubation time for H_2O_2 is 15 min and that for 5-FU is 24 h. The IC_{50} for H_2O_2, 5-FU and camptothecin were found to be 257.20 µM 83.25 µM and 88.65 µM respectively. For further assays, concentrations of 250 µM H_2O_2, 50 µM 5-FU and 50 µM camptothecin were fixed as positive control, which were below IC_{50} value.

4.3.2.2 Effect of fermentation supernatant on DNA damage

Morphological alterations indicating apoptosis were tested by Hoechst 33342 staining. Nuclear changes such as chromatin condensation and DNA fragmentation are hallmarks of apoptotic cells. Effect of LS and BS on nuclear morphology of HT29 colon cancer cells was investigated by DNA-binding fluorescent dye (Hoechst 33342 stain). As shown in Figure 4.11, cells treated for 24 h with LS, BS, and H_2O_2 showed remarkable changes in the chromatin structure including fragmentation, uniform condensation and forming apoptotic bodies when compared to untreated control. In contrast, the untreated cells remained uniformly stained. More damage in the DNA was observed when treated with BS. The morphological analysis indicated that LS and BS could induce apoptosis in HT29 cells. However the activity of PIF fermentation supernatant is less when compared to the positive control H_2O_2 treated group.

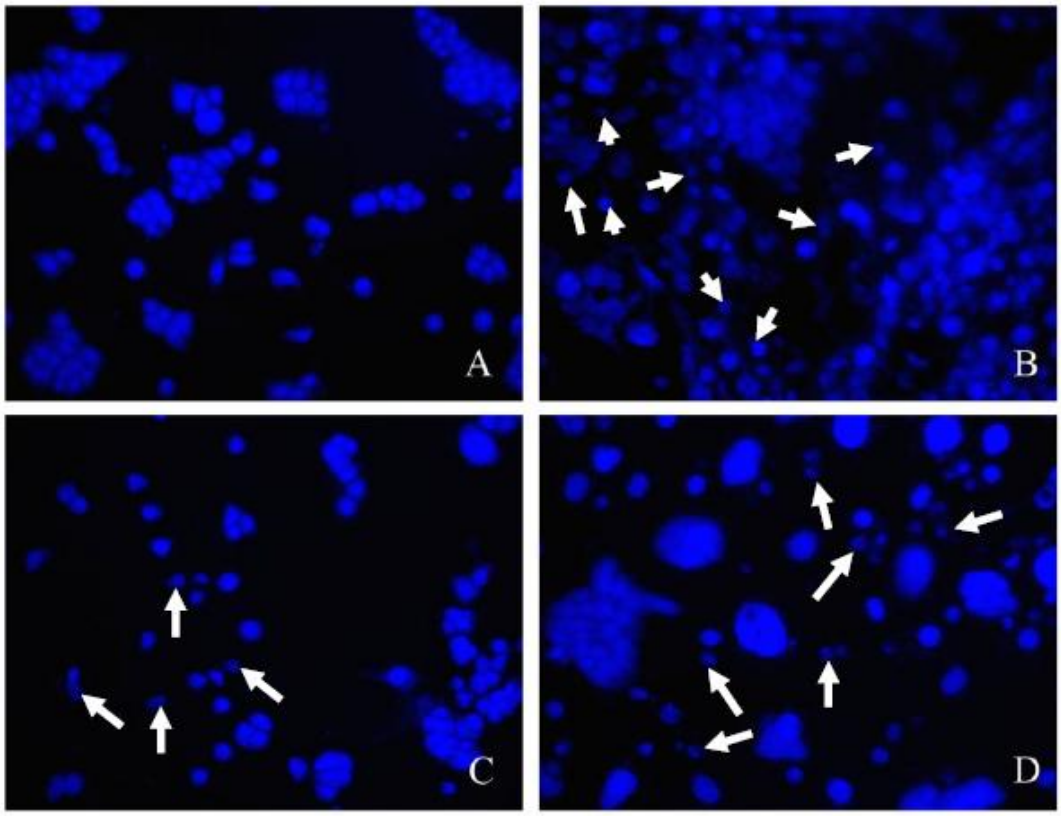


Figure 4.11. Effect of PIF fermentation supernatant – LS and BS on DNA damage in HT29 colon cancer cells. The arrow indicates chromatin condensation which is a hallmark of apoptosis. (A) Untreated control, (B) H_2O_2 (250 µM), (C) LS (750 µL), (D) BS (450 µL).

4.3.2.3 Effect of fermentation supernatant on apoptosis

Cell death is an essential part of the normal development and maturation (Jacobson et al., 1997), and a homeostatic balance between the rates of cell proliferation and cell death is critical for maintaining normal physiological processes. Apoptosis is a very tightly programmed cell death and contributes to elimination of unnecessary and unwanted cells to maintain the healthy balance between cell survival and cell death (Cotter, 2009). Cancer is one of the scenarios where too little apoptosis occurs, resulting in malignant cells that will not die. The mechanism of apoptosis is complex and involves many pathways (Wong, 2011) and defects in apoptosis play important roles in

tumor pathogenesis. Therefore one of the main aims in cancer research is the induction of apoptosis in cancer cells.

Since LS and BS is having cytotoxicity and DNA damaging effect, the ability to induce apoptosis was also analyzed. The representative histogram for apoptosis from flow cytometry was shown in Figure 4.12[I].

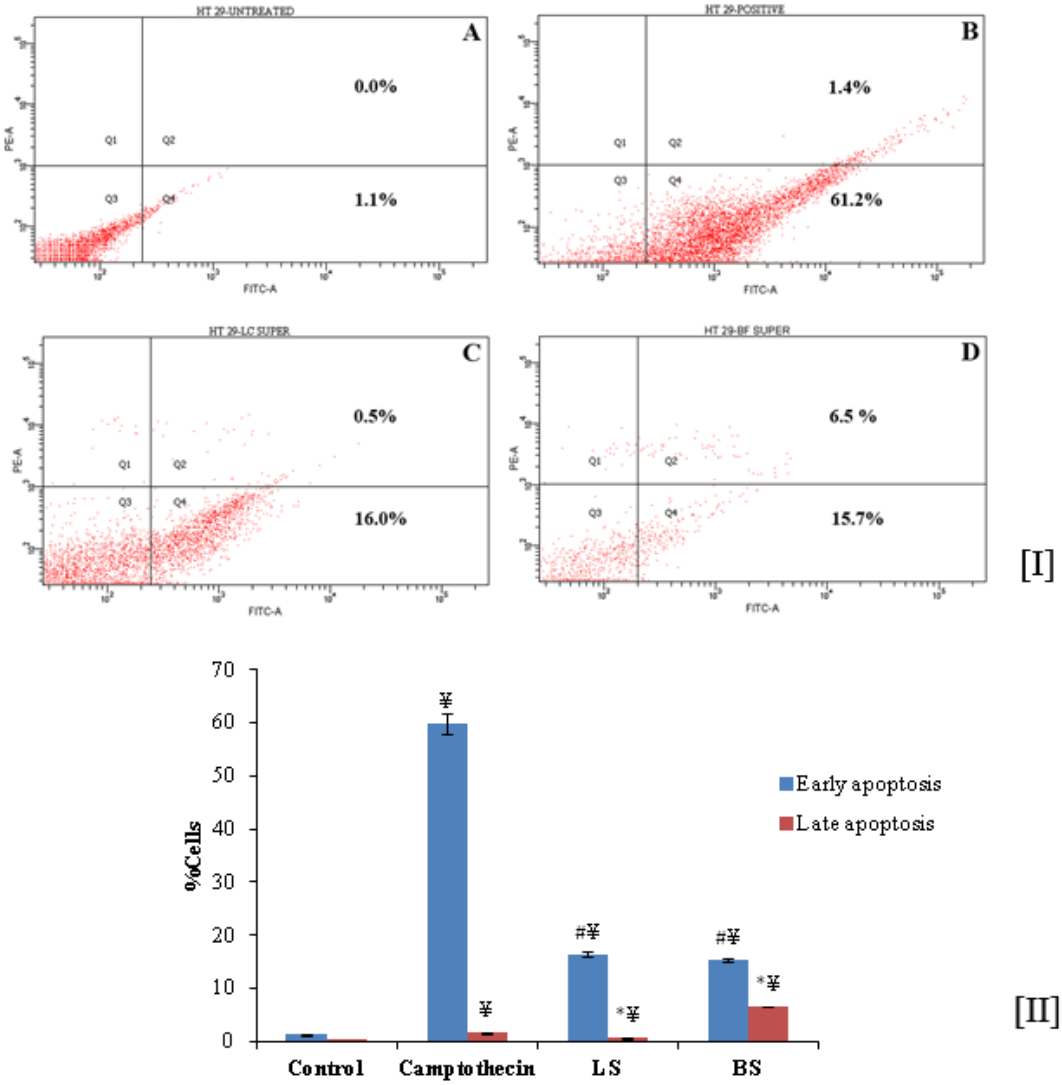


Figure 4.12. Apoptotic effect of PIF fermentation supernatant LS and BS. [I] Representative image of induction of apoptosis by PIF fermentation supernatant. (A) Untreated control, (B) Camptothecin (50 µM), (C) LS (750 µL), (D) BS (450 µL). Four quadrants (Q1, Q2, Q3 and Q4) represent dead cells, late apoptotic cells, live cells and early apoptotic cells respectively. [II] Graphical representation of apoptotic effect of

PIF fermentation supernatant LS and BS. Each value represents mean ± SD from triplicate measurements. #No significant difference found between LS and BS in early apoptotic stage. *Significant difference found between LS and BS in late apoptotic stage. ¥Significantly different from control group. $p \leq 0.05$ considered as significantly different.

From the graph (Figure 4.12[II]) it is clear that in late apoptosis stage, the BS (6.55 ± 0.07) is having better effect in inducing apoptosis than LS (0.6 ± 0.49). But, the activity is not worth to compare with standard camptothecin as it has better activity than LS and BS particularly in early apoptosis stage. However our results confirm that supernatant obtained from fermentation of dietary fibre by probiotic bacteria is able to induce apoptosis in colon cancer cells and is comparable with that of earlier reports explaining the apoptotic effect of fermentation supernatant from various sources such as inulin, oligofructose and wheat (Munjal et al., 2009; Borowicki et al., 2010).

4.3.2.4 Effect of fermentation supernatant on intracellular reactive oxygen production

Apoptosis can be initiated by a variety of stimuli. ROS and oxidative damage have been implicated in the induction of apoptosis (Dimmeler et al., 1998; Tamarit et al., 1998). Hence we analyzed whether ROS production was increased in the cells after the treatment with the supernatant. The ROS level in HT-29 cells was measured using DCFH-DA in a flow cytometry analysis system (Figure 4.13[I]).

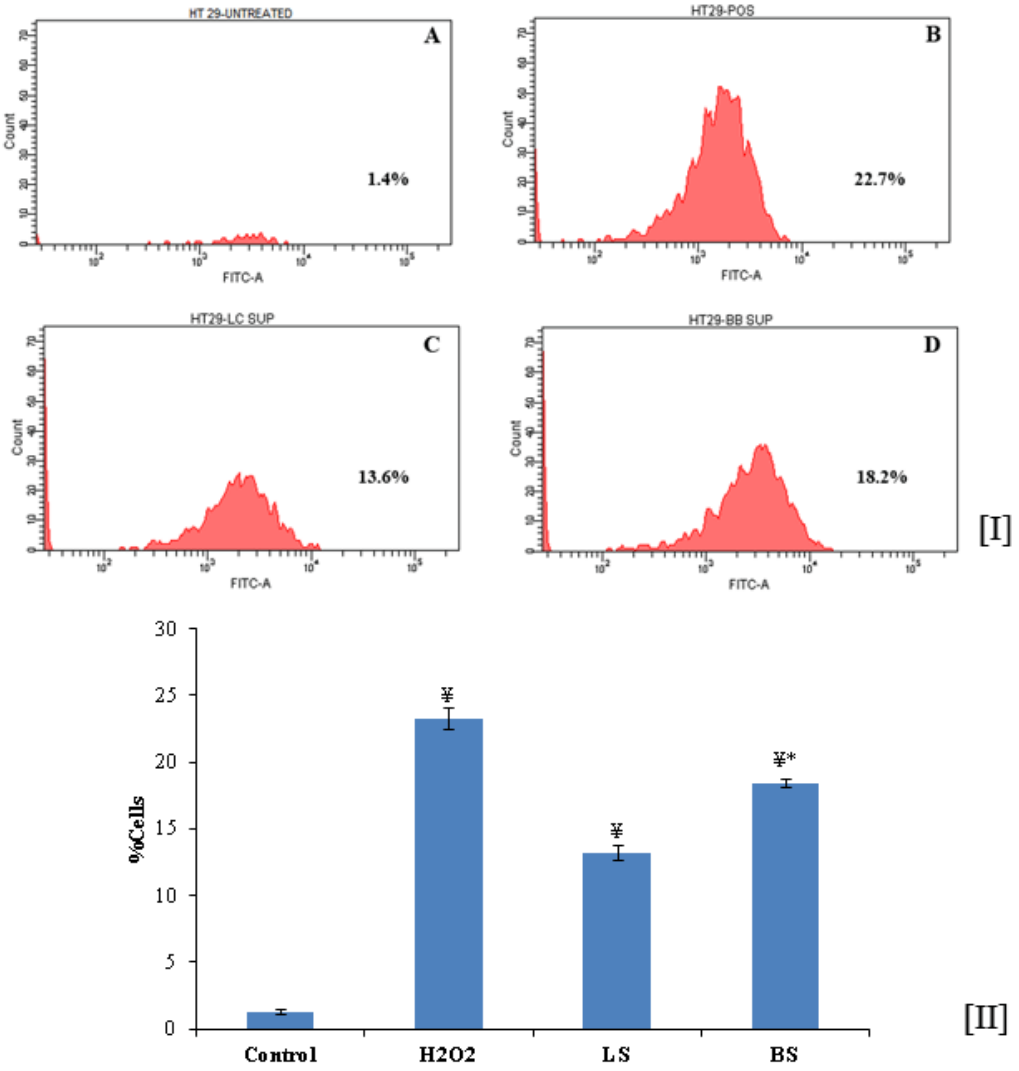


Figure 4.13. ROS production by PIF fermentation supernatant LS and BS. [I] Representative image for ROS production by fermentation supernatant. (A) Untreated control, (B) H_2O_2 (250 µM), (C) LS (750 µL), (D) BS (450 µL). [II] Graphical representation of ROS production by fermentation supernatant LS and BS. Each value represents mean ± SD from triplicate measurements. $p \leq 0.05$ considered as significantly different. ¥Significantly different from control group. *BS significantly different from LS. $p \leq 0.05$ considered as significantly different.

Compared to untreated control cells, the ROS levels in HT-29 cells significantly increased after the treatment with supernatant, for 24 h. Among the supernatant, BS (18.45 ± 0.35) was found to induce more ROS production than LS (13.2 ± 0.56) (Figure

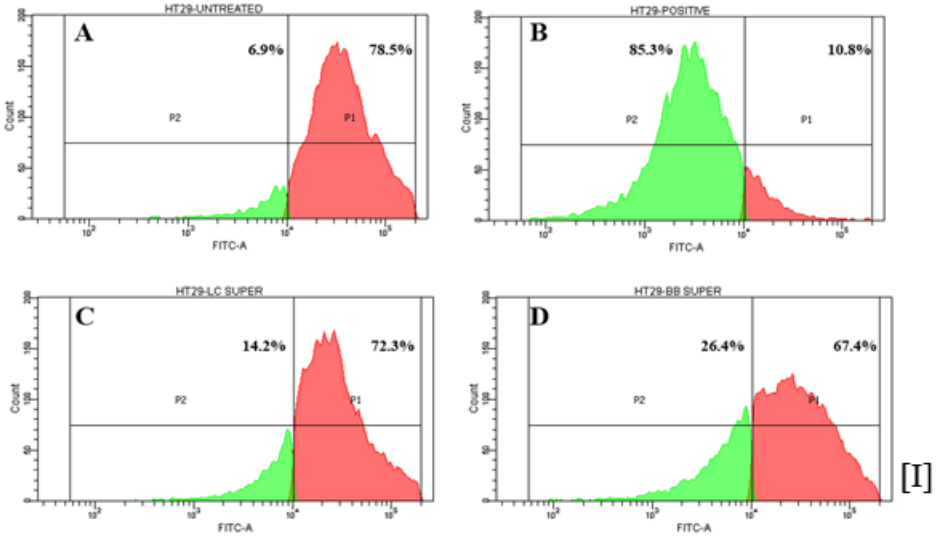
4.13[II]). Various natural compounds (Nakazato et al., 2005; Antoslewicz et al., 2008) and some anticancer drugs (Meshkini and Yazdanparast, 2012; Berndtsson et al., 2007) are known to up regulate ROS production in cancer cells and induce apoptosis. Among the different short chain fatty acids, butyrate is also reported to induce apoptosis in HT29 cells through the production of ROS (Domokos et al., 2010). Hence the apoptotic effect of the PIF fermentation supernatant can be related to the presence of butyrate. The higher butyrate content in BS induces increased ROS production which in turn enhances apoptosis in HT29 cells.

4.3.2.5 Effect of fermentation supernatant on mitochondrial membrane potential ($\Delta\psi_m$)

Cancer cells have a more hyperpolarized mitochondrial membrane potential than normal cells. Abnormalities in mitochondrial structure and function and a general elevation in the mitochondrial membrane potential have been linked to colonic carcinoma cells (Heerdt et al., 2005). The apoptotic cascade includes the translocation of the proapoptotic BAX and/or BAK from the cytosol to the mitochondria, dissipation of the $\Delta\psi_m$, and the release of Cytochrome C and other factors (Shimizu et al., 1999), which drives the recruitment and processing of pro-caspase-9, ultimately leading to terminal apoptosis (Ly et al., 2003). Interfering with $\Delta\psi_m$ dissipation through its elevation, stabilization, or collapse results in delayed, decreased, or blocked apoptosis and the majority of colonic tumors exhibit elevations in the mitochondrial membrane potential (Chen and Rivers, 1990).

Mitochondrial membrane potential of HT29 colon cells were determined by rhodamine 123 dye. Rhodamine 123 selectively enters mitochondria with an intact membrane potential and is retained in the mitochondria. Once the membrane potential is

lost, the dye is washed out of the mitochondria. The histograms obtained from flow cytometric analysis were shown in Figure 4.14[I].



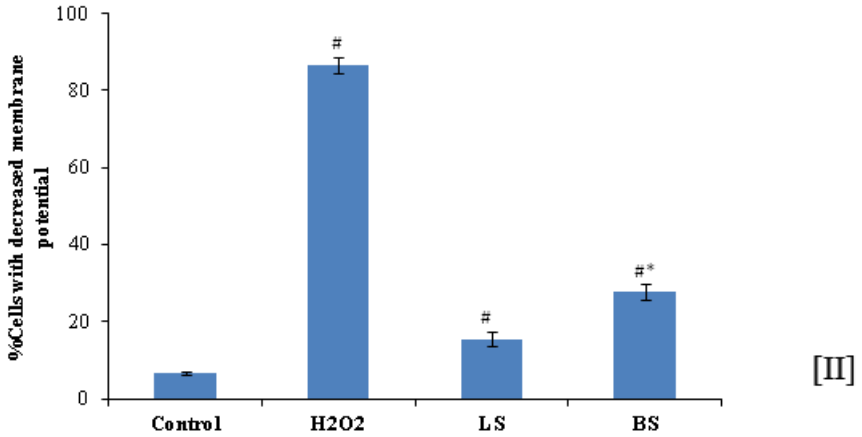


Figure 4.14. Reduction in mitochondrial membrane potential by PIF fermentation supernatant LS and BS in HT29 colon cancer cells. [I] Representative histogram for the effect of fermentation supernatant on mitochondrial membrane potential. (A) Untreated control, (B) H_2O_2 (250 µM), (C) LS (750 µL), (D) BS (450 µL). [II] Graphical representation of reduction in mitochondrial membrane potential. Each value represents mean ± SD from triplicate measurements. $p \leq 0.05$ considered as significantly different. $^{\#}$Significantly different from control group. *BS significantly different from LS. $p \leq 0.05$ considered as significantly different.

The results showed that the mitochondrial membrane potential of HT29 colon cancer cells was decreased when treated with fermentation supernatant of LS and BS. However the activity of fermentation supernatant is less when compared to the positive control H_2O_2 used in this assay. It was found that the loss of mitochondrial membrane potential was 86.75 ± 2.05% when treated with H_2O_2 (250 µM) where as 15.5 ± 1.83 and 27.75 ± 1.90% of cells lose mitochondrial membrane potential when treated with LS and BS respectively (Figure 4.14[II]). The results are promising as the loss of mitochondrial membrane potential in cancer cells affects the production of ATP and may facilitate the initial steps of apoptosis like release of Cytochrome C. From the results it is obvious that BS significantly exhibits better activity than LS.

4.3.2.6 Effect of fermentation supernatant on ATP production

ATP is the main energy currency of the cell and uncontrollably dividing cancer cells have a high demand for ATP. ATP molecule is involved in the processes that mediate all types of cell death – apoptosis, autophagy and necrosis (Lemasters et al., 2002). During late-stage apoptosis, ATP levels sharply drop, mostly because of the loss of mitochondrial function and consumption by ATP-dependent proteases. Thus ATP production plays an important role in the survival of cancer cells (Zhou et al., 2012).

The effect of fermentation supernatant on ATP production in HT29 cells were analyzed by HPLC at 259 nm. The representative chromatogram was shown in Figure 4.23. The retention time was found to be 3.808, 4.858 and 7.119 min respectively for ATP, ADP and AMP (Figure 4.15[I]).

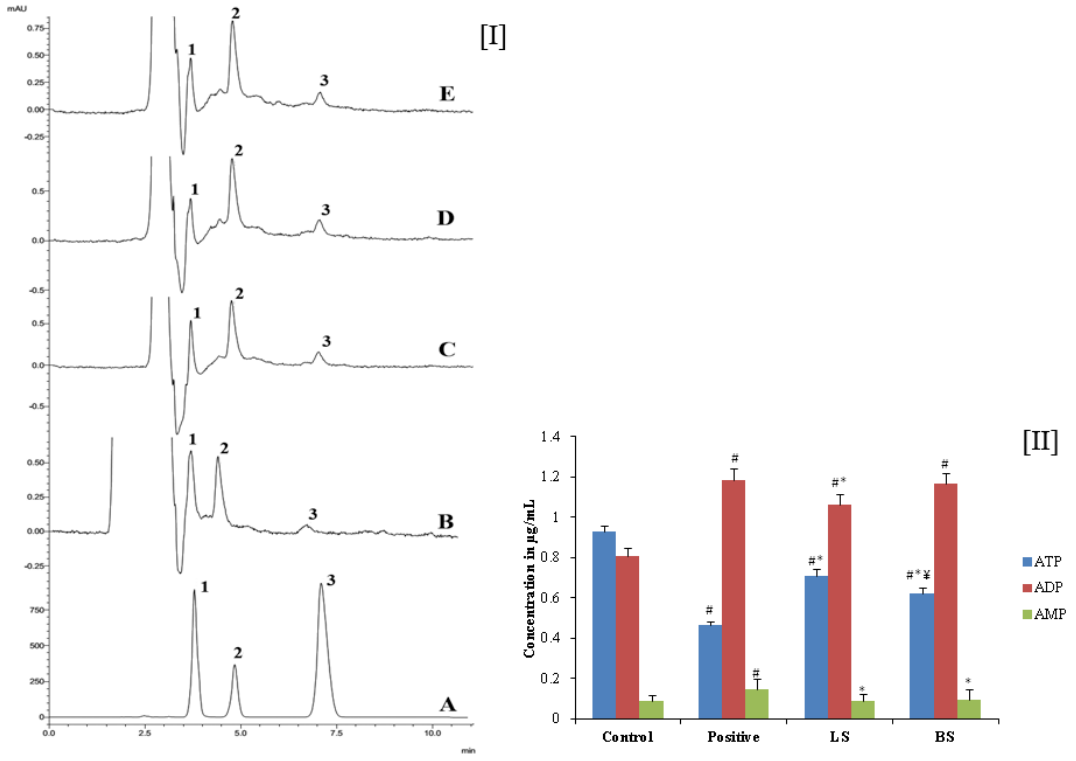


Figure 4.15. Reduction in ATP production by PIF fermentation supernatant LS and BS in HT29 colon cancer cells. [I] Representative HPLC chromatogram showing the effect of fermentation supernatant LS and BS on ATP production. (1) ATP, (2) ADP and (3) AMP. (A) Standard ATP, ADP and AMP at 1 mg/mL concentration, (B) Untreated cells, (C) Positive control- 250 µM H_2O_2 treated, (D) LS (750 µL) treated and (E) BS (450 µL) treated. [II] Graphical representation of reduction in ATP production by fermentation supernatant LS and BS. Each value represents mean ± SD from triplicate measurements. $p \leq 0.05$ considered as significantly different. $^{\#}$Significantly different from control group. *Significantly different from positive group. $^{¥}$BS significantly different from LS. $p \leq 0.05$ considered as significantly different.

The results clearly indicate that after the treatment of HT29 colon cancer cells with fermentation supernatant, the ATP production was reduced. The ATP content in control group was found to be 0.931 ± 0.024 µg/mL, which was reduced to 0.465 ± 0.017 µg/mL, 0.709 ± 0.033 µg/mL and 0.619 ± 0.031 µg/mL respectively for cells treated with H_2O_2, LS and BS (Figure 4.15[II]). Thus these results confers that the

decreased mitochondrial potential has affected the production of ATP in cancer cells after treating with fermentation supernatant – LS and BS. The fermentation supernatant may also have affected enzymes of glycolytic pathway which consecutively reduce the ATP production. The results showed that BS has better activity than LS in reducing the ATP production.

4.3.2.7 Effect of fermentation supernatant on Cytochrome C release

Release of Cytochrome C from mitochondria is a key initiative step in the apoptotic process (Renault et al., 2013). Following exposure of cells to stimuli that trigger apoptosis, Cytochrome C is rapidly released from mitochondria into the cytoplasm where it binds with APAF-1 protein and activates proteolytic molecules known as caspases (Eskes et al., 1998) that are crucial for the execution of apoptosis. The antiapoptotic BCL2 proteins are widely over expressed in human cancers including colorectal cancer (Koehler et al., 2013), which are mainly involved in the inhibition of Cytochrome C release from mitochondria and thereby preventing the cells to enter apoptosis.

Hence we analyzed the effect of fermentation supernatant LS and BS on Cytochrome C release from mitochondria. The Cytochrome C was analyzed by HPLC method at 393 nm. The retention time was found to be 4.492 min. The representative chromatogram was shown in Figure 4.16[I]. From the results it was obvious that the release of Cytochrome C was increased after the treatment of LS and BS. The Cytochrome C release was increased from 0.761 ± 0.016 µg/mL (Control) to 1.074 ± 0.023 and 1.292 ± 0.027 µg/mL respectively for LS and BS (Figure 4.16[II]). BS exhibited significantly better activity than LS, however the activity of both LS and BS

is less when compared to the positive control H_2O_2 (1.815 ± 0.039 µg/mL) used in the assay.

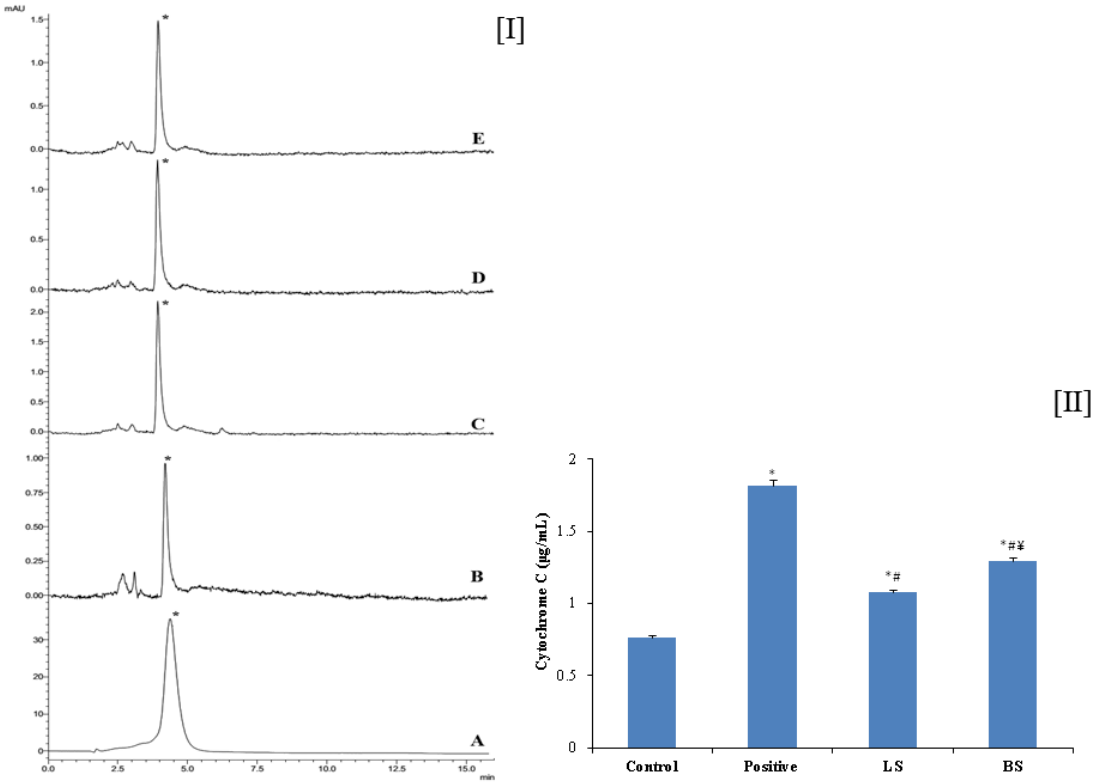


Figure 4.16. Cytochrome C release by PIF fermentation supernatant LS and BS in HT29 colon cancer cells. [I] Representative HPLC chromatogram showing the effect of fermentation supernatant LS and BS on Cytochrome C release in HT29 cells. (*) Cytochrome C. (A) Cytochrome C standard, (B) Untreated cells, (C) Positive control- H_2O_2 (250 µM) treated, (D) LS (750 µL) treated and (E) BS (450 µL) treated. [II] Graphical representation of Cytochrome C release by PIF fermentation supernatant. Each value represents mean ± SD from triplicate measurements. $p \leq 0.05$ considered as significantly different. *Significantly different from control group. $^{\#}$Significantly different from positive group. $^{¥}$BS significantly different from LS. $p \leq 0.05$ considered as significantly different.

Thus the preliminary anticancer assays depicted that PIF fermentation supernatant is able to initiate ROS induced apoptosis in HT29 colon cancer cells. The supernatant is able reduce mitochondrial membrane potential which in turn reduces

ATP production as well as increases Cytochrome C release which is an indication for the initiation of apoptosis.

4.3.2.8 Western blot analysis

The results from various assays proved that the fermentation supernatant LS and BS has significant anticancer effect on HT29 colon cancer cells. It was obvious from the studies that the fermentation supernatant of LS and BS exhibit potential anticancer effect by inducing apoptosis. In order to understand mechanism of action in depth, the expression some of the key proteins involved in apoptosis after treating HT29 cells with LS and BS were analyzed.

The BCL2 family of proteins which is involved in the regulation of apoptotic cell death consists of anti-apoptotic and pro-apoptotic members (Tsujimoto, 1998). The anti-apoptotic members of this family, such as BCL2, prevent apoptosis either by preventing the release of mitochondrial apoptogenic factors such as Cytochrome C into the cytoplasm or by sequestering caspases. On the contrary, pro-apoptotic members of this family, such as BAX, elicit the release of Cytochrome C from mitochondria leading to caspase activation. Caspases play important roles in the induction of apoptosis and are classified as either initiator (Caspase-8 and Caspase-9) or effecter caspases (Caspase-3 and Caspase-7) (Vishchuk et al., 2013). Activated initiator caspases can cleave and activate effecter caspases, which in turn cleave a variety of cellular substrates, most notably poly (ADP-ribose) polymerase (PARP). PARP helps to repair single-strand DNA nicks; hence, cleaved PARP is a useful marker for apoptosis (Jin and El-Deiry, 2005).

The expression levels of cleaved PARP (c-PARP), cleaved caspase (c-Caspase),

BCL2 and BAX in HT29 cells were analyzed, after the treatment with fermentation supernatant LS and BS. The results are shown in Figure 4.17. It was found that LS and BS had no effect on the expression of anti apoptotic protein BCL2 (Figure 4.17 A). However, the expression of pro-apoptotic protein BAX increased after the treatment with LS and BS (Figure 4.17 B). The BS is found to induce BAX more than that of LS. The fermentation supernatant LS and BS were able to induce activation of c-Caspase 3 and c-PARP (Figure 4.17 C and 4.17 D). The activation of BAX may have initiated the release of Cytochrome C which in turn leads to the cleavage of Caspase 3 and PARP which sequentially initiates the cell death. The activity of LS and BS was less when compared to the standard 5-Fluorouracil (50 µM) used in the assay.

Short chain fatty acids (SCFA), principally butyrate, propionate, and acetate, are produced in the gut through the fermentation of dietary fiber by the colonic microbiota. Butyrate has been demonstrated to inhibit proliferation and induce apoptosis and differentiation in numerous cell lines, including colorectal cancer cell lines (Hamer et al., 2008). Butyrate has been reported to initiate Caspase 3 activation and leads to cell death in HCT116 colon cancer cells (Fung et al., 2011). In Caco2 cells, butyrate is reported to induce apoptosis through mitochondrial pathway (Ruemmele et al., 2003). Summarizing the role of SCFA, especially butyrate, from these reports, it can be understood that the apoptotic effect of LS and BS fermentation supernatant is due to the presence of SCFA. It was already confirmed that the fermentation supernatant contains significant amount of SCFA (Table 4.1). BS exhibits better activity than LS as the SCFA content is more in BS.

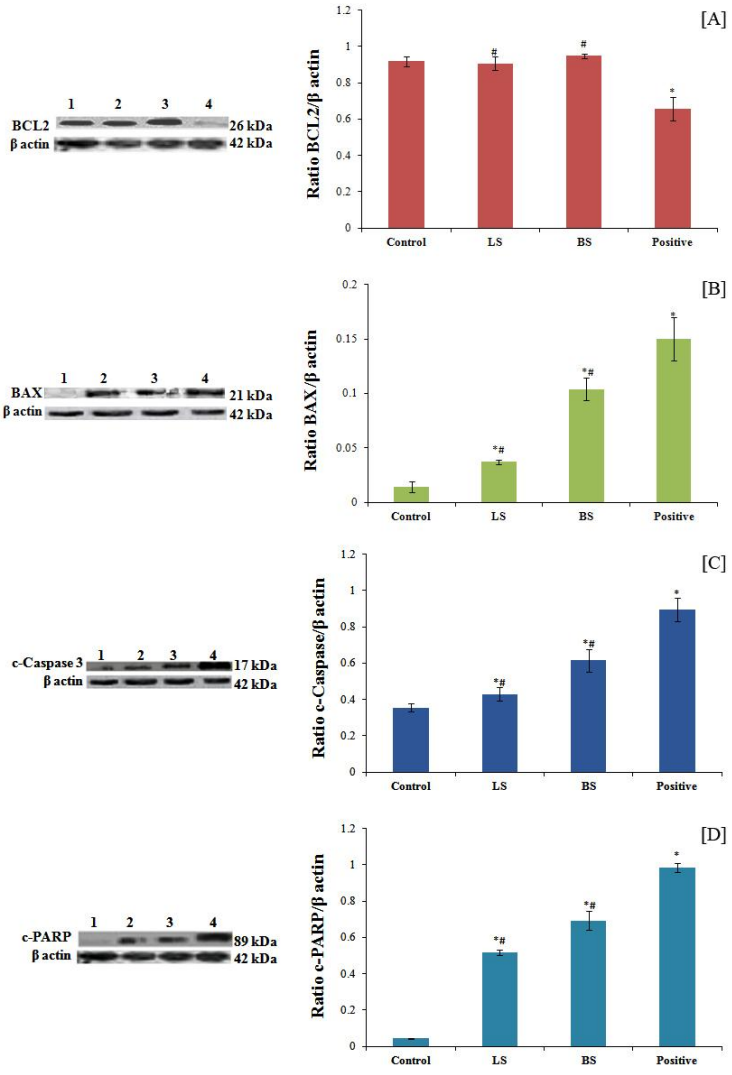


Figure 4.17. Effect of PI dietary fibre fermentation supernatant LS and BS on expression of various proteins involved in apoptosis in HT29 colon cancer cells. The results are compared with that of protein obtained from untreated cells (first lane) as well as cells treated with positive control 5-Fluorouracil (50 µM) (fourth lane). Protein from cells treated with LS (750 µL) and BS (450 µL) were loaded in second and third lane. The ratio of intensity of various protein bands against intensity of β actin bands were plotted in the graphs. Expression of BCL2, BAX, c-Caspase 3 and c-PARP are shown respectively in A, B, C and D. Each value represents mean ± SD from triplicate measurements. $p \leq 0.05$ considered as significantly different. *Significantly different from control group. $^{\#}$Significantly different from positive group.

4.3.2.9 Changes in the protein expression profile of HT-29 human colon cancer cells after treatment with BS fermentation supernatant

Based on the above anticancer assays it was clear that the supernatant obtained from the fermentation of plantain inflorescence dietary fibre by *Bifidobacterium bifidum* (BS); possess better activity than that of *Lactobacillus casei* (LS). Among the two fermentation supernatant, BS was found to be more active against HT29 cells; and was able to induce ROS mediated apoptosis in HT29 colon cancer cells. Hence we were eager to know the effect of BS on total protein profile of HT29 cells. For this, we have used a proteomic approach in HT-29 cells by performing 2D-PAGE and peptide mass fingerprinting (MALDI-TOF/MS/MS) for identification of differentially expressed proteins.

HT-29 human colon cancer cells were treated for 24 h with fermentation supernatant BS. Total proteins were isolated and then separated by 2D gel electrophoresis using 3–10 nonlinear IPG strips and 12% gels. After Coomassie staining, the gel patterns were scanned and computer-assisted image analysis was performed by means of the PDQuest 8.0 software (Bio-Rad Laboratories GmbH, Munich, Germany) to compare the stained protein spots from treated and untreated samples. The two dimensional map of protein expression of HT29 cells after the treatment with BS, were shown in Figure 4.18. Five differentially expressed proteins were selected (P1-P5, denoted with arrows), based on the changes in expression profile on 2D gel after checking the reproducibility of the expression profile (experiments were repeated three times to confirm the reproducibility of the results).

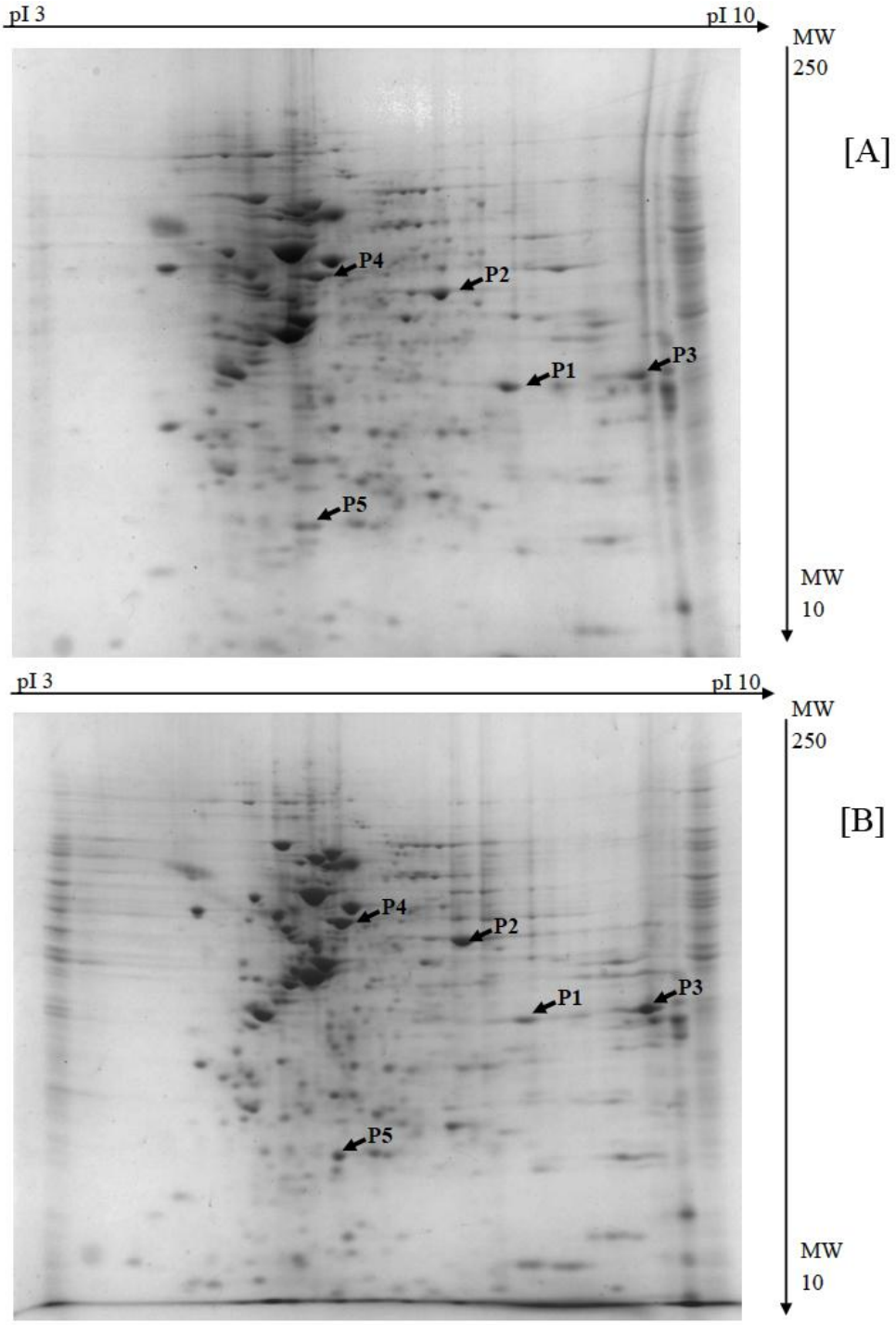


Figure 4.18. Two-dimensional map of protein expression profile of HT-29 human colon cancer cells after treatment with PIF fermentation supernatant of *Bifidobacterium bifidum*. (A) Untreated and (B) BS treated. Arrows and spot numbers indicate the proteins identified. Proteins were separated based on isoelectric point (pI) on a linear pH 3–10 IPG-strip in the first dimension and on molecular weight (MW in kDa) on a 12% SDS-polyacrylamide gel in the second dimension.

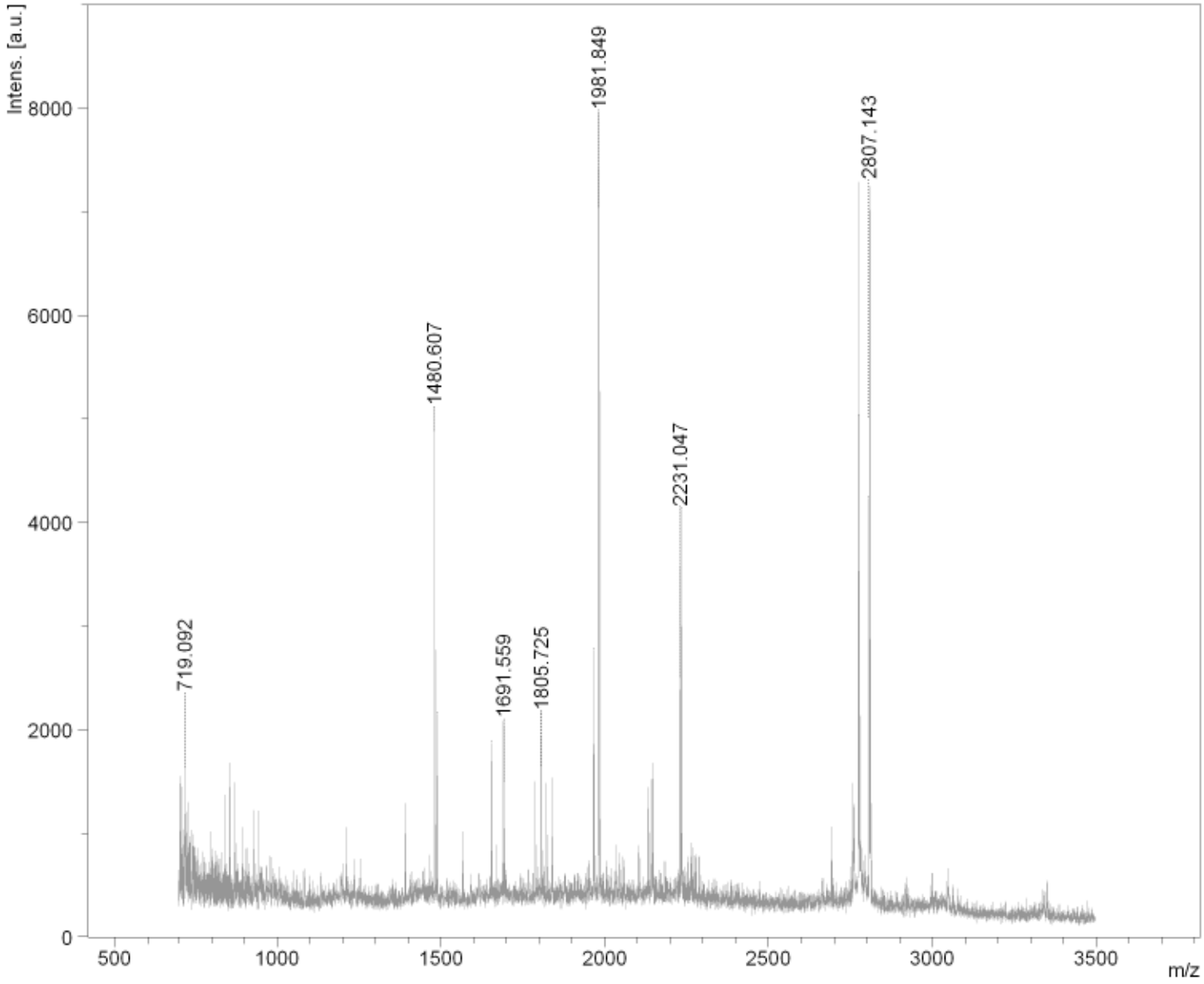
Five proteins excised manually from the gels and were subjected to peptide mass fingerprinting (PMF) for identification of proteins. For this, selected proteins were subjected to in-gel tryptic digestion, followed by MALDI TOF MS/MS of the tryptic peptide mixtures and database search. Based on the data, the proteins identified were Apoptosis inducing factor-mitochondria associated 1 isoform 6 (AIFM1), Annexin A2 isoform 2, Solute carrier family 25 member 35 (SLC25A35), Heat shock cognate 71 kDa protein isoform 2 (HSPA8) and Triose phosphate isomerase isoform 3 (TPI 3). The PMF data and MASCOT search result of a representative protein - Heat shock cognate 71 kDa protein isoform 2 is shown (Plate 1 and Plate 2) for reference.

PLATE 1

D:\Data\nisst\pnisha\SAMPLE H6\0_K6\1\1SRef

Comment 1

Comment 2



Acquisition Parameter

Date of acquisition	2015-12-03T12:39:02.703+05:30
Acquisition method name	D:\Methods\flexControlMethods\Specification\RP_700-3500_Da.par
Aquisition operation mode	Reflector
Voltage polarity	POS
Number of shots	500
Name of spectrum used for calibration	
Calibration reference list used	

Instrument Info

D:\Data\nisst\pnisha\SAMPLE H6\0_K6\1\1SRef

User	BDALDE
Instrument	FLEX-PC
Instrument type	ultraflexTOF/TOF

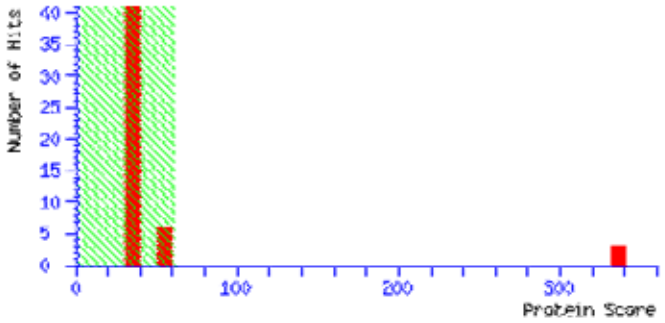
Mascot Search Results

PLATE 2

```
User            : SARAVANAKUMAR
Email           : msaravana@rgcb.res.in
Search title    : HUMAN SAMPLE 6
MS data file    : DATA.TXT
Database        : NCBI_Homosapiens HumanDB_NCBI (71338 sequences; 45882706 residues)
Timestamp       : 4 Dec 2015 at 18:42:15 GMT
Warning         : A Peptide summary report will usually give a much clearer picture of MS/MS search results.
Top Score       : 336 for gi|24234686, heat shock cognate 71 kDa protein isoform 2 [Homo sapiens]
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 61 are significant (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Protein Summary Report

Format As | Protein Summary (deprecated) | Help

Significance threshold p< 0.05 Max. number of hits AUTO

Re-Search All | Search Unmatched

Index

	Accession	Mass	Score	Description
1.	gi|24234686	53598	336	heat shock cognate 71 kDa protein isoform 2 [Homo sapiens]
2.	gi|5729877	71082	333	heat shock cognate 71 kDa protein isoform 1 [Homo sapiens]
3.	gi|578822169	71082	333	PREDICTED: heat shock cognate 71 kDa protein isoform X1 [Homo sapiens]
4.	gi|47419930	251067	48	chondroitin sulfate proteoglycan 4 precursor [Homo sapiens]

Results List

1. gi|24234686 **Mass:** 53598 **Score:** 336 **Expect:** 1.8e-029 **Matches:** 3
heat shock cognate 71 kDa protein isoform 2 [Homo sapiens]

Observed	Mr(expt)	Mr(calc)	Delta	Start	End	Miss	Ions	Peptide
1487.5712	1486.5639	1486.6940	-0.1301	37	49	0	12	R.TTPSYVAFTDTER.L
1981.8513	1980.8440	1980.9905	-0.1465	138	155	0	140	K.TVTNAVVTVPAYFNDSQR.Q
2774.1621	2773.1548	2773.3195	-0.1647	424	447	0	149	K.QTQTFTTYSDNQPGVLIQVYEGER.A

No match to: 2145.0574

2. gi|5729877 **Mass:** 71082 **Score:** 333 **Expect:** 3.6e-029 **Matches:** 3
heat shock cognate 71 kDa protein isoform 1 [Homo sapiens]

Observed	Mr(expt)	Mr(calc)	Delta	Start	End	Miss	Ions	Peptide
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1981.8513	1980.8440	1980.9905	-0.1465	138	155	0	140	K.TVTNAVVTVPAYFNDSQR.Q
2774.1621	2773.1548	2773.3195	-0.1647	424	447	0	149	K.QTQTFTTYSDNQPGVLIQVYEGER.A

No match to: 2145.0574

3. gi|578822169 **Mass:** 71082 **Score:** 333 **Expect:** 3.6e-029 **Matches:** 3

PREDICTED: heat shock cognate 71 kDa protein isoform X1 [Homo sapiens]

Observed	Mr(expt)	Mr(calc)	Delta	Start	End	Miss	Ions	Peptide
1487.5712	1486.5639	1486.6940	-0.1301	37	49	0	12	R.TTPSYVAFTDTER.L
1981.8513	1980.8440	1980.9905	-0.1465	138	155	0	140	K.TVTNAVVTVPAYFNDSQR.Q
2774.1621	2773.1548	2773.3195	-0.1647	424	447	0	149	K.QTQTFTTYSDNQPGVLIQVYEGER.A

No match to: 2145.0574

4. gi|47419930 **Mass:** 251067 **Score:** 48 **Expect:** 1.1 **Matches:** 3

chondroitin sulfate proteoglycan 4 precursor [Homo sapiens]

Observed	Mr(expt)	Mr(calc)	Delta	Start	End	Miss	Ions	Peptide
1487.5712	1486.5639	1486.7569	-0.1929	2310	2322	1	---	R.TPNPALKNGQYWV.-
1981.8513	1980.8440	1981.0091	-0.1651	1416	1431	1	24	R.TLSAFSWRMVEEQLIR.Y + Oxidation (M)
2774.1621	2773.1548	2773.4035	-0.2487	296	319	1	8	R.LEISVDQYPTHTSNRGVLSYLEPR.G

No match to: 2145.0574

Search Parameters

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Type of search         : MS/MS Ion Search
Enzyme                 : Trypsin
Fixed modifications    : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values            : Monoisotopic
Protein Mass           : Unrestricted
Peptide Mass Tolerance : ± 0.7 Da
Fragment Mass Tolerance: ± 1.2 Da
Max Missed Cleavages   : 1
Instrument type        : MALDI-TOF-TOF
Query1  (1487.5712,1+) : <no title>
Query2  (1981.8513,1+) : <no title>
Query3  (2145.0574,1+) : <no title>
Query4  (2774.1621,1+) : <no title>
```

Among these five proteins, one get up-regulated and four are down-regulated after the treatment. The identified proteins with their theoretical molecular weight (Mw), isoelectric point (pI) and fold change with respect to untreated control (ratio of band intensity of sample to that of untreated control) were summarized in Table 4.2. AIFM1 was found to be up-regulated with a fold difference of 2.315 ± 0.105. The down-regulated proteins are Annexin A2 isoform 2, SLC25A35, HSPA8 and TPI 3. Among the selected down-regulated proteins, HSPA8 is having better significant fold change (0.514 ± 0.017).

Table 4.2 Proteins identified by 2D electrophoresis with altered expression after treating with BS

	Proteins	**Molecular Weight (kDa)**	**Isoelectric point (pI)**	**Fold change (treated/control)**
Spot	**Up regulated proteins identified**			
P1	Apoptosis inducing factor, mitochondria associated 1 isoform 6	26.03	9.26	2.315 ± 0.105
Spot	**Down regulated proteins identified**			
P2	Annexin A2 isoform 2	40.41	8.53	0.787 ± 0.025
P3	Solute carrier family 25 member 35	32.43	9.21	0.929 ± 0.018
P4	Heat shock cognate 71 kDa protein isoform 2	53.52	5.61	0.514 ± 0.017
P5	Triose phosphate isomerase isoform 3	17.958	5.39	0.579 ± 0.019

AIFM1function both as NADH oxidoreductase and as regulator of apoptosis. In response to apoptotic stimuli, it is released from the mitochondrion intermembrane space into the cytosol and to the nucleus, where it functions as a proapoptotic factor in a caspase-independent pathway. In contrast, it functions as an antiapoptotic factor in normal mitochondria via its NADH oxidoreductase activity. It interacts with EIF3G, and thereby inhibits the EIF3 machinery and protein synthesis, and activates casapse-7

to amplify apoptosis. The 2D profiling in the present study showed that after BS treatment, AIFM1 isoform 6 is up-regulated. Since the treatment with BS reduces the mitochondrial membrane potential (section 4.2.6.2.5), the signal triggers the release of AIFM1 which may have played a key role in inducing apoptosis. This is in accordance with apoptosis results discussed earlier in section 4.2.62.3. Methanol extract of Gienseg has been reported to up-regulate AIFM1 protein in HepG2 human hepatocarcinoma cells (Jang et al., 2013).

Annexin A2 is a calcium-dependent, phospholipid-binding protein found on various cell types. It is up-regulated in various tumor types and plays multiple roles in regulating cellular functions, including angiogenesis, proliferation, apoptosis, cell migration, invasion and adhesion. Annexin A2 binds with plasminogen and tissue plasminogen activator on the cell surface, which leads to the conversion of plasminogen to plasmin. Plasmin is a serine protease which plays a key role in the activation of metalloproteinases and degradation of extracellular matrix components essential for metastatic progression (Lokman et al., 2011). Xiu et al. (2016) has reported the up-regulation of Annexin A2 promoting the proliferation, migration, and invasion of Caco-2 cells *in vitro*. Our study reveals that BS treatment down-regulates the expression of Annexin A2 which may be playing a role in the anticancer effect of BS. Thus by down-regulating Annexin A2 isoform 2, BS can play an important role in inhibiting proliferation, migration and invasion of colon cancer cells. Further confirmation studies are required for this.

SLC25A35 is a mitochondrial carrier protein belonging to family of SLC25 (solute carrier family 25) family of mitochondrial carrier proteins (Haitina et al., 2006).

For efficient metabolism, mitochondria depend on compartmentalization of certain enzymes, ions and metabolites. Numerous carriers are expressed, targeted and folded in the inner mitochondrial membrane in order to fulfill its activities. Among these carriers, the six-transmembrane-helix mitochondrial SLC25 proteins facilitate transport of solutes with disparate chemical identities across the inner mitochondrial membrane (Gutierrez-Aguilar and Baines, 2013). As discussed in section 4.3.1.2.5, the drop of mitochondrial membrane potential after the treatment of BS may have down regulated SLC25A35 as evident from proteomics analysis. The down-regulation of SLC25A35 will certainly affect the functioning of mitochondria and thereby the cancer cells.

HSPA8 isoform 2 are expressed under cellular stress conditions, and heat shock cognate proteins (Hsc70), which are constitutively expressed without any stress stimulation (Dworniczak and Mirault, 1987). Fan et al. (2015) verified that HSPA8 proteins were over-expressed in CRC tissue and seven colon cancer cell lines - DLD1, HCT116, SW480, SW620, LoVo, RKO, and SW1116. HSPA8 regulates cell survival (Powers et al., 2008) and confers protection from several forms of cellular stresses, such as viral infection (Yan et al., 2010) metabolic stress (Williams et al., 1993), and oxidative stress (Chong et al., 1998). Our results suggest that BS treatment down-regulates heat shock cognate 71 kDa protein. HSPA8 down-regulation will make the cancer cells incapable of withstanding oxidative and metabolic stress, hence affecting the survival of these cells. Since the BS treatment down-regulates HSPA8, HT29 cells fails to withstand the ROS induced oxidative stress (section 4.2.6.2.4) and were forced to undergo apoptosis. Further analyses are required for authentication.

Triose phosphate isomerase catalyzes the isomerization of glyceraldehyde 3-

phosphate and dihydroxy-acetone phosphate in glycolysis and gluconeogenesis. Glycolysis has been shown to be elevated in almost all cancers, the so-called "Warburg effect" (Warburg, 1956). The increased aerobic glycolysis for ATP generation in cancer cells is frequently associated with mitochondrial respiration defects and hypoxia (Altenberg and Greulich, 2004). An *in vivo* metastatic experiment using SW620 showed the expressions of α-enolase and triose phosphate isomerase to increase in the liver metastases in comparison to those in the splenic implanted lesion (Katayama et al., 2006). Glycolytic enzymes including triose phosphate isomerase were found to be up-regulated in colon cancer tissues (Bi et al., 2006). Up-regulation of glycolysis is an adaptive response of cancer cells to increase ATP production in an oxygen deprived environment. BS treatment down-regulates triose phosphate isomerase isoform 3 and there by prevents HT29 cells depending on glycolytic pathway for ATP requirement. We have already identified that the total ATP production was reduced after treating HT29 cells with BS (section 4.2.6.2.6). As ATP is the energy currency of the cell, its reduction will affect functioning of the cells.

Thus proteomic analysis of proteins from HT29 cells after exposure to BS showed that it has got significant ability to alter the expression of different proteins of HT29 cells. BS is able to upregulate apoptosis inducing factor, mitochondria associated 1 protein which is a key protein involved in apoptotic pathway. By downregulating triose phosphate isomerase protein BS will be able to inhibit the glycolitic pathway and thereby interepet the HT29 cells acquiring ATP from glycolitic pathway.

PART B

Evaluation of anticancer potential of plantain inflorescence extracts

4.4 Materials and Methods

4.4.1 Chemicals

Chemicals used have been explained in Part A under section 4.2.1.

4.4.2 Sample

PI sample preparation explained in chapter 3 under section 3.2.2.

4.4.3 Preparation of PI extracts

The extract preparation was already described in chapter 3 under section 3.2.4.

4.4.4 Cell line and culture medium

Details explained under section 4.2.4.1.

4.4.5 MTT assay for cytotoxic activity

Cytotoxicity of the PI extracts in HT29 cell lines was determined by the MTT assay as described by Mosmann (1983). The protocol is same as described in section 4.2.4.2.

4.4.6 LDH release assay for cytotoxic activity

For the LDH release cells were grown in 24-well plates at a density of 1×10^4 cells/mL and were treated with PI extracts (100, 150, 200, 250 and 300 µg/mL) and control cells (100% DMSO) grown in the same condition. After 24 h exposure the plated cells were assayed for LDH release assay. The amount of LDH released into the media after exposure to each extract plant was detected using a LDH assay kit (Cayman Chemical Company, USA). The optical density was measured at 490 nm and the results were compared with that of positive control (DMSO treated).

4.4.7 Detection of cell death by acridine orange/ethidium bromide staining

The effect of PI extract to induce cell death in HT29 cells was determined by AO/EtBr dual staining (Pitchai et al. 2014). The cells were grown on the 96 well black plate, and then treated with the different concentration of PIMET for 24 h. After incubation, 5 µL of AO (1 mg/mL) and 5 µL of EtBr (1 mg/mL) were added, and the induction of cell death was observed by using confocal microscopy (BD Pathway 855, BD Bioscience, USA).

4.4.8 Analysis of cell cycle distribution profile

The effect of extract on HT29 cell cycle distribution was assessed by flow cytometry after propidium iodide staining (Lin et al., 2007) with slight modification. 1×10^4 cells/well was treated with different concentration of PIM for 24 h. Cells in the control group received only media containing 0.1% DMSO and Taxol (50 nm) was used as positive control. Cells were harvested, washed with PBS, fixed with ice-cold 70% ethanol and kept at 4°C for 12 h. The cells were again washed with cold PBS and stained with propidium iodide (5 µg/mL) solution containing 0.1 mg/mL RNase and incubated in dark for 30 min. The cellular DNA content was analyzed by using Fluorescence Activated Cell Sorting (BD FACS Aria II, USA) and percentage of cells determined in G0/G1, S, and G2/M phases of cell cycle using BD FACSDiva™ Software v6.1.2.

4.4.9 Hoechst 33342 staining

Apoptotic activity of extract on HT29 cells were determined by Hoechst 33342 staining as described earlier in section 4.2.4.3.

4.4.10 Apoptosis assay by flow cytometry

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining was used to detect the effect of PI extracts on apoptosis in HT29 cells. The protocol was described in section 4.2.4.4.

4.4.11 Detection of mitochondrial membrane potential in HT29 cells

The effect of PI extracts on mitochondrial membrane potential was identified by staining with Rhodamine 123. The flowcytometry procedure for detecting change in membrane potential was described in section 4.2.4.6.

4.4.12 Adenisone triphosphate (ATP) production by HPLC analysis

The ATP levels in HT29 cells after treatment with extracts were determined using HPLC method (section 4.2.4.7).

4.4.13 Detection of Cytochrome C release

Cytochrome C release due to mitochondrial damage was assessed as described in section 4.2.4.8.

4.4.14 Western blot analysis

The effect of PI extracts on expression of apoptotic (pro/anti – apoptotic) proteins were studied by western blot analysis. The protocol was described in section 4.2.4.9.

4.4.15 Two dimensional electrophoresis

The change in total proteomics of HT29 cells after treating with PI extracts were analyzed by 2D electrophoresis following the procedure described in section 4.2.4.10.

4.4.16 Statistical analysis

The experimental results were expressed as mean ± SD (standard deviation) of triplicate measurements. The data were subjected to one-way analysis of variance (one-way

ANOVA) and the significance of differences between means were calculated by Duncan's multiple range test using SPSS for windows, standard version 7.5.1, SPSS (SPSS Inc., USA) and the significance accepted at $p \leq 0.05$.

4.5 Results and Discussion

4.5.1 Evaluation of anticancer potential of PI extracts

As discussed earlier in the introduction chapter biologically active molecules are associated with dietary fibre matrix, hence the extracts from PI are also analyzed for anticancer potential against HT29 colon cancer cells.

4.5.2 Cytotoxic effect of plantain inflorescence extracts

The cytotoxic effect of ethyl acetate and methanol extracts were determined by MTT assay and LDH release assay. The cellular reduction of MTT occurs in mitochondria by the enzyme succinate dehydrogenase and outside the mitochondria by the action of NADH and NADPH (Berridge and Tan, 1993). Therefore, only live cells in a population can reduce MTT. The LDH release assay is based on the fact that the cytosolic LDH leaks out of the cells with damaged membrane.

The MTT assay showed that among the extracts of PI, methanol extract showed better cytotoxic effect against HT29 colon cancer cells. The results are shown in Figure 4.19[A]. The IC_{50} values were found to be 259.84 and 130.26 µg/mL respectively for PIETH and PIMET extracts. The cytotoxic effect of extracts increases in a concentration dependent manner. From the results it is clear that PIMET is exhibiting better activity than PIETH.

The cytotoxic effects of the PI extracts were again confirmed with LDH release assay. The results are shown in Figure 4.19[B]. The results indicated that the viability of HT29 cells decrease significantly as the concentration of PI extracts increases. The

results were in accordance with that of MTT assay and PIMET exhibited better activity than PIETH. The IC_{50} values were found to be 268.62 and 195.31 µg/mL respectively for PIETH and PIMET extracts.

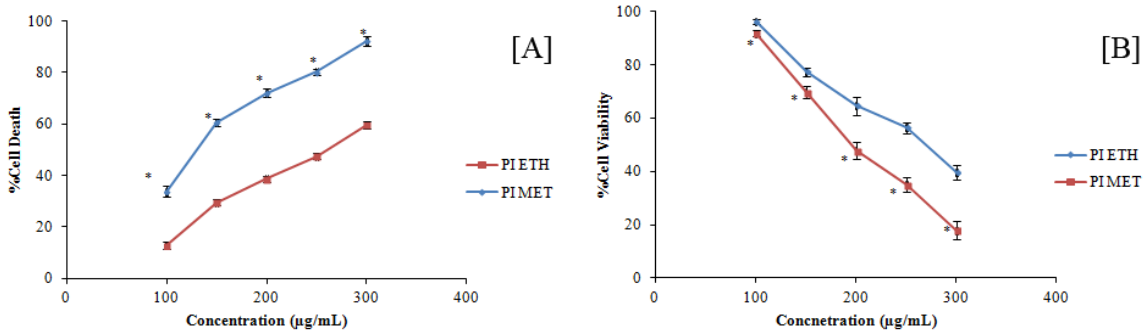


Figure 4.19. Cytotoxic effect of ethyl acetate and methanol extracts of PI against HT29 colon cancer cells. [A] Cytotoxic effect determined by MTT assay. [B] Cytotoxic effect determined by LDH release assay. Each value represents mean ± SD from triplicate measurements. $p \leq 0.05$ considered as significantly different. *Methanol extract (PIMET) significantly different from ethyl acetate extract (PIETH).

This cytotoxic activity of PI extracts may be related to the higher phenolic content of PIMET as the phenolic compounds are reported to have anticancer activity (Dai and Mumper, 2010). The polyphenols determined in PI extracts such as gallic acid, syringic acid, catechol, ferulic acid (Table 2.2) etc. are reported to have anticancer activity on various cancer cell lines (Glei and Klenow, 2009; Karthik et al., 2014; Deng and Fang, 2013; Whent et al., 2012). The extracts may be affecting the membrane integrity of HT29 cells which acts as a trigger for inducing cell death. Since from the MTT and LDH release assay it was clear that PIMET is exhibiting better activity, for further anticancer assays methanol extract was selected (PIMET 1 – 50 µg/mL and PIMET 2 – 100 µg/mL).

4.5.3 Changes in morphology of cells on treatment with methanol extract of PI using AO/EtBr double staining

AO/EtBr double staining, allow rapid and easy recognition of live and dead cells when visualized by fluorescence microscopy. AO is a crucial dye which can stain cells that have lost membrane integrity. Under fluorescent emission, AO is taken up by both the viable and non-viable cells emitting a green fluorescence, whereas EtBr is taken up only by the non-viable cells emitting red fluorescence when intercalated into the DNA strand. Hence, the live cells have a normal green nucleus whereas the early apoptotic cells have a bright green-yellow nucleus with condensed or fragmented chromatin and late apoptotic cells show condensed or fragmented orange-red chromatin.

The morphological observation of HT29 cells treated with PIMET showed various morphological changes when compared to the untreated control cells. The control cells appeared to be intact oval shape and the nucleus were stained uniformly green due to the AO dye. However, PIMET treated HT29 cells showed signs of apoptosis such as cell shrinkage, formation of apoptotic bodies and membrane blebbing (Saraste and Pulkki, 2000). The representative images are shown in Figure 4.20. The number of apoptotic cells increases as the concentration of PIMET increases. However the activity is less than the standard H_2O_2 used for the assay.

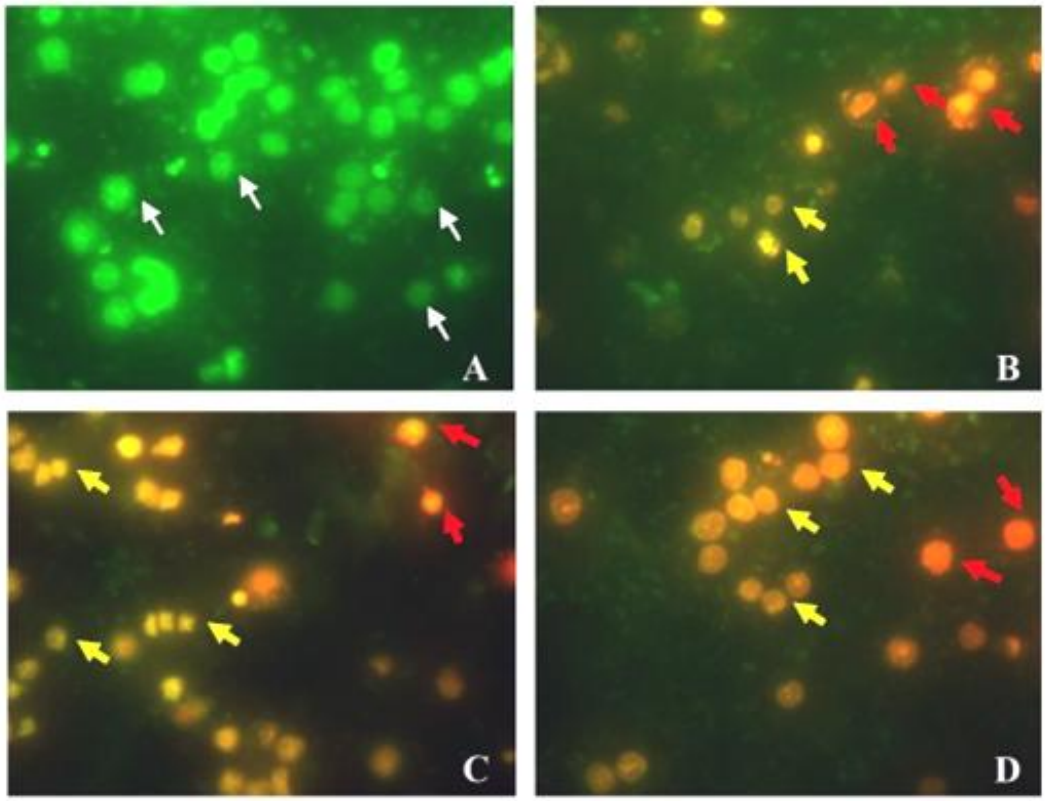


Figure 4.20. Detection by fluorescent microscopy of AO/EtBr double stained HT29 cells treated with PI methanol extracts for 24 h. (A) Untreated control cells, (B) H_2O_2 (250 µM) treated cells, (C) PIMET – 50 µg/mL and (D) PIMET – 100 µg/mL. Untreated viable cells are uniformly green (white arrow); early apoptotic cells stained greenish yellow (yellow arrow); late apoptotic cells stained yellow-orange or red colour (red arrow). Magnification 40×.

4.5.4 Effect of PI methanol extract on DNA damage

DNA damage induced by PIMET extract on HT29 cells were determined by Hoechst staining. Hoechst 33342 binds to adenine-thymine-rich regions of DNA in the minor groove. On binding to DNA, the fluorescence greatly increases which helps to identify characteristic changes. After cells treated with PIMET extracts for 24 h, marked morphological changes of cell apoptosis such as condensation of chromatin and nuclear fragmentations were found clearly using Hoechst 33342 staining. Morphological changes of cell apoptosis such as condensation of chromatin and nuclear fragmentations

were found clearly (Figure 4.21). The morphological analysis indicates that PIMET will be able to induce apoptosis in HT29 cells. Apoptotic cells gradually increased in concentration-dependent manner when treated with PIMET extract. However the activity of supernatant is less when compared to the positive control H_2O_2 treated group.

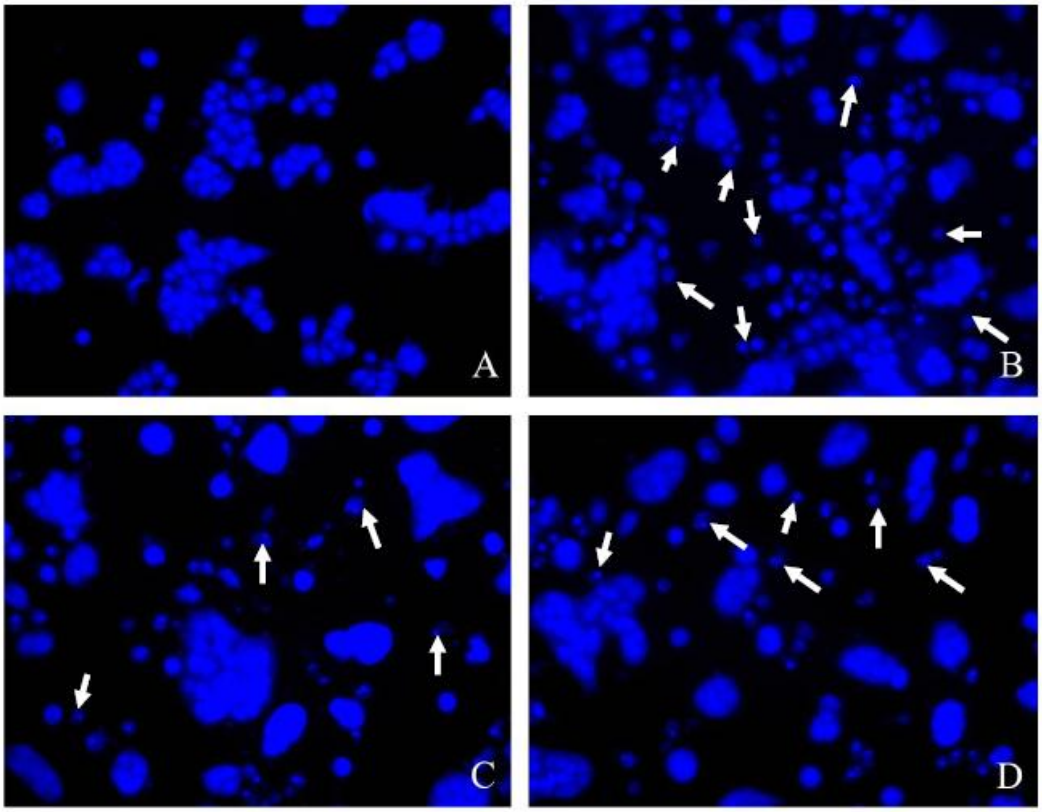


Figure 4.21. Hoechst staining for the detection of DNA damage in HT29 cells treated with PI methanol extracts for 24 h. (A) Untreated control cells, (B) H_2O_2 (250 µM) treated cells, (C) PIMET – 50 µg/mL and (D) PIMET – 100 µg/mL. The arrow indicates chromatin condensation which is a hallmark of apoptosis.

4.5.5 Effect of PI methanol extract on cell cycle

Normal cell usually transformed into a cancerous cell, when the proteins involved in regulating cell division events fails to drive progression from one cell cycle stage to the next appropriately. Cancer cells reproduce at a rate far beyond the normally tightly

regulated limits of the cell cycle (Chow, 2010). To determine the cellular mechanism of growth inhibition of PIMET in HT29 cells, we investigated cell cycle progression after PIMET treatment. As shown in Figure 4.22, the distribution of cells in all the three phases of HT29 changed significantly as PIMET concentration increases from 50 µg/mL to 100 µg/mL. It was noted that the number of G2/M phase cells increased significantly, whereas the number of G0/G1 phase cells decreased. However the activity of PIMET is less when compared to standard taxol (50 nM) used in the assay.

Polyphenols are reported to possess cancer preventive and therapeutic activity (Ross and Kasum, 2002; Chen and Dou, 2008). The cell cycle is controlled by a group of regulatory proteins named cyclins. Reports showed that cyclin B1 expression are decreased when G2/M phase arrest occurs which prevents cells entering to mitosis (Lindqvist et al., 2009). Previous studies have confirmed that grape seed, Longon seed, or Longon flower extract increases S phase cells in the CRC (Hsu et al., 2010; Chung et al., 2010; Chung et al., 2012). In the present study, HT29 cells exhibited significant increases in the number of G2/M phase cells following treatment with PIMET, which differed from previous reports. G2/M phase cells increases from 26.2 ± 1.2% (untreated control) to 53.2 ± 1.6% (PIMET 100 µg/mL). These findings suggested that the anti-proliferative effect induced by polyphenols from naturally occurring products could occur through a different cell cycle controlling mechanism. The different composition of the polyphenols in each natural product might induce different expressions of cyclin proteins to control the cell cycle in CRC cells. The molecular mechanism responsible for the perturbation of the M to G1 phase of the cell cycle in HT29 cells needs further investigation.

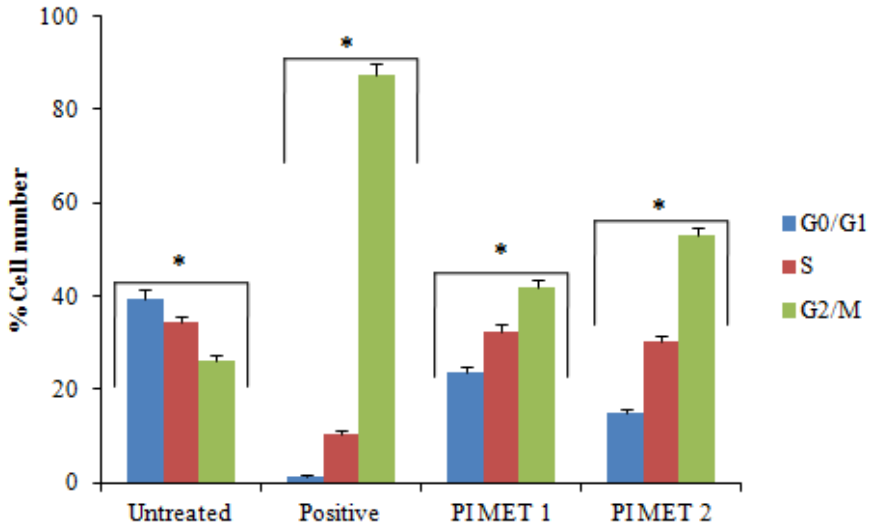


Figure 4.22. Cell cycle analysis of HT29 cells treated with PIMET extract. Propidium iodide stained cells were analyzed using a flow cytometre and the distribution of cells in each phase of cell cycle was determined. Data are expressed as percentage of total cell number, represent the average of three independent experiments and are expressed as mean±SD. $p \leq 0.05$ considered as significantly different. *Each phase of different groups is significantly different from that of untreated group.

4.5.6 Effect of PI methanol extract on apoptosis

The death of aged cells in our body is programmed by a sequence of events known as apoptosis. Cancer research has developed much better in exploring in the role of oncogenic mutation which rattles the apoptotic pathway that advances to tumor initiation, progression or metastasis. Polyphenols are known to overcome this oncogenic mutation and prompt apoptosis in cancer cells (Chen and Kong 2005). Hence the effect of PIMET extract on apoptosis was investigated using the Annexin V-FITC and PI combination staining method.

The flow cytometry results (Figure 4.23[I]) depicted that PIMET extracts can induce apoptosis in HT29 colon cancer cells. About, 23.7 ± 1.54% of cells were in the early stage of apoptosis after treating with 50 µg/mL of PIMET which was increased to

27.8 ± 0.78% when treated with 100 µg/mL of PIMET (Figure 4.23[II]). Further studies are required to identify specific compounds from PI extracts which can be used as a treatment strategy to induce apoptosis in colon cancer cells.

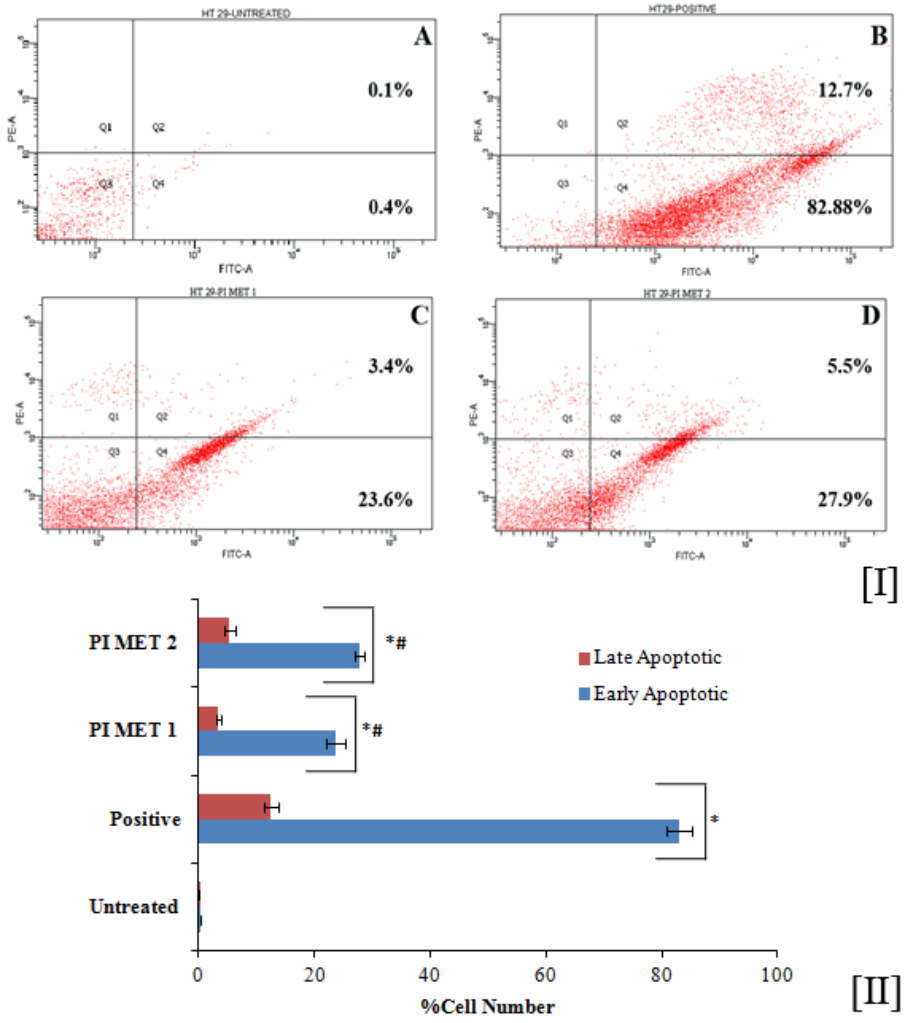


Figure 4.23. Apoptotic effect of PI methanol extract on HT29 cells. [I] Representative image of induction of apoptosis by PI Methanol extract. (A) Untreated control, (B) Camptothecin (50 µM), (C) PIMET – 50 µg/mL, (D) PIMET – 100 µg/mL. Four quadrants (Q1, Q2, Q3 and Q4) represent dead cells, late apoptotic cells, live cells and early apoptotic cells respectively. [II] Graphical representation of apoptotic effect of PI Methanol extract on HT29 cells. Each value represents mean ± SD from triplicate measurements. *Significantly different from untreated goup. #Significantly different from positive group. $p \leq 0.05$ considered as significantly different.

4.5.7 Effect of PI methanol extract on mitochondrial membrane potential

Mitochondria are vital for life, mainly due to their key role in processes such as ATP generation. In the mid-1990s, evidence emerged implicating a central role for mitochondria in apoptotic cell death (Lopez and Tait, 2015). Mitochondrial membrane potential plays an important role in inducing apoptosis. This membrane potential is relevant, since decrease in mitochondrial membrane triggers the release of Cytochrome C and other pro-apoptotic proteins. Another important factor is that cancer cells exhibits higher mitochondrial membrane potential (Chen and Rivers, 1990; Houston et al., 2011; He et al., 2015), which in turn helps them to inhibit apoptosis. General elevations in the mitochondrial membrane potential have been linked to colonic carcinoma cells (Heerdt et al., 2005). Hence we analyzed the effect of PIMET extract on mitochondrial membrane potential.

Mitochondrial membrane potential of HT29 colon cells were determined by rhodamine 123 dye. Rhodamine 123 selectively enters mitochondria with an intact membrane potential and is retained in the mitochondria. Once the membrane potential is lost, the dye is washed out of the mitochondria. The histograms obtained from flow cytometric analysis are shown in Figure 4.24[I]. The results showed that the mitochondrial membrane potential of HT29 colon cancer cells was decreased when treated with PI methanol extract. However the activity of PIMET is less when compared to the positive control H_2O_2 used in this assay. 82.7 ± 2.551% cells lose their mitochondrial membrane potential when treated with H_2O_2 (250 µM) where as 33.73 ± 2.254 and 64.13 ± 1.75% of cells lose mitochondrial membrane potential when treated with 50 and 100 µg/mL of PIMET extract respectively (Figure 4.24[II]). The results are

promising as the loss of mitochondrial membrane potential in cancer cells affects the production of ATP and may facilitate the initial steps of apoptosis e.g., release of Cytochrome C.

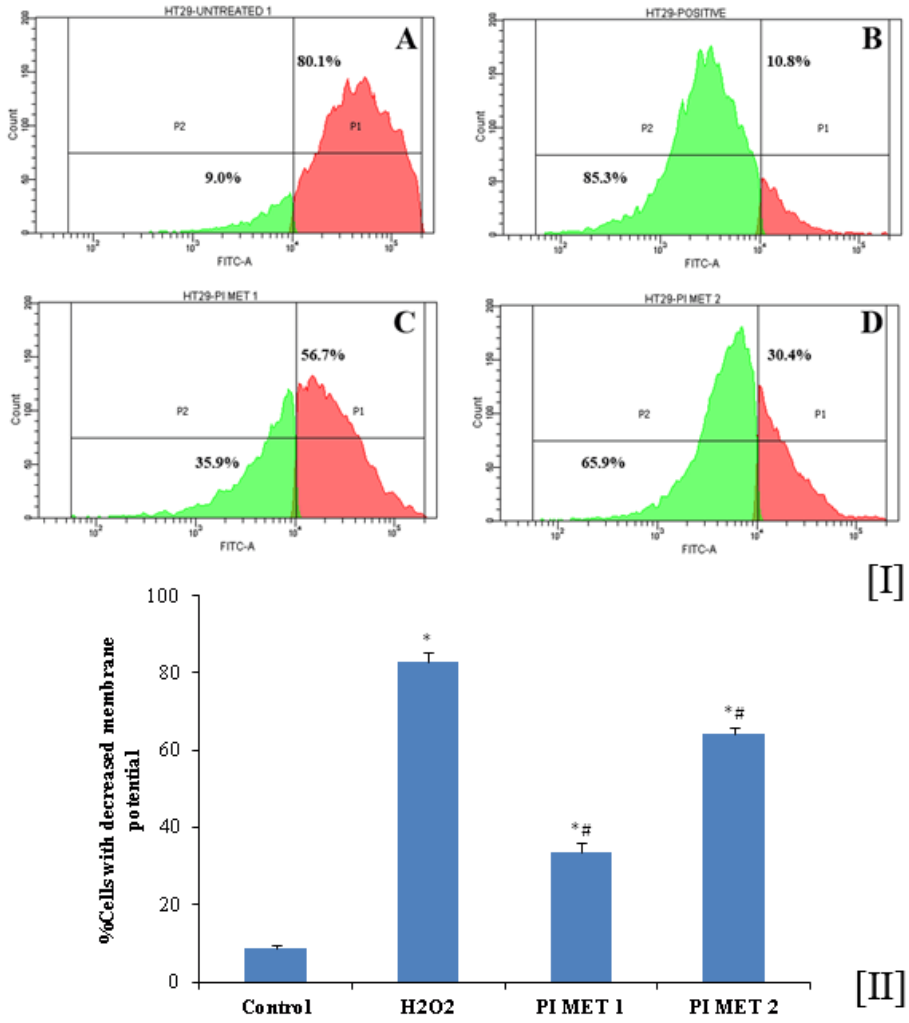


Figure 4.24. Reduction in mitochondrial membrane by PIMET extract in HT29 colon cancer cells. [I] Representative image for the effect of PIMET on mitochondrial membrane potential. (A) Untreated control, (B) H_2O_2 (250 µM), (C) PIMET – 50 µg/mL, (D) PIMET – 100 µg/mL. [II] Graphical representation of reduction in mitochondrial membrane by PIMET extract in HT29 colon cancer cells. Each value represents mean ± SD from triplicate measurements. $p \leq 0.05$ considered as significantly different. *Significantly different from control group. $^{\#}$Significantly different from H_2O_2. $p \leq 0.05$ considered as significantly different.

4.5.8 Effect of PI methanol extract on ATP production

During late-stage apoptosis, ATP levels sharply drop, mostly because of the loss of mitochondrial function and consumption by ATP-dependent proteases. Thus ATP production plays an important role in the survival of cancer cells (Zhou et al., 2012). Cancer cells are reported to rely mostly on glycolytic pathway rather than oxidative phosphorylation for ATP production (Dang et. al., 2011; Zhao et al., 2013) Cancer cells exhibit this altered metabolism to meet energy need during tumor progression; which is known as the Warburg effect (Jang et al., 2013). Hence cancer research has been focused on targeting ATP production.

The effect of PIMET extract on ATP production in HT29 cells were analyzed by HPLC at 259 nm. The representative chromatogram was shown in Figure 4.25[I]. The retention time was found to be 3.808, 4.858 and 7.119 min respectively for ATP, ADP and AMP. The results clearly indicate that after the treatment of HT29 colon cancer cells with PIMET extract, the ATP production was reduced. The ATP content in control group was found to be 0.931 ± 0.024 µg/mL, which was reduced to 0.465 ± 0.017 µg/mL, 0.69 ± 0.029 µg/mL and 0.543 ± 0.03 µg/mL respectively for cells treated with H_2O_2, PIMET-50 and PIMET-100 µg/mL (Figure 4.25[II]). Thus these results confers that the decreased mitochondrial potential as well has affected the production of ATP in cancer cells after treating with PIMET extract. Cancer cells have an adaptive response to increase ATP production by enhancing the glycolytic pathway (Cairns et al., 2011). The extract may also have affected enzymes of glycolytic pathway which consecutively reduce the ATP production. Further studies are required to confirm this.

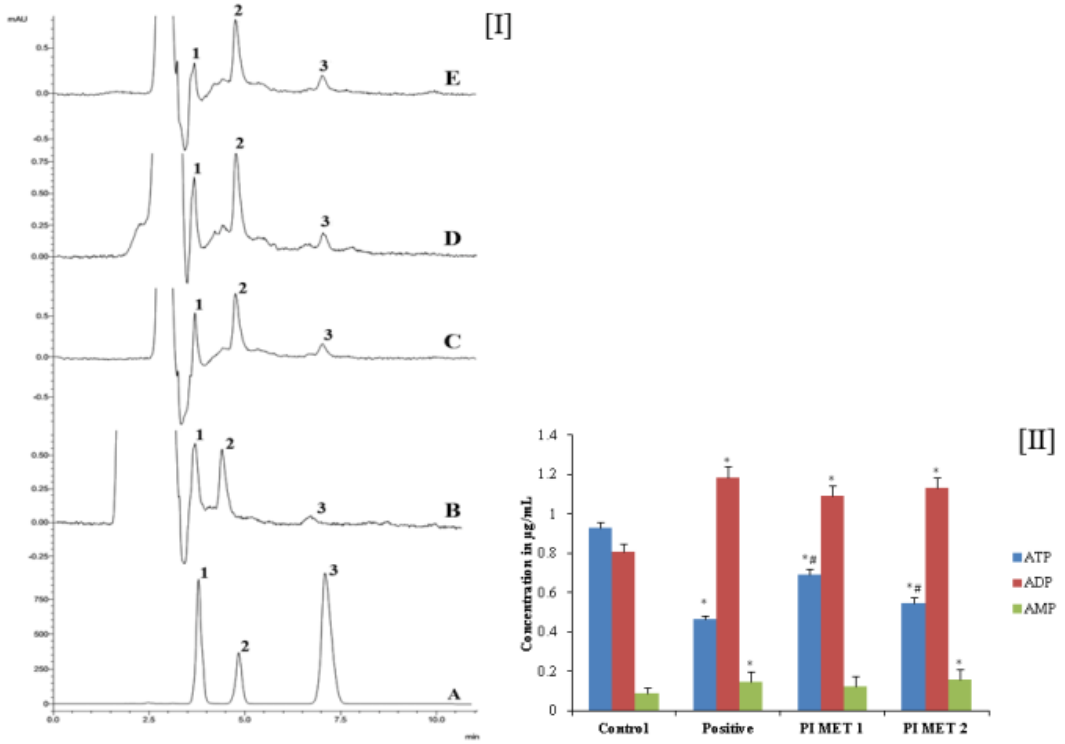


Figure 4.25. Reduction in ATP production by PIMET extract in HT29 colon cancer cells. [I] Representative HPLC chromatogram showing the effect of PIMET extract on ATP production in HT29 cells. (1) ATP, (2) ADP and (3) AMP. (A) Standard ATP, ADP and AMP at 1 mg/mL concentration, (B) Untreated cells, (C) Positive control- 250 µM H_2O_2 treated, (D) PIMET – 50 µg/mL treated and (E) PIMET – 100 µg/mL treated. [II] Graphical representation of reduction in ATP production by PIMET extract in HT29 colon cancer cells. Each value represents mean ± SD from triplicate measurements. $p \leq 0.05$ considered as significantly different. *Significantly different from control group. $^{\#}$Significantly different from positive group. $p \leq 0.05$ considered as significantly different.

4.5.9 Effect of PI methanol extract on Cytochrome C release

Many pro-apoptotic signals trigger mitochondrial Cytochrome C release, leading to Caspase activation and ultimate cellular breakdown. Mitochondrial Cytochrome C release and Caspase activation are often impaired in tumors with BCL2 over expression or BAX defective status (Seervi et al., 2011).

Hence we analyzed the PIMET extract on Cytochrome C release from mitochondria. The Cytochrome C was analyzed by HPLC method at 393 nm. The retention time was found to be 4.492 min. The representative chromatogram was shown in Figure 4.26[I]. From the results it was obvious that the release of Cytochrome C was increased after the treatment of PIMET. The Cytochrome C release was increased from 0.761 ± 0.016 µg/mL (Control) to 1.461 ± 0.031 and 1.734 ± 0.037 µg/mL respectively for 50 and 100 µg/mL of PIMET (Figure 4.26[II]). The activity of PIMET was comparable that of the positive control H_2O_2 (1.815 ± 0.039 µg/mL) used in the assay.

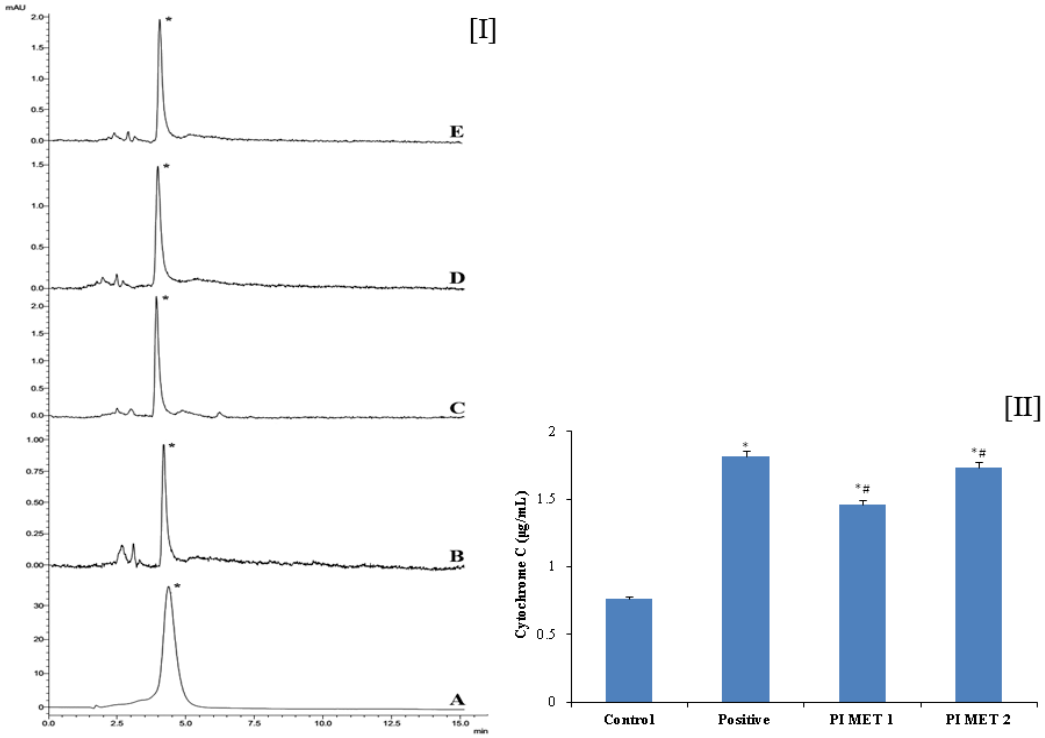


Figure 4.26. Cytochrome C release by PIMET extract in HT29 colon cancer cells. [I] Representative HPLC chromatogram showing the effect of PIMET extract on Cytochrome C release in HT29 cells. (*)Cytochrome C (A) Cytochrome C standard, (B) Untreated cells, (C) Positive control- 250 µM H_2O_2 treated, (D) PIMET – 50 µg/mL treated (E) PIMET – 100 µg/mL treated. [II] Graphical representation of Cytochrome C release by PIMET extract in HT29 colon cancer cells. Each value represents mean ± SD from triplicate measurements. $p \leq 0.05$ considered as significantly different.

*Significantly different from control group. #Significantly different from positive group. $p \leq 0.05$ considered as significantly different.

4.5.10 Western blot analysis

The effect of PI methanol extract on the expression of various proteins involved in apoptosis was analyzed by western blot analysis. Anti apoptotic protein BCL2, pro apoptotic protein BAX, Caspase 3, c-Caspase 3, PARP and c-PARP were analyzed. The results are shown in Figure 4.27. The PIMET was able to reduce the expression of anti apoptotic protein BCL2 and increase the expression of pro apoptotic protein BAX in a dose dependent manner (Figure 4.27 A and B). The results indicate that the PIMET extract induce activation of cleavage of Caspase 3 and PARP (Figure 4.27 C and D). The change in expression of above mentioned proteins may be the reason for apoptotic effect of methanol extract of PI. The activity of PIMET is almost comparable to that of 5-fluorouracil (50 µM) in case of the activation of c-Caspase 3 and c-PARP.

The relationship between natural polyphenols, apoptosis and cancer was evaluated by different studies and the capacity of these compounds to perform as cancer chemopreventive and/or chemotherapeutic agents were also investigated (Kelloff et al., 1994; Stoner and Mukhtar, 1995; Jang et al., 1997). Polyphenols, in addition to their antioxidant activity, have been reported to exhibit many potential anticancer mechanisms including regulation of the expression of cell survival and proliferation genes (Kang et al., 2011), induce different mechanisms associated with cell death (Giovannini and Masella, 2012), or inhibit matrix metalloproteinases (Katiyar, 2006) and vascular endothelial growth factor (Oak et al., 2005), thus counteracting angiogenesis and affecting to metastasis development (Rosa et al., 2016). Gallic acid has been reported to induce apoptosis in HCT15 and HT29 colon cancer cells

(Subramanian et al., 2016; Tan et al., 2015). Jaganathan et al. (2013) has reported that p-coumaric acid is able to induce apoptosis in HCT15 colon cancer cells. All these results suggests that the ability of PIMET extract to induce activation and deactivation of pro and anti apoptotic proteins can be attributed to the polyphenols present in the extract.

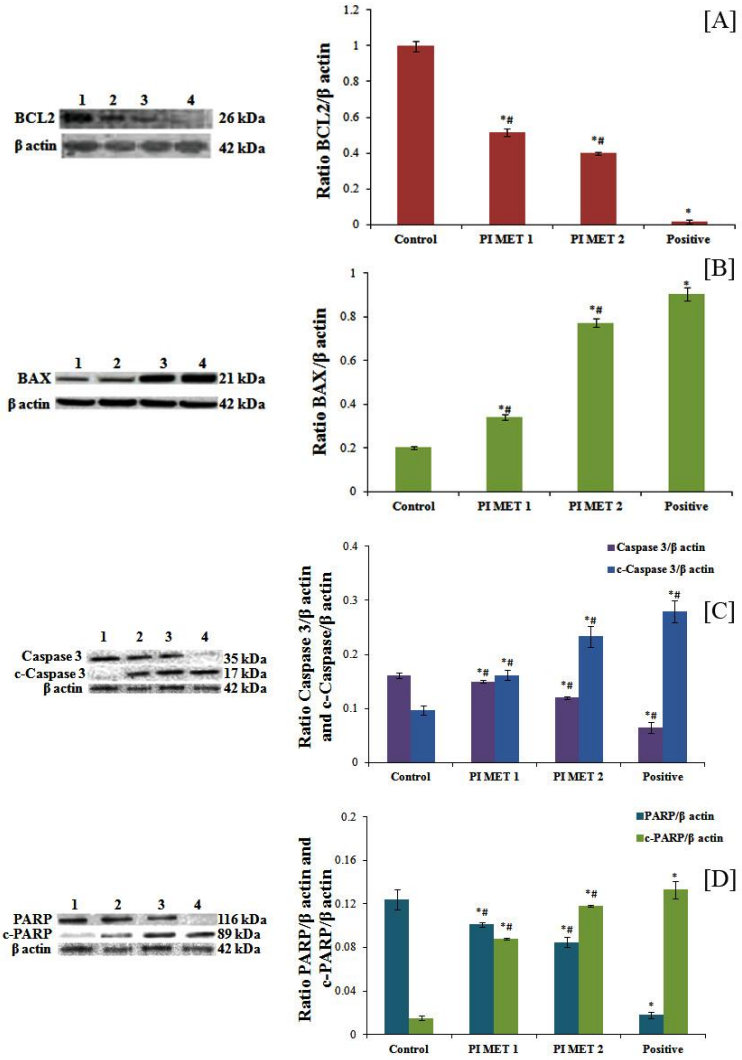


Figure 4.27. Effect of PIMET extract on expression of various proteins involved in apoptosis in HT29 colon cancer cells. The results are compared with that of protein obtained from untreated cells (first lane) as well as cells treated with 5-Fluorouracil (50 µM) (fourth lane). Protein from cells treated with PIMET 50 and 100 µg/mL were loaded in third and fourth lane. The ratio of intensity of various protein bands against

intensity of β actin bands were plotted in the graphs. (A) Expression of BCL2, (B) Expression of BAX, (C) Expression of Caspase 3 and c-Caspase 3 (D) Expression of PARP and c-PARP are shown respectively in A, B, C and D. Each value represents mean ± SD from triplicate measurements. $p \leq 0.05$ considered as significantly different. *Significantly different from control group. #Significantly different from positive group.

4.5.11 Changes in the protein expression profile of HT-29 human colon cancer cells after treatment with methanol extract of PI

Above studies confirmed that the methanol extract of PI exhibits anticancer property by its ability to induce cell cycle arrest and apoptosis in HT29 colon cancer cells. For better understanding of the implementation of this anticancer potential, it is important to know the mechanisms of action as well as the cellular response towards the extract. Hence proteomic profiling, using 2D-PAGE and peptide mass fingerprinting for identification of differentially expressed proteins, was performed after treating HT-29 cells with PIMET extract.

Total proteins isolated from HT29 cells after treatment were separated by 2D gel electrophoresis using nonlinear IPG strips (3-10) and 12% gels. After Coomassie staining, the gel patterns were scanned and computer-assisted image analysis was performed by means of the PDQuest 8.0 software (Bio-Rad Laboratories GmbH, Munich, Germany) to compare the stained protein spots from treated and untreated samples. The two dimensional map of protein expression of HT29 cells after the treatment with PIMET, were shown in Figure 4.28. Eight differentially expressed proteins were selected (P1-P8, denoted with arrows), based on the changes in expression profile on 2D gel after checking the reproducibility of the expression profile (Reproducibility of the results were confirmed by performing the experiments thrice).

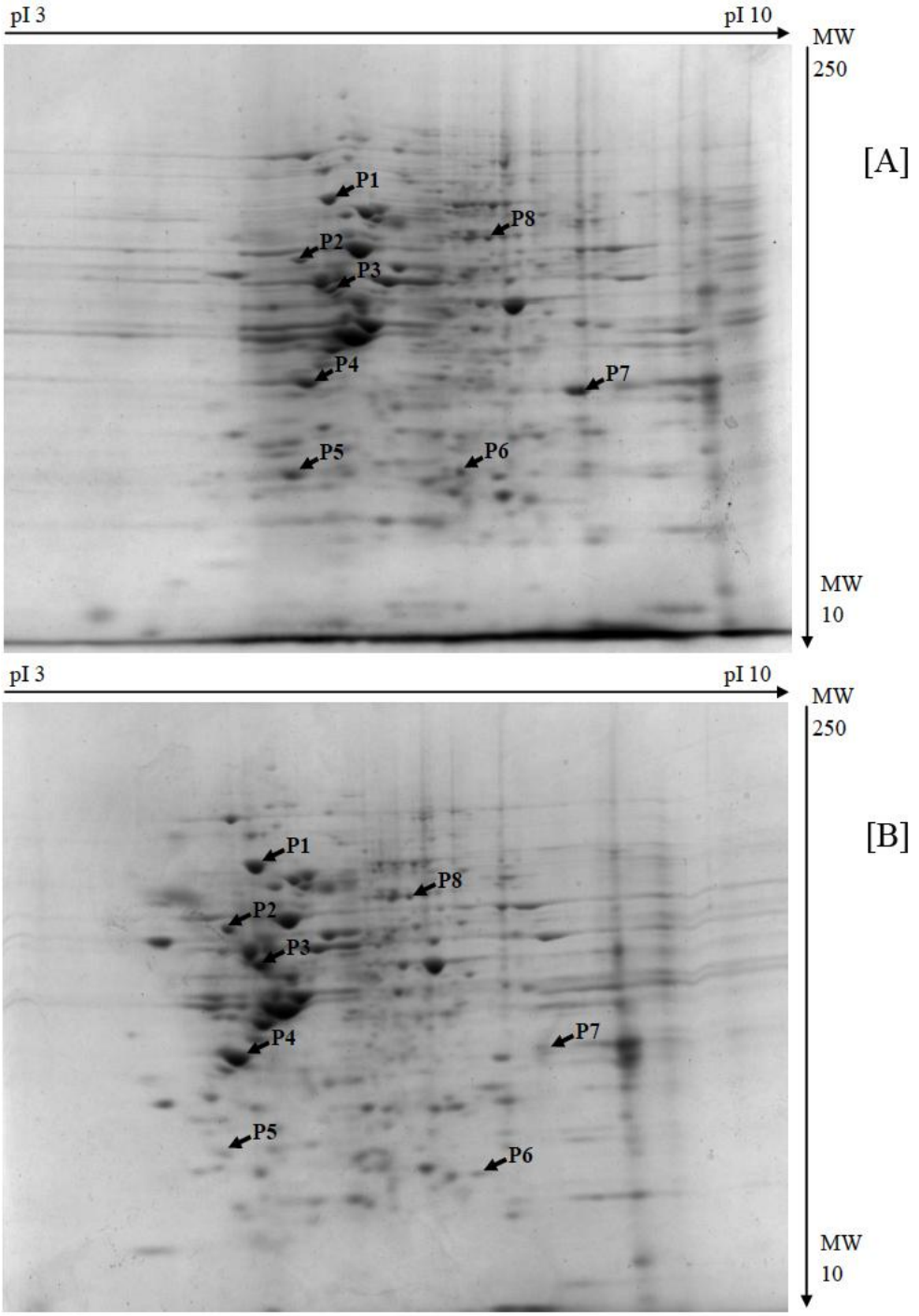


Figure 4.28. Two-dimensional map of protein expression profile of HT-29 human colon cancer cells. (A) Untreated and (B) PIMET treated. Arrows and spot numbers indicate the proteins identified. Proteins were separated based on isoelectric point (pI) on a linear pH 3–10 IPG-strip in the first dimension and on molecular weight (Mw inkDa) on a 12% SDS-polyacrylamide gel in the second dimension.

Gels with stained protein spots were scanned and computer-assisted image analysis was performed by means of the PDQuest 8.0 software (Bio-Rad Laboratories GmbH, Munich, Germany). Selected differentially expressed proteins were excised manually from the gels and subjected to peptide mass fingerprinting for identification of proteins. The two dimensional map of protein expression of HT29 cells after the treatment with PIMET were shown in Figure 4.28. Eight proteins were identified in this experiment.

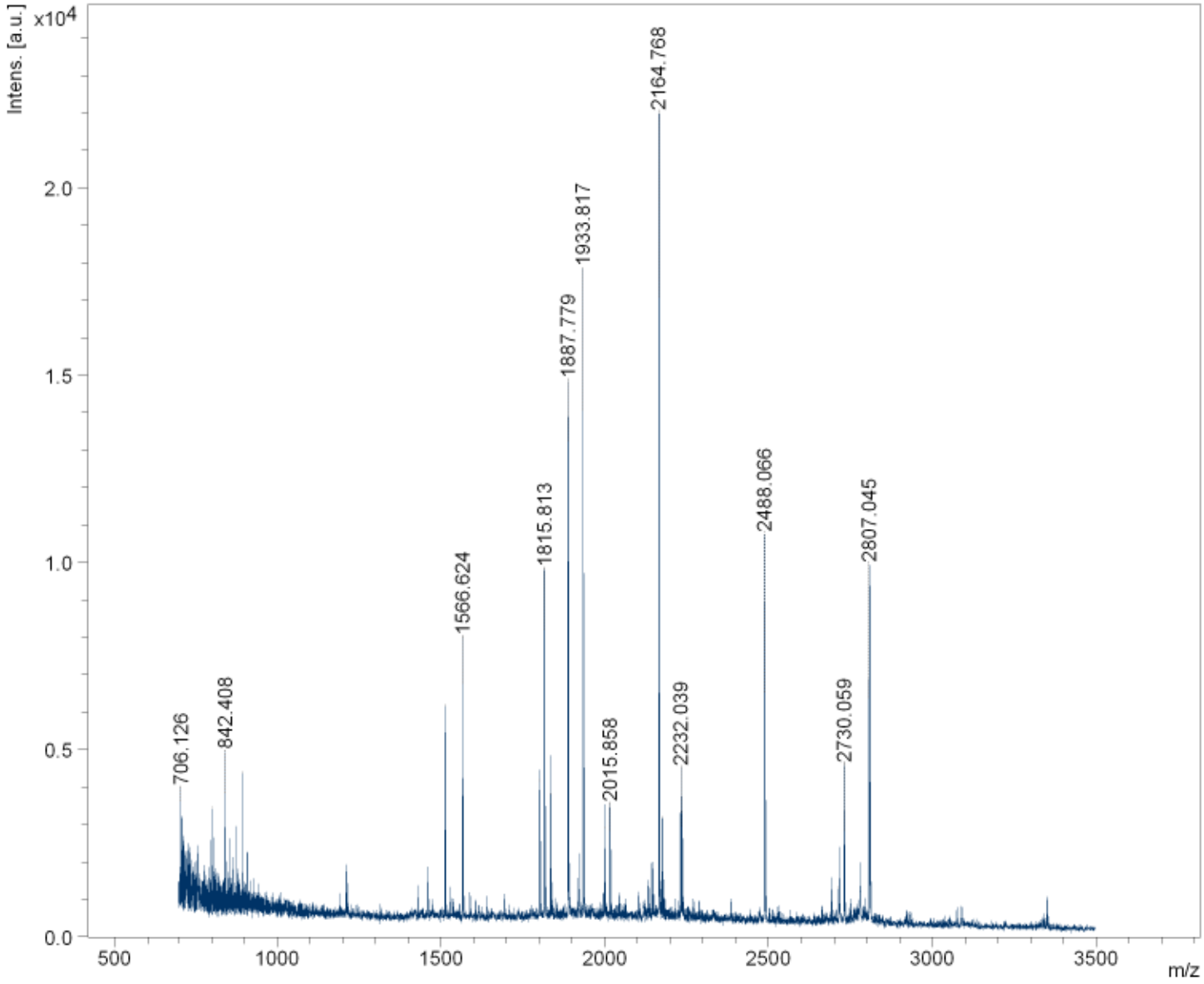
Eight proteins excised manually from the gels and were subjected to peptide mass fingerprinting (PMF) for identification of proteins. For this, selected proteins were subjected to in-gel tryptic digestion, followed by MALDI TOF MS/MS of the tryptic peptide mixtures and database search. Based on the data, the selected protein spots were identified as 78 kDa glucose-regulated protein precursor, calreticulin precursor, COP9 signalosome complex subunit 4, pyruvate dehydrogenase (lipoamide) beta, Polymerase (RNA) II subunit C, peroxiredoxin 6, solute carrier family 25 member 35 and X-linked inhibitor of apoptosis The PMF data and MASCOT search result of a representative protein - 78 kDa glucose-regulated protein precursor is shown (Plate 3 and Plate 4) for reference.

PLATE 3

D:\Data\nisst\pnisha\SAMPLE H 1\0_K1\1\1SRef

Comment 1

Comment 2



Acquisition Parameter

Date of acquisition	2015-12-03T12:37:34.125+05:30
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Aquisition operation mode	Reflector
Voltage polarity	POS
Number of shots	500
Name of spectrum used for calibration	
Calibration reference list used	

Instrument Info

D:\Data\nisst\pnisha\SAMPLE H 1\0_K1\1\1SRef

User	BDALDE
Instrument	FLEX-PC
Instrument type	ultraflexTOF/TOF

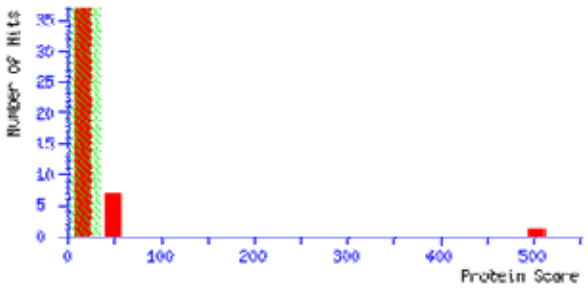
Mascot Search Results

PLATE 4

```
User            : SARAVANAKUMAR
Email           : msaravana@rgcb.res.in
Search title    : NCBI HUMAN
MS data file    : DATA.TXT
Database        : NCBI_Homosapiens HumanDB_NCBI (71338 sequences; 45882706 residues)
Timestamp       : 4 Dec 2015 at 18:22:31 GMT
Protein hits    : gi|16507237   78 kDa glucose-regulated protein precursor [Homo sapiens]
                  gi|27886653   potassium voltage-gated channel subfamily H member 7 isoform 1 [Homo sapiens]
                  gi|103472005  antigen KI-67 isoform 1 [Homo sapiens]
                  gi|190341093  fibroblast growth factor-binding protein 3 precursor [Homo sapiens]
                  gi|55770860   T-box transcription factor TBX15 [Homo sapiens]
```

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 34 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Peptide Summary Report

Format As: Peptide Summary — Help

Significance threshold p< 0.05 — Max. number of hits AUTO

Standard scoring ◉ MudPIT scoring ○ — Ions score or expect cut-off 0 — Show sub-sets 0

Show pop-ups ◉ Suppress pop-ups ○ — Sort unassigned: Decreasing Score — Require bold red ☐

Select All | Select None | Search Selected | ☐ Error tolerant | Archive Report

1. gi|16507237 **Mass:** 72402 **Score:** 503 **Matches:** 4(4) **Sequences:** 4(4)
78 kDa glucose-regulated protein precursor [Homo sapiens]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
☑ 1	1512.5929	1511.5856	1511.7442	-0.1586	1	91	1.4e-007	1	U	R.AKFEELNMDLFR.S
☑ 2	1815.8156	1814.8083	1814.9890	-0.1807	1	109	1.6e-009	1	U	R.IINEPTAAAIAYGLDKR.E
☑ 3	1933.8212	1932.8139	1933.0058	-0.1918	0	158	2.1e-014	1	U	K.DNHLLGTFDLTGIPPAPR.G
☑ 4	2164.7737	2163.7664	2163.9848	-0.2184	0	145	6.3e-013	1	U	R.IEIESFYEGEDFSETLTR.A

---

2. gi|27886653 **Mass:** 136226 **Score:** 41 **Matches:** 1(1) **Sequences:** 1(1)
potassium voltage-gated channel subfamily H member 7 isoform 1 [Homo sapiens]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
1	1512.5929	1511.5856	1511.6852	-0.0996	1	41	0.016	2	U	K.RYNDSDSSSGPSIK.D

Proteins matching the same set of peptides:
gi|27886665 **Mass:** 83707 **Score:** 41 **Matches:** 1(1) **Sequences:** 1(1)
potassium voltage-gated channel subfamily H member 7 isoform 2 [Homo sapiens]

---

3. gi|103472005 **Mass:** 360698 **Score:** 40 **Matches:** 1(1) **Sequences:** 1(1)
antigen KI-67 isoform 1 [Homo sapiens]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide

3	1933.8212	1932.8139	1932.8371	-0.0232	1	40	0.014	2	U	K.DPAAGHTEESMTDDKTTK.I

Proteins matching the same set of peptides:
gi|225543215 **Mass:** 321301 **Score:** 40 **Matches:** 1(1) **Sequences:** 1(1)
antigen KI-67 isoform 2 [Homo sapiens]
gi|578819493 **Mass:** 266350 **Score:** 40 **Matches:** 1(1) **Sequences:** 1(1)
PREDICTED: antigen KI-67 isoform X1 [Homo sapiens]

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4. gi|190341093 **Mass:** 28086 **Score:** 38 **Matches:** 1(1) **Sequences:** 1(1)
fibroblast growth factor-binding protein 3 precursor [Homo sapiens]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
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---

5. gi|55770860 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
T-box transcription factor TBX15 [Homo sapiens]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
1	1512.5929	1511.5856	1511.7191	-0.1335	0	35	0.06	3	U	K.WMVAGNADSPVPPR.V + Oxidation (M)

Proteins matching the same set of peptides:
gi|122937456 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
gi|530363242 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
gi|530363244 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
gi|578813065 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)

Peptide matches not assigned to protein hits: (no details means no match)

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
☑ 5	2488.0706	2487.0633	2487.1807	-0.1174	1	26	0.28	1		NKMTAYITELSDMVPTCSALAR + Oxidation (M)

Search Parameters

```
Type of search         : MS/MS Ion Search
Enzyme                 : Trypsin
Fixed modifications    : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values            : Monoisotopic
Protein Mass           : Unrestricted
Peptide Mass Tolerance : ± 0.7 Da
Fragment Mass Tolerance: ± 1.2 Da
Max Missed Cleavages   : 1
Instrument type        : MALDI-TOF-TOF
Number of queries      : 5
```

Among these eight proteins, four are up regulated and another four are down regulated after the treatment. The identified proteins with their theoretical molecular weight (Mw), isoelectric point (pI) and fold change with respect to untreated control (ratio of band intensity of sample to that of untreated control) were summarized in Table 4.3. 78 kDa glucose-regulated protein precursor, calreticulin precursor, COP9 signalosome complex subunit 4 and pyruvate dehydrogenase (lipoamide) beta are the up-regulated proteins. Among the selected up-regulated proteins, COP9 signalosome complex subunit 4 is having highest fold difference (2.832 ± 0.141). The down regulated proteins are polymerase (RNA) II subunit C, peroxiredoxin 6, Solute carrier family 25 member 35 and X-linked inhibitor of apoptosis. Among the selected down-regulated proteins, Solute carrier family 25 member 35 is having significant fold change (0.534 ± 0.125).

Table 4.3 Proteins identified by 2D electrophoresis with altered expression

	Proteins	**Molecular Weight (kDa)**	**Isoelectric point (pI)**	**Fold change (treated/control)**
Spot	**Up regulated proteins identified**			
P1	78 kDa glucose-regulated protein precursor	72.33	5.07	2.417 ± 0.204
P2	Calreticulin precursor	48.14	4.29	1.346 ± 0.105
P3	COP9 signalosome complex subunit 4	46.26	5.57	2.832 ± 0.141
P4	Pyruvate dehydrogenase (lipoamide) beta	37.19	5.64	2.723 ± 0.108
Spot	**Down regulated proteins identified**			
P5	Polymerase (RNA) II subunit C	31.44	4.79	0.831 ± 0.031
P6	Peroxiredoxin 6	25.03	6.00	0.849 ± 0.019
P7	Solute carrier family 25 member 35	32.43	9.21	0.534 ± 0.125
P8	X-linked inhibitor of apoptosis	56.68	6.22	0.965 ± 0.067

Glucose-regulated protein 78 (GRP78) is a key chaperone and stress response protein (Munro and Pelham, 1986). GRP78 resides primarily in the endoplasmic reticulum and plays a role in facilitating the assembly of multimeric protein complexes inside the endoplasmic reticulum. Increased expression of the ER stress protein GRP78 has been found in several types of cancer, including breast cancer, gastric cancer, and colon cancer (Fu et al., 2007; Zheng et al., 2008; Xing et al., 2006). However, the role of GRP78 expression in the prognosis of colon cancer is controversial. GRP78 silencing has been found to enhance apoptosis in colon cancer cells and suppressed colon cancer growth through the down-regulation of the VEGF/VEGFR2 signaling pathway (Chang et al., 2012; Kuo et al., 2013). Although over expression of GRP78 was noted in colon cancers, its expression level was negatively correlated with lymphatic invasion (Takahashi et al., 2011). In another study a low level of GRP78 was found to increase in metastasis ability in colon cancer cells by altering E-cadherin and vimentin expression and activating the NRF-2/HO-1 signaling pathway (Chang et al., 2015). From our results we had found that 78 kDa glucose-regulated protein precursors are increasing after treatment with PIMET. By the up-regulation of GRP78 after PIMET treatment we are unable to reach in a conclusion whether the PIMET is enhancing the formation of GRP78 or it is inhibiting the conversion of GRP78 precursors to the active molecule. Further studies are required to confirm this.

Calreticulin is a calcium-binding chaperone that promotes folding, oligomeric assembly and quality control in the endoplasmic reticulum (ER) via the calreticulin/calnexin cycle. It is also found in nucleus and can act as an important modulator of the regulation of gene transcription by nuclear hormone receptors.

Calreticulin in human colon adenocarcinomas to that of normal epithelium by proteomic analysis on whole tissue samples containing both neoplastic and non-neoplastic cells, show divergent results i.e. an up-regulation (Cole et al., 2000) or a down-regulation (Brunagel et al., 2003) of Calreticulin in human colon adenocarcinomas. Toquet et al. (2007) has reported that Calreticulin expression was down-regulated in 51.7% human colon adenocarcinomas. Our results showed that Calreticulin was up regulated after the treatment.

COP9 signalosome complex (CSN), a complex involved in various cellular and developmental processes. CSN is a potential player in tumorigenesis and its subunits are often overexpressed in tumors (Sui et al., 2001; Kouvaraki et al., 2003; Hsu et al., 2008). Recently, a reduced expression of different CSN subunits in HT29 and HeLa cells and, as a potential consequence, led to increased levels of the CDK inhibitor p27 and of the tumor suppressor p53 in HT29 cells has been reported (Feist et al., 2014; Leppert et al., 2011). Our results agreed with later results as the COP9 signalosome complex subunit 4 was found to up-regulated after the treatment with PIMET. The relevance of this up-regulation is yet to study further.

The pyruvate dehydrogenase (PDH) complex is a nuclear-encoded mitochondrial multienzyme complex that catalyzes the overall conversion of pyruvate to acetyl-CoA and carbon dioxide, and provides the primary link between glycolysis and the tricarboxylic acid (TCA) cycle. The PDH complex is composed of multiple copies of three enzymatic components: pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2) and lipoamide dehydrogenase (E3). The E1 enzyme is a heterotetramer of two alpha and two beta subunits. From the proteomics data we found

that the Pyruvate dehydrogenase (lipoamide) beta subunit is upregulating after PIMET treatment. The relevance of up-regulation is unclear; no such studies are reported so far.

Polymerase (RNA) II subunit C (POLR2C) is the subunit of RNA polymersase II which is involved in the synthesis of mRNA. The protein was found to be down-regulated after PIMET treatment. By down-regulating POLR2C, PIMET extract may be inhibiting the transcription process in cancer cells and there by affecting the functioning of cancer cells.

Peroxiredoxin 6 (PRDX6) is a member of peroxidases, and has glutathione peroxidase and calcium-independent phospholipase A2 (iPLA2) activities (Jo et al., 2014). As peroxiredoxins are antioxidants, they support survival and tumor maintenance by protecting cells from oxidative stress-induced apoptosis (Neumann and Fang, 2007). In a recent study, over expression of PRDX 6 attenuates cisplatin-induced apoptosis in human ovarian cancer cells (Pak et al., 2011). In contrast, reduction of PRDX6 expression increased peroxide-induced cell death in liver cancer cells (Walsh et al., 2009). Peroxiredoxin 6 was found to be down-regulated in the present study, after the treatment with PIMET, which may be initiating the death of HT29 cells.

Solute carrier family 25 member 35 (SLC25A35) belongs to family of SLC25 family of mitochondrial carrier proteins (Haitina et al., 2006). The mitochondrion relies on compartmentalization of certain enzymes, ions and metabolites for the sake of efficient metabolism. In order to fulfill its activities, numerous carriers are expressed, targeted and folded in the inner mitochondrial membrane. Among these carriers, the six-transmembrane-helix mitochondrial SLC25 (solute carrier family 25) proteins facilitate transport of solutes with disparate chemical identities across the inner mitochondrial

membrane (Gutierrez-Aguilar and Baines, 2013). The decrease in membrane potential after the treatment of PIMET (as discussed in section 4.3.17.7) may have down regulated SLC25A35 as evident from proteomics analysis. This down-regulation will negatively affect the proper functioning of mitochondria and there by the HT29 cells.

X-linked inhibitor of apoptosis belongs to a family of apoptotic suppressor proteins. This protein functions through binding to tumor necrosis factor receptor-associated factors TRAF1 and TRAF2 and inhibits apoptosis. This protein also inhibits Caspase 3, 6 and 7 by blocking the activity of Caspase 9 (Yang et al., 2003). The expression of XIAP has been shown to be up-regulated in many human colon carcinomas (Yang et al., 2003; Yagihashi et al., 2003; Endo et al., 2004). However in our study, this apoptotic inhibitor protein was down-regulated after the treatment of HT29 cells with PIMET extract and thus supports our earlier observation that PIMET is able to induce apoptosis in HT29 colon cancer cells (as discussed in section 4.3.17.6).

Thus proteomic analysis of proteins from HT29 showed that exposure to methanol extract of PI is able to siginificant alter the expression of different proteins of HT29 cells. The downregulation of peroxiredoxin 6 have made the cells failed to withstand the stress, similarly the downregulation of solute carrier protein affect the normal functioning of mitochondria of HT29 cells. The downregulation of X-linked inhibitor of apoptosis in particular might have forced the HT29 cells to enter apoptosis. Further, the identification of remaining protein spots is necssary which is not in the scope of this study.

4.6 Conclusion

The dietary fibre isolated from Plantain Inflorescence were analyzed for its prebiotic

potential. The prebiotic efficacy studies reveals that PI dietary fibre efficiently promotes the growth of the selected probiotic species – *Lactobacillus casei and Bifidobacterium bifidum*. Upon analysis of the fermentation supernatant we found that its rich in short chain fatty acids. Since short chain fatty acids are known for exhibiting anticancer effects against colon cancer, we analyzed the fermentation supernatant for its anticancer potential. We have got promising results from various anticancer assays performed. Among the supernatants analyzed, BS was found to possess better anticancer activity. BS initiates anticancer effect by triggering ROS induced apoptosis, affects the mitochondrial membrane potential, decrases ATP production and increases Cytochrome C release. In addition to this western blot and proteomic analysis showed that BS is able to alter the expression of relevant proteins involved in apoptosis and other vital biological functions.

Since dietary fibre usually act as a matrix holding antioxidant molecules with releavnt biological properties, the extracts from PI were studied for anticancer potential. The methanol extract of PI was found to be more cytotoxic against HT29 colon cancer cells. The PI MET extract is able to arrest cell cycle at G2/M phase, reduces mitochondrial membrane potential which results in decreased ATP production and Cytochrome C release and induces apoptosis in HT29 cells. Apart from this the effect of PI MET on protein expression level were analyzed by western blotting and 2D electrophoresis, which shows that expression of some of the siginificant proteins were altered in favour of inducing apoptosis, especially down-regulating one of the key apoptotic inhibitor protein XIAP.

Thus we conclude that plantain infloresence is a good source of prebiotic dietary fibre along with compounds having significant anticancer potential aginst HT29 colon cancer cells. Further studies are required to isolate the potential compound/compounds, which can be related to the anticancer effect, from methanol extract of plantain inflorescence.

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Chapter 5

Development of synbiotic components by encapsulation and spray drying methods

5.1 Introduction

Synbiotics is the co-administration of probiotics and prebiotics with the expectation that the prebiotics will enhance the survival and growth of the probiotics (Grimoud et al., 2010). During the development of new synbiotic products it is very vital to determine prebiotic and probiotic interactions and influence of prebiotic on probiotic growth and antibacterial activity (Pranckute et al., 2014). The prebiotic potential of PI was studied and explained in Chapter 4. Based on those results synbiotic components were prepared integrating soluble dietary fibre and probiotic species (*Lactobacillus casei* and *Bifidobacterium bifidum*) which is summarized in Chapter 5 along with literature support.

The human gut is dominated by several bacterial phyla and is estimated that the human microbiota contains as many as 10^{14} bacterial cells (Ley et al., 2006). The gut microbiota has a crucial role in human health and disease. *Lactobacillus* and *Bifidobacterium*, have been shown to positively influence health. The colon or the large intestine is the organ which is the preferred site for bacterial colonization. The imbalanced gut bacteria have been studied in colon cancer and other gut related diseases (Usha and Natarajan, 2012). Probiotics, prebiotics and synbiotics are the new concepts that have been developed to modulate the target gastrointestinal microflora balance.

Probiotics are viable live microorganisms when administered in adequate amounts confer a health benefit on the host (Fuller, 1989). The idea in the beneficial properties of probiotics is based on the facts that the intestinal flora can protect humans against infection and disturbance of this flora can increase susceptibility to infection. Various *in vivo* and *in vitro* studies have depicted that the normal intestinal flora is

really an effective barrier against pathogenic and opportunistic microorganisms (Fuller 1991). Probiotics are usually targeted for use in intestinal disorders in which specific factors (such as antibiotics, medication, diet or surgery) disrupt the normal flora of the gastrointestinal tract, making the host animal susceptible to disease. Many microorganisms have been used or considered for use as probiotics (Rolfe, 2000). The most commonly used probiotics are strains of *Lactobacillus* and *Bifidobacterium* which have been studied for decades (Saxelin et al., 2005; Boesten and De Vos, 2008; Kleerebezem and Vaughan, 2009; Didari et al., 2014). These bacteria resist adverse gastric conditions, adhere and readily colonize the intestinal tract. Lactic acid bacteria have been demonstrated to inhibit the in vitro growth of many enteric pathogens and have been used in both humans and animals to treat a broad range of gastrointestinal disorders (Meurman et al. 1995, Silva et al. 1987).

Prebiotics can be defined as a non- digestible food ingredient which beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improving host health (Gibson and Roberfroid, 1995). For a prebiotic to survive it must pass through the upper gastrointestinal tract undigested and then be metabolized primarily by probiotic bacteria in the lower intestine as an energy source where glucose is limited (Ozer et al., 2005). Presently a number of indigestible carbohydrates (disaccharides, fructooligosaccharides, oligosaccharides and polysaccharides) are used as prebiotics (Gibson and Roberfroid, 1995; Kontula, et al., 1999; Saarela et al., 2003). Inulin, a naturally occurring fructooligosaccharide, is commonly used as a prebiotic for both *Lactobacilli* and *Bifidobacteria* because of the proven ability to increase microbial mass and lower the

pH of the colonic content (Akin et al., 2007; Gibson and Roberfroid, 1995). The growth promoting effect of another prebiotic lactulose has been mainly reported for *Bifidobacteria* (Watanabe et al., 2008).

Synbiotics is nothing but the synergy between probiotic and prebiotic effect in the gastrointestinal tract or in other words, synbiotics is the usage of both probiotics and prebiotics in combinations. Indeed synbiotic combinations are considered to have more beneficial effects on human health than probiotics or prebiotics alone (Bandyopadhyay and Mandal, 2014). Reports showed that synbiotics stimulated the fecal bacterial counts of *lactobacilli* and *bifidobacteria* in human subjects, but little effect was seen when only the probiotic or the prebiotic was given (Gopal et al., 2003; Ghanem et al., 2004).

Because of the increase in awareness on probiotics and prebiotics and their health benefits, there is increase in demand for developing functional foods with these components among the consumers. Microencapsulation of probiotic species with prebiotics is one of the promising techniques for the development of synbiotic components for functional food applications. Encapsulation is considered as one of the plausible techniques for ensuring probiotic stability when incorporated in food products that enhances the viability of the organisms, facilitates handling of cells and allows controlled dosage. It is reported that when prebiotics are incorporated in the encapsulating media for encapsulation of probiotics, it protects probiotics against gastro intestinal conditions and other adverse environments (Desmond et al., 2002; Rajam et al., 2012; Bustos and Borquez, 2013). Currently available different scientific studies reveal that most popular targets for prebiotic use are *lactobacilli* and *bifidobacteria* and different scientific laboratories have been screening on most efficient synbiotic

combination for maintaining sustained human health. We have already studied the prebiotic effect of soluble dietary fibre from plantain inflorescence in promoting the growth of *Lactobacillus casei* and *Bifidobacterium bifidum* which was explained in chapter 4. This chapter details about the synbiotic preparation utilizing the prebiotic plantain inflorescence and probiotic species.

5.1.1 Objectives

The main objectives of this chapter are summarized as follows:

- To develop synbiotic components by incorporating probiotic culture and prebiotic soluble dietary fibre from PI by spray drying and lyophilization techniques
- Assessment of encapsulation capacity, moisture content and water activity of synbiotic formulations
- To assess short chain fatty acid production after assimilating synbiotics in media
- Evaluate the viability of bacteria in synbiotics during storage at different conditions of temperature

The outline of Chapter 5 is shown in Figure 5.1.

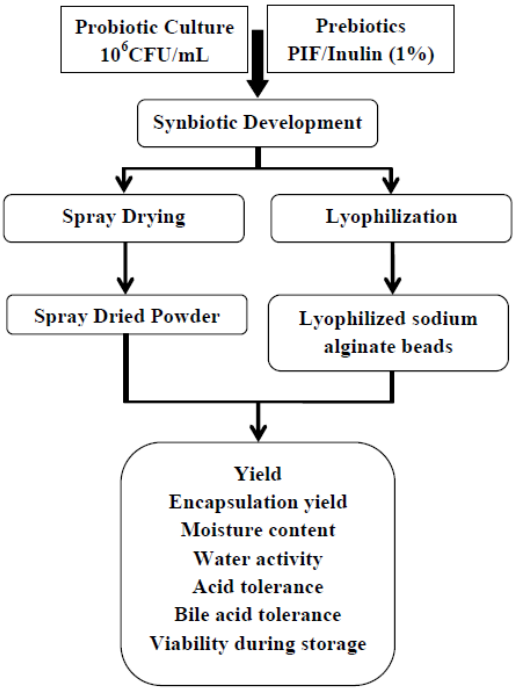

Figure 5.1. Outline of chapter 5

5.2 Materials and Methods

5.2.1 Chemicals

Sucrose, calcium chloride, and starch were obtained from Sisco Research Laboratories, India. Bile salts, pepsin, sodium alginate was obtained from Sigma Aldrich, St Louis, USA. All microbiological media were obtained from Himedia Laboratories Pvt. Ltd, India and L-cysteine was obtained from Merck Chemicals, India. Inulin (Orfati®Inulin) was supplied by Beneo Asia Pacific Pte. Ltd., Singapore. All reagents used were of analytical grade.

5.2.2 Micro Organisms

Lactobacillus casei (NCDC17) cultured in MRS broth, *Bifidobacteria bifidium* (NCDC255) cultured in MRS broth and L-cysteine media. The freeze dried cultures were supplied by National Dairy Research Institute, Karnal, Haryana, India.

5.2.3 Extraction of soluble dietary fire (SDF) from PI

The soluble dietary fibre from plantain inflorescence (PIF) was isolated as described in Chapter 1.

5.2.4 Development synbiotic components

5.2.4.1 Encapsulation by spray drying

Solutions of 10 g of maltodextrin (control), 10 g of maltodextrin + 1 g inulin (positive control) and 10 g of maltodextrin + 1 g SDF from PI (sample) was prepared in 100 mL of distilled water and stirred for 1 h to hydrate. They were then autoclaved at 121°C for 15 min. The selected probiotic culture was inoculated into MRS broth and incubated at 37°C for 48 h under anaerobic conditions. The cells were harvested by centrifugation at 14500×g for 10 min at 4°C, washed with sterilized distilled water. These organisms were resuspended in the sterilized encapsulating media (control, positive control and sample) at the rate of 10^6 CFU/mL approximately and stirred with a magnetic stirrer till the solution becomes homogeneous.

These feed solutions were directly used for spray dried application as described by Madhu et al. (2011) with slight modifications. A bench top spray dryer (LU – 228 ADVANCED, LabUltima, Mumbai, India) was used for spray drying the feed solution. The inlet and outlet temperatures were 140 ± 2°C and 60 ± 2°C respectively with a flow rate of 1.5 mL/min. The feed solution was pumped in to the drying chamber by a peristaltic pump. The feeding solutions were constantly stirred during the spray drying process. The aspiration rate was about 55% while the feeding rate of the solution was 2 mL/min. The microcapsules were separated by cyclone and gathered in the collection vessel. The residual viability of spray dried culture was determined and the samples

were kept for storage studies at room temperature (27-30°C) and refrigerated condition (4°C).

5.2.4.2 Encapsulation in alginate gel micro beads followed by lyophilization

The freeze dried synbiotic formulation was developed on the basis of the method followed earlier by Collins and Hall (1984) and Crittenden et al. (2006) with suitable modifications. The cells were harvested as described in the case of spray drying and re-suspended at the rate of 10^6 CFU/mL in a sterilized solution containing 10% sucrose, 4% starch, 100 mM $CaCl_2$ and 1% PIF. A control (without any prebiotic) and positive control (1% inulin) group was also prepared for comparison. The uniform distribution of components was ensured by proper mixing. Then the solution was added drop by drop using a sterile glass syringe to sodium alginate solution (0.6%) under mild stirring. The beads were filtered, washed with distilled water and immersed in calcium chloride solution (1.22%) for 30 min. The beads were again filtered out, washed with the distilled water and placed in skim milk (5%) for 30 min. After that the beads were filtered out, washed with distilled water and freeze dried using lyophilizer (VirTis genesis, USA). The residual viability of lyophilized beads was determined and the samples were kept for storage studies at room temperature (27 - 30°C) and refrigerated condition (4°C).

5.2.5 Product yield (PY)

The product yield was calculated as the percentage of the ratio of weight of collected samples (g dry matter) to weight of feed solution (g dry matter).

5.2.6 Water activity and moisture content

Water activities of the samples were measured by using a water activity meter (Rotronic

HygroPalm - HP23-AW-A, Bassersdorf, Switzerland) at 25°C. The moisture content of the samples was analyzed by oven dry method at 105°C.

5.2.7 Encapsulation efficiency/Encapsulation yield

Encapsulation efficiency was analyzed according to Shah et al. (2016) with slight modifications. Synbiotic formulation (1 g) was re-suspended in 9 mL phosphate buffer (pH 7) followed by homogenization for 15 min and following the colony formation units were determined. Encapsulation efficiency refers to the number of bacterial cells that survived the process and encapsulated inside the microcapsules was calculated as follows

$$EE = \left({}^{N}/_{N_0}\right) \times 100$$

where N is the number of viable entrapped bacteria (Log CFU/g) released from the synbiotic formulation just after its preparation and N_0 is the number of free cells (Log CFU/mL) added to the encapsulation matrix during the production of synbiotic formulation.

5.2.8 Morphological analysis of synbiotic components

The topographical properties of prepared synbiotics were investigated by scanning electron microscopy (SEM) according to Lotfipour et al., (2012) with slight modifications. Prior to examination, samples were stuck on to aluminium stub with the help of a carbon tape. Then the sample whose SEM images have to be captured is shadow casted with thin layer of gold (30-40 nm). SEM analysis was carried out using JEOL JSM-5600LV (Italy). Prior to examination, samples were sputter coated with gold–palladium to render them electrically conductive by using HUMMLE VII Sputter

Coating Device (Anatech Electronics, Garfield, N.J., USA). The micrographs were taken at magnification of 3000X and 5000X.

5.2.9 Survival of microcapsules in simulated gastric juice (SGJ)

Fresh simulated gastric juice (SGJ) was prepared by suspending 3 g/L of pepsin in sterile saline (9 g/L) and adjusted to pH 3 with 0.1 N HCl, in order to study the viability at gastric conditions. The studies were also carried out at pH 2 and 4 for evaluating the acid tolerance of the micro capsules. The encapsulated synbiotic components (0.1 g) were mixed in 10 mL of SGJ at various pH and incubated for 1 h at 37°C with constant agitation at 50 rpm. The viable count was enumerated by pour plate method and the percentage survival rate was determined by comparing with viability at neutral pH (pH 7).

$$Survival\ rate\ (\%) = \frac{(Log\ CFU/mL\ \ at\ experimental\ pH)}{(Log\ CFU/mL\ \ at\ neutral\ pH)} \times 100$$

5.2.10 Survival of microcapsules in simulated intestinal juice (SIJ)

Simulated intestinal juice (SIJ) was prepared by dissolving bile salts at different concentrations of 0.3, 0.5 and 1% (to study the effect of bile salts) in intestinal solution (6.5 g/L NaCl, 0.835 g/L KCl, 0.22 g/L $CaCl_2$ and 1.386 g/L $NaHCO_3$) and pH adjusted to 7.5 with 0.1 N NaOH. Tolerance of the synbiotic component in SIJ was evaluated by mixing 0.1 g of the synbiotic component in 10 mL of SIJ followed by incubation at 37°C for 1 h with constant agitation at 50 rpm. The viability of strains were enumerated by pour plate method and the percentage survival rate was determined by comparing with viability at neutral condition (without bile acid).

$$Survival\ rate\ (\%) = \frac{(Log\ CFU/mL\ \ at\ experimental\ bile\ acid\ \%)}{(Log\ CFU/mL\ \ without\ bile\ acid)} \times 100$$

5.2.11 Production of short chain fatty acids by the probiotic organisms in the synbiotic preparation

Short chain fatty acid (SCFA) production was analyzed and quantified by HPLC following the method of Guerrant et al. (1982) with some modifications. One gram of synbiotic formulations were transferred to 100 mL respective broth for *Lactobacillus casei* and *Bifidobacterium bifidum* and incubated for 48 h. The supernatant was collected by centrifuging the media at 10000 rpm for 10 min. The supernatant and the standard SCFA solutions were filtered through 0.45 µm PTFE filter; 20 µL was injected into the HPLC system. The analysis was performed on a Prominence UFLC system (Shimadzu, Japan) containing LC-20AD system controller, Phenomenex Gemini C18 column (250 × 4.6 mm, 5µm), a column oven (CTO-20A), a Rheodyne injector (USA) with a loop of 20 µL volume and a diode array detector (SPD-M20A). The mobile phase used was 10% acetonitrile with pH 2 (pH adjusted using ortho phosphoric acid). The flow rate was 0.5 mL/min; the injection volume was 20 µL and column was kept at 37°C. The fractions were monitored at 200 nm. Sample peaks were identified by comparing with retention times of standard peaks and also by spiking the sample with standard SCFA. LC LabSolutions software was used for data acquisition and analysis.

5.2.12 Storage stability of encapsulated probiotics during storage

The synbiotic formulations were stored at two different conditions, *i.e*., room temperature and 4°C for 45 days and the viability was assessed at specific time intervals (1, 15, 30 and 45^{th} days). The viability of encapsulated bacteria in the synbiotic components were determined by dissolving 100 mg of product in respective media

followed by incubation for 72 h at 37°C and the bacterial viability was enumerated using pour plate method.

5.2.13 Statistical analysis

The experimental results are expressed as the mean ± standard deviation of triplicate measurements. The data were analyzed by one-way ANOVA with one factor using SPSS software version 11.5. The level of significance was set at $p \leq 0.05$.

5.3 Results and Discussion

5.3.1 Development of synbiotic components

Encapsulation of probiotic species with dietary fibre not only provides protection against adverse processing and gastrointestinal conditions (Haghshenas et al., 2011), it also promotes the growth of these organisms in colon (Rao, 2002). As described earlier along with PIF, maltodextrin and sodium alginate were used respectively for spray drying and lyophilization techniques for the preparation of synbiotic components. Maltodextrin is reported to increase the glass transition temperature during spray drying and enhances stability during storage (Avila et al., 2015). Encapsulation with alginate is a promising technique for probiotics protection against adverse conditions to which probiotics can be exposed (Khosravi et al., 2014). Therefore in order to understand the efficacy of incorporation of PIF in the encapsulating matrix for the encapsulation of *Lactobacillus casei* and *Bifidobacterium bifidum* intended for gastrointestinal delivery, using spray drying and freeze drying, were attempted.

The lyophilized and spray dried synbiotic formulations with sodium alginate and maltodextrin respectively was developed by incorporating the selected probiotic culture and PIF. The synbiotic formulations were kept under refrigerated (4°C) as well as room

temperature (27°C) in air tight packs for viability studies. The photographic images of synbiotic formulations were shown in Figure 5.2.

Figure 5.2 Photographic images of synbiotic components. [I] Lyophilized beads and [II] Spray dried powder. (A) Control- *Lactobacillus casei*, (B) Inulin + *Lactobacillus casei*, (C) PIF + *Lactobacillus casei*, (D) Control- *Bifidobacterium bifidum*, (E) Inulin + *Bifidobacterium bifidum*, (F) PIF + *Bifidobacterium bifidum*. PIF- Soluble dietary fibre from plantain inflorescence.

5.3.2 Product yield, encapsulation efficiency, moisture content and water activity

Synbiotic components were prepared by encapsulation using spray drying and by freeze drying of alginate beads in the present study. In order to understand the protective effect of PIF during encapsulation process and storage thereafter, it was incorporated (1%) in the encapsulating matrix along with other components. A positive control was also prepared where PIF was substituted with inulin (1%) in the encapsulation matrix and the properties of the encapsulated synbiotic components were evaluated and compared.

The product yield, encapsulation efficiency, moisture content and water activity of synbiotic formulations are shown in Figure 5.3.

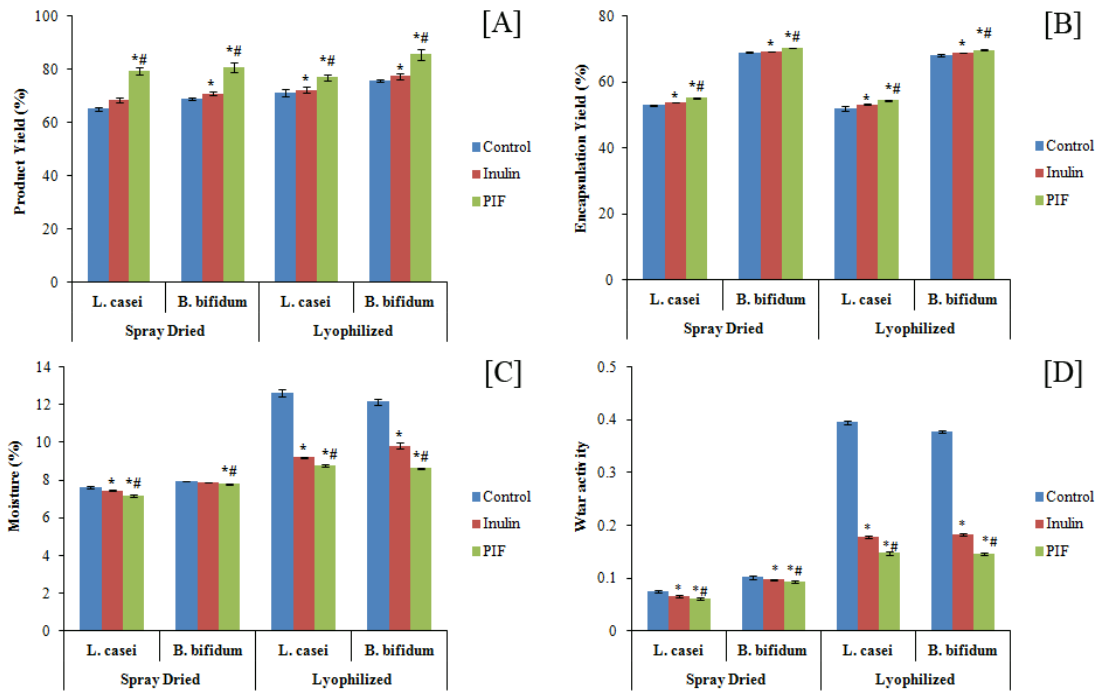

Figure 5.3. Yield (A), Encapsulation yield (B), Moisture content (C) and Water activity (D) of spray dried and lyophilized synbiotic formulations. Each value represents mean ± SD (standard deviation) from triplicate measurements. *Values are significantly different from control group. #Values are significantly different from inulin group. ($p \leq 0.05$).

The product yield of various synbiotic formulations are given in Figure 5.3[A]. The encapsulation matrix with PIF exhibited significantly higher yield as compared to inulin and control. The lyophilized beads with *Bifidobacterium bifidum* prepared by freeze drying showed highest yield (85.63 ± 2.1%) where as the yield for the corresponding control was 75.59 ± 0.45%. The yield of spray dried formulation of *Saccharomyces cervisiae* with whey protein (g/100 g feed dry matter) and gum Arabic

was reported to be 54.65 and 46.63 g/100 g feed dry matter respectively (Arslan et al. 2015).

Encapsulation techniques have been employed to improve acid and bile tolerance of probiotics and also to inhibit unwanted reactions during storage which result in loss of activity. The efficacy of entrapment and the survival of viable cells during the microencapsulation procedure were expressed in terms of encapsulation efficiency/yield (Figure 5.3[B]). As can be seen, the synbiotic formulations with PIF showed better encapsulation efficiency than that the control and inulin group ($p \leq 0.05$). *Bifidobacterium bifidum* exhibited better viability to the microencapsulation conditions as compared to *Lactobacillus casei*. The spray dried powder with *Bifidobacterium bifidum* prepared by freeze drying showed highest encapsulation yield (70.34 ± 0.01%) where as the encapsulation yield for the corresponding control was 68.93 ± 0.19%. Our results are in agree with earlier reports that prebiotics when incorporated in the encapsulating matrix for probiotics encapsulation, it protects the organisms against the adverse conditions during the encapsulation process (Sathyabama et al., 2014).

Moisture content and water activity significantly influences the viability of probiotics as well as the microbial and physico-chemical properties of probiotic containing foods during storage (Jixian and Mittal, 2013). Hence we analyzed the moisture content and water activity of synbiotic components (Figure 5.3[C] and 5.3[D]). It was observed that the moisture content and water activity of the spray dried formulations were lower than the lyophilized beads. The synbiotic formulations prepared using PIF were found to have significantly lower moisture content and water activity than that of the inulin and the control group. The lowest moisture content and

water activity were observed for spray dried formulation with *Lactobacillus casei* (7.19 ± 0.051% and 0.161 ± 0.002, respectively). It is reported that the microbial stability of spray dried microcapsules can be ensured if the moisture content and water activity is kept in the range of 4-9% (Chavez and Ledeboer, 2007; Ghandi et al., 2012; Rajam and Anadharamakrishnan, 2015; Poddar et al., 2014) and 0.15 – 0.3 respectively (Corcoran et al., 2004; Viernstein et al., 2005; Chavez and Ledeboer, 2007; Manojlovic et al., 2010). The results we obtained for PIF incorporated synbiotics are within the recommended range for better microbial stability.

5.3.3 Morphological study by SEM

The morphological analysis was done by scanning electron microscopy. SEM images for the probiotics immobilized in the alginate matrix and spray dried form are shown in Figure 5.4. As can be seen, the morphology of lyophilized beads with PIF, inulin and control were different from each other, in the cases of both the probiotic species studied. The results showed that the morphology of lyophilized beads are a little bit rough and is not uniform. However, the external surfaces of the lyophilized beads are smoother in the case of PIF and inulin as compared to the control. It can be noted that there was no disruption of gelation of alginates in the present study as earlier reported by Sathyabama et al., (2014) where prebiotics from sugar beet, and chicory were used as encapsulating medium along with 2% alginate. It can also be noted that the organisms are more densely packed on the surface of the beads with SDF from PI and inulin. However the spray dried particles were soft and maintained uniform spherical shape. The encapsulation, incorporated probiotic species efficiently as none of them is evident at outer surface in both freeze dried and spray dried synbiotic formulations.

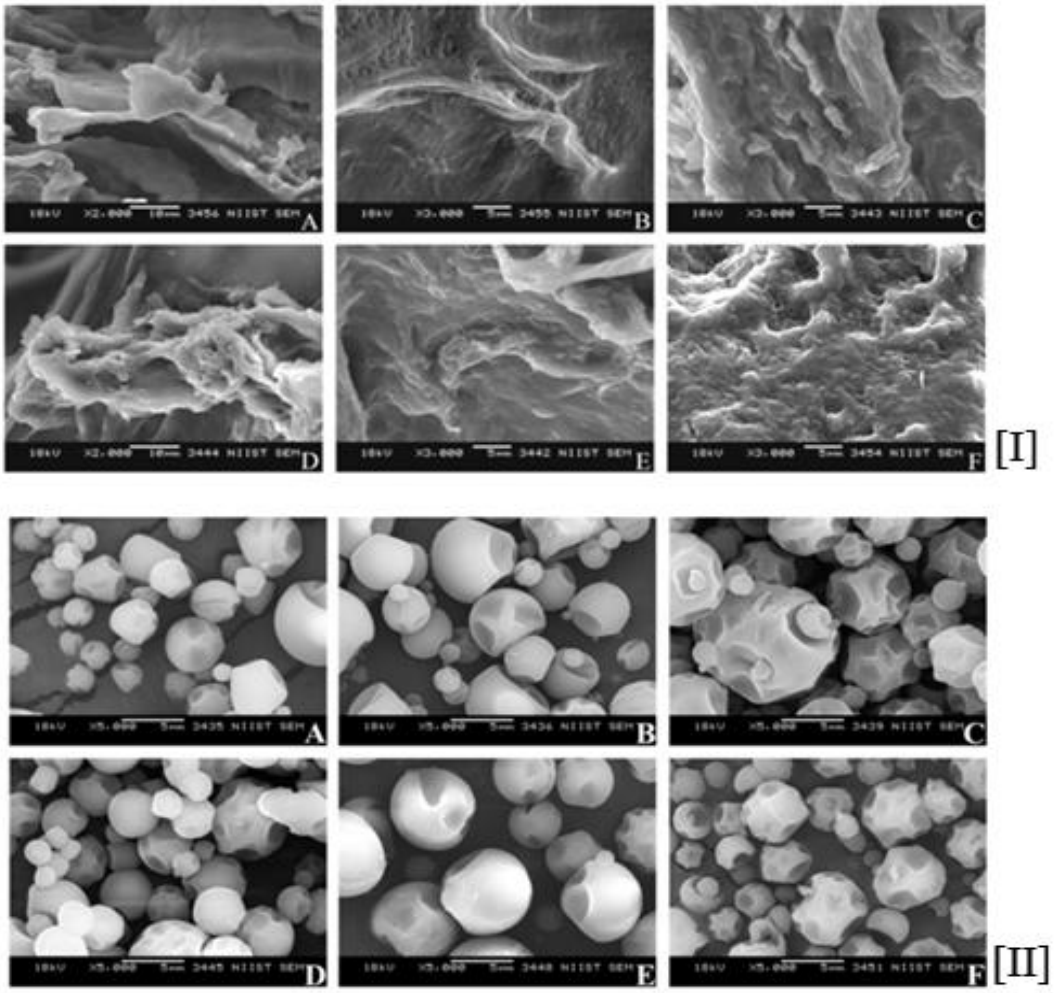

Figure 5.4 SEM images of Lyophilized beads [I] and Spray dried powders [II]. (A) Control-*Lactobacillus casei*, (B) Inulin + *Lactobacillus casei*, (C) PIF + *Lactobacillus casei*, (D) Control-*Bifidobacterium bifidum*, (E) Inulin + *Bifidobacterium bifidum*, (F) PIF + *Bifidobacterium bifidum*. PIF- Soluble dietary fibre from plantain inflorescence.

5.3.4 Survival of entrapped bacteria in simulated gastric juice

Studies on the survival of encapsulated bacteria at low pH conditions (pH 2, 3 and 4) were carried out, as pH is an important factor that determine the survival of bacterial strains and low pH is a constrain for the survival of most of the strains. It is reported that microencapsulation protects bacterial cells from the harsh conditions of the simulated gastirc juice (Anal and Singh, 2007; Ding and Shah, 2007; Heidebach et al.,

2010). The results (Figure 5.5[A]) indicated that encapsulating media with PIF exhibited better survival rate when compared to the known prebiotic inulin and control group at all the pH studied. At pH 2, *Lactobacillus casei* and *Bifidobacterium bifidum* were more stable in lyophilized beads incorporated with PIF with a survival rate of 72.0 ± 1.8% and 70. 0 ± 2.2% respectively than the corresponding spray dried products (survival rate of 28.0 ± 1.8% and 25.0 ± 1.2% respectively). Microencapsulated synbiotic formulation in the form of lyophilized alginate beads demonstrated better viability as compared to the spray dried one. The increased viability of the freeze dried product may be due to the rough nature of lyophilized beads along with the gelation of alginates which might have protected the organisms against the adverse acidic conditions.

It is reported that a membrane is formed on the surface of the granules due to strong bonding of biopolymers with alginates resulting from the electrostatic interactions. This might reduce the probability of migration of coating material protecting cells from the adverse acidic conditions (Peredo et al., 2016). In the case of spray dried synbiotics, the cell wall contains maltodextrin. The low-molecular weight sugars present in maltodextrin act as plasticizers and reduce the polymer chain contacts, which in turn decrease the rigidity of the three-dimensional film structure of the micro capsule (Villacrez, Carriazo, & Osorio, 2014). This may be correlated with the lesser stability of spray dried synbiotics in SGJ leading to the exposure of organisms to the gastric juice and hence less viability.

5.3.5 Survival of entrapped bacteria in simulated intestinal juice

The viability of bacteria in synbiotic formulation at three bile salt concentrations (0.3,

0.5 and 1%) conditions were studied in order to understand the efficacy of microencapsulation on the viability of bacterial cells against the harsh conditions of high bile acid condition similar to intestine. As can be seen, the viability of the encapsulated organisms was inversely proportional to bile concentration studied. The lyophilized alginate beads demonstrated significantly higher survival rate in simulated intestinal juice as compared to spray dried microcapsules (Figure 5.5[B]). Significantly higher survival rate was demonstrated when the probiotics were encapsulated with PIF. Among the beads (at 1% bile salt), the survival rate for *Lactobacillus casei* and *Bififdobacterium bifidum* with PIF was found to be 23 ± 1.4% and 30 ± 1.8% respectively, whereas the same for spray dried formulations were 18 ± 1.3% and 20 ± 1.3% respectively. The uneven and rough nature of lyophilized beads may have helped to resist the higher bile salt condition. This may be due to the membrane formation tendency of alginate with biopolymers as described earlier (Peredo et al., 2016). Apart from this different studies have shown that the addition of starch as a filler material in the alginate capsule matrix improved the viability of probiotic cultures (Donthidi et al., 2010).

Stability of the probiotic bacteria are important considerations, because the bacteria must survive in the synbiotic formulation during shelf life and during transit through the acidic conditions of the stomach, and resist degradation by hydrolytic enzymes and bile salts in the small intestine. Beneficial effects of probiotic species can be expected only when viable cells of these organisms are able to survive passage through the human stomach and digestive system and colonize in the human gut (Kailasapathy and Chin, 2000). The stomach and the surroundings of the human

gastrointestinal tract have the highest acidity and the pH of these areas may fall to as low as 1.5. Gastrointestinal systems have varying concentrations of bile. The rate of secretion of bile acid and its concentration depend on the type of food consumed. Bile concentrations range from 0.5 to 2.0% in the first hour of digestion and the levels may decrease during the second hour (Alm, 1991). Hence we analyzed the viability of bacteria in synbiotic formulation at low pH and high bile salt concentrations. The results clearly proved that our synbiotic formulations are found to withstand lower pH and high bile salt concentration.

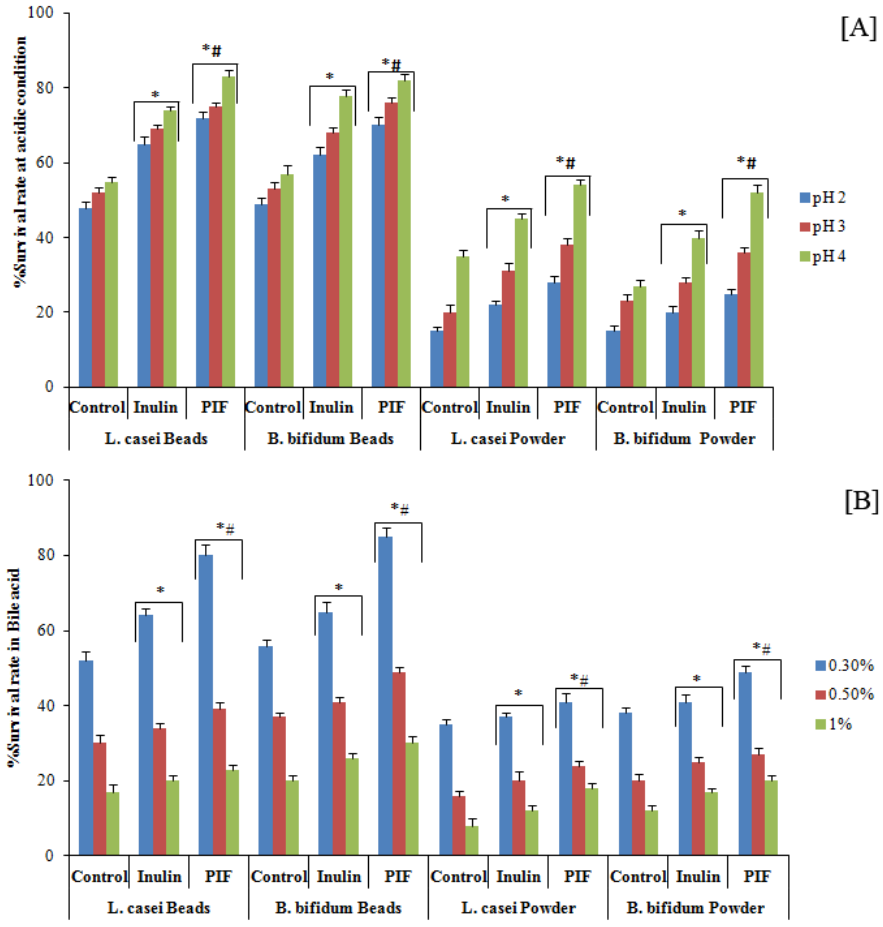

Figure 5.5 Survival of entrapped bacteria in adverse conditions. [A] Survival of entrapped bacteria in simulated gastric juice at different pH conditions. [B] Survival of in simulated intestinal juice at different concentrations of bile acid. Each value

represents mean ± SD (standard deviation) from triplicate measurements. *Significantly different from control in corresponding pH and beads/powder. #Significantly different from positive control (Inulin) at corresponding pH and beads/powder ($p \leq 0.05$).

5.3.6 Short chain fatty acid production by the probiotic organisms in the synbiotic formulation

We analyzed whether the bacteria entrapped in encapsulation matrix is able to metabolize the dietary fibre incorporated in the synbiotic formulation. The results showed that better production of SCFA was found for PIF incorporated synbiotics (Table 5.1). After 48 h incubation, among the two probiotic species incorporated with PIF, *Bifidobacterium bifidum* integrated spray dried powder shows the better yield of butyrate (4.94 ± 0.041 µg/mL). The corresponding butyrate yield for control and inulin group was only 2.07 ± 0.071 and 3.45 ± 0.061 µg/mL respectively. The results showed that the entrapped bacteria are viable and are able to metabolize the dietary fibre used for preparing synbiotic formulations.

Table 5.1 Short chain fatty acid production by entrapped bacteria in synbiotic formulations

	Lactobacillus casei			*Bifidobacterium bifidum*			*Lactobacillus casei*			*Bifidobacterium bifidum*		
	Spray dried			Spray dried			Lyophilized			Lyophilized		
	AA	PA	BA	AA	PA	BA	AA	PA	BA	AA	PA	BA
Control	9.42± 0.034^{a}	4.65± 0.021^{a}	2.06 ± 0.046^{a}	13.64± 0.043^{a}	4.58± 0.06^{a}	2.07± 0.07^{a}	7.52± 0.43^{a}	2.54± 0.035^{a}	1.16± 0.046^{a}	8.89± 0.057^{a}	2.58± 0.03^{a}	1.60± 0.025^{a}
Inulin	9.67± 0.029^{b}	6.17± 0.027^{b}	3.16 ± 0.042^{b}	16.08± 0.063^{b}	7.04 ± 0.052^{b}	3.45± 0.61^{b}	12.57± 0.045^{b}	7.63 ± 0.029^{b}	3.28 ± 0.221^{b}	12.88± 0.057^{b}	7.44 ± 0.045^{b}	3.20± 0.039^{b}
Fibre	15.55± 0.074^{c}	11.34± 0.045^{c}	4.84± 0.043^{c}	17.16± 0.043^{c}	11.66± 0.034^{c}	4.94± 0.041^{c}	14.34± 0.043^{c}	9.46± 0.186^{c}	4.51± 0.027^{c}	14.78± 0.056^{c}	9.83± 0.052^{c}	4.70± 0.033^{c}

AA- Acetic acid, PA- Propionic acid, BA- Butyric acid. The results are expressed in µg/mL. Each value represents mean ± SD from triplicate measurements $p \leq 0.05$ considered significantly different. [a,b,c] Values with different alphabets in same row are significantly different.

5.3.7 Viability of entrapped probiotics during storage

At present the environmental sensitivities of many potential probiotic strains limit their sensible use in non-refrigerated foods and pharmaceutical-type supplements. Hence, technologies that can protect the viability of probiotics during manufacture, storage, and gastrointestinal transit are highly desired. For this reason we analyzed the effect of time and temperature on the viability of entrapped probiotics in synbiotic formulations and the results are shown in Figure 5.6.

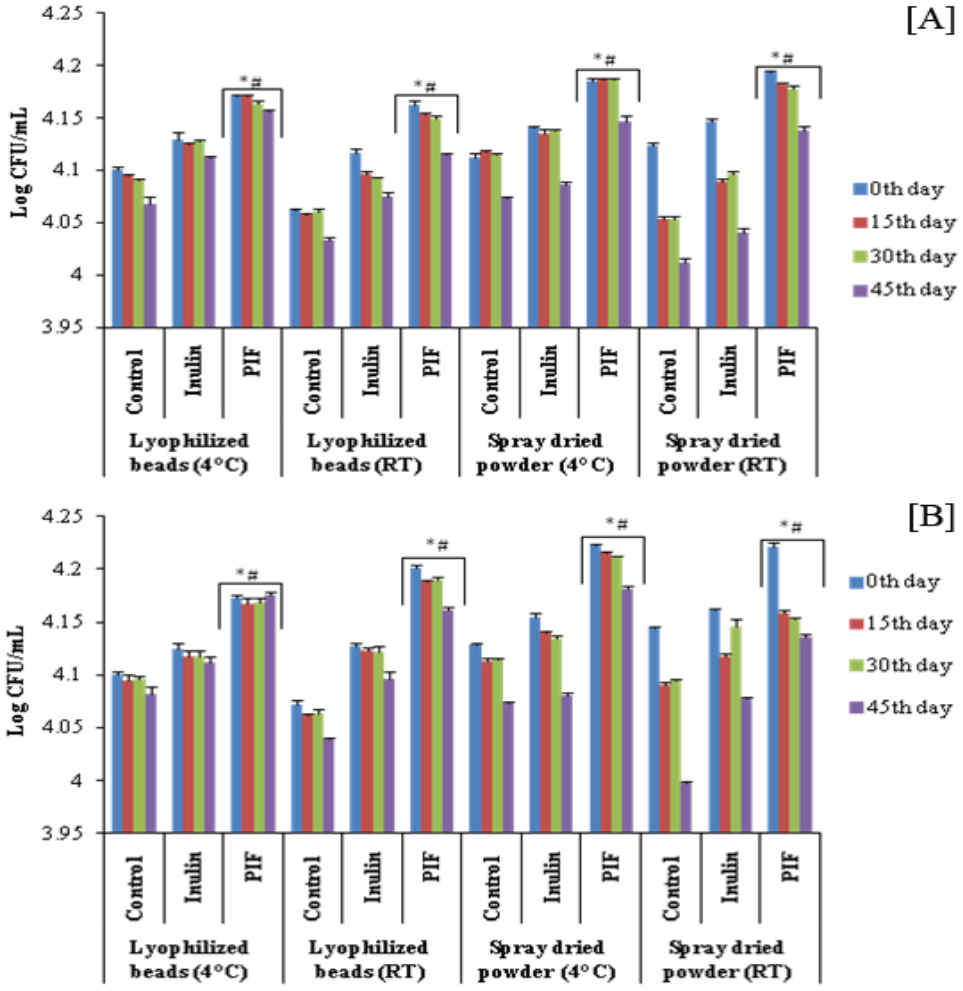

Figure 5.6 Viability of probiotic bacteria incorporated in synbiotic formulations. [A] *Lactobacillus casei* incorporated beads and powder. [B] Viability of *Bifidobacterium bifidum* incorporated beads and powder. Each value represents mean ± SD (standard

deviation) from triplicate measurements.*Significantly different from control in corresponding temperature and beads/powder. #Significantly different from positive control (Inulin) at corresponding temperature and beads/powder ($p \leq 0.05$).

For this, the synbiotic formulations were kept in room temperature and refrigerated conditions up to 45 days and the viability assessed during 0^{th}, 15^{th}, 30^{th} and 45^{th} day of storage. From the results it is obvious that synbiotic formulation with PIF have better viability in room temperature as well as refrigerated condition when compared to control and inulin. The survival rate of *Lactobacillus casei* in the beads with PIF after 45 days of storage under refrigerated and room temperature condition were 4.156 ± 0.0015 and 4.115 ± 0.0016 Log CFU/mL respectively. However in *Lactobacillus casei* spray dried product 4.147 ± 0.0046 Log CFU/mL and 4.138 ± 0.0047 Log CFU/mL organisms were viable at refrigerated and room temperature condition respectively on 45^{th} day. In case of *Bifidobacterium bifidum* beads with PIF, 4.176 ± 0.0029 Log CFU/mL and 4.161 ± 0.0028 Log CFU/mL organisms were viable at refrigerated and room temperature condition respectively on 45^{th} day. However in *Bifidobacterium bifidum* spray dried product 4.182 ± 0.0028 Log CFU/mL and 4.136 ± 0.0031Log CFU/mL organisms were viable at refrigerated and room temperature condition respectively on 45^{th} day. Considering the 0^{th} day values in both *Lactobacillus casei* and *Bifidobacterium bifidum*, it is clear that the lyophilized beads kept at refrigerated condition contain more number of viable bacteria.

The better protective effect of alginate beads over spray dried components can be related to the nature of maltodextrin and alginate. During increased temperature at spray drying, maltodextrin tends to form crystal structures which induce disruption of structural integrity of wall matrix resulting in release of encapsulated actives and

degradation during storage period (Sansone et al., 2011). Different studies have shown that alginate microcapsules are better protected in the presence of prebiotics, with the increase in survival of bacteria, under different conditions than when bacteria were tested in free state (Donthidi et al., 2010; Brinques & Ayub, 2011). Thus our results evidently agree with these earlier reports.

5.4 Conclusion

The prebiotic potential of soluble dietary fibre from plantain Inflorescence was studied in Chapter 4. Based on that we have developed two synbiotic components comprising *Lactobacillus casei*/*Bifidobacterium bifidum* and soluble dietary fibre from plantain inflorescence. Two well known food preservation methods - lyophilization (freeze drying) using sodium alginate and spray drying using maltodextrin were used for the preparation of synbiotic components. The developed synbiotic components withstand adverse gastric conditions and maintain the viability of probiotic bacteria up to 45^{th} day. The results are promising and further studies are required to develop nutraceutical/functional food/nutrient supplement that can be utilized for the microbial gut modulation and thereby preventing the risk of developing colorectal cancer.

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Chapter 6

Summary and Conclusion

6.1 Background

Lifestyle diseases characterize those diseases whose occurrence relies primarily on the daily habits of people and are a result of an inappropriate relationship of people with their environment. The main factors contributing to lifestyle diseases include unhealthy food habits, physical inactivity, consumption of alcohol, smoking and disturbed biological clock. The four main types of lifestyle diseases are cardiovascular diseases, cancers, chronic respiratory diseases and diabetes. In India, eight out of every ten deaths are caused by lifestyle diseases.

Dietary fibre (DF) and antioxidants provide properties associated with slow glucose absorption, high colonic fermentability, lower serum cholesterol levels, enhancement of immune functions and protection against oxidative damage. Epidemiological evidence suggests a strong correlation between the consumption of diets rich in dietary fibre and lifestyle associated diseases especially in the prevention and management of colorectal cancer (CRC). Prebiotic dietary fibre (nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one, or a limited number of, bacteria in the colon) is one of the most promising current targets for maintaining the homeostasis of human gastrointestinal tract and protecting colon from the development of any malignancy. The gut microbiota (microbes that reside in the human gut - Probiotics) have extensive metabolic functions that influence human health. Recently, the functional food research has moved progressively towards the development of dietary supplementation, introducing the concept of probiotics, prebiotics and their combination (synbiotics), which may affect gut microbial composition and activities.

Colorectal cancer (CRC) is the third most common form of cancer. Current treatments are associated with high risk of complications and low success rate. There is accumulating evidence describing the ability of probiotic strains, prebiotics and a combination of both –synbiotic- to prevent CRC and recently, synbiotics have been proposed as a new preventive and therapeutic option. The mechanisms by which probiotics may inhibit colon cancer are not yet fully characterized. However, there are evidences for alteration of the metabolic activities of intestinal microflora, alteration of physicochemical conditions in the colon, elimination of potential carcinogens, production of short chain fatty acid, production of anti-tumorigenic or antimutagenic compounds, elevating the hosts' immune response and altering the hosts' physiology. Therefore, the mechanisms by which pro-, pre- and synbiotics may inhibit colon cancer are gaining a lot of research attention. In this context, there is an increasing demand to find new sources of dietary fibers with specific bioactive constituents that can be formulated into functional foods and nutraceutical products.

Hence the present study was designed to develop a synbiotic component by the combination of probiotics and dietary fibre from agro produces that can be effectively used for the prevention and management of lifestyle associated diseases. The prebiotic efficiency of the isolated dietary fibre and its possible mechanism in prevention and management of CRC were also attempted to gain a better understanding of its nutraceutical potential.

6.2 Screening of agro-industrial for antioxidant dietary fibre

Different agro-industrial residues were screened for antioxidant dietary fibre based on soluble dietary fibre content and antioxidant potential. The antioxidant potential was

assessed by ability to scavenge free radicals- DPPH, NO, Hydroxyl and Superoxide. Prior to this total phenolic and flavonoid content in the extracts were analyzed. Plantain inflorescence was also found to be a very good source of dietary fibre with SDF/IDF ratio better than many reported sources of dietary fibre. Based on the results from preliminary experiments, plantain (*Musa paradisiaca*) inflorescence was chosen for further studies.

6.3 Efficacy of plantain inflorescence against diabetes and associated complications

As natural antioxidants and dietary fibre possess potential protective effect in managing diabetes and associated complications, further studies were carried out to evaluate the antidiabetic potential of *Musa paradisiaca* (Plantain) inflorescence by exploring the underlying mechanisms in terms of glucose absorption capacity of dietary fibre and antioxidant activity, carbohydrate digesting enzyme inhibition (α glucosidase and α amylase) potential, antiglycation and glucose uptake capacity of extracts prepared from the inflorescence. The study also focused on the cardiovascular protecting efficacy of plantain inflorescence as it is one of the major complications associated with diabetes mellitus. The results indicated that the methanolic extract of plantain inflorescence exhibits (PIMET) better activity in inhibiting α amylase and α glucosidase enzymes and glycation process. The PIMET showed an IC_{50} value of 166.14, 106.37 and 142.27 µg/mL respectively for α-amylase and α-glucosidase inhibition assay and antiglycation assay.

Over production of reactive oxygen species (ROS) plays an important role in the onset of diabetes and the methanol extract of plantain inflorescence was found to reduce the oxidative stress in L6 myoblasts by scavenging the H_2O_2 induced ROS. The

antiglycation properties shown by the extracts can play major role in delaying the onset of some of the diabetic related complications like cardiomyopathy. Further to this, the bioactives from PI also exhibited excellent cardio vascular protection by inhibiting LDL oxidation and Angiotensin Converting Enzyme, which is very important in modulating the cardiovascular complications associated with diabetes. Dietary fibre from PI demonstrated potential glucose and cholesterol binding properties. The antidiabetic and cardiovascular protection potential is summarized in Figure 6.1. Further in vivo studies are required to confirm the glucose and cholesterol lowering property of dietary fibre. In addition to this active compounds should be isolated from methanol extract of PI for further antidiabetic and cardiovascular protection studies.

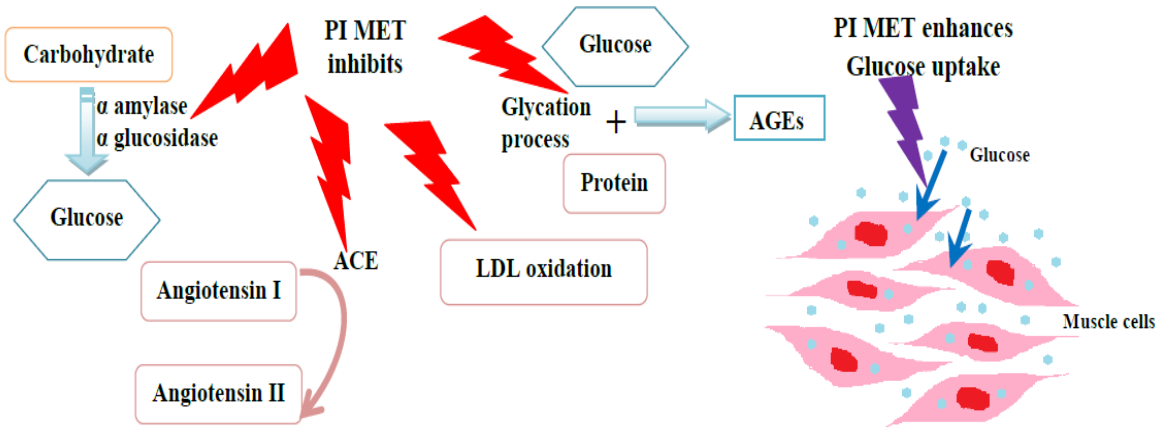

Figure 6.1. Schematic diagram showing the antidiabetic and cardiovascular protection effect of methanol extract of PI

6.4 Prebiotic efficacy and anticancer potential of fermentation metabolites of soluble dietary fibre from PI

Dietary fibre (prebiotics) is known to promote the growth of helpful microorganisms (probiotics) which is essential for the normal functioning of colon cells. The prebiotic potential of soluble dietary fibre from PI was assessed by analyzed by

evaluating its ability for promote the growth of two selected probiotic species – *Lactobacillus casei* and *Bifidobacterium bifidum* in terms of pH change, optical density and dry mass measurement, colony count and short chain fatty acid (SCFA) production analysis. The results are very promising and showed that PI SDF effectively promotes the growth of the two selected probiotic species. The number of colony formation units increased from 4.2 to 12.45 and 4.2 to 12.46 log CFU/mL respectively for *L. casei* and *B. bifidum* respectively after 72 h incubation in media containing PI SDF. 37.60 µg/mL and 45.60 µg/mL of butyric acid were produced in presence of PI SDF by *L. casei* and *B. bifidobacterium* respectively. The presence of fibre enhances the aggregation property, and inhibits pathogenic *E.coli* bacteria and the β glucuronidase enzyme produced by *E.coli*.

The fermented supernatant containing SCFA by *L. casei* (LS) and *B. bifidobacterium* (BS) were analyzed anticancer potential against HT29 colon cancer cells. The supernatant were found to be cytotoxic and further analysis revealed that exposure of HT29 cells to LS and BS increases the ROS production in HT29 cells. This ROS may be inducing the apoptosis in HT29 cells. BS is able to induce early and late stage apoptosis in 15.7% and 6.5% cells respectively. The mitochondrial membrane potential was decreased which resulted in the reduction of ATP production. At the same time cytochrome c was also released from mitochondria to cytosol. The western blot analysis showed that expression of BAX, c-caspase 3 and c-PARP, the proteins which are apoptosis inducing factors, over expressed, after the treatment of cells with the fermentation supernatant. However, no effect was found on the expression of antiapoptotic protein BCL2. Among the supernatants, BS was found to be more

effective against HT29 cells. Therefore, the effect of BS on total protein profile of HT29 cells was evaluated. Five proteins were identified by MALDI TOF analysis. The results showed that BS was able to induce apoptosis as well as inhibit the availability of ATP from glycolysis in HT29 cells respectively by up-regulating apoptosis inducing factor, mitochondria associated 1 protein and down-regulating triose phosphate isomerase.

6.5 Anticancer potential PI extracts

Since dietary fibres are known to entrap biologically active molecules, we analyzed the anticancer effect of extracts of PI. The cytotoxicity study reveals that methanol extract is found to be exhibiting better anticancer potential. Further studies were carried out with PI MET extract. PI MET is able to induce damage in DNA as evident from AO/EtBr and Hoechst staining of PI MET treated HT29 cells. The methanol extract was able to arrest HT29 cells at G2/M phase of cell cycle and induces early stage apoptosis in 27.8% cells at 100 µg/mL concentration. The PI MET treatment decreased the mitochondrial membrane potential which in turn reduces ATP production and increased the release of Cytochrome C from mitochondria to cytosol which initiates apoptosis. The western blot analysis showed that the treatment HT29 cells with PI MET increases the expression of BAX, c-caspase 3 and c-PARP; and decreases the expression of BCL2, PARP and caspase 3, which may lead to the induction of apoptosis. Further to this, proteomics analysis was performed and eight proteins were identified. Among them X-linked inhibitor of apoptosis – an anti apoptotic protein, was found to be down-regulated after treatment with PIMET which might have triggered apoptosis in HT29 cells.The schematic representation of possible anticancer mechanism

exerted by PIMET and SCFA from fermentation of soluble dietary fibre from PI was shown in Figure 6.2.

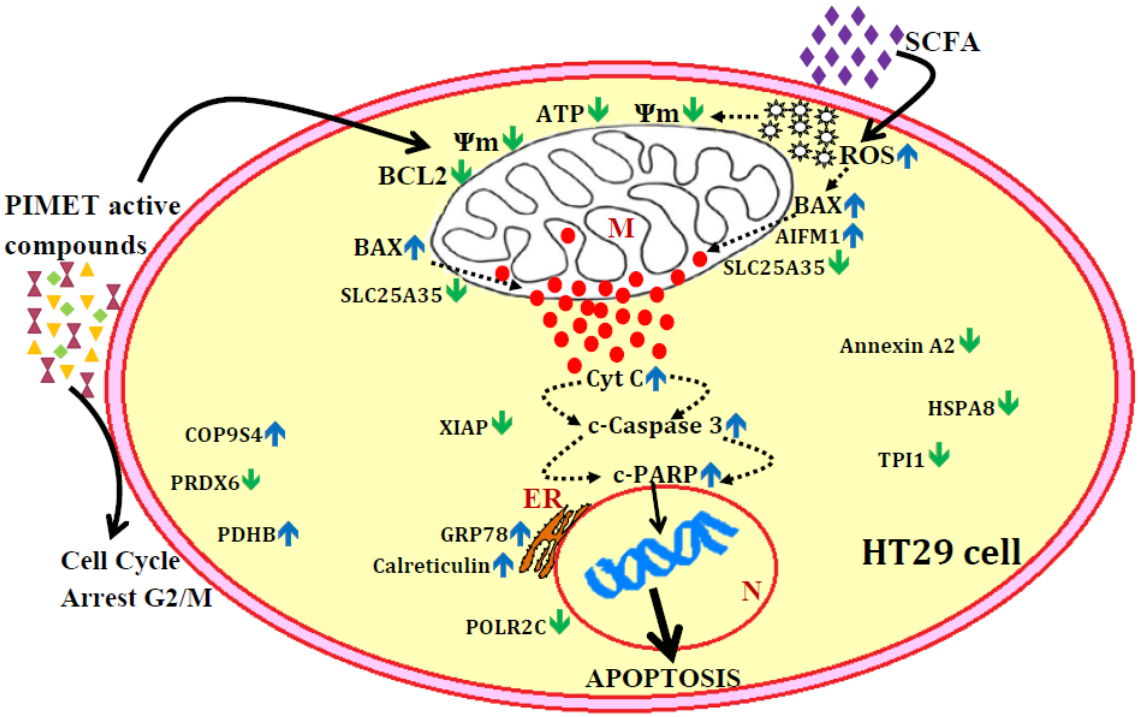

Figure 6.2. Schematic diagram showing the proposed mechanism for anticancer property exhibited by methanol extract of PI and SCFA produced by the fermentation of soluble dietary fibre from PI. SCFA – short chain fatty acids, PIMET methanol extract of PI - M – mitochondria, ER – endoplasmic reticulum, N – nucleus. Blue upward arrow and green downward arrow indicates up regulation and down regulation respectively.

6.6 Development of synbiotic components

Synbiotics are the combination of prebiotics and probiotics which imparts the beneficial effects of both the components. The results for the study indicated that PI is a good source of dietary fibre with prebiotic activity. Based on this, synbiotic components were developed that can be used as a nutrient supplement for the prevention and management of lifestyle associated diseases especially colon cancer. Spray driying and lyophilization techniques were adopted to prepare synbiotics by combining *L. casei*/*B. bifidum* with

SDF from PI. The water activity and moisture content were analyzed. The components exhibit better tolerance against acidic and bile acid environment. They were analyzed for SCFA, and results showed that the encapsulated organisms are still viable and able to ferment dietary fibre to produce SCFA. The prepared synbiotic components were stored at refrigerated and room temperature conditions to assess the viability and the results showed that the organisms are viable up to 45 days and better viability is retained in synbiotic formulations kept in refrigerated condition. The results are promising and further studies are required to develop nutraceutical/functional food/nutrient supplement that can be employed for the microbial gut modulation and thereby preventing the risk of developing colorectal cancer. Studies can be done to improvise these synbiotics which can be used as targeted drug delivery system for the treatment of colon cancer

6.7 Conclusion

In conclusion, it is evident from the study that agro-industrial residues can be exploited as good source of dietary fibre as well as biologically potent molecules that can be used for the treatment and management of many chronic diseases. Plantain inflorescence is remarkably a good source of antidiabetic and anticancer components. More work in characterization of active/purified compounds, incorporation of synbiotics in functional foods and studies in animal model has to be done that could further substantiate these findings. The methodologies employed here could be used for screening of agro-industrial residues for dietary fibre and other potent molecules for target diseases such as colon cancer, diabetes and cardiovascular diseases. The efficient reuse of agro-industrial residues will benefit the agro-industrial sector and moreover the information

generated in this study is expected to create aid to rejuvenate the agro-industrial by products to isolate dietary fibre and/or natural antioxidants for use by food and/or pharmaceutical industries.

Abbreviations

µg	-	Micro gram
µM	-	Micro molar
2-NBDG	-	2-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2-deoxyglucose
5-FU	-	5- Fluorouracil
AA	-	Acetic acid
AARs	-	Annual incidence rates
ABTS	-	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACE	-	Angiotensin converting enzyme
ADP	-	Adenisone Diphosphate
AGE	-	Advanced glycated end products
AIFM1	-	Apoptosis inducing factor, mitochondria associated 1 isoform 6
AMP	-	Adenisone Monophosphate
AO	-	Acridine Orange
APC	-	Adenomatous polyposis coli
ATP	-	Adenisone Triphosphate
a_w	-	Water activity
B. bifidum	-	*Bifidobacterium bifidum*
BA	-	Butyric acid
BS	-	PIF fermentation supernatant of *B. bifidum*
c-Caspase 3	-	Cleaved caspase 3
CFU	-	Colony formation unit
CHAPS	-	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
COP9S4	-	COP9 signalosome subunit 4
c-PARP	-	Cleaved PARP
CRC	-	Colorectal cancer
$CuCl_2$	-	Copper chloride
CUPRAC	-	Cupric ion reducing antioxidant capacity
CVD	-	Cardio vascular diseases
DAB	-	3,3′-Diaminobenzidine
DCF	-	Dichlorofluorescein
DCFDA	-	2',7' –dichlorofluorescin diacetate
DCFH	-	Dichloro dihydro fluorescein
DMEM	-	Dulbecco's Modified Eagle's medium
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribonucleic acid
DPPH	-	2,2-diphenyl-1-picrylhydrazyl
DPPH•	-	2,2-Diphenyl-2-picrylhydrazyl radical
DTT	-	Dithiothreitol
E. coli	-	*Escherichia coli*
EDTA	-	Ethylene diamine tetra acetic acid
EtBr	-	Ethidium Bromide
FAP	-	familial adenomatous polyposis

FAP	-	Furanacryloyl-L-phenylalanine
FAPGG	-	Furanacryloyl-L-phenylalanylglycylglycine
$FeCl_2$	-	Ferric chloride
FU	-	Fluorouracil
GAE	-	Gallic acid equivalence
GI	-	Gastro intestine
GRP78	-	Glucose regulated protein 78
H_2O_2	-	Hydrogen peroxide
H_2S0_4	-	Sulphuric acid
HCl	-	Hydro chloric acid
HNPCC	-	Hereditary nonpolyposis colon cancer
HPLC	-	High pressure liquid chromatography
HSPA8	-	Heat shock cognate 71 kDa protein
HT29	-	Human colorectal adenocarcinoma cell line
IC_{50}	-	50% Inhibition concentration
IDF	-	Insoluble dietary fibre
KH_2PO_4	-	Potassium dihydrogen phosphate
KOH	-	Potassium hydroxide
L. casei	-	*Lactobacillus casei*
L6	-	Rat myoblast cell line
LDH	-	Lactate dehydrogenase
LDL	-	Low density lipoprotein
LS	-	PIF fermentation supernatant of *L. casei*
mg	-	Milli gram
mL	-	Milli litre
mM	-	Milli molar
MMR	-	Mismatch repair genes
MP	-	Mature potato peel
MPE	-	Ethyl acetate extract of mature potato peel
MPM	-	Methanol extract of mature potato peel
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2CO_3	-	Sodium carbonate
NADH	-	Nicotineamide adenine dinucleotide
NaOH	-	Sodium hydroxide
NBT	-	Nitro blue tetrazolium chloride
NCD	-	Non communicable diseases
Nm	-	Nano molar
NO	-	Nitric oxide
NO	-	Nitric oxide
OD	-	Optical Density
OH	-	Hydroxyl radical
OHC	-	Oil holding capacity
PA	-	Propionic acid
PARP	-	Poly(ADP-ribose) polymerase
PBS	-	Phosphate buffered saline

PBS	-	Phosphate buffered saline
PDH	-	Pyruvate dehydrogenase
PDHB	-	Pyruvate dehydrogenase (lipomaide) beta
PI	-	Plantain Inflorescence
PIE	-	Ethyl acetate extract of Plantain Inflorescence
PIF	-	Soluble dietary fibre from Plantain Inflorescence
PIM	-	Methanol extract of Plantain Inflorescence
PMF	-	Peptide mass fingerprinting
PMS	-	Phenazine methosulphate
POLR2C	-	Polymerase RNA II subunit C
PRDX 6	-	Peroxiredoxin 6
QE	-	Quercetin equivalence
RB	-	Rice bran
RBE	-	Ethyl acetate extract of Rice bran
RBM	-	Methanol extract of Rice bran
RC	-	Raw Cumin
Rh123	-	Rhodamine 123
ROS	-	Reactive oxygen species
SC	-	Spent Cumin
SCFA	-	Short chain fatty acid
SD	-	Standard deviation
SDF	-	Soluble dietary fibre
SEM	-	Scanning electron microscopy
SGJ	-	Simulated gastric juice
SIJ	-	Simulated intestinal juice
SLC25A35	-	Solute carrier family 25 member 35
SNP	-	Sodium nitroprusside
TBS	-	Tris-buffered saline
TBST	-	Tris-buffered saline containing 0.1% Tween 20
TCA	-	Tri chloro acetic acid
TFC	-	Total flavonoid content
TPC	-	Total phenolic content
TPI 3	-	Triose phosphate isomerase 3
TR	-	Trolox
WB	-	Wheat bran
WBE	-	Ethyl acetate extract of Wheat bran
WBM	-	Methanol extract of Wheat bran
WHC	-	Water holding capacity
WHO	-	World Health Organization
WRC	-	Water retention capacity
XIAP	-	X-linked inhibitor of apoptosis
YP	-	Young potato peel
YPE	-	Ethyl acetate extract of young potato peel
YPM	-	Methanol extract of young potato peel
$\Delta\psi_m$	-	Mitochondrial membrane potential