Development of novel natural food preservatives from phenolics of peels of five selected varieties of potato with special emphasis on omega-3 fatty acids fortified functional foods

THESIS SUBMITTED FOR THE DEGREE OF

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BY

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled ***"Development of novel natural food preservatives from phenolics of peels of five selected varieties of potato with special emphasis on omega-3 fatty acids fortified functional foods"*** *submitted by* ***Abhishek Bhattacharya*** *who got his name registered on February 9, 2018 for the award of Ph.D. (Science) degree of* ***Jadavpur University****, is absolutely based upon his own work under the supervision of* ***Prof. Rabi Ranjan Chattopadhyay*** *and that neither this thesis nor any part of it has been submitted for either any degree/diploma or any other academic award anywhere before.*

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DECLARATION

I hereby declare that the research embodied in the thesis entitled "**Development of novel natural food preservatives from phenolics of peels of five selected varieties of potato with special emphasis on omega-3 fatty acids fortified functional foods***"* is carried out by me at Agricultural and Ecological Research unit, Indian Statistical Institute, Kolkata, India under the supervision of Professor Rabi Ranjan Chattopadhyay. This work is original and not submitted in part or full for any degree or diploma to this or any other university. All the ideas and references have been duly acknowledged.

Date: 19th September 2022

Place: Kolkata (ABHISHEK BHATTACHARYA)

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"The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day."

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(ABHISHEK BHATTACHARYA)

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and following AOCS guidelines.

PREFACE

Oxidation of lipids has long been recognized as a major problem in the storage of fatty acids in foods. Oxidation of unsaturated fatty acids in foods give rise to formation of unhealthy compounds which is directly related to economic, nutritional and storage problems of food (Jacobsen et al., 2013). Omega-3 fatty acids have immense health benefits and in recent years to increase the nutritional value of foods and to reduce the risk of chronic diseases, omega-3 fatty acids enriched oils are added to foods. But developing omega-3 fatty acids fortified stable functional food is challenging, because the two principle omega-3 fatty acid components eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are highly susceptible to auto-oxidation even at room temperature due to high degree of unsaturation in their molecular structures (Anbudhasan et al, 2014). Besides, foodborne diseases caused by consumption of spoiled and contaminated food is a major public health problem worldwide (Lorenzo et al., 2018). These problems should be addressed. To overcome these problems, synthetic antioxidants and antimicrobials are generally used in the food industry. But these synthetic preservatives have accumulated evidence that they could be toxic and carcinogenic (Sharma 2015). Therefore, innovation should continue to seek a more potent, safe and effective novel natural antioxidants and antimicrobials as preservatives especially from plant origin for protecting foods both from oxidative deterioration as well as microbial spoilage and contamination to extend the shelf life of foods especially omega-3 fatty acids fortified functional foods.

Plant polyphenols may serve as a potential source of such compounds due to their strong antioxidant and antimicrobial potential with multimodal action (Chibane et al, 2019; Martinengo et al, 2021). In recent years, the use of fruit and vegetable waste has becoming more popular to be studied by taking into account that these residues are one of the important sources of polyphenols. Therefore, proper re-cycling and use of phenolic rich fruit and vegetable waste could generate economic gains for the food industry, contribute to reducing food nutritional problems, would produce beneficial health effects and would reduce environmental pollution (Torres-Leon et al, 2018;Kumar et al, 2020). Potato peels are rich in phenolic compounds. The phenolic content in potato peels is higher than potato tuber. The

potato processing industries generate substantial quantities of phenolic rich potato peels as waste byproduct and has become a major disposal problem for the industry concerned (Habeebullah et al, 2010). Potato peels are also substantially generated as waste byproduct by households. But, these phenolic rich potato peels may serve as a potential source of natural food preservatives because of their strong antioxidant and antimicrobial potential (Akyol et al, 2016; Geberchristos et al, 2020). The growing interest in replacing synthetic antioxidant and antimicrobial compounds with natural ones has stimulated a search for these bioactive compounds from waste potato peels. Therefore, the present work aims to project the waste potato peels as a potential source of natural antioxidants and antimicrobials and may serve as a promising alternative to synthetic food preservatives for preserving foods especially omega-3 fatty acids fortified functional foods in the food pharmaceutical industries.

To achieve the goal, in the present work a detailed and systematic study on (i) individual and combined antioxidant and antimicrobial potential of phenolic extracts of peels of five selected varieties of potato (*Kufri Chipsona-1, Kufri Chipsona-3, Kufri Chipsona-4, Kufri Jyoti* and *Kufri Chandramukhi*) in various *in vitro* models and in omega-3 fatty acids fortified food model systems were evaluated, (ii) bioactive compounds responsible for antioxidant and antimicrobial potential were isolated, identified and chemically characterized , (iii) cytotoxic potential of active extracts of potato peels as well as their bioactive compounds if any was evaluated , (iv) possible modes of antioxidant and antimicrobial action of bioactive compounds from active potato peel extracts were elucidated. The ultimate goal of this study was to develop a more potent, safe and effective, novel natural antioxidants and antimicrobials from potato peel waste as preservatives for protecting foods especially omega-3 fatty acids fortified functional foods both from oxidative deterioration and microbial spoilage and contamination which may serve as a promising alternative to synthetic food preservatives.

The thesis contains 9 Chapters. **Chapter 1** deals with a brief background of the present work along with objectives and rationale of this study. **Chapter 2** reviews the available literature. **Chapter 3** describes the materials and methods used to fulfil the objectives of the study. **Chapter 4** reported individual effect on antioxidant and antimicrobial

potential of phenolic extracts of peels of selected varieties of potato in various *in vitro* models (Objective 1). **Chapter 5** deals with individual antioxidant potential of phenolic extracts of peels of selected varieties of potato against oxidation of omega-3 fatty acids enriched food supplement and omega-3 fatty acids fortified various functional foods (Objective 2). **Chapter 6** describes the combined antioxidant potential of active extracts of potato peels in various *in vitro* models (Objective 3). **Chapter 7** deals with isolation, identification and chemical characterization of bioactive compounds responsible for antioxidant and antimicrobial efficacy alone and in combination along with cytotoxicity evaluation (Objective 4). **Chapter 8** describes possible modes of antioxidant and antimicrobial action of bioactive compounds alone (Objective 5) and in combination and Chapter 9 deals with summary, conclusive remarks and recommendations for future research.

SYMBOLS AND ABBREVIATIONS

ATCC	American Type Cell Culture
ATP	Adenosine triphosphate
β	Beta
BHIA	Brain-Heart Infusion Agar
BHIB	Brain-Heart Infusion Broth
Cytochrome C	Cytochrome Complex
C	Celsius
CFU	Colony Forming Unit
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DPPH	1, 1-diphenyl-2-picrylhydrazyl
e.g.	for example
et al.	and others
h	Hour
HaCaT cells	Human Keratinocyte cells
IZD	Inhibition Zone Diameter
IC_{50}	50% Inhibitory Concentration
i.e.	that is
L	Liter
mg/ml	milligram per millilitre
MTCC	The Microbial Type Culture Collection and Gene Bank
µg/ml	microgram per millilitre
mm	Millimetre
mM	Millimolar

mg/L	milligram per liter
ml/min	milliliter per minute
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
M	Molar
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
n	number of samples
nm	Nanometer
OD	Optical Density
PBS	phosphate-buffered saline
R^2	Regression squared
R_f	Retention factor
RPMI	Rosewell Park Memorial Institute Medium
SPSS	Statistical Package for the Social Sciences
SD	Standard Deviation
TLC	Thin Layer Chromatography
TSA	Tryptic Soy Agar
v/v	Volume by volume
UV/Vis	Ultraviolet/Visible
w/v	weight/volume

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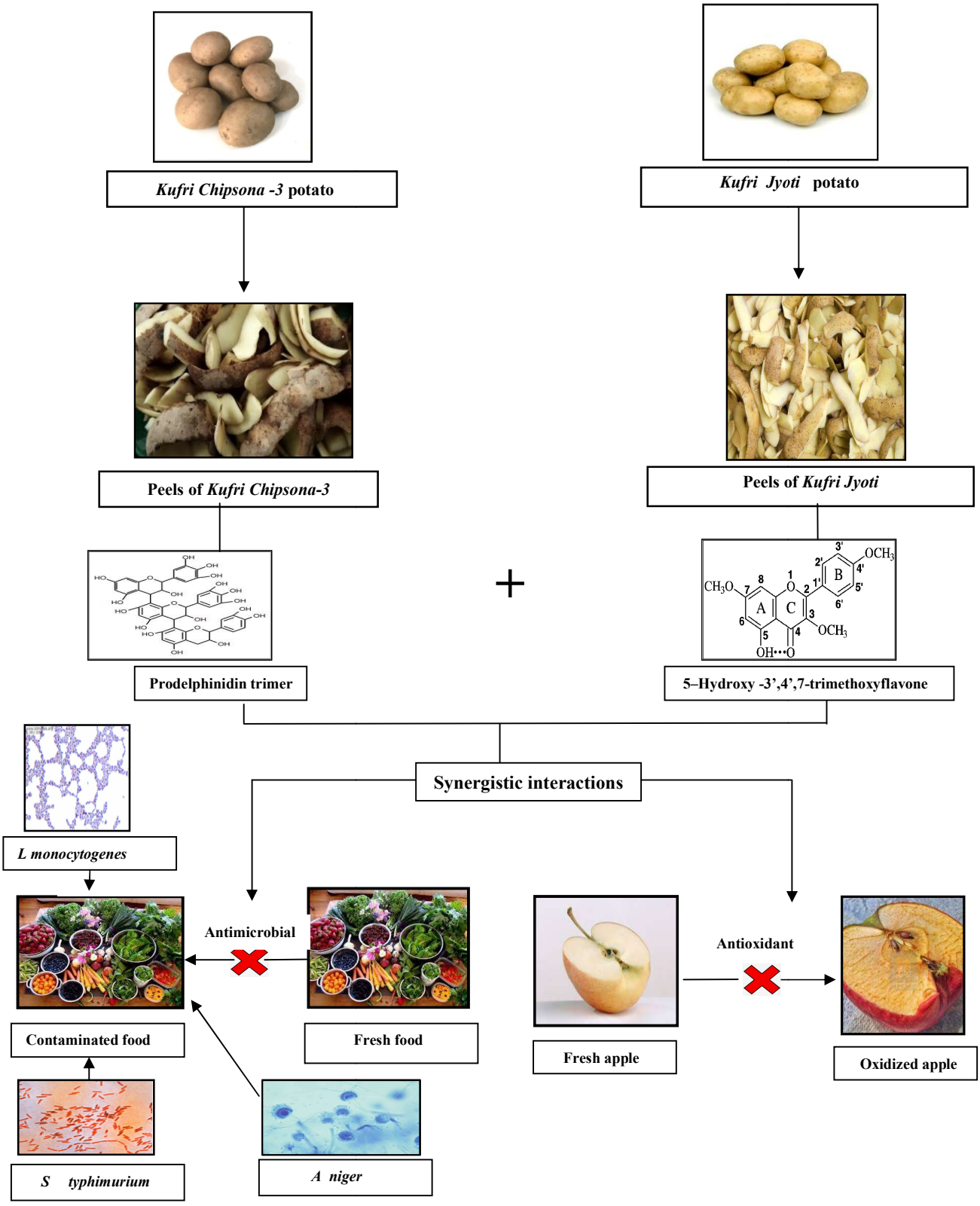
GRAPHICAL ABSTRACT



CHAPTER 1

INTRODUCTION

Background Information

Both omega-6 and omega-3 polyunsaturated essential fatty acids (PUFAs) are important components of cell membrane and human body needs them for many functions from building healthy cells to maintaining brain and nerve functions (Djuricic and Calder, 2021) . But they have opposite effects when it comes to cardiovascular health and inflammatory responses, e.g. eicosanoids derived from omega-6 fatty acids (viz. arachidonic acid) have pro-inflammatory property , and due to their pro-inflammatory property they can induce a number of diseases viz. cardiovascular disease, arthritis, diabetes, Alzheimer's disease and even cancer whereas eicosanoids derived from omega-3 fatty acids (viz. pentaenoic acid) have anti-inflammatory property and due to their anti-inflammatory property, they can reduce these diseases (Schmitz and Ecker, 2008). Western diets contain high level of omega-6 fatty acids but very low level of omega-3 fatty acids leading to omega-6/omega-3 fatty acids ratio of 15:1 to 20:1 or even higher. Ideally this ratio should be around 1:1 or to a maximum of 4:1. A lower ratio of omega-6/omega-3 fatty acids in our diet is more desirable in reducing the risk of many of the cardiovascular diseases and other chronic diseases (Simopoulas, 2008)**.** Therefore, to increase the nutritional value of foods and reduce the risk of chronic diseases, in recent years, omega-3 fatty acids enriched oils are added to foods. But, developing omega-3 fatty acids fortified stable functional foods is challenging because, the two principal omega-3 fatty acid components eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are highly susceptible to auto-oxidation due to high degree of unsaturation in their molecular structure (Nogueria et al, 2019). Besides foodborne diseases caused by consumption of spoiled and contaminated foods, oxidation of lipids is another major public health problem worldwide (Hussain, 2016; Gizaw, 2019)**.** The synthetic antioxidants and antimicrobials are generally used to overcome these problems but they have a number of adverse health effects. Hence, special emphasis should be given to seek safe and effective novel, natural antioxidant and antimicrobial agents as preservatives from other sources especially from plant origin to protect lipid bearing foods especially omega-3 fatty acids fortified functional foods both from oxidative deterioration and microbial spoilage and contamination with a view to extend the shelf life of omega-3 fatty acids fortified functional foods and also to reduce the risk of chronic diseases. Among the natural sources, plant polyphenols may serve as a potential source of such compounds because they contain diverse group of phytocompounds with preservative properties i.e. strong antioxidant and antimicrobial properties in addition to their well-established health promoting benefits

(Chibane et al, 2019; Martinengo et al, 2021; Shahidi and Ambigaipalan, 2015). Potato peels are rich in phenolic compounds and the concentration of phenolic compounds is higher in potato peels than in potato tuber (Akyol et al, 2016). Potato processing industries generate substantial quantities of potato peels as waste by-product which creates a major disposal problem for the industry concerned. Besides, potato peels are also produced as waste by-product by households. But these waste by-product phenolic rich potato peels could be a valuable source of natural antioxidants and antimicrobials for protecting foods both from oxidative deterioration and microbial spoilage and contamination (Samarin et al, 2012; Prasad and Pushpa, 2007; Gebrechritos and Chen, 2018) . Although, a number of studies on antioxidant and antimicrobial potential of various potato peel extracts have been reported by several workers (Prasad and Pushpa, 2007; Samarin et al, 2012; Hussain, 2016), detailed knowledge about their food preservative properties i.e. antioxidant and antimicrobial efficacy against lipid bearing foods especially omega-3 fatty acids fortified functional foods is lacking. This knowledge is particularly important for developing a more potent, safe and effective, novel natural antioxidants and antimicrobials for preserving foods especially omega-3 fatty acids fortified functional foods. With a view to fulfil the lacunae mentioned above, the present work has been designed accordingly to seek safe and effective novel, natural antioxidant and antimicrobial agents from phenolics of peels of five selected varieties of potato (*Kufri Chipsona-1, Kufri Chipsona-3, Kufri Chipsona-4, Kufri Jyoti* and *Kufri Chandramukhi*) for protecting foods especially omega-3 fatty acids fortified functional foods both from oxidative deterioration and microbial spoilage and contamination which subsequently may help to develop omega-3 fatty acids fortified stable functional foods.

Objectives

- Objective 1: To evaluate *in vitro* antioxidant and antimicrobial efficacy of phenolic extract of peels of selected varieties of potato at their individual effect.
- Objective 2: To evaluate antioxidant potential of phenolic extract of peels of selected varieties of potato against oxidation of omega-3 fatty acids enriched food supplement and omega-3 fatty acids fortified various functional foods at their individual effect.
- Objective 3: To evaluate combined antioxidant and antimicrobial potential of active phenolic extracts of peels of selected varieties of potato in various *in vitro* models.

- Objective 4: To isolate, identify and characterize the bioactive antioxidant and antimicrobial compounds from active phenolic extracts of peels of selected varieties of potato and evaluation of their cytotoxic potential, if any.
- Objective 5: To elucidate possible mode of antioxidant and antimicrobial action of bioactive compounds isolated from active extracts of potato peels alone and in combination.

Rationale of present work

Functional foods provide important nutrients that can help to protect human health from different types of diseases. Omega-3 fatty acids have immense health benefits. In recent years, to increase the nutritional value of foods and reduce the risk of chronic diseases viz. cardiovascular disease, arthritis, Alzheimer's disease, diabetes, and cancer, omega-3 fatty acids enriched oils are added to foods. But, developing omega-3 fatty acid fortified stable functional food is challenging because two principal omega-3 fatty acid components eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are highly susceptible to oxidation even at room temperature due to high degree of unsaturation in their molecular structures. Besides, all food products including omega-3 fatty acids fortified functional foods can be contaminated by a variety of pathogenic and spoilage microorganisms, the former causing foodborne diseases and the latter causing significant economic losses for the food industry due to undesirable effects on the food properties. Food preservatives play a vital role in preventing oxidative deterioration and protecting against microbial spoilage and contamination of foods. Consumers demand food products that are safe and preferably free of synthetic preservatives because synthetic preservatives have a numbers of adverse effects. These problems demand that a renewed effort should be made to seek a more potent, safe, effective, novel and natural preservatives from other sources especially from plant origin. The use of a combination of preservation factors has been suggested as a viable alternative to synthetic preservatives to preserve foods both from oxidative deterioration and microbial spoilage and contamination for extended periods of time. Because it is well established, that a single molecule or agent of plant origin having both antioxidant and antimicrobial properties is considered to be more effective than synthetic preservatives. Therefore, innovation should continue to seek a more potent safe and effective, novel, natural food preservatives having dual preservative properties i.e. both antioxidant and antimicrobial efficacy at sufficiently low concentration from other sources especially from plant origin for extending the shelf life

of food products especially omega-3 fatty acids fortified functional foods. In food processing industries particularly potato processing industries, a huge volume of potato peels are generated as waste byproduct. The most common cause of environmental pollution is associated with organic wastes decomposition of potato peels by microbes and has become a major disposal problem for the industry concerned. But, these potato peels are rich in phenolic compounds and may serve as a potential source of novel natural food preservatives for protecting foods both from oxidative deterioration and microbial spoilage and contamination and detailed knowledge about it is lacking. Keeping these in mind, in the present work an attempt has been made for a detailed and systematic study on antioxidant and antimicrobial potential of peel extracts of five selected varieties of potato (*Kufri Chipsona-1*, *Kufri Chipsona-3*, *Kufri Chipsona-4*, *Kufri Jyoti* and *Kufri Chandramukhi*) to seek safe and effective, novel natural antioxidant and antimicrobial agents which may serve as a promising natural alternative to synthetic food preservatives especially for extending the shelf life of omega-3 fatty acids fortified functional foods. The important findings obtained from this study may significantly contribute in the field of study which subsequently may help in developing a more potent, safe and effective, novel, natural antioxidant and antimicrobial agent from peel extracts of selected varieties of potato for extending the shelf life of foods especially omega-3 fatty acids fortified functional foods in food and pharmaceutical industries and also may help in converting potato peels from waste by-product to value added products.

REVIEW OF LITERATURE

- ***Food Safety and Human Health***

Food safety is an important issue that affects people of every age, race, gender, and income level all over the world (Gizaw, 2019). Food safety deals with safeguarding food products from microbial growth and hazardous chemical agents (Radovanovic, 2011; Uyttendaele et al., 2016). Safe and nutritious food is the source of human energy and the first defense against diseases. Therefore, for sustaining life and promoting good health, access to sufficient amounts of safe and nutritious food is essential. Unsafe food containing harmful microbes or toxic chemicals cause more than 200 diseases—ranging from diarrhea to cancer (WHO, 2019). Ensuring food safety to protect public health remains a significant challenge in both developing and developed countries (WHO, 2006).

- ***Importance of food preservation***

Foods are perishable by nature and require protection at all points in its journey from its source until it reaches the consumers. Foods are spoiled and contaminated by various factors of which oxidative spoilage or deterioration and microbial spoilage and contamination are most important. Deleterious changes in foods caused by lipid oxidation include loss of flavour, development of off-flavours, loss of colour, nutrient value and functionality, and the accumulation of toxic compounds which may be detrimental to the health of consumer (Addis, 1986). Microbial spoilage is caused by microorganisms like fungi (moulds, yeasts) and bacteria. They spoil food by growing in it and producing substances that change the colour, texture and odour of the food. Eventually the food will be unfit for human consumption mainly due to oxidative deterioration and microbial spoilage and contamination. Food loss by either spoilage or contamination, affects food industry and consumers leading to economic losses and increased hospitalization costs respectively (Lorenzo et al., 2018). Food preservation is the maintenance of safe and nutritious food for an extended period of time. The central objectives of food preservation are : (i) to prevent food spoilage until it can be consumed, (ii) to improve and maintain nutritional value, (iii) to enhance quality, (iv) to reduce wastage, (v) to enhance customer acceptability, (vi) to make food more readily available, (vii) to offer the opportunity to have a wide variety of foods year-round, (viii) to reduce risk of diseases and deaths caused by consumption of unsafe food, (ix) to reduce burden of financial losses due to foodborne illnesses, and (x) to facilitate processing food

items (Rahaman, 2007; Amit et al., 2017). Therefore, preservation of foods is vital both for human health and food industry and it should be done from production to consumption.

- ***Food spoilage***

Food spoilage is the process owing to which food edibility diminishes. Oxidative deterioration and microbial spoilage and contamination are considered to be mainly responsible for food spoilage. The initial stages of food spoilage caused by oxidative deterioration and food spoilage microbes are being detected by colour, smell, flavour, and texture of food. On the other hand, foods contaminated with pathogenic microorganisms usually do not look bad, taste bad, or smell bad. It is impossible to determine whether a food is contaminated with pathogenic microorganisms without microbiological testing. To avoid potential problems in foods, it is very important to control oxidative deterioration and inhibit the growth of these microorganisms in food products. Various microbiological, chemical, enzymatic or physical actions lead to food spoilage and rendering the food product unacceptable to consumers (Steele, 2004; Amit et al., 2017).

(a) Microbial spoilage

The common type of microorganisms responsible for the spoilage of foods and food products are bacteria, yeast, and molds (Lianou et al., 2016). When they reach in food products they grow by utilizing the nutrients and produce metabolites that cause food spoilage (Parlapani et al., 2013). Foodborne disease caused by consumption of contaminated food is another pervasive food safety problem and has become a major public health problem worldwide (Kirk et al., 2017). There are two types of foodborne diseases: infection and intoxication. Foodborne infection is caused by the ingestion of food containing live pathogenic bacteria which grow and establish themselves in the human intestinal tract. On the other hand, foodborne intoxication is caused by ingesting food containing toxins formed by bacteria which resulted from the bacterial growth in the food item (Addis and Sisay, 2015).

- ***Factors affecting microbial spoilage***

The factors that can affect the microbial spoilage of food can be divided into two classes: Intrinsic factors and Extrinsic factors. Intrinsic factors are those that are characteristic of the food itself (e.g. pH, water activity, nutrient content, and oxidation–reduction potential) (Steele, 2004; Doyle, 2009). Extrinsic factors are those that refer to the environment surrounding the food (mainly temperature, humidity and oxygen) (Jay, 2000; Steele, 2004).

By understanding the factors affecting the growth of microorganisms in food we can know how to keep food safe to eat. This knowledge can also help us to work out how to preserve food for longer.

➢ ***Intrinsic factors***

- **$_P$H**

Most microorganisms grow best at close to the neutral pH value (pH 6.6 to 7.5). Only a few microorganisms grow in very acid conditions below a pH of 4.0. Bacteria grow at a fairly specific pH for each species, but fungi grow over a wider range of pH values.

- **Water activity (a_w)**

Microorganisms need a moist environment for their growth. The water requirements of microorganisms are described in terms of water activity (a_W), a measure of how much water is present. The water activity of pure water is $a_W = 1.00$. Most foodborne pathogenic bacteria require a_W to be greater than 0.9 for growth and multiplication; however, *Staphylococcus aureus* may grow with a_W as low as 0.86. But *Staphylococcus aureus* cannot grow and multiply in drier food like bread, which has $a_W = 0.7$, although fungi can.

- **Nutrient content**

In order to grow, multiply and function normally, microorganisms require a range of nutrients such as nitrogen, vitamins and minerals. Microorganisms therefore grow well on nutrient-rich foods.

- ***Structure of food***

The natural covering of some foods provides excellent protection against the entry and subsequent damage by spoilage organisms. Examples of such protective structures are the skin of fruits and vegetables such as tomatoes and bananas.

➢ ***Extrinsic factors***

Extrinsic factors are factors in the environment *external* to the food, which affect both the microorganisms and the food itself during processing and storage. Extrinsic factors include temperature, humidity and oxygen.

- ***Temperature***

Temperature plays a significant role in the spoilage of fruits and vegetables. Different microorganisms grow over a wide range of temperatures. Some microorganisms like to grow in the cold, some like to grow at room temperature and others like to grow at high temperatures. This is of paramount importance in food safety, because if we know the temperature growth ranges for dangerous microorganisms, it helps us to select the proper temperature for food storage to make them less able to grow and reproduce.

- ***Humidity***

The humidity of the storage environment is an important factor for the growth of microorganisms at the food surfaces. If we store food in a dry atmosphere, microorganisms are less able to grow than if the food is stored in a humid (moist) environment. Therefore, dry conditions are better for food storage than moist conditions.

- ***Oxygen***

Many microorganisms need oxygen in order to develop and reproduce: these are called aerobic microorganisms (e.g. *Escherichia coli*). If we keep food in a low oxygen environment, aerobic bacteria cannot grow and multiply. Conversely, there are some microorganisms that grow without oxygen, called anaerobic microorganisms e.g. *Clostridium botulinum*.

(b) Chemical spoilage

Chemical reactions in food are responsible for changes in the colour and flavour of foods during processing and storage. Foods are of best quality when they are fresh, but after fruits and vegetables are harvested or animals are slaughtered, chemical changes begin automatically within the foods and lead to deterioration in quality. During autooxidation, fats in food break down, yield various oxidation products and become rancid (smell bad). Enzymatic mechanisms also catalyze the oxidation of lipids in foods by lipoxygenase and cytochromes P450 to produce hydro-peroxides (Kowbow, 1992; Feussner and Wasternack, 2002). Some of the chemical spoilage responsible for food quality deterioration are listed below.

- ***Food quality deterioration caused by oxidation of lipids in foods***

Lipid oxidation is a major cause of food quality deterioration and also great problem in the storage of fatty acids in foods. Oxidation affects many interactions among food constituents, leading to undesirable products that are detrimental to human health. Lipid oxidation reduces the shelf life of many complex food products and nutritive value of food by limiting the content of essential polyunsaturated fatty acids (Bottcher et al., 2015). Lipid oxidation are mainly of two types: autoxidation and enzymatic oxidation.

(i) Autoxidation

Autoxidation of lipids in food leads to breakdown of lipids and formation of wide range of oxidation products (Kowbow, 1992). When lipid substrate exposed to heat, light or metal ions their hydrogen atom of double bond is extracted and free or alkyl radical is formed (Lee et al., 2004). These free radicals reacts with oxygen and yield peroxy radicals by subtracting hydrogen atom from another unsaturated fatty acids. It leads to the formation of primary oxidation products called hydro-peroxides (Julia et al., 2015). The length of propagation cycle is directly proportional to the degree of lipid unsaturation (Kowbow, 1992). These primary oxidation products are not stable and further break down into carbonyl compounds such as aldehyde, ketones and alcohols which are toxic to human health (Tirosh et al., 2015). Oxidation of omega-3 fatty acids is an example of autoxidation.

Oxidation of omega -3 fatty acids

Marine fish oil, walnut oil, flaxseed oil are rich sources of omega-3 fatty acids. Omega-3 fatty acids enriched oils without having any antioxidant undergoes rapid autoxidation due to high degree of unsaturation in their molecular structures (Umesha and Naidu, 2015). Different fatty acids have different rate of oxidation. During omega-3 fatty acid oxidation, the peroxide value (PV) which is the primary oxidation product of omega-3 fatty acid increases and omega-3 fatty acids content decreases (Turner et al., 2006). The two principal omega-3 fatty acids components EPA and DHA have five and six double bonds respectively. These are highly susceptible to autooxidation by three phases called initiation, propagation and termination (Kerrihard, 2015). When molecular oxygen reacts with these polyunsaturated fatty acids, during initiation phase free radicals and peroxides are produced. But in propagation phase some other reactive chemical products are produced from peroxides such as dihydroperoxides, because peroxides are unstable and have active methylene group. In

termination phase non-reactive secondary oxidation products such as hydrocarbon, aldehydes and ketones are formed and cause off-flavour (Kolanowski et al., 2007). In these polyunsaturated fatty acids peroxide value is not a good indicator of autoxidation because their hydro-peroxides are not stable and secondary oxidation products play important role in oxidative deterioration in these fatty acids.

(ii) Enzymatic Oxidation

Enzymatic mechanisms also catalyze the oxidation of lipids in foods by lipoxygenase and cytochromes P450 to produce hydro-peroxides. But oxidation of polyunsaturated fatty acids initiated by lipoxygenases via a free radical mechanisms to form specific hydro-peroxides (Kowbow, 1992; Feussner and Wasternack, 2002). Lipoxygenase enzyme molecules contain one heme iron, 1, 4-pentadiene system that desired in the fatty acid as a substrate for lipoxygenase. Ferric form of lipoxygenase enzyme oxidized 1,4 – pentadiene fatty acid moiety into pentadienyl radical, which react with molecular oxygen to form peroxyl radical and finally produce hydroperoxides (Gajera et al., 2015; Mandal et al., 2014). These hydro-peroxides are precursor of further transformation by enzymatic chemical reaction, because lipoxygenase reaction initiates the synthesis of a single fatty acid hydro-peroxide from substrate of fatty acids (Mandal et al., 2014).

In order to prevent lipid oxidation in food, the mechanisms of lipid oxidation should be comprehensively understood and in particular the control of free radical formation is very important because free radical is able to initiate free radical chain reactions. Iron and copper ions also play an important role in lipid peroxidation and act as pro-oxidant which can directly and indirectly catalyze the initiation of lipid peroxidation by extracting hydrogen atom from lipid molecule. Antioxidants and chelating agents are therefore the most helpful inhibitors of lipid oxidation.

(c) Physical Spoilage

Food can also be spoiled by physical changes. The factors responsible for physical spoilage are moisture content, temperature, glass transient temperature, crystal growth, and crystallization (Amit et al., 2017).

- ***Moisture content***

A major cause of degeneration of food products is the change in their moisture content. Moisture transfer depends upon the water activity (a_w) of food item. Generally, water activity of foods at normal temperature is 1.0 **(**Fabunmi, 2015).

- ***Temperature***

Temperature plays a significant role in the spoilage of fruits and vegetables. Low temperature can have a detrimental impact on foods that are susceptible to damage by freezing. When temperature is lower, food products become partially frozen, this leads to disruption of cells which in turn damages the product (Steele, 2004).

- ***Glass transition temperature***

Glass transition (Tg) is a physical property of food polymers. It is the temperature range where food polymers undergo a phase change from rigid/glassy to soft. Food polymers can be proteins, starch and non-starch polysaccharides (Ahmed and Rahman, 2014). The success of freeze drying, spray drying, and extrusion and the stability of dehydrated foods against flow, collapse, and crystallization is based on the control of the glassy state during the dehydration process and storage. Glass transition data is also important in manufacturing of various cereal products and snacks by extrusion (Roos, 1991).

- ***Crystal growth and crystallization***

Freezing can maintain a low-temperature environment inside food, reducing water activity and preventing microorganism growth. However, when ice crystals are large, present in high amounts, and/or irregularly distributed, irreversible damage to food can occur. Therefore, ice growth is a vital parameter that needs to be controlled during frozen food processing and storage. Freezing can also lead to degradation of food. Foods, which go through slow freezing or multiple freeze, can severely suffer owing to crystal growth. They undergo extracellular ice growth. Rapid freezing facilitates the growth of ice within food cells, and these foods are more stable as compared to slow freezing processed foods (Reid, 1990). For minimizing large ice crystal growth, emulsifiers and other water binding agents are added to food during freezing cycles. Foods having high sugar content can undergo sugar crystallization either by virtue of moisture accumulation or by application of increased temperature (Levine and Slade, 1988). Consequently sugar comes to the surface from inside,

which is readily visible as a gray or white appearance. Staling of sugar cookies, graininess in candies and ice creams results from sugar crystallization (Steele, 2004).

- ***Food preservation processes***

Foods are perishable or deteriorative by nature and therefore undergo spoilage due to microbial, chemical, or physical actions. The growth of most microorganisms as well as oxidative deterioration in foods can be hindered or delayed by manipulating storage temperature, decreasing water activity, lowering pH, adding preservatives, and also by virtue of proper packaging (Tianli et al., 2014). Based on the mode of action, major food preservation techniques can be categorized as: slowing down or inhibiting chemical deterioration and microbial growth; directly inactivating bacteria, yeast, moulds and enzymes and avoiding recontamination before and after processing. Different traditional categories or techniques, such as drying, chilling, freezing, and fermentation, had been evolved in the past to preserve foods and to maintain their nutrition value and texture. With time and growing demands, preservation techniques have been improved and modernized. Irradiation, high pressure food preservation, and pulsed electric field effect, hurdle technology are the latest innovations used to increase shelf life of foods. Different chemical reagents have also been introduced as food additives and preservatives. However, there are growing concerns of using chemical additives and preservatives in food items because of possible health hazards. Some of these categories or techniques for food preservation are:

(a) Physical processing

- ***Drying***

Drying or dehydration is the process of removing water from a solid or liquid food by means of evaporation. The purpose of drying is to obtain a solid product with sufficiently low water content. It is one of the oldest methods of food preservation (Berk, 2013). In this method, the moisture content is lowered to the point where the activities of these microorganisms are inhibited (Jay, 2000). Most microorganisms are inactive at water activity below 0.9. Most of the microorganisms cannot grow at water activity below 0.88 (Syamaladevi et al, 2016).

- ***Pasteurization***

Pasteurization is a physical preservation technique in which food is heated up to a specific temperature to destroy spoilage-causing microorganisms and enzymes **(**Baker et al., 1997;

Shenga et al, 2010). Almost all the pathogenic bacteria, yeasts, and molds are destroyed by this process and as a result, the shelf life of food increases (Laudon, 2009; Cavazos, 2016).

- ***Thermal sterilization***

Thermal sterilization is a heat treatment process that completely destroys all the viable microorganisms resulting in a longer period of shelf life. Retorting and aseptic processing are two categories of thermal sterilization (Rahman, 2007).

(i) Retorting

Retorting is defined as the packaging of food in a container followed by sterilization (Knechtges, 2012). Foods with pH above 4.5 require more than 100 °C as sterilization temperature.

(ii) Aseptic packaging

Aseptic packaging involves placing commercially sterilized food in a sterilized package which is then subsequently sealed in an aseptic environment. Conventional aseptic packaging utilizes paper and plastic materials. Sterilization can be achieved either by heat treatment, by chemical treatment, or by attributing both of them (Potter and Hotchkiss, 1999). Aseptic packaging is highly used to preserve juices, dairy products.

- ***Preservation by low temperatures***

(i) Chilling

Food preservation by cooling and freezing are the oldest methods using natural low temperatures. Chilling is used to reduce the rate of biochemical and microbiological changes and hence to extend the shelf life of fresh and processed foods. Chilling also reduces the rate of enzymatic changes and retards respiration of fresh foods. Chilling prevents the growth of thermophilic and many mesophilic microorganisms. The main microbiological concerns with chilled foods are a number of pathogens that can growth during extended refrigerated storage below 5˚C or as a result of any increase in temperature (temperature abuse) and this may cause food poisoning (Saravacos and Kostaropoulous, 2002; Indira and Sudheer, 2007).

(ii) Freezing

Freezing is a method of food preservation in which the temperature of food is reduced below it freezing point and a proportion of the water undergoes a change in state to form ice crystals, resulting concentration of dissolved solutes in unfrozen water respectively reducing the water activity (a_w) and pH values. In freezing, preservation is achieved by a combination of low temperatures and reduced water activity (Velez-Ruiz and Rahman, 1999).

- **Irradiation**

Irradiation is a physical process in which substance undergoes a definite dose of ionizing radiation (IR) (Arvanitoyannis, 2010). IR can be natural and artificial. Natural IR generally includes X-rays, gamma rays, and high-energy ultraviolet (UV) radiation; artificially generated IR is accelerated electrons and induced secondary radiation (Sommers, 2010). The effects of IR include: (a) disinfestations of grains, fruits, and vegetables, (b) improvement in the shelf life of fruits and vegetables by inhibiting sprouting or by altering their rate of maturation and senescence, and (c) improvement in shelf life of foods by the inactivation of spoilage organisms and by inactivating foodborne pathogens (Heldman and Moraru, 2010; Kanat et al, 2006).

- **High-pressure food preservation**

High hydrostatic pressure or ultra-high pressure processing (HPP) technology involves pressure attribution up to 900 MPa to kill microorganisms in foods. This process also inactivates spoilage of foods, delays the onset of chemical and enzymatic deteriorative processes, and retains the important physical and physiochemical characteristics of foods. HPP has the potential to serve as an important preservation method without degrading vitamins, favors, and colour molecules during the process (Dunne, 2007; Koutchma et al., 2016).

- ***Pulsed electric field***

Pulsed electric field food processing is defined as a technique in which food is placed between two electrodes and exposed to a pulsed high voltage field (20– 40 kV/cm). Generally, the pulsed electric field problem treatment time is less than one second (Brennan, 2006). Low processing temperature and short residence time of this process allow a highly effective inactivation of microorganisms (Sun, 2014). Pulsed electric field processing is

much effective to destroy Gram-negative bacteria than Gram-positive bacteria. Vegetative cells are much more sensitive than spores to this process (Jay, 2000). Pulsed electric field technology retains taste, flavor, and colour of the foods. Furthermore, this technique is not toxic (Mohammed et al., 2016).

(b) Biological process

- ***Fermentation***

Fermentation method uses microorganisms to preserve food. This method involves decomposition of carbohydrates with the action of microorganisms and/or the enzymes (Shivsankar, 2002). Bacteria, yeasts, and molds are the most common groups of microorganisms involved in fermentation of a wide range of food items, such as dairy products, cereal-based foods, and meat products (Battock and Azam-Ali, 1998; Katz, 2001).

(c) Chemical processes

Food preservation using chemical reagents is one of the traditional methods. Effectiveness of this method depends on the concentration and selectivity of the chemical reagents, spoilage-causing organisms, and the physical and chemical characteristics of food items (Frank and Paine, 1993). However, using chemical reagents as food additives and preservatives is a sensitive issue because of health concerns (Mursalat et al, 2013).

❖ ***Preservatives***

Preservatives are defined as the substances capable of inhibiting, retarding, or arresting the growth of microorganisms or any other deterioration resulting from their presence**.** Food preservatives extend the shelf life of food products. Preservatives retard degradation caused by microorganisms and autoxidation of food components and therefore maintain the colour, texture, and flavor of the food item (Adams, 2008). Food preservatives can be classified as natural and artificial. Animals, plants, and microorganisms contain various chemicals which have potential to preserve foods and are considered as natural preservatives. Artificial or synthetic preservatives are produced industrially. These can be classified as antimicrobial, antioxidant, and antienzymatic (Sati and Sati, 2013).

- ***Use of chemical preservatives***

- ***Synthetic antimicrobials as preservatives***

Antimicrobial food preservatives acts by inhibiting the growth of undesirable microorganisms (fungi, bacteria, yeast). They develop unfavorable environment for microorganisms by reduction of moisture content and by increasing acidity. Sorbic acid (2,4-hexadienoic acid) and potassium sorbet are synthetic antimicrobial preservatives readily used for the preservation of cheese, bakery products, vegetable based products, dried fruits, beverages, and other products as well as smoked fish, margarine, salad cream, and mayonnaises (Lucera et al., 2012)

- ***Synthetic antioxidants as preservatives***

Antioxidants are used as food preservatives to inhibit atmospheric oxidation. It is primarily used for the products containing unsaturated fatty acids, oils, and lipids. Oxidation of unsaturated fats results in generation of free radicals which can start chain reactions. In this reaction, aldehyde and ketones are produced resulting in the rancid taste of foods. Antioxidants as food preservatives disrupt these chain reactions by removal of free radical intermediates and inhibit other oxidation reactions. Butylated hydroxylanisole (BHA), butylated hydroxytoluene (BHT) are examples of synthetic preservatives used for the preservation of butter, lard, meats, beer, baked goods, snacks, potato chips, nut products, dry mix for beverages (Lobo et al.,2010).

- ***Synthetic antienzymatic preservatives***

Anti-enzymatic food preservatives prevent natural ripening process and oxidative deterioration of food by inhibiting the bacteria, parasite, and fungi. It hinders enzymatic processes in the food that are responsible for metabolizing after harvest. Metal chelating agents are able to remove the metal cofactors which are required by enzymes. Citric acid is an example of anti-enzymatic preservatives used for the preservation of foods, beverages, dairy products and pharmaceuticals (Inetianbor, 2015).

- **Natural antimicrobials and antioxidants as preservatives**

Growing interest in using antimicrobial plant-derived extracts is caused by the need to reduce the use of synthetic additives in food (Mendonca et al., 2018). Antioxidant capacity usually

joins the antimicrobial characteristics of these natural products; both properties together in one molecule makes the compound even more effective (Pisoschi et al, 2018). Plants and herbs (oregano, garlic, parsley, sage, coriander, rosemary, and lemongrass), spices (cinnamon, clove), oils (citral) and their bioactive compounds have been used alone for their antimicrobial and antioxidant properties or in combination with other techniques for food preservation (Proestos et al, 2008). Besides, Nisin, a well known antimicrobial peptide is widely used as natural antimicrobial and α-tocopherol is used as natural antioxidant in food preservation system (Gharsallaoui et al, 2016). Natural preservatives are chemical agents derived from plants, animals, microbes and their metabolites that prevent the decomposition of food products by inhibition of microbial growth, oxidation and certain enzymatic reactions occurring in the foodstuffs. They are generally considered safe (GRAS).

- ***Combined methods for Food Preservation (hurdle technology)***

The microbial and oxidative stability and safety of most traditional and novel food is based on the combination of several preservative factors commonly known as hurdle effect. The hurdle effect is of fundamental importance for the preservation of foods, since hurdles in a stable product control microbial spoilage, oxidation, food poisoning, as well as desired fermentation processes. In fact, the hurdle concept illustrates only the well-known fact that complex interaction of temperature, water activity, pH, redox potential, etc. are significant for the microbial and oxidative stability of foods. The relation between technology and homeostasis of microorganisms as well as oxidation in food products is well established Foods preserved by this method are safe, stable, nutritious, tasty and economical. Hurdle technology was derived, which allows improvements in the safety and quality of foods using deliberate and intelligent combination of hurdles. In industrialised countries, hurdle technology is currently of practical interest for minimally processed foods, whereas in developing countries foods storable without refrigeration, due to stabilization by hurdle technology, are at present of paramount importance (Leistner and Gorris, 1995). Therefore, in food preservation the combined effect of preservative factors must be taken into account, which is illustrated by the hurdle effect.

- ***Possible adverse effects of synthetic food preservatives***

Chemical food preservatives/additives are mostly considered safe, but several of them have negative and potentially life-threatening side effects. For example, nitrates, upon ingestion, are converted to nitrites that can react with hemoglobin to produce met-hemoglobin, a

substance that can cause loss of consciousness and death, especially in infants. Different artificial food colorings, such as tartrazine, allura red, ponceau, and benzoate preservatives, have adverse effects on the behaviour of infants; these additives are credited as the cause of the hyperactive behaviours of infants. Preservatives also have intolerances among people who have asthma. Sulfates (including sodium bisulfate, sodium meta-bisulfate, and potassium bisulfate) found in wine, beer, and dried fruits are known to trigger asthmatic syndromes and cause migraines in people who are sensitive to them. Sodium nitrate and sodium nitrite used in food products as preservatives are also classified as 'probable carcinogenic elements' to humans by International Agency for Research of Cancer (IARC) (Nogrady, 2013). Nitrites and benzoates preservatives may have adverse effects on pregnant women. Sodium nitrite intake lowers hemoglobin and hematocrit values of pregnant women. Both benzoate and nitrite induce decrease in serum bilirubin and increase in serum urea. Consequently, the mean weight and length of the fetus get lowered (Mowfy et al., 2001). Nitrites, after ingestion, get converted into nitrosamines, which could be harmful to a fetus (Linsha et al, 2018).

- **Beneficial effects of plant-based preservatives**

Plant-based preservatives are generally regarded as safe (GRAS) without any documented detrimental impact and have been reported to contain diverse groups of bioactive compounds that play an important role in the preservation of foods through their multimodal action. Several bioactive compounds naturally occurring in plants have antioxidant and antimicrobial properties and may play crucial roles in the preservation system (Beya et al., 2021). A single molecule of plant origin having both antioxidant and antimicrobial properties is more effective than synthetic preservatives and may play a promising alternative to synthetic food preservatives (Pisoschi et al, 2018).

Among the plant secondary metabolites, plant polyphenols are most important phytocompounds for their powerful antimicrobial and antioxidant properties in addition to their number of beneficial effects on human health. Their bioactivity is based on their capability to: scavenge free radicals and reactive oxygen/nitrogen species (ROS/N), reduce oxidized intermediates, induce metal chelation, and inhibit enzymes responsible for the formation of free radicals, and inhibit microbial growth (Efenberger-Szmechtyk, 2021). These molecules differ in terms of their physiochemical structure and molecular weight (Leopoldini et al., 2011). The bioactivity of polyphenols is dependent on the configuration of

the molecules, and the position and number of the hydroxyl group(s) in that molecule (Michalak, 2006).

- ***Potato peels may serve as a potential source of antioxidant and antimicrobial agents in food preservation system***

Food processing industries generate a substantial quantity of waste byproducts which are a major disposal problem for the industry concerned and may cause environmental pollution. But, these byproducts of food processing are inexpensive, affordable, and valuable starting material for the extraction of value added products (Chiellini et al, 2004; Bildstein et al, 2009). The plant-originated food processing byproducts offer unlimited opportunities for development of different types of value-added products because of their diversified chemical property (Kujala et al, 2000).

Potato (*Solanum tuberosum* L.) is one of the most important agricultural crops for human consumption. It is the fourth main crop behind rice, wheat and maize (Zhang et al, 2017). Food processing industries particularly potato chips manufacturing industries generate a huge volume of potato peel as waste by-product. Disposal of waste potato peels is one of the major problems for the industry concerned and may cause environmental pollution (Wu, 2016). Potato peel as a byproduct of food processing industry poses to be totally inexpensive, valuable and affordable starting material for the production of economically important substances (Bildstein et al, 2009). Although potato peel waste is used for low-value animal feed, pig feed, fertilizer, biogas, and amylase enzyme production (Bhusan et al., 2008; Gebrechristos and Chen, 1018), this could also be used as a promising replacement for the current synthetic antioxidant and antimicrobial in food preservation system due to its high phenolic contents (Gebrechristos and Chen, 2018; Grunert, 2005; Seplev and Galoburda, 2015; Wu, 2016). Therefore, the effective utilization of potato peels both as an antioxidant and antimicrobial agents may help in developing a more potent, safe and effective novel natural alternative to synthetic food preservatives for preserving foods especially omega-3 fatty acids fortified functional foods.

- ***Antioxidant property of potato peels***

The oxidative deterioration of fats and oils in food is responsible for rancid odours and flavours, with a consequent decrease in nutritional quality and safety of foods caused by the formation of secondary, potentially toxic compounds. Lipid oxidation products have

mutagenic, carcinogenic and cytotoxic properties and considered to be risk factor for human health (Julia et al., 2015). Thus, addition of antioxidants is an important step in food processing to prevent oxidative deterioration and to avoid nutrient loss in foods. Due to the concerns of synthetic antioxidants in humans' foods, potato peel extracts may serve as a possible potential antioxidant in food preservation systems because they contain a high concentration of phenolic compounds (Gebrechristos and Chen, 2018; Ito et al., 1986; Perelman et al., 1998; Sebedioo et al, 1991). A strong positive correlation between total polyphenolic compounds in potato peel extracts and their antioxidant potency was observed by several workers (Kim et al., 2019; Brown, 2005). A number of studies in antioxidant potential of potato peel extract on various *in vitro* models and in marine fish oil as well as vegetable oils were conducted by several workers. Based on the results of their work, they suggested potato peel extract exhibited strong antioxidant activity both in various *in vitro* models as well as in vegetable and marine fish oils (Oneyeneho and Hettiarchy, 1993). The antioxidant activity measurements of potato peel extract did emphasis it's strong reducing power activity, superoxide scavenging ability as well as its ion chelating potency (Javed et al, 2019). More recently, potato peel extract were studied as a natural antioxidant to prevent deteriorations of food lipids. They suggested that potato peel extract in oils, fats and other food products can safely be used as natural antioxidant to suppress lipid oxidation (Rehman et al, 2004; Mohdaly et al., 2010; Farvin et al, 2012). The *in vitro* results suggest the possibility that potato peel waste could be effectively employed as an ingredient in health or functional food, to alleviate oxidative stress (Singh and Rajini, 2004).

- ***Antimicrobial properties of potato peels***

In addition to antioxidant efficacy of potato peel extract, it also exhibited antimicrobial effects. Potato peel extracts have antimicrobial compounds against bacterial and fungal organisms. It has also been reported that potato peel has bacteriostatic nature with nonmutagenic behaviour and safe to use in food processing industries (Amanpour, 2015; Sotillo et al, 2007). The antibacterial activity of the potato peel was found to be species dependent and the water extract of potato peel exhibited antibacterial efficacy against Gram-negative and one Gram-positive bacteria only at high concentration (Sotillo et al, 2007). The main polyphenolic compound detected in potato peel extract which showed antibacterial efficacy is found to be chlorogenic acid (Sotillo et al., 2007). Besides, some other workers have also reported that antimicrobial nature could be due to the presence of flavonoids and terpenes organic compounds (Nostro et al., 2000). Therefore, in addition to their antioxidant

property, potato peel extract may also serve as a potential source of natural antimicrobial agents for inhibiting the growth of foodborne microbes.

- ***Chemical composition of potato peels***

Potato peel contains various polyphenolic compounds which are found to be responsible for its antioxidant and antimicrobial activities (Maldonado et al., 2014; Jeddou et al, 2016; Sotillo et al., 2007). Potato peel also contains starch, non-starch polysaccharide, protein, acid-soluble and acid-insoluble lignin, lipids and ash (Liang and McDonald, 2014; Liang et al, 2015). Potato peel also contains lipids. The lipid fraction of potato peels includes long-chain fatty acids, alcohols, triglycerides and sterol esters. Elemental analysis of potato peel shows that it contains carbon (C), hydrogen (H), nitrogen (N) and oxygen (O). The C/N ratio of potato peel is 10.7 and its pH is 6.5 (Liang et al, 2015). Besides different types of flavonoids and tannins, various phenolic acids such as gallic acid, protocatechuic acid, vanillic acid, caffeic acid, chlorogenic acid, p-hydroxybenzoic acid and p-coumaric acid are also found in the potato peel extract (Javed et al, 2019).

Thus, strong antioxidant as well as broad spectrum antimicrobial properties of potato peels with diverse groups of chemical compounds may help to discover new chemical classes of antioxidant and antimicrobial substances that could serve as a safe and effective novel natural food preservative in food processing industries (Gebrechristos et al, 2020).

- ***Basis of selection of five selected varieties of potato as starting plant material***

Among the Indian processing varieties of potato, based on increased demands of processed potato products, suitability and availability of indigenously developed potato processing varieties, only three processing varieties (*Kufri Chipsona-1*, *Kufri Chipsona-3* and *Kufri Chipsona-4*) are widely used by the potato processing industry for production of quality potato chips and French fries. Besides these three processing varieties of potato, two table varieties of potato (*Kufri Chandramukhi and Kufri Jyoti)* are also widely used by households. A large volume of peels of these five varieties of potato are generated day by day.

In my present work I, therefore, used peels of these widely used five varieties of potato, three from processing varieties (*Kufri Chipsona-1*, *Kufri Chipsona-3* and *Kufri Chipsona-4*) and two from table varieties (*Kufri Chandramukhi* and *Kufri Jyoti*) as starting plant material.

CHAPTER 3

MATERIALS AND METHODS

Materials and Methods

- **Materials**

- ***Collection and processing of plant materials***

The five selected varieties of potato (*Kufri Chipsona-1, Kufri Chipsona-3, Kufri Chipsona-4, Kufri Chandramukhi* and *Kufri Jyoti*) , used in the present work were obtained from the Vegetable farm of Bidhan Chandra Krishi Viswavidyalaya (BCKV), West Bengal, India and Central Potato Research Institute , Shimla, India and were identified by a botanist. The selected varieties of potato were washed thoroughly in tap water and peeled manually to a depth of ~ 1 mm. The peels were then dried at room temperature and ground into a coarse powder using manual grinder.

- ***Extraction of phenolic fraction of potato peels***

Phenolic extract of peels of selected varieties of potato was prepared following the method of Abu-Reidah et al. (2013). Briefly, the coarse powder of potato peels (25 g) was macerated with 250 ml of water: ethanol (20:80 v/v) solvent mixture with occasional stirring for 24 h and filtered using Whatman No. 1 filter paper. The obtained residue was then further re-extracted twice following the same procedure as mentioned above and the filtrates were pooled. Per 100 ml of pooled filtrate, Carrez reagent A (4 ml) was added, vortexed for 2 min and left for one minute; then Carrez reagent B (4 ml) was added and vortexed for 20 s. These two reagents were added in order to precipitate out the polysaccharides and proteins respectively. The mixture was then centrifuged at 2000 × g for 20 min. The supernatant was drawn off and centrifuged again at 2000 × g for 10 min and filtered. The filtrate was then evaporated to dryness in a rotary evaporator (bath temperature 40°C) and finally dried in a desiccator using anhydrous sodium sulphate as dehydrating agent. The dried mass of the potato peel extract of selected potato varieties were then kept at −20 °C until used [yield: *Kufri Chipsona-1:* 3.05%; *KufriChipsona-3*: 3.92%; *Kufri Chipsona-4*: 2.98%; *Kufri Chandramukhi*: 3.46% and *Kufri Jyoti*: 3.72%].

- ***Test microorganisms***

The foodborne bacterial and fungal pathogens used in the present investigation were selected based on their relevance and importance in the food industry. The bacterial strains used were pure reference standard foodborne bacteria *Listeria monocytogenes* (MTCC 657) and

Salmonella typhimurium (MTCC 3224) as indicator strains of Gram-positive and Gram-negative foodborne bacteria, respectively. The fungal strain used was *Aspergillus niger* (ATCC 16404). Bacterial strains were collected from the Institute of Microbial Technology, Chandigarh, India and fungal strain was obtained from National Chemical Laboratory, Pune, India. The bacterial strains were maintained following the standard CLSI guidelines for bacteria (CLSI, 2005) and fungal strain was maintained following CLSI M38-A2 guidelines for fungi (CLSI, 2008).

- ***Standardization of inoculum size***

In order to standardize bacterial inoculum size, test bacterial strains were incubated in nutrient broth (Hi-Media, Mumbai, India) for 3–6 h at respective temperature (*Listeria monocytogenes* at 30ºC and *Salmonella typhimurium* at 37ºC) until the culture attained a turbidity of 0.5 McFarland Unit. The final bacterial inoculum size was adjusted to 5×10^5 CFU/ml (CLSI, 2005). For standardization of fungal inoculums size, initially inoculum suspension of *A. niger* was prepared by taking the sporulated fungus with a loop from fresh, matured (3–4 day old) culture of *A. Niger* which were grown on Sabouraud Dextrose Agar slant at 35ºC and suspending it in 10ml of sterile water. The fungal suspension was then filtered once through a sterile gauze to remove hyphae. The resulting suspension of *A. niger* was then vortexed and was adjusted to a concentration of 5×10^5 CFU/ml by addition of sterile distilled water and using a hemocytometer cell counting chamber (CLSI, 2008).

- ***Omega-3 fatty acids enriched oil***

Marine fish oil (Sigma-Aldrich) was used as omega-3 fatty acids enriched food supplement and for preparation of omega-3 fatty acids fortified various functional foods in the present investigation.

- ***Chemical and Reagents***

All chemicals and reagents used in the present investigation were obtained from E. Merck KGaA, Darmstadt, Germany unless stated otherwise.

❖ Methods

▪ Methods used to fulfil Objective 1

[Objective 1: ***To evaluate in vitro antioxidant and antimicrobial potential of phenolic extract of peels of selected varieties of potato at their individual effect***]

A. Methods used to evaluate *in vitro* antioxidant potential of phenolic extract of peels of selected varieties of potato at their individual effect

- ***DPPH radical scavenging assay method***

Free radical scavenging activity of phenolic extract of potato peels of selected varieties of potato was evaluated quantitatively by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay method (Wang et al., 1998). Briefly, 100 µl of potato peel extract in varying concentrations (31.25 µg/ml-1000 µg/ml) was taken in test tubes and 3.9ml of 0.1mM solution of DPPH in methanol was added to these tubes. Tubes were shaken vigorously and allowed to stand in dark at room temperature for 30 min. The control was prepared as above without the peel extract and methanol was used for zero adjustment. BHT (butylated hydroxytoluene) was used as positive control. Absorbance of the samples was measured at 517 nm. Inhibition of the DPPH radical by the peel extract was calculated according to the following formula:

$$(\%)\ Free\ radical\ scavenging = \left(\frac{A_{blank} - A_{Sample}}{A_{blank}}\right) \times 100$$

Where, A_{sample} is the absorbance of the sample in DPPH solution after reacting with a given concentration of potato peel extract and A_{blank} is the absorbance of DPPH solution with methanol blank instead of potato peel extract. The percentage of DPPH radical scavenging capacity was plotted against the concentration of potato peel extract and their IC_{50} values (the concentration required for scavenging 50% of the DPPH) were calculated. All tests were performed in triplicate.

- ***β-Carotene- linoleic acid bleaching activity study***

β-Carotene-linoleic acid bleaching activity of potato peel extract was measured by the method of Velioglu et al (1998). Briefly, a solution of β-carotene was prepared by dissolving 2mg of β-carotene in 10ml of chloroform. 2ml of this solution was the pipetted into a 100ml round bottom flask. After the removal of chloroform under vacuum, 40mg of purified linoleic

acid, 400mg of Tween 20 and 100 ml of distilled water were added to the flask with vigorous shaking. Aliquots (4.8ml) of this emulsion were then transferred into test tubes containing 100µl of potato peel extract at different concentrations ranging from 31.25µg/ml to 1000µg/ml and the zero time absorbance was measured at 470 nm using a spectrophotometer (ThermoFisher Scientific, USA). The tubes were then placed in water bath at 50°C and measurement of absorbance was recorded after 2 h. A control was prepared without β-carotene in order to facilitate background subtraction. BHT was used as a positive control. Inhibition percentage was calculated using the following formula:

$$\%\ inhibition = \left[\frac{(A_{S120} - A_{C120})}{(A_{C0} - A_{C120})}\right] \times 100$$

Where, A_{S120} is the absorbance of the sample at t = 120 min,

Ac_{120} is the absorbance of the control at t = 120 min, and A_{C0} is the absorbance of the control at t = 0 min. Each experiment was repeated thrice.

- ***Ferrous (Fe^{2+}) ion chelating activity***

Ferrous (Fe^{2+}) ion chelating activity of potato peel extract was determined following the method of Dinis et al. (1994). Briefly, 50 µl of 2 mM $FeCl_2$ solution was added in tubes containing 100µl of varying concentrations (31.25µg/ml–1000µg/ml) of potato peel extract. The reaction was started by the addition of 0.2 ml of 5 Mm ferrozine solution. The mixture was then vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was subsequently measured at 562 nm using a spectrophotometer (ThermoFisher Scientific, USA). Sample control was prepared without adding potato peel extract. Ascorbic acid was used as a positive control. The percentage inhibition of ferrozine–Fe^{2+} complex formation was calculated as follows:

$$\%\ Inhibition = \left[\frac{A_c - A_s}{A_s}\right] \times 100$$

Where, Ac was the absorbance of the control, and As was the absorbance of the sample. Each experiment was repeated thrice.

B. Methods used to evaluate *in vitro* antimicrobial potential of phenolic extract of peels of selected varieties of potato at their individual effect

- ***Determination of inhibition zone diameter (IZD)***

Inhibition zone diameter (IZD) of potato peel extract of selected varieties of potato at their individual effect against the studied bacterial pathogens was accessed by agar well diffusion method (Okeke et al., 2001). Briefly,1ml of inoculum (5×10^5 CFU/ml) was spread uniformly on nutrient agar (HiMedia, Mumbai, India) plates with the help of a glass rod spreader and 6 mm diameter wells were bored on the surface of agar plates. 100µl (1000 µg/ml) of each potato peel extract reconstituted in nutrient broth was pipetted into wells. After keeping the plates at room temperature for 2h in order to allow diffusion of peel extracts into the agar, they were subsequently incubated at respective temperature (30ºC/37ºC) for 24h.

For fungal strain, Sabouraud Dextrose Agar (SDA) plate was employed instead of nutrient agar plate and the same procedure was followed as done for IZD determination against bacterial strains and the plates containing fungal strain were incubated at 35ºC for 48 h. Inhibition zone diameter (IZD) was measured in millimetre (mm). Each experiment was repeated thrice.

- ***Determination of minimum inhibitory concentration (MIC)***

Minimum inhibitory concentration (MIC) values of potato peel extracts against the studied microbes were determined at their individual effect following the CLSI (2005) guidelines. Briefly, 100µl of potato peel extract reconstituted in nutrient broth at various concentrations (1.56µg/ml –200µg/ml) was added in wells of microtiter plates containing 90µl of nutrient broth (HiMedia, Mumbai, India). After that, 10µl of inoculum (5×10^5 CFU/ml) was added to each well. Negative control wells received nutrient broth instead of potato peel extract. Plates were then incubated at 30ºC for *L. monocytogenes* and 37ºC for *S. typhimurium* for 24h. Then, 40 µl (0.4 mg/ml) of INT solution was added to wells and they were further incubated for 6h. The lowest concentration of potato peel extract with no visible colour change was considered as MIC. Each experiment was repeated thrice.

- ***Determination of minimum fungicidal concentration (MFC)***

Minimum fungicidal concentrations (MFCs) of potato peel extracts at their individual effect against fungal strain *A. niger* were determined following CLSI M38-A2 guidelines (CLSI,

2008). Briefly, 100µl of reconstituted solution of potato peel extract with RPMI 1640 medium at various concentrations (1.56µg/ml–200 µg/ml) was added in wells of the microtiter plate. Then, 100µl of *A. niger* inoculum (5×10^5CFU/ml) was added in each well and they were incubated for 48h at 35ºC. Wells having *A. niger* inoculum and RPMI 1640 instead of potato peel extract were served as negative control. After incubation period, 40µl of INT solution (0.4 mg/ml) was added and further incubated for 6h. MFC was defined as the lowest concentration of potato peel extract that exhibited no visible colour change. Each experiment was repeated thrice.

- **Methods used to fulfil Objective 2**

[Objective 2: ***To evaluate individual antioxidant potential of phenolic extract of peels of selected varieties of potato against oxidation of omega-3 fatty acids enriched food supplement and omega-3 fatty acids fortified functional foods at their individual effect***]

(A) Evaluation of antioxidant potential of phenolic extract of peels of selected varieties of potato against oxidation of omega-3 fatty acids enriched food supplement at their individual effect

Antioxidant potential of potato peel extract against oxidation of omega-3 fatty acids-enriched food supplement (marine fish oil) was evaluated in two lipid model systems (bulk oil and oil-in-water emulsion) and for this before going to evaluate antioxidant potential of potato peel extract in bulk oil and oil-in-water emulsion model systems, at first oil-in-water emulsion model was prepared as follows.

- ***Preparation of oil-in-water emulsion***

A 10% oil-in-water emulsion was prepared with 1% Tween-20 as emulsifier. In brief, 5 g of Tween-20 and 5 g of marine fish oil were weighed into a glass beaker and thoroughly mixed together by a magnetic stirrer. Following this, deionized water (44.5ml) was taken into a 250 ml beaker in which fish oil and Tween-20 mixture was added in drop-wise manner and homogenized at 8000 rpm for 3 min in order to form stable oil-in-water emulsion for antioxidant activity study.

- ***Evaluation of antioxidant potential of phenolic extract of peels of selected varieties of potato in bulk oil and oil-in-water emulsion model systems at their individual effect***

Antioxidant potential of potato peel extract both in bulk oil and oil-in-water emulsion model systems was evaluated following the method of Habeebullah et al. (2010). Briefly, both in bulk oil and oil-in-water emulsion model systems, 200µg/ml concentration of peel extract was mixed separately and homogenized. BHT (200µg/ml) (the regulatory limit allowed in oils) was used as positive control. Bulk oil and oil-in-water emulsion without potato peel extract or BHT served as negative control. Samples were cooled in an ice bath and homogenized. Both peel extract treated and control samples were transferred to amber bottles with aluminium foil wrapping and kept in an oven at 60°C for 15 days. The lids of amber bottles were kept loosely attached in order to allow free passage of air out of the head space above the samples. Aliquots were removed at 0, 3, 6, 9, 12 and 15 day intervals for analysis. The oxidative state of samples was monitored by determining the peroxide value (PV), *p*-anisidine value (*p*-AV) and TOTOX value for the determination of primary oxidation, secondary oxidation and total oxidation of oils in two lipid model systems respectively. The PV was determined iodometrically and the *p*-AV was determined following AOCS guidelines. TOTOX value was calculated using the following formula:

TOTOX = 2PV +*p*-AV (Poiana et al., 2010)

(B) Evaluation of antioxidant potential of phenolic extract of peels of selected varieties of potato in omega-3 fatty acids fortified various functional foods at their individual effect

To evaluate the antioxidant efficacy of the potato peel extract at their individual effect on the primary , secondary and total oxidation of omega-3 fatty acids fortified various functional foods, their effects on omega-3 fortified whole milk, paneer and yoghurt foods models were evaluated. For this before going into antioxidant activity study, omega-3 fatty acids fortified various functional foods were prepared as follows.

- ***Preparation of omega-3 fatty acid fortified various functional foods (whole milk, yoghurt and paneer)***

Fresh whole milk, yoghurt and paneer were purchased from local market (Baranagar Bazar, Kolkata, India) on the day of experiment and 10g of each of foods (whole milk, yoghurt and paneer) were taken separately and battered with a mortar and pestle and then mixed with 10 ml phosphate buffered saline (pH 7.2) and 200 µg/ml of omega-3 fatty acids enriched marine

fish oil (Sigma-Aldrich). The mixtures were further homogenised at 3000 rpm for 5 min to prepare omega-3 fatty acids fortified various functional foods.

Now, the antioxidant activity of potato peel extracts at their individual effect against oxidation of omega-3 fatty acids fortified various functional food model systems was evaluated as follows.

- ***Determination of antioxidant efficacy of potato peel extract on primary, secondary and total oxidation in omega-3 fatty acids fortified various functional food model systems at their individual effect***

Antioxidant potential of potato peel extract in omega-3 fatty acids fortified various functional food model systems was evaluated as described by Habeebullah et al (2010). Briefly, in each test food model media, 200 μg/ml concentration of peel extract was mixed separately and homogenized. BHT (200 μg/ml) (the regulatory limit allowed in oils) was used as positive control. Omega-3 fatty acids fortified various food models (whole milk, yoghurt and paneer) without antioxidants (peel extract or BHT) served as negative control. Samples were cooled in an ice bath and then homogenized. Both control as well as peel extract or BHT treated omega-3 fatty acids fortified functional foods were transferred to amber bottles with aluminium foil wrapping and kept in an oven at 60°C for 15 days. The lids of amber bottles were loosely attached so that air could pass in and out of the headspace above the samples. Aliquots were removed at 0, 3, 6, 9, 12 and 15 day intervals for analysis. The oxidative state of samples (primary oxidation, secondary oxidation and total oxidation) was monitored following the same procedure as described before for evaluating antioxidant efficacy of peel extract in omega-3 fatty acids enriched food supplement.

➢ ***Methods used to fulfil Objective 3***

[**Objective 3**: ***To evaluate combined antioxidant and antimicrobial potential of active phenolic extracts of peels of selected varieties of potato in various in vitro models***]

Based on the threshold limits of antioxidant efficacy and antimicrobial susceptibility testing, only three extracts of potato peels were found to be active and subjected to further studies.

(A) Evaluation of combined antioxidant potential

Combined antioxidant potential of active potato peel extracts was evaluated by (i) DPPH radical scavenging method and (ii) Briggs-Rauscher Oscillation reaction method.

Determination of combined antioxidant potential by DPPH radical scavenging method

(i) DPPH radical scavenging method

- ***Determination of IC_{50}***

Combined antioxidant potential of active extracts of potato peels was evaluated by DPPH radical scavenging method followed by isobologram analysis. For this, at first IC_{50} values of antioxidant potential of active potato peel extracts were determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay method (Wang et al., 1998). Briefly, 100 µl of potato peel extract alone and in combination (1:1) at different concentrations (3.12µg/ml to 200 µg/ml) were taken in test tubes and 3.9 ml of 0.1mM solution of DPPH solution in methanol was added to tubes and shaken vigorously. The tubes were then allowed to stand in dark at room temperature for 30 min. The control was prepared as above without potato peel extract. Methanol was used for zero adjustment. Absorbance of the samples was measured at 517 nm. Per cent radical scavenging activity by potato peel extract alone and in combination was evaluated as follows:

$$(\%)\ Free\ radical\ scavenging = \left(\frac{A_{blank} - A_{Sample}}{A_{blank}}\right) \times 100$$

Where, A_{sample} is the absorbance of DPPH solution after reacting with a given concentration of potato peel extract and A_{blank} is the absorbance of DPPH solution with methanol blank instead of potato peel extract. The IC_{50} values (the concentration required for scavenging 50% of the DPPH radical) of potato peel extracts alone and in combination were determined from dose-response curve.

- ***Isobologram analysis for determination of combination index (CI) based on IC_{50} values***

Based on IC_{50} values, the antioxidant combination index (CI) of potato peel extracts in combination was assessed using isobologram analysis. The classical isobologram combination index (CI) equation was used for analysing the data (Rodea-Palomares et al., 2010).

$$CI = \frac{D_1}{D_{x1}} + \frac{D_2}{D_{x2}}$$

where (D_1) and (D_2) are the doses (IC_{50} values) of two different potato peel extracts in combination; (Dx_1) and (Dx_2) are the doses (IC_{50} values) of two individual potato peel extracts that are in combination (Rodea-Palomares et al., 2010).

- ***Determination of type of antioxidant interactions based on combination index (CI)***

On the basis of CI values, the type of antioxidant interactions in combination were interpreted as follows: CI < 1: synergistic; CI = 1: additive; CI > 1: antagonistic (Chou, et al., 1994).

(ii) Briggs–Rauscher oscillation reaction method

The combined antioxidant potential of active extracts of potato peels was evaluated using Briggs-Rauscher oscillation reaction method (Briggs & Rauscher, 1973) .The following three steps were used to evaluate antioxidant efficacy using this method.

Step I: At first following three stock solutions were prepared. **Solution A**: 43 g potassium iodate and 15.9 g perchloric acid were dissolved in distilled water and diluted to 1L. **Solution B**: 15.6g malonic acid, 3.4g manganese sulfate monohydrate, and 3g starch were dissolved in distilled water and diluted to 1L and **Solution C**: 400 ml of 30% hydrogen peroxide was diluted to 1L in distilled water.

Step II: To determine the antioxidant potential of potato peel extracts, 5 ml of each solution of A and B were poured into 100 ml thermostated beaker which were equipped with a magnetic stirrer bar and placed on a stirring plate. Following this, 5ml of solution C was used to initiate oscillation. After the completion of the third oscillation, 1ml of 200 µg/ml concentration of potato peel extract alone and in combination (1:1) was added to the BR mixture and just after the immediate quenching of oscillation, the stopwatch was started and the oscillating behaviour of the reaction mixture was monitored visually. The inhibition time ($t_{inhibition}$; the duration of time in seconds from cessation of oscillation by antioxidant in BR mixture to subsequent regeneration of oscillations) were then determined. Each experiment was repeated thrice.

Step III: The antioxidant potential of potato peel extracts in combination was determined on the basis of inhibition time ($t_{inhibition}$). The types of antioxidant interactions (synergistic, additive, or antagonistic) were interpreted based on $t_{inhibition}$ values as follows. “Synergistic”:

if $t_{inhibition}$ in combination increases significantly ($p < 0.05$) in comparison to the average $t_{inhibition}$ of individual components in combination; "Antagonistic": if $t_{inhibition}$ in combination decreases significantly ($p < 0.05$) in comparison to average $t_{inhibition}$ of individual component in combination and "Additive": if $t_{inhibition}$ in combination does not increase or decrease significantly ($p \geq 0.05$) in comparison to the average $t_{inhibition}$ of their individual components in combination (Milos and Makota, 2012).

(B) Evaluation of combined antimicrobial potential

Combined antimicrobial potential of active potato peel extracts was determined by checkerboard titration method followed by kill-kinetics assay.

(a) Checkerboard titration method

- ***Determination of fractional inhibitory concentration index (FICI) by checkerboard titration method***

For the determination of fractional inhibitory concentration index (FICI) values of active extracts of potato peels at first, MIC and MFC values of potato peel extracts in combination (1:1) were determined following the guidelines of CLSI (2005) for bacteria and CLSI (2008) for fungi. In order to determine the MIC in combination against bacterial pathogens, 10µl of bacterial inoculum (5×10^5 CFU/ml) was added in wells of the microtiter plate containing 90 µl of nutrient broth. Then, potato peel extract (100 µl) in combination (1:1) at various concentrations (1/32 × MIC to 4 ×MIC) were added in wells and incubated for 24 h at 30ºC for *L. monocytogenes* and at 37ºC for *S. typhimurium.* For the determination of MFC in combination against fungal pathogen,100 µl of fungal suspension (5×10^5 CFU/ml) and 100 µl of potato peel extract in combination (1:1) at various concentrations (1/32 × MFC to 4 × MFC) were added in wells of the microtiter plate and incubated for 48h at 35ºC. The lowest concentration of potato peel extract that showed no visible colour change was considered as MIC for bacterial and MFC for fungal strains.

Based on MIC and MFC values, fractional inhibitory concentration index (FICI) values were determined as follows:

FICI = (MIC or MFC of AE_A in the presence of AE_B/MIC or MFC of AE_A alone) + (MIC or MFC of AE_B in the presence of AE_A/MIC or MFC of AE_B alone).

Where, AE_A and AE_B are two different potato peel extracts. The antimicrobial interactions between two potato peel extracts in combination were interpreted as follows. FICI ≤ 0.5 (Synergy); 0.5 < FICI ≤4: Additive; and FICI > 4 (antagonistic) (Leclercqet al., 1991).

(b) Kill-kinetics assay

To confirm the synergistic antimicrobial efficacy of active potato peel extracts in combination against the studied microbes obtained from checkerboard titration method, kill-kinetics assay was performed following the method of Levinson (2004). Briefly, in microtiter plate wells, 90 µl of respective broth, 10 µl of bacterial or fungal suspension (5×10^5 CFU/ml) and 100 µl of active potato peel extracts at different concentrations (0.5×MIC, 1×MIC, 2×MIC, and 4×MIC) were added and mixed thoroughly. The plates were then incubated for 24 h at respective temperature (30 ºC/37 ºC). Then, 10 µl sample was removed from wells at 0, 3, 6, 12, and 24h of incubation, and diluted serially with respective broth. Viable counts were determined by plating 100µl of diluted aliquots on fresh selective agar plates and incubated at respective temperature (30ºC/37ºC) for 24h. Agar plates with 30 to 300 colonies were used for CFU counting. Kill-kinetics curves were constructed by plotting Log_{10} CFU/ml against time. Each experiment was repeated thrice. Active potato peel extracts in combination that showed a reduction in colony count by >$2log_{10}$ CFU/ml at 24h in comparison to their most active single component's effect was considered synergistic antimicrobial interaction (Chlipala et al., 2010).

(C) Determination of total phenolic contents of active extracts of potato peels

To determine whether there is any association between total phenolic content of active extracts of potato peels and their antioxidant as well as antimicrobial efficacy, the total phenolic content of active extracts of potato peels was estimated by Folin–Ciocalteu method (McDonald et al, 2001) as follows.

Briefly, a volume of 0.5 ml of potato peel extract (100 µg/ml) was mixed with 1ml of Folin–Ciocalteu reagent (diluted 1:10 with deionized water), shaken vigorously and kept for 3 min at room temperature. Thereafter, 3ml of 2% Na_2CO_3 solution was added and the mixture was allowed to stand for 2h with intermittent shaking in order to develop colour. The absorbance of the resulting blue colour was measured at 760nm. The total phenolic content was determined from the linear equation of a standard curve prepared with different

concentrations of gallic acid. The content of total phenolic compounds was expressed as mg Gallic acid equivalent /g of dry extract (mg GAE/g of dry extract).

- **Methods used to fulfil Objective 4**

[Objective 4: ***To isolate, identify and characterize the bioactive antioxidant and antimicrobial compounds from active phenolic extracts of peels of selected varieties of potato and evaluation of their cytotoxic potential, if any***]

(A) **Isolation, identification and chemical characterization of bioactive compounds**

For isolation, identification and chemical characterization of bioactive compounds responsible for antioxidant and antimicrobial efficacy, only peel extract that showed synergistic antioxidant and antimicrobial interactions in combination were chemically analyzed by TLC-bioautography guided separation followed by isobologram analysis, checkerboard titration and spectrometric characterization methods as follows.

(a) **TLC bioautography guided detection and isolation of antioxidant components**

For TLC bioautography guided detection and isolation of bioactive compounds, following three steps were performed.

Step I: Analytical TLC for determination of R_f value of separated components of active extracts of potato peels

Analytical TLC separation technique was used for the determination of R_f values of separated components of active extracts of peels of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties (that showed synergistic antioxidant and antimicrobial interactions in combination) following the method of Gu et al., (2009). Analytical TLC was performed in aluminium backed TLC plate (5 × 10 cm, 0.25 mm thickness, Silica gel G 60 F_{254}, Merck, Darmstadt, Germany) preconditioned by heating at 120˚C for 1h, then 5µl (10 mg/ml) of active extracts of peels of *Kufri Chipsona*-3 and *Kufri Jyoti* potato varieties were applied to the TLC plate with a capillary pipette and allowed to dry for a few minutes. Afterwards, the plate was developed with butanol: ethyl acetate: formic acid (5:4:1) (v/v) solvent mixture in presaturated glass chamber at room temperature for 3-4 hours. After the development of TLC plate, the absorbent layer was dried under airflow to remove the solvent mixture completely. Visualization was performed by spraying with *p*-anisaldehyde-sulphuric acid reagent followed by heating at 110°C for 5 min. The R_f value of the spots of separated components of

active peel extracts in the plate were calculated using the following equation (Ali et al., 2013).

$$R_f = \frac{Distance\ traveled\ by\ solute}{Distance\ traveled\ by\ solvent}$$

Simultaneously, TLC plates for the detection of antioxidant compounds in TLC bioautography were also prepared following the same procedure as mentioned above without spraying visualizing reagent, in order to avoid possible interference of visualizing reagent on antioxidant activity of test samples in bioautograms (Suleimana et al., 2010).

Step II: ***TLC bioautography guided detection of antioxidant components***

The developed air-dried plate obtained from analytical TLC prepared for detection of antioxidant compounds was then placed in sterile petri dish and was sprayed with DPPH solution (0.02% w/v in methanol) followed by heating at 110°C for 5 min and observed for development of bright yellow to pink colour for confirmation of antioxidant components (Hafid et al., 2014). The R_f values of the antioxidant spots were determined from the corresponding R_f values of the components spot in analytical TLC plate.

Step III: ***Preparative TLC for isolation of antioxidant components***

For isolation of antioxidant components of active extracts of *Kufri chipsona*-3 and *Kufri Jyoti* potato varieties, a streak of test active peel extracts was applied manually on a preparative TLC glass plate (20 × 20cm, 1 mm thickness) (Sigma-Aldrich, USA) and air dried. After air drying, the plate was developed using the same solvent mixture in a presaturated glass chamber as used in analytical TLC. Bioautograms developed were sprayed with 0.02% (w/v) DPPH solution in methanol. The bands that showed antioxidant activity were scratched off carefully from the silica gel and dissolved in 80% methanol. The scratched antioxidant materials in methanol were then centrifuged at 10000 × g for 15 min to remove the silica gel and the supernatants were collected. The supernatants were filtered through 0.22 µm membrane filter and dried in vacuum. The dried antioxidant components with different R_f values were then isolated and kept in a refrigerator until further use.

(B) Isobologram analysis and checkerboard titration of isolated antioxidant components from TLC bioautography for combined antioxidant and antimicrobial efficacy study

The isolated antioxidant components from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties were subjected to both combined antioxidant and antimicrobial efficacy study following the isobologram analysis and checkerboard titration method respectively using same experimental protocol as described earlier for combined antioxidant and antimicrobial efficacy study of peel extracts of selected varieties of potato (objective -3).

(C) Spectrometric analysis

Bioactive components of peel extract of *Kufri Chipsona-3* (R_f : 0.57) and *Kufri Jyoti* (R_f : 0.61) potato varieties that showed synergistic antioxidant and antimicrobial interactions in combination obtained from isobologram analysis and checkerboard titration method were then subjected to spectrometric (UV/Vis, FTIR, HR-LCMS/MS) analysis for chemical characterization of bioactive compounds.

Only one component from peel extract of *Kufri Chipsona-3* (R_f : 0.57) and *Kufri Jyoti* (R_f: 0.61) in combination showed synergistic antioxidant and antimicrobial interactions were subjected to spectrometric analysis for chemical characterization as follows.

(i) UV/Vis absorption spectra

UV/Vis absorption spectra of two components one from *Kufri Chipsona-3* (R_f: 0.57) and the other from *Kufri Jyoti* (R_f: 0.61) that showed synergistic antioxidant and antimicrobial interactions in combination were recorded using a Perkin Elmer Lambda 950 UV-Vis spectrometer in the range 200-850 nm. UV/Vis absorption spectral analysis of samples was performed at Sophisticated Analytical Instruments Facility (SAIF) Unit, IIT Madras, India.

(ii) FT-IR analysis

The FT-IR spectral analysis of bioactive components from *Kufri Chipsona-3* (R_f : 0.57) and *Kufri Jyoti* (R_f : 0.61) was done at SAIF, IIT Bombay using the Fourier-transform infrared instrument (make and model:3000 Hyperion Microscope with Vertex 80 FTIR System, Burker, Germany) in the scan range 450–4000 cm^{-1}.

(iii) HR-LCMS/MS analysis

The HR-LCMS/MS spectrometric analysis of bioactive components from *Kufri Chipsona-3* (R_f : 0.57) and *Kufri Jyoti* (R_f : 0.61) was done at SAIF , IIT Bombay using HR-LCMS instrument (make and model : Agilent Technologies, USA, 1290 Infinity UHPLC System,1260 infinity Nano HPLC with Chipcube, 6550 iFunnel Q-TOFs) in the mass range 50-32200 amu and resolution 40000 FWHM. The instrument was equipped with (i) UHPLC & NanoHPLC for small molecules as well as for large molecules; (ii) Direct Infusion Mass with ESI & APCI (Positive & Negative mode ionization; (iii) UHPLC PDA Detector -Mass spectrometer and (iv) Nano HPLC with Chipcube (Microfluidic column) -Mass spectrometer.

(D) Evaluation of cytotoxic potential of active extracts of potato peels and their bioactive compounds

The cytotoxic potential of active peel extracts of selected varieties of potato and their bioactive compounds that showed synergistic interactions in combination was evaluated using various methods (*Allium cepa* root growth inhibition assay and WST-1 based colorimetric assay).

(i) *Allium cepa* root growth inhibition assay

Allium cepa root growth inhibition assay was used to evaluate cytotoxic potential of active extracts of potato peels of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties (that showed synergistic antioxidant and antimicrobial action in combination) following the method described by Fiskesjo (1985). Briefly, healthy onion bulbs (20–25g) were purchased from local supermarket (Burabazar market, Kolkata, India). Prior to the experiment, they were dried and the outer scales of the dried bulbs were removed away avoiding destruction of the root primordial. The bulbs were then thoroughly rinsed under running tap water in order to remove traces of herbicides and chemicals, if any. Thereafter, bulbs were allowed to germinate in tap water at room temperature in dark for 3–4 days until the new roots were 3–4 cm in length. Just after the emergence of new roots, nine groups of bulbs having six bulbs in each group were taken in glass tubes containing 10 ml of tap water in each tube. Now, seven different concentrations (15.62, 31.25, 62.5, 125, 250, 500, and 1000 µg/ml) of peel extracts of *Kufri Chipsona-3/Kufri Jyoti* in combination (1:1) was given in tubes of seven groups of onion bulbs. Negative control group received no treatment and positive control group received sodium azide (10µg/ml). Both control as well as peel extracts treated groups were

then kept in the dark at room temperature for 96h. At the end of the exposure period (96h), the best developed 10 roots of *Allium cepa* bulbs in each group were measured and mean root length (cm) was determined. Taking the average root length of the negative control group onion bulbs as 100% growth, decrease in root length of potato peel extract treated groups and positive control group with respect to negative control group were determined. Also, at the end of the exposure period, visible morphological verifications, such as changes in root consistency and colour as well as the presence of swelling, hooks, or twists in the roots, if any, were observed.

(ii) WST -1 based colorimetric assay

The cytotoxicity study of bioactive compounds isolated from active potato peel extracts that exhibited synergistic antioxidant and antimicrobial interactions in combination was evaluated by WST-1 assay using human normal keratinocyte cell line (HaCaT cells). Briefly, the immortalized human keratinocyte cell line was used in the Human Genetics Unit, Indian Statistical Institute, Kolkata, India. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM glutamine, and 1% penicillin/streptomycin (growth medium). Cytotoxicity study was performed in 96-well plates using WST-1based colorimetric assay. For this, 100 µl (1×10^4cells/well) of cell suspension was seeded for 24 h in a 96-well culture plate. The cells were then exposed to different concentrations (6.25, 12.5, 25, 50, 100 µg/ml) of isolated bioactive compounds in combination (1:1) and incubated at 37 °C for 72 h in a humidified atmosphere containing 5% CO_2. After incubation, 10 µl WST-1 reagent (Roche, Mannheim, Germany) was added. Plates were incubated for further 24 h in the previously mentioned conditions, and absorbance was measured at 450 nm. The positive control tube received 1% Triton X-100, and culture medium was used as negative control. Percentage of cell viability was determined as follows.

Cell viability (%) = (Absorbance of treated sample/Absorbance of negative control) × 100

This experiment was carried out at Human Genetics Unit, Indian Statistical Institute, Kolkata, India.

➢ Methods used to fulfil Objective 5

[**Objective 5:** ***To elucidate possible modes of antioxidant and antimicrobial action of bioactive compounds isolated from active extracts of potato peels alone and in combination***]

(A) Methods used to elucidate mode of antioxidant action

After chemical characterization of bioactive compounds of peel extracts of *Kufri Chipsona-3* (R_f: 0.57) and *Kufri Jyoti* (R_f: 0.61) potato varieties that were found to be responsible for synergistic antioxidant and antimicrobial interactions in combination were subjected to evaluate their antioxidant potential in various *in vitro* methods with a view to elucidate their possible modes of synergistic antioxidant action.

➢ ***Methods used to evaluate their individual antioxidant effect***

- ***DPPH radical scavenging assay method***

Free radical scavenging activity of test bioactive compounds at their individual effect was evaluated quantitatively by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay method (Wang et al., 1998). Briefly, 100 µl of each bioactive in varying concentrations (6.25µg/ml-200 µg/ml) was taken in test tubes and 3.9ml of 0.1mM solution of DPPH in methanol was added to these tubes. Tubes were shaken vigorously and allowed to stand in dark at room temperature for 30 min. The control was prepared as above without the bioactive compound and methanol was used for zero adjustment. BHT (butylated hydroxytoluene) was used as positive control. Absorbance of the samples was measured at 517 nm. Inhibition of the DPPH radical by bioactive components was calculated according to the following formula:

$$(\%)\ Free\ radical\ scavenging = \left(\frac{A_{blank} - A_{Sample}}{A_{blank}}\right) \times 100$$

Where, A_{sample} is the absorbance of the sample in DPPH solution after reacting with a given concentration of bioactive compound and A_{blank} is the absorbance of DPPH solution with methanol blank instead of potato peel extract. The percentage of DPPH radical scavenging capacity was plotted against the concentration of bioactive compound and their IC_{50} values

(the concentration required for scavenging 50% of the DPPH) were calculated. All tests were performed in triplicate.

- ***ABTS •+ radical cation decolourization assay***

Free radical scavenging activity of chemically characterized bioactive compounds of *Kufri Chipsona-3* (R_f: 0.57) and *Kufri Jyoti* (R_f: 0.61) potato peel extracts at their individual effect was determined by ABTS •+ radical cation decolorization assay. For this, at first ABTS •+ radical cation was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate (1:1), stored in the dark at room temperature for 12-16 h before use. ABTS •+ solution was then diluted with methanol to obtain an absorbance of 0.700 at 734 nm. After that, 100 µl of various concentrations (6.25 µg/ml- 200 µg/ml) of reconstituted bioactive compounds was added to 3.995 ml of diluted ABTS•+ solution, the absorbance was measured 30 min after the initial mixing at 734 nm. Methanol was used as blank. Each experiment was repeated thrice. Trolox was used as reference standard (Re et al., 1999).

Percent inhibition was calculated using the following formula,

$$\text{ABTS } \bullet + \text{ scavenging effect (\%)} = \frac{A_B - A_A}{A_B} \times 100$$

Where, A_A is absorbance of ABTS •+ radical cation + bioactive compound and A_B is the absorbance of ABTS •+ radical cation + methanol. IC_{50} values of bioactive compounds at their individual effect against ABTS •+ radical cation decolourization were determined using dose response curve.

- ***Trolox equivalent antioxidant power (TEAC)***

After determining the antioxidant activity of bioactive compounds at their individual effect in ABTS •+ radical cation decolourization assay, their inhibitory activity (IC_{50}) was compared with Trolox as reference standard (Re et al., 1999). From Trolox standard curve the Trolox equivalent antioxidant power (TEAC) of the bioactive compounds were determined following the method of Pellegrini et al. (2003).

- ***Ferrous (Fe^{2+}) ion chelating activity***

Ferrous (Fe^{2+}) ion chelating activity of the bioactive compounds at their individual effect was determined following the method of Dinis et al. (1994). Briefly, 50µl of 2mM $FeCl_2$ solution was added in tubes containing 100µl of varying concentrations (6.25 µg/ml–200 µg/ml) of

bioactive compounds. The reaction was started by the addition of 0.2 ml of 5 Mm ferrozine solution. The mixture was then vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was subsequently measured at 562 nm using a spectrophotometer (ThermoFisher Scientific, USA). Sample control was prepared without adding bioactive compounds. EDTA was used as a positive control. The percentage inhibition of ferrozine–Fe^{2+}complex formation was calculated as follows:

$$\% \, Inhibition = \left[\frac{A_c - A_s}{A_s}\right] \times 100$$

Where, Ac was the absorbance of the control, and As was the absorbance of the sample. Each experiment was repeated thrice.

- ***Ferric reducing antioxidant power (FRAP)***

The ferric reducing antioxidant power (FRAP) of the bioactive compounds isolated from peel extracts of *Kufri Chipsona-3* (R_f : 0.57) and *Kufri Jyoti* (R_f : 0.61) potato peel extracts at their individual effect was determined spectrophotometrically following the procedure of Benzie and Strain (1996) with slight modification. The method is based on the reduction of Fe^{3+} TPTZ complex (colourless complex) to Fe^{2+}-tripyridyltriazine (blue coloured complex) formed by the action of electron donating antioxidants at low pH. This reaction is monitored by measuring the change in absorbance at 593 nm. The Ferric reducing antioxidant power (FRAP) reagent was prepared by mixing 300 mM acetate buffer, 10 ml TPTZ in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in the proportion of 10:1:1 at 37°C. Then 3.995 ml of freshly prepared working FRAP reagent was mixed with 5 µl (200µg/ml) of reconstituted bioactive compounds and shaken vigorously. An intense blue colour complex was formed when ferric tripyridyl triazine (Fe^{3+} TPTZ) complex was reduced to ferrous (Fe^{2+}) form and the absorbance at 593nm was recorded against a reagent blank (3.995 ml FRAP reagent+5 µl distilled water) after 30 min incubation at 37°C. All the determinations were performed in triplicates. Ferric reducing antioxidant power (FRAP) of bioactive compounds at their individual effect was calculated using the following formula. BHT was used as reference standard.

$$\% \, Activity = \left[\frac{A_c - A_s}{A_s}\right] \times 100$$

Where, Ac was the absorbance of the control, and As was the absorbance of the sample.

- ***Soybean lipoxygenase (soyLOX) inhibitory activity***

Before going to evaluate lipoxygenase inhibitory activity of isolated bioactive compounds at their individual effect, crude lipoxygenase from soybean was prepared following the method of Leelaprakash et al. (2012). Briefly, pulverized soybean was defatted and decolorized with cold acetone. The defatted sample was dried overnight. Exactly 10 g of the pulverized sample was added into 30 mL of 50 mM sodium phosphate buffer (pH 6.8) and constantly stirred for 5 hours at 40 °C to form slurry. The slurry was filtered through mesh cloth. The filtrate was centrifuged at 12000 g for 15 minutes and the supernatant was subsequently used as LOX source. The anti-lipoxygenase activity was studied using linoleic acid as the substrate and soyLOX as the enzyme according to the method described by Shinde et al. (1999). For this, 100 µl of test bioactive compounds at their individual effect at various concentrations (6.25 µg/ml-250 µg/ml) was mixed with 250 µl of 0.1 M phosphate buffer (pH 9.0) and 150 µl of soyLOX and vortexed. The mixture was allowed to stand at 30 °C for 5 minutes. Then, 500 µl of 0.6 mM linoleic acid solution was added and mixed gently. The absorbance was read at 234 nm. Indomethacin was used as reference standard. The percent inhibition was calculated using the following equation:

$$\% \, Inhibition = \left[\frac{A_0 - A_1}{A_0}\right] \times 100 \, \%$$

Where A_0 is the absorbance of control and A_1 is the absorbance of the sample. IC_{50} values were calculated using dose response curve.

- **Methods used to evaluate their combined antioxidant effect**

- **Anti-lipid peroxidation potential**

Anti-lipid peroxidation potential of isolated bioactive compounds from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties alone and in combination were evaluated against oxidation of omega-3 fatty acids enriched oil in bulk oil model system.

- ***Evaluation of anti-lipid peroxidation potential in bulk oil (marine fish oil)***

The anti-lipid peroxidation potential of bioactive compounds of test bioactive compounds at their individual and combined effects was evaluated in bulk oil following the method of Habeebullah et al (2010). Briefly, 200 µg/ml of test bioactive compounds alone and combination (1:1) were separately mixed in 1 ml of bulk oil and homogenized. BHT (200

µg/ml) (the regulatory limit allowed in oils) was used as positive control. Bulk oil without test bioactive compounds or BHT served as negative control. Samples were cooled in an ice bath and homogenized. Both treated and control samples were transferred to amber bottles with aluminum foil wrapping and kept in an oven at 60°C for 15 days. The lids of amber bottles were loosely attached so that air could pass in and out of the headspace above the samples. Aliquots were removed at 0, 3, 6, 9, 12 and 15 day intervals for analysis. The oxidative state of samples was monitored by analysis of peroxide value (PV) for the determination of primary oxidation, *p*-anisidine value (*p*-AV) for the determination of secondary oxidation. The PV was determined iodometrically and the *p*-AV was determined following AOCS guidelines (AOCS, 1998). Total oxidation (TOTOX) value was calculated using the following formula:

TOTOX = 2PV +*p*-AV (Poina et al., 2010)

All experiments were repeated thrice.

- ***Briggs–Rauscher oscillation reaction method***

The combined antioxidant potential of bioactive compounds of active extracts of potato peels was also evaluated using Briggs-Rauscher oscillation reaction method (Briggs & Rauscher, 1973) in following three steps.

Step I: At first following three stock solutions were prepared as follows. **Solution A**: 43 g potassium iodate and 15.9 g perchloric acid were dissolved in distilled water and diluted to 1L. **Solution B**: 15.6g malonic acid, 3.4g manganese sulfate monohydrate, and 3g starch were dissolved in distilled water and diluted to 1L and **Solution C**: 400 ml of 30% hydrogen peroxide was diluted to 1L in distilled water.

Step II: To determine the antioxidant potential of bioactive compounds, 5 ml of each solution of A and B were poured into 100 ml thermostated beaker which were equipped with a magnetic stirrer bar and placed on a stirring plate. Following this, 5ml of solution C was added to initiate oscillation. After the completion of the third oscillation, 1ml of 200 µg/ml concentration of bioactive compounds alone and in combination (1:1) was added to the BR reaction mixture and just after the immediate quenching of oscillation, the stopwatch was started and the oscillating behaviour of the reaction mixture was monitored visually. The inhibition time ($t_{inhibition}$; the duration of time in seconds from cessation of oscillation by

antioxidant in BR mixture to subsequent regeneration of oscillations) were then determined. Each experiment was repeated thrice.

Step III: The antioxidant potential of potato peel extracts in combination was determined on the basis of inhibition time ($t_{inhibition}$). The types of antioxidant interactions (synergistic, additive, or antagonistic) were interpreted based on $t_{inhibition}$ values as follows. "Synergistic": if $t_{inhibition}$ in combination increases significantly ($p < 0.05$) in comparison to the average $t_{inhibition}$ of their single components in combination; "Antagonistic": if $t_{inhibition}$ in combination decreases significantly ($p < 0.05$) in comparison to the average $t_{inhibition}$ of their single components in combination and "Additive": if $t_{inhibition}$ in combination does not increase or decrease significantly ($p \geq .05$) in comparison to the average $t_{inhibition}$ of their single components in combination (Milos and Makota, 2012).

(B) Methods used to elucidate possible mode of antibacterial action

The following methods were used to elucidate possible mode of synergistic antibacterial action of bioactive compounds isolated from *Kufri Chipsona-3* and *Kufri Jyoti* potato peel extracts

- ***Effects on cell viability (Kill-kinetics assay)***

The kill kinetics assay was used to investigate the antibacterial efficacy of the bioactive compounds isolated from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties responsible for synergistic antimicrobial interactions in combination. To achieve this, 100 µl (5×10^5 CFU/ml) of overnight culture of both *L. monocytogenes* (MTCC 657) and *S. typhimurium* (MTCC 3224) as indicator strains of Gram-positive and Gram-negative foodborne bacterial pathogens respectively were treated with 100 µl of test bioactive compounds alone at their 0.5×MIC and 1×MIC concentrations and in combination at their 0.5×MIC concentrations. The control group received respective broth instead of bioactive compounds. Both control and treated cells were then cultivated at respective temperature (30°C/37 °C) for 24h. Then 10 µl of control and bioactive compound treated samples were removed from wells at 0, 3, 6, 12 and 24 h of incubation and diluted serially with respective broth. Viable counts were determined by plotting 100 µl of diluted aliquots on fresh selected agar plates and incubated at respective temperature for further 24 h. Agar plates with 30 to 300 colonies were used for CFU/ml counting. Kill kinetics curves were constructed by plotting Log_{10} CFU/ml against time. Each experiment was repeated thrice.

- **Effect on cell membrane integrity**

- ***Leakage of 260 nm absorbing materials***

The effect of bioactive compounds alone and in combination (1:1) on cell membrane integrity was monitored by the release of 260 nm absorbing materials (DNA and RNA) following method of Chen and Cooper (2002). For this, 100 µl (5×10^5 CFU/ml) of overnight culture of both the studied Gram-positive *L. monocytogenes* (MTCC 657) and Gram-negative *S. typhymurium* (MTCC 3224) foodborne bacterial pathogens in respective broth were treated with 100 µl of test bioactive compounds alone at their 0.5×MIC and 1×MIC concentrations and in combination at their 0.5×MIC concentrations and incubated for 24h at respective temperature (30°C/37°C). After incubation, both control and treated samples were centrifuged at 10000 g for 5 min and filtered. The absorbance of the filtrate was measured at 260 nm in a spectrophotometer.

- ***Release of extracellular proteins through bacterial cell membrane***

The effect of test bioactive compounds alone and in combination on bacterial cell membrane integrity was also evaluated by the measurement of release of extracellular proteins of studied bacterial pathogens following the method of Bradford (1976). Briefly, 100 µl (5×10^5 CFU/ml) of overnight culture of both the studied Gram-positive and Gram-negative foodborne bacteria *L. monocytogenes* (MTCC 657) and *S. typhimurium* (MTCC 3224) in respective broth were treated with 100 µl of test bioactive compounds alone at their 0.5×MIC and 1×MIC concentrations and in combination at their 0.5×MIC concentrations and incubated for 24h at respective temperature (30°C/37°C). After incubation, both control and treated cells were centrifuged at 10000 g for 5 min and filtered. The absorbance of the filtrate was measured at 595 nm in a spectrophotometer.

- **Effect on cell membrane permeability**

The effect of test bioactive compounds isolated from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties alone and in combination on cell membrane permeability of studied foodborne bacteria *L. monocytogenes* (MTCC 657) and *S. typhimurium* (MTCC 3224) was monitored by using ortho-Nitrophenyl-β-galactoside (ONPG) following the method of Li et al. (2016). Briefly, 100 µl (5×10^5 CFU/ml) of overnight culture of test bacterial pathogens were treated with 100 µl of bioactive compounds alone at their 0.5×MIC and 1×MIC concentrations and in combination at their 0.5×MIC concentrations. Then 10 µl

of ONPG (25 mmol/mL) was added to the suspension, mixed thoroughly and incubated at respective temperature (30°C/37°C) for 0, 1, 2, 4 and 8 h. After incubation, samples were centrifuged at 10000 g for 5 min and the absorbance of sample supernatants was measured by a spectrophotometer at 420 nm.

- ***Effect on cellular morphology (Atomic Force Microscopy Analysis)***

Effects of bioactive compounds alone and in combination on cellular morphology against foodborne bacterial pathogens *L. monocytogenes* (MTCC 657) and *S. typhimurium* (MTCC 3224) were monitored by atomic force microscopy (AFM) analysis following the method described by Bolshakova et al. (2001) with slight modification. Briefly, 100 µl of an overnight culture of bacterial cells (5 $\times 10^5$ CFU/ml) were treated with bioactive compounds alone at their 1 ×MIC and in combination (1:1) at their 0.5×MIC concentrations and incubated for 24h at respective temperature (30°C/37°C). After incubation, cells were centrifuged at 10000×g for 5 min, after which the pellet was washed slowly with 50 mM phosphate buffer solution (pH 7.2), mounted over N type silicon wafer (Ultrananotech, Bangalore, India, Diameter :100mm ± 0.3m (4inch), Orientation: <100>, Thickness: 525±25µm,Resistivity: 1-10 ohm-cm) and fixed with 100 ml glutaraldehyde (2.5%). The specimen was then dehydrated using different concentrations of ethanol (50%–100%). Finally, ethanol was replaced by t-butanol and incubated at room temperature for 2h. The samples were then observed for possible changes in cellular morphology of treated cells compared to their controls by XE 70, Park Systems (South Korea). The scan rate was set at 0.5 Hertz, Z servo gain at 0.457, with set point at 5.99 nm and scan size of 1µm for *Salmonella* and 0.8 µm for *Listeria* samples.

- **Effect on biofilm (anti-quorum sensing activity)**

Anti-quorum sensing activity of test bioactive compounds was carried out by the determination of inhibitory effect of test bioactive compounds alone and in combination against the formation of biofilm (the major phenotype of quorum sensing) of studied foodborne bacterial pathogens. For this, biofilms of test bacterial strains [*L. monocytogenes* (MTCC 657) and *S. typhimurium* (MTCC3224)] were formed by aliquoting 100 µl of test microorganisms (5 × 10^5 CFU/ml) in wells of 96 wells microtiter plate along with 100 µl of bioactive compounds alone at their 0.5×MIC and 1×MIC concentrations and in combination at their 0.5×MIC concentrations. The microtiter plates were incubated at respective temperature (30°C/37°C) for 24 h. After incubation period, plates were removed and each

well of microtiter plates was washed thoroughly with sterile distilled water to remove free-floating cells; thereafter plates were air-dried for 30 min and the biofilm formed was stained with 100 µl of 0.1% aqueous solution of crystal violet (CV) and kept for 15 min at room temperature. The excess of stain was removed by washing the plate three times with sterile distilled water and air-dried. Finally, the dye bound to the cells was solubilized by adding 200 µl of 95% ethanol to each well and after 15 min of incubation at room temperature absorbance of wells was determined at 595nm. Control wells received 100 µl of respective broth in place of test bioactive compounds and percent inhibition of biofilm formation was calculated using the following formula (Rogers and Melander 2008).

$$\% \, Inhibition = \left[\frac{OD_{control} - OD_{treatment}}{OD_{control}}\right] \times 100$$

Each experiment was repeated thrice.

Statistical analysis

The experimental data were expressed as mean ± SD of triplicate experiments. Data were statistically analysed using SPSS software: Version 18.0. A one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test was applied for analysis of data with the level of significance set at $p < 0.05$.

CHAPTER 4

OBJECTIVE 1

TO EVALUATE *IN VITRO* ANTIOXIDANT AND ANTIMICROBIAL POTENTIAL OF PHENOLIC EXTRACT OF PEELS OF SELECTED VARIETIES OF POTATO AT THEIR INDIVIDUAL EFFECT

INTRODUCTION

RESULTS

DISCUSSION

Introduction

Food products require protection during their preparation, storage and distribution to give the desired shelf life (Horbanczuk et al, 2019; Lucera et al., 2012). To protect food products, generally two types of synthetic preservatives (a) antimicrobials and (b) antioxidants are used in the food industry. Antimicrobials are used to inhibit the growth of spoilage and pathogenic microorganisms and antioxidants are used to prevent or retard the auto-oxidation of fats and oils in foods. But, these synthetic food preservatives have accumulated evidence that they could be toxic and carcinogenic (Gultekin et al, 2015). Therefore, innovation should continue to seek a more potent, safe and effective novel natural food preservatives from other sources that have both antioxidant and antimicrobial potential. Because, natural antioxidants and antimicrobials especially from plant origin seem to be the most promising answer to many of the increasing concerns regarding food safety and could yield better results than synthetic food preservatives. The presence of both antioxidant and antimicrobial properties in a single molecule of plant origin makes them more effective and better suited as food preservatives (Ngwoke et al., 2011; Tiwari et al, 2009). Therefore, novel types of effective and healthy antioxidant and antimicrobial blend from plant origin that could protect food against oxidative deterioration and microbial spoilage and contamination as well as consumer against infection is highly demanded (Papuc et al., 2017; Stojkovic et al., 2013). Upgrading of food processing residues is currently a topic of global interest as scientists and industries have focused on using natural sources of antioxidants and antimicrobials as alternatives to synthetic food preservatives (Gupta et al., 2015; Mohdaly et al., 2010; Samotyja, 2019).

Potatoes (*Solanum tuberosum* L.) are one of the most important agricultural crops for human consumption all over the world. Potato peels are considered as waste material in many households as well as in the potato processing industries. Each year, potato processing industries generate substantial quantities of potato peel waste as by-products which create a major waste disposal problem for the industry concerned. But these potato peels are rich in phenolic compounds and may serve as a potential source of natural antioxidant and antimicrobial agents in food and pharmaceutical industries (Habeebullah et al. 2012; Gebrechristos and Chen, 2018). Although antioxidant and antimicrobial potential of potato peel extracts of a few varieties of potato have been reported by several workers (Franco et al., 2016; Habeebullah et al, 2012; Prasad & Pushpa, 2007; Sinha and Dua, 2016), their studies

were restricted only to evaluate their either antioxidant or antimicrobial efficacy individually. Detailed knowledge about potato peel extracts having both antioxidant as well as antimicrobial potential is lacking. This knowledge is particularly important for designing a more potent, safe and effective novel natural preservatives that have dual action antioxidant and antimicrobial for protecting food products. Besides, this will also help to upgrade potato peels from waste by-product to value added products. The present investigation has therefore been designed to evaluate antioxidant and antimicrobial efficacy of phenolic extract of peels of five selected varieties of potato (three from processing varieties: *Kufri Chipsona-1*, *Kufri Chipsona-3*, and *Kufri Chipsona-4*; and two from table varieties: *Kufri Chandramukhi* and *Kufri Jyoti*) at their individual effect with a view to screen active extracts of potato peels that have both antioxidant and antimicrobial efficacy to fulfil objective 1.

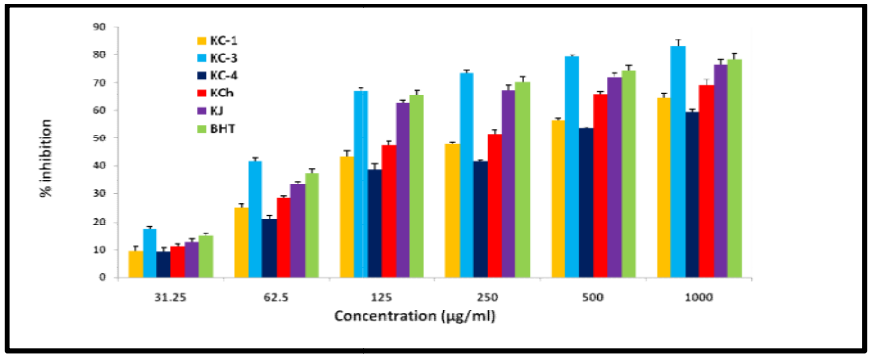
Results

➢ *Results on individual antioxidant efficacy*

The *in vitro* antioxidant potential of phenolic extract of peels of five selected varieties of potato at their individual effect was evaluated by (i) DPPH radical scavenging, (ii) β-carotene-linoleic acid bleaching and (iii) ferrous (Fe^{2+}) ion chelating methods.

- ***DPPH radical scavenging***

Figure 4.1 shows the results of free radical scavenging activity of peel extract of five selected varieties of potato in DPPH radical scavenging method.



Peel extract	IC50 (µg/ml)
KC-1	301.47
KC-3	100.70
KC-4	411.20
KCh	113.80
KJ	107.68
BHT	119.96

Figure 4.1 DPPH radical scavenging activity of potato peel extracts

KC-1 : *Kufri Chipsona*-1; KC-3 : *Kufri Chipsona* -3; KC-4 : *Kufri Chipsona* – 4; KCh : *Kufri Chandramukhi*; KJ : *Kufri Jyoti*; BHT : Butylated hydroxytoluene

From figure 4.1 it was observed that all the peel extracts tested exhibited varying degrees of concentration dependent antioxidant efficacy. Peel extract of *Kufri Chipsona-3* variety exhibited highest DPPH radical scavenging activity (IC_{50}:100.7µg/ml) followed by peel extracts of *Kufri Jyoti* (IC_{50}:107.68µg/ml) and *Kufri Chandramukhi* (IC_{50}:113.80µg/ml). Peel extracts of *Kufri Chipsona-1* (IC_{50}: 301.47 µg/ml) and *Kufri Chipsona-4* (IC_{50}: 411.20 µg/ml) potato varieties showed weak DPPH radical scavenging activity compared to BHT (IC_{50}:119.96 µg/ml) (shown in side table) (Figure 4.1), a reference standard antioxidant agent.

- ***β-carotene-linoleic acid bleaching***

Table 4.1 shows the results on inhibitory effect of peel extracts of selected varieties of potato on β-carotene-linoleic acid bleaching at their individual effect. Peel extract of *Kufri Chipsona-3* potato variety exhibited highest inhibitory effect in β-carotene–linoleic acid bleaching (IC_{50}: 185.6µg/ml) followed by peel extracts of *Kufri Jyoti* (IC_{50}:380.5 µg/ml) and *Kufri Chandramukhi* (IC_{50}: 442.3 µg/ml). On the other hand, peel extracts of both *Kufri Chipsona-1* and *Kufri Chipsona-4* potato varieties were found to be inactive (IC_{50}> 1000µg/ml). Peel extract of Kufri Chipsona-3 was found to be more effective than reference standard antioxidant BHT (IC_{50}: 255.9 µg/ml)

Table 4.1 Effect of potato peel extracts on β-carotene-linoleic acid bleaching activity

Treatment	Concentration (µg/ml)						IC_{50} (µg/ml)
	31.25	62.5	125	250	500	1000	
	% inhibition						
KC-1	9.87 ± 1.24	12.53 ± 1.22	14.37 ± 1.21	21.64 ± 2.17	36.54 ± 3.24	42.67 ± 4.20	>1000
KC-3	21.83 ± 1.44	27.46 ± 1.41	41.63 ± 2.74	52.76 ± 2.43	67.45 ± 3.87	76.24 ± 4.15	185.6
KC-4	7.24 ± 1.05	8.66 ± 1.12	9.84 ± 1.32	17.35 ± 1.68	30.49 ± 3.69	43.45 ± 3.90	>1000
KCh	16.67 ± 1.05	19.21 ± 1.72	25.83 ± 1.45	34.74 ± 2.36	52.37 ± 3.64	59.63 ± 4.32	442.3
KJ	18.81 ± 1.21	23.57 ± 1.32	34.47 ± 1.73	39.84 ±2.41	56.53 ± 3.46	67.70 ± 3.69	380.5
BHT	20.84 ± 1.52	25.65 ± 1.43	37.65 ± 1.86	47.48 ± 3.05	58.75 ± 3.56	71.46 ± 3.87	255.9

Results are Mean ± S.D. of triplicate experiments.
KC-1: *Kufri Chipsona* -1; KC-3: *Kufri Chipsona* -3; KC-4: *Kufri Chipsona* – 4; KCh: *Kufri Chandramukhi*; KJ: *Kufri Jyoti*; BHT: Butylated hydroxytoluene

- ***Ferrous (Fe^{2+}) ion chelating activity***

The results of ferrous (Fe^{2+}) ion chelating activity of peel extracts of selected varieties of potato at their individual effect are shown in Figure 4.2. It was observed that Fe^{2+} ion chelating activity of peel extracts of selected varieties of potato were in the following decreasing order : *Kufri Chipsona*-3 (IC_{50}: 304.60µg/ml) >*Kufri Jyoti* (IC_{50}: 434.8 µg/ml) >ascorbic acid (**IC_{50}**: 512.20 µg/ml) >*Kufri Chandramukhi* (IC_{50}: 587.79µg/ml) >*Kufri Chipsona*-1 (IC_{50}: 928.3µg/ml). Peel extract of *Kufri Chipsona*-4 potato variety was found to be inactive (IC_{50}> 1000 µg/ml).

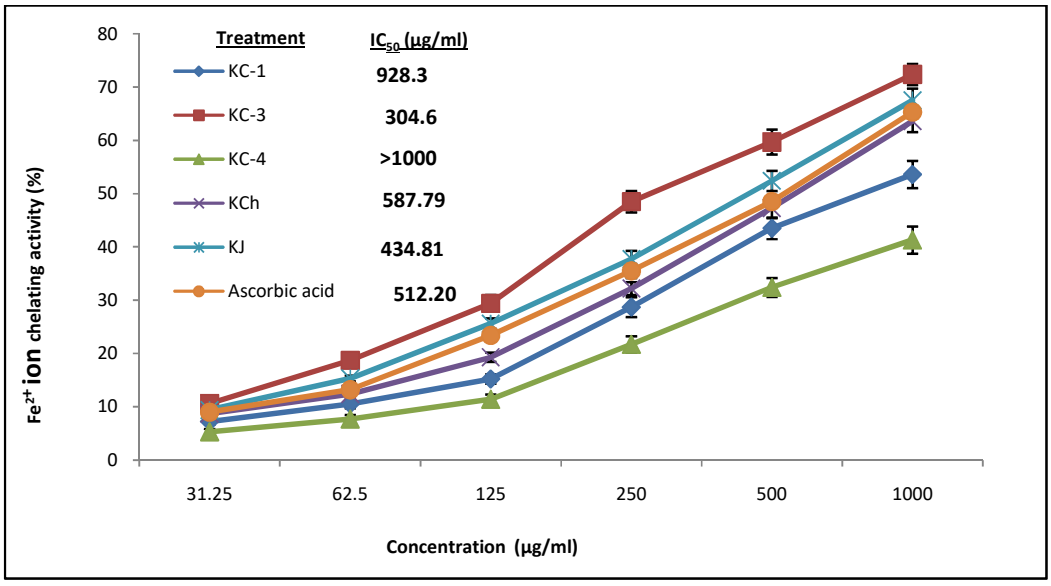


Figure 4.2 Fe^{2+} ion chelating activity of potato peel extracts

KC-1 :*Kufri Chipsona*-1; KC-3 : *Kufri Chipsona* -3; KC-4 : *Kufri Chipsona* – 4; KCh :*Kufri Chandramukhi.*

- ***Antimicrobial results at their individual effect***

- ***Inhibition zone diameter***

The results of inhibition zone diameter (IZD) of test potato peel extracts at their individual effect against the studied foodborne bacterial and fungal pathogens are shown in Table 4.2. It was observed from Table 4.2, that among the potato peel extracts tested, only peel extracts of *Kufri Chipsona*-3, *Kufri Jyoti* and *Kufri Chandramukhi* were found to be active (IZD ≥ 11 mm) against all the studied Gram-positive and Gram-negative bacterial pathogens as well as

fungal pathogen. Peel extracts of *Kufri Chipsona*-1 and *Kufri Chipsona*-4 were found to be inactive (IZD < 8 mm) against all the studied microbes.

Table 4.2 Inhibition zone diameter (IZD) in mm of potato peel extracts against the studied foodborne bacterial and fungal pathogens

Treatment	*L. monocytogenes* (MTCC 657)	*S. typhimurium* (MTCC 3224)	*A. niger* (ATCC 16404)
KC-1	7.63±0.28	5.72±0.63	6.42±0.91
KC-3[a]	21.44±1.07	18.44±1.29	19.37±0.87
KC-4	5.21±0.63	6.74±0.35	4.76±0.53
KJ[a]	17.14±0.82	14.82±1.64	19.43±1.06
KCh[a]	14.75±1.47	12.38±1.21	11.58±1.43
Ciprofloxacin[a]	27.83±1.43	24.70±1.53	23.32±1.74
DMSO	–	–	–

KC-1*: Kufri Chipsona*-1; KC-3: *Kufri Chipsona*-3; KC-4: *Kufri Chipsona*-4; KJ: *Kufri Jyoti;* KCh: *Kufri Chandramukhi.* Values are mean ± SD of triplicate experiments. [a]Active (IZD ≥ 11 mm against all the studied foodborne bacterial and fungal strains (Bauer et al., 1966)

Figure 4.3 (a,b,c) show a few photographs of inhibition zone diameter (IZD) of peel extracts of *Kufri Chipsona-3* , *Kufri Jyoti* , *Kufri Chandramukhi* against *L. monocytogenes* (MTCC 657) in agar well diffusion method.

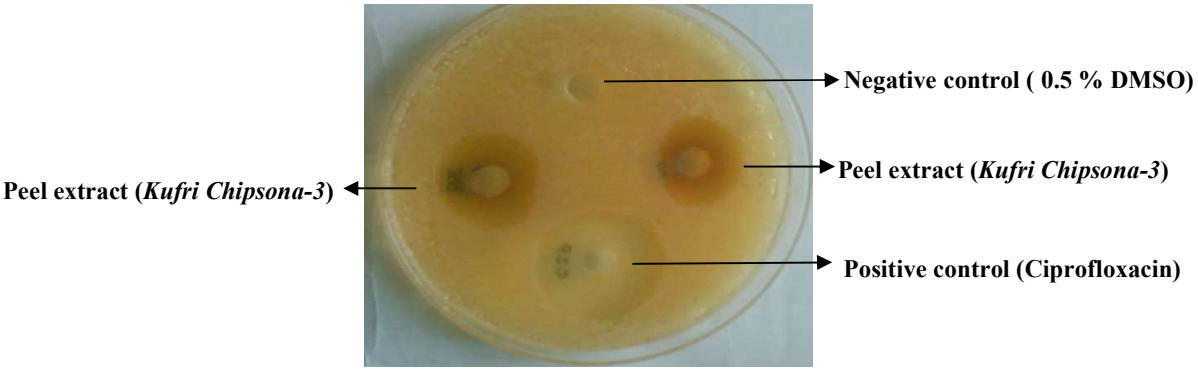


Figure 4.3 (a) Inhibition Zone Diameter (IZD) of *Kufri Chipsona-3* peel exract against *Listeria monocytogenes (MTCC 657)*

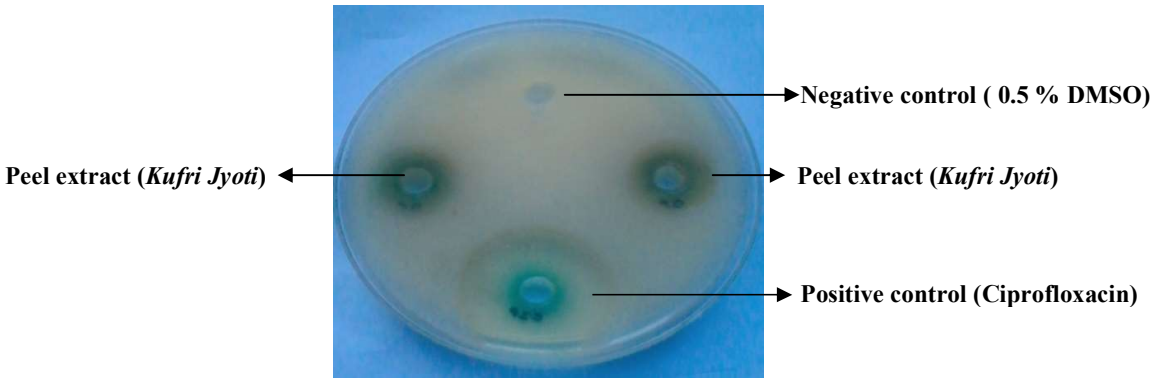


Figure 4.3 (b) Inhibition Zone Diameter (IZD) of *Kufri Jyoti* peel exract against *Listeria monocytogenes (MTCC 657)*

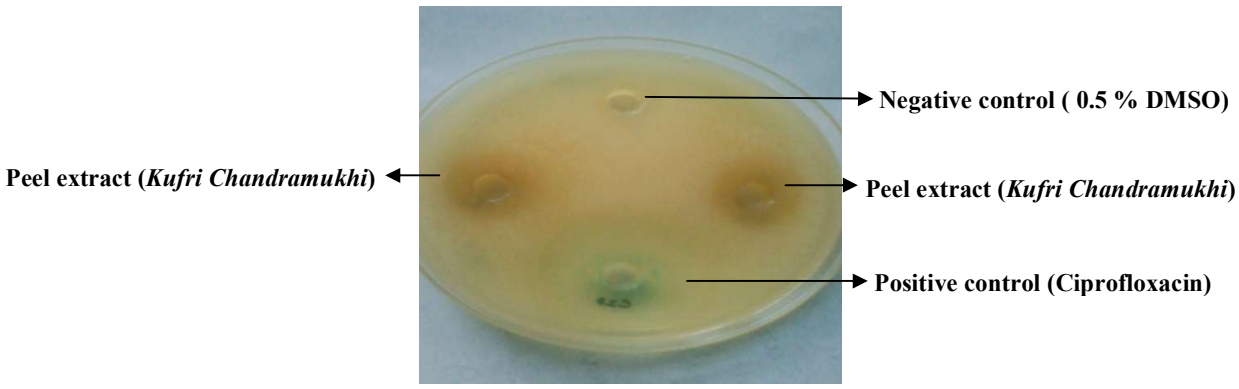


Figure 4.3 (c) Inhibition Zone Diameter (IZD) of *Kufri Chandramukhi* peel exract against *Listeria monocytogenes (MTCC 657)*

Table 4.3 shows the MIC and MFC values of test potato peel extracts against the studied bacterial and fungal pathogens at their individual effect. Peel extract of *Kufri Chipsona*-3 showed highest antimicrobial activity against all the studied microbes with MIC : 44.38±4.77 µg/ml against *L. monocytogenes* and 63.54±3.12 µg/ml against *S. typhimurium* and MFC : 57.64±2.43 µg/ml against *A. niger* followed by *Kufri Jyoti* (MIC : 55.34±3.80 µg/ml and

74.87±4.53 µg/ml against *L. monocytogenes* and *S. typhimurium* respectively and MFC : 62.27±2.16 µg/ml against *A. niger*) and *Kufri Chandramukhi* (MIC: 79.21±4.37 µg/ml and 98.32±5.67 µg/ml against *L. monocytogenes* and *S. typhimurium* respectively and MFC: 87.75±3.71 µg/ml against *A. niger*). But, peel extracts of *Kufri Chipsona*-1 and *Kufri Chipsona*-4 were found to be inactive against all the studied microbes (MIC or MFC > 100 µg/ml).

Table 4.3 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of test potato peel extracts at their individual effect

Treatment	*L. monocytogenes* (MTCC 657) (MIC: µg/ml)	*S. typhimurium* (MTCC 3224) (MIC: µg/ml)	*A. niger* (ATCC 16404) (MFC: µg/ml)
KC-1	107.44±3.41	117.83±4.54	112.21±3.47
KC-3[a]	44.38±4.77	63.54±3.12	57.64±2.43
KC-4	114.45±3.63	128.64±4.70	119.83±3.64
KJ[a]	55.34±3.80	74.87±4.53	62.27±2.16
KCh[a]	79.21±4.37	98.32±5.67	87.75±3.71
Ciprofloxacin[a]	32.40±1.63	37.38±2.84	29.43±3.18
DMSO	–	–	–

KC-1*: Kufri Chipsona*-1; KC-3: *Kufri Chipsona*-3; KC-4: *Kufri Chipsona*-4; KJ: *Kufri Jyoti;* KCh: *Kufri Chandramukhi.* Values are mean ± SD of triplicate experiments. [a]Active (MIC or MFC ≤ 100 µg/ml against all the studied foodborne bacterial and fungal pathogens (Chlipala et al., 2010)

Figure 4.4 (a,b,c) show a few photographs of MIC determination of peel extracts of *Kufri Chipsona-3* , *Kufri Jyoti* and *Kufri Chandramukhi* against *L. monocytogenes* (MTCC 657) in microbroth dilution method.

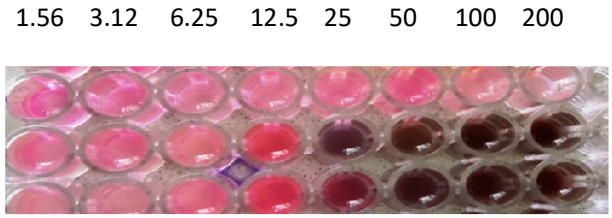


Figure 4.4 (a) Antibacterial activity of *Kufri Chipsona-3* peel extract at different concentrations (µg/ml) against *Listeria monocytogenes (*MTCC 657)

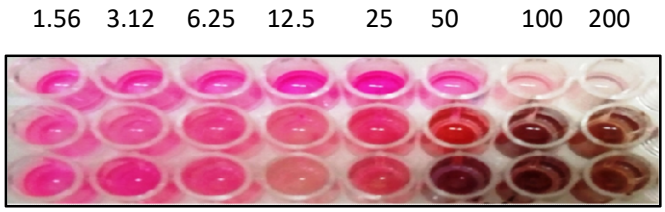


Figure 4.4 (b) Antibacterial activity of *Kufri Jyoti* peel extract at different concentrations (µg/ml) against *Listeria monocytogenes* (MTCC 657)

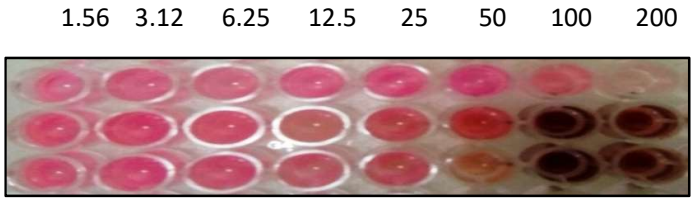


Figure 4.4 (c) Antibacterial activity of *Kufri Chandramukhi* peel extract at different concentrations (µg/ml) against *Listeria monocytogenes* (MTCC 657)

Discussion

In vitro antioxidant efficacy

The relevant literature reveals that the antioxidant activity of phenolic compounds comprises of their capacity of scavenging free radicals, donating hydrogen atoms, electrons, or chelate metal cations (Afanasev et al., 1989). Therefore in this present investigation , antioxidant efficacy of peel extracts of selected varieties of potato was evaluated in DPPH radical scavenging , β- carotene –linoleic acid bleaching and Fe^{2+} ion chelating methods with a view to observe their hydrogen donating, electron transfer and metal ion chelating ability.

DPPH radical scavenging method was used because this method is widely used routinely as a quick, reliable and reproducible parameters in order to screen *in-vitro* antioxidant activity of crude plant extracts or purified compounds. The effect of antioxidants

on DPPH radical scavenging can be attributed to their hydrogen donating ability. The reduction capacity of DPPH radical is determined by the decrease in absorbance which is being triggered by antioxidants (Minatel et al., 2017). Therefore, in the present investigation to evaluate the hydrogen donating ability of test potato peel extract in the scavenging of stable free radicals, in the present investigation DPPH radical scavenging method was used. It was observed that all the phenolic extracts of potato peels of selected varieties of potato tested were capable of reducing the stable radical DPPH to the yellow coloured diphenylpicrylhydrazine at varying degrees (Figure 4.1). The findings suggest that tested peel extracts exhibited varying degrees of hydrogen donating ability and peel extract of *Kufr*i *Chipsona-3* potato variety showed highest hydrogen donating ability followed by peel extracts of *Kufri Jyoti* and *Kufri Chandramukhi*. Peel extracts of *Kufri Chipsona-1* and *Kufri Chipsona-4* showed weak DPPH radical scavenging activity compared to other extracts tested (Figure 4.1) suggesting their varying degrees of hydrogen donating ability for scavenging stable free radicals and peel extract of *Kufri Chipsona -3* exhibited highest hydrogen donating ability whereas peel extract of *Kufri Chipsona-4* showed lowest hydrogen donating ability.

The antioxidant efficacy of test potato peel extracts at their individual effect was also evaluated by β-carotene-linoleic acid bleaching assay. The principle of this method is that β-carotene undergoes rapid discolouration in absence of antioxidant, which gives rise to the reduction in absorbance of the test solution with increasing reaction time. The presence of antioxidant inhibits the extent of bleaching by neutralizing the linoleic hydroperoxyl radicals formed (Kulisica, 2004). Thus, the degradation rate of β-carotene depends on the antioxidant activity of crude plant extracts or purified antioxidant compounds. To evaluate the propagated radical scavenging activity of test potato peel extract in oxidative chain reaction, either by donating hydrogen atom or electrons, their antioxidant efficacy in β-carotene-linoleic acid bleaching reaction was evaluated. From Table 4.1, it was observed that test potato peel extracts inhibited β-carotene-linoleic acid bleaching activity at varying degrees. *Kufri Chipsona-3* potato peel extract showed highest antioxidant potential whereas *Kufri Chandramukhi* peel extract demonstrated lowest antioxidant potential in β-carotene linoleic acid bleaching assay (Table 4.1). On the basis of IC_{50} values, the propagated radical scavenging activity in oxidative chain reaction by peel extracts was found to be in the following decreasing order : *Kufri Chipsona-3* > *Kufri Jyoti* > *Kufri Chandramukhi.* Peel extracts of *Kufri Chipsona-1* and *Kufri Chipsona-4* were found to be inactive (IC_{50} > 1000 μg /ml).

It is well documented that iron is an essential mineral for normal physiology, but an excess of it can be detrimental for human health and may lead to cellular injury. If they undergo Fenton reaction, the reduced metal may form reactive hydroxyl radicals as a prooxidant and therefore triggers oxidative stress (Hippeli and Elstner, 1999). An important mechanism of antioxidant activity is the ability to chelate/deactivate transition metals, which possess the ability to catalyze hydroperoxide decomposition and Fenton-type reactions. Consequently, it is considered important to screen the iron (II) chelating ability of the peel extracts. Therefore, in the present investigation, Fe^{2+} ion chelating ability of test potato peel extracts were evaluated at their individual effect. It was observed that Fe^{+2} ion chelating ability of peel extract of *Kufri Chipsona-3* was found to be highest followed by peel extracts of *Kufri Jyoti* and *Kufri Chandramukhi*. Peel extracts of *Kufri Chiposona-1* and *Kufri Chipsona-4* were found to be inactive.

- ***In –vitro antimicrobial efficacy***

For screening antimicrobial efficacy of crude plant extract or purified compound, a number of techniques or methods are used. Among these, the first and foremost method used for antimicrobial susceptibility testing is agar well diffusion or disk diffusion method. The agar well diffusion or disk diffusion method offers many advantages over other methods viz. simplicity, low cost, the ability to test enormous numbers of microorganisms and antimicrobial agents, and the ease to interpret results. The above-mentioned advantages of this method, mainly simplicity and low cost, have contributed to its common use for the antimicrobial screening of plant extracts, essential oils and other drugs (Das et al., 2010). Besides, this method provides qualitative results by categorizing bacteria as susceptible, intermediate or resistant (Bauer et al., 1966; Jorgensen and Ferraro, 2009). In this work, I therefore, evaluated the antimicrobial efficacy of peel extracts of selected varieties of potato in agar well diffusion method. The results revealed that among the peel extract of selected varieties of potato tested, only peel extracts of *Kufri Chipsona-3*, *Kufri Chandramukhi* and *Kufri Jyoti* potato varieties were found to be active antimicrobial agent against the studied microbes (IZD ≥ 11 mm). Peel extract of *Kufri Chipsona-3* was found to be more effective antimicrobial agent followed by peel extracts of *Kufri Jyoti* and *Kufri Chandramukhi* (Table 4.2).

Since agar well diffusion method assay provides only the qualitative data for categorizing the bacteria as susceptible, intermediate or resistant (Bauer et al., 1966; Jorgensen and Ferraro, 2009), I have also evaluated antimicrobial susceptibility of test potato peel extracts against the studied microbes by broth dilution method. Broth dilution method was used because this method is the most appropriate ones for the determination of MIC values, since they offer the possibility to estimate the concentration of the tested antimicrobial agent in broth medium (macrobroth or microbroth dilution). Results of antimicrobial susceptibility testing of test potato peel extracts against the studied microbes in broth microdilution method revealed that among the peel extracts tested, only peel extracts of *Kufri Chipsona-3*, *Kufri Chandramukhi* and *Kufri Jyoti* potato varieties exhibited strong antimicrobial activity (MIC < 100 µg/ml) against the studied microbes (Table 4.3).

Now, a comparative study on antioxidant and antimicrobial efficacy of peel extracts of selected varieties of potato was made. Based on threshold limits of antioxidant efficacy [IC_{50}< 100 µg/ml (Nordin et al., 2018)]; and antimicrobial susceptibility testing [IZD > 11 mm (Bauer et al., 1966) and MIC or MFC < 100 µg/ml (Chlipala et al., 2009), among the peel extracts tested, only peel extracts of *Kufri Chandramukhi*, *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties were found to have strong antioxidant as well as antimicrobial potential at their individual effect.

CHAPTER 5

OBJECTIVE 2

TO EVALUATE INDIVIDUAL ANTIOXIDANT POTENTIAL OF PHENOLIC EXTRACT OF PEELS OF SELECTED VARIETIES OF POTATO AGAINST OXIDATION OF OMEGA-3 FATTY ACIDS ENRICHED FOOD SUPPLEMENT AND OMEGA-3 FATTY ACIDS FORTIFIED FUNCTIONAL FOODS AT THEIR INDIVIDUAL EFFECT

INTRODUCTION

RESULTS

DISCUSSION

Introduction

Lipid oxidation has long been recognized as a major problem in the storage of fatty acids in foods. Oxidation of lipids in food can cause rancidity such as off flavours, loss of colour, altered nutrient value, and may produce toxic compounds, which can be detrimental to the health of consumers (Habeebullah et al., 2010). Thus, lipid oxidation is one of the most important issues related to food quality in term of shelf life of food containing lipids and nutritional values of lipid bearing foods (Ahmed et al., 2016). Omega-3 fatty acids have immense health benefits. More recent studies assessed that omega – 3 polyunsaturated fatty acid supplementation could be helpful against many inflammatory diseases (Meydani et al., 1993). Their anti-inflammatory effects is mainly related to its competition as substrates for cyclooxygenase (COX) and lipoxygenase (LOX) leading to the formation of less active prostaglandins and leukotrienes (James et al., 2000; Ziboh et al., 2000). Therefore, in recent years, to increase the nutritional value of foods and reduce the risk of chronic diseases, omega-3 fatty acids enriched oils (marine fish oil, walnut oil) are added to foods (Anbudhasan et al., 2014). But developing omega-3 fatty acids fortified stable functional food is challenging because the two principal omega-3 fatty acid components eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are highly susceptible to auto-oxidation due to high degree of unsaturation in their molecular structures (Keogh et al., 2001). Therefore, in this work an attempt has been made to evaluate possible antioxidant potential of phenolic extracts of peels of selected varieties of potato against oxidation of omega-3 fatty acids enriched food supplement and omega-3 fatty acids fortified various functional foods (whole milk, yoghurt and paneer) at their individual effect to extend the shelf life of lipid bearing foods especially omega-3 fatty acids fortified functional foods to fulfil objective 2 , with a view to shed some light on these important aspects.

- **Results**

(A) Results on individual antioxidant potential of peel extracts in omega-3 fatty acids enriched food supplement (marine fish oil) in two lipid model systems (Bulk oil and oil-in-water emulsion)

- ***Results on primary and secondary oxidation***

Table 5.1 and Table 5.2 show the results of primary and secondary oxidation products of omega-3 fatty acids in two lipid model (bulk oil and oil-in-water emulsion) systems before and after treatment of peel extracts of selected varieties of potato at their individual effect. It was observed that among the peel extracts of selected potato varieties tested, peel extract of *Kufri Chipsona-3*, *Kufri Chandramukhi* and *Kufri Jyoti* potato varieties showed varying degrees of anti-lipid peroxidation potential against both primary oxidation (Table 5.1) as well as secondary oxidation (Table 5.2) of fatty acids in two lipid model systems. These active three peel extracts of potato varieties (*Kufri Chipsona-3*, *Kufri Jyoti* and *Kufri Chandramukhi*) were also found to be more effective in bulk oil than oil-in-water emulsion model systems. On the other hand, peel extracts of *Kufri Chipsona-1* and *Kufri Chipsona-4* potato varieties showed very weak anti-lipid peroxidation potential against primary and secondary oxidation of fatty acids in both the test lipid model systems (Table 5.1 and Table 5.2).

Table 5.1 Effects of potato peel extracts on primary oxidation (peroxide value; PV) in omega-3 fatty acids enriched bulk oil and oil-in -water emulsion model systems

Treatment	Bulk oil (meq/kg oil)						Oil-in-water emulsion (meq/kg oil)					
	Days						Days					
	0	3	6	9	12	15	0	3	6	9	12	15
Control	1.26 ± 0.25	9.98 ± 0.69	11.02 ± 1.10	15.25 ± 1.27	18.56 ± 1.66	21.08 ± 2.98	1.42 ± 0.14	10.56 ± 1.98	12.25 ± 1.66	16.54 ± 2.25	20.43 ± 2.02	22.63 ± 2.62
KC-1	1.28 ± 0.27	9.64 ±1.45 (3.44)	10.21 ± 0.79 (7.43)	12.80± 0.51 (16.08)	16.63 ± 0.82 (22.39)	16.20± 2.18 (23.14)	1.47 ± 0.12	10.3 ± 0.89 (2.53)	11.59± 0.73 (5.40)	14.19 ± 1.62 (14.21)	16.50 ± 1.44 (19.26)	18.14 ± 2.54 (19.84)
KC-3	1.24 ± 0.24	8.72 ± 0.32 (12.64)	8.63 ± 0.27 (21.72)	9.43 ± 0.21* (38.17)	9.02 ± 0.42* (51.43)	7.92 ± 1.85* (62.45)	1.39 ± 0.15	9.54 ± 0.24 (9.67)	10.00± 0.56 (18.44)	10.64 ± 0.80* (35.71)	11.02 ± 0.74* (46.05)	9.07± 0.73* (59.89)
KC-4	1.25 ± 0.24	9.77 ± 0.78 (2.1)	10.61± 0.25 (5.74)	13.21 ± 0.56 (13.44)	15.35± 0.65 (17.33)	16.62 ± 1.02 (21.16)	1.46 ± 0.14	10.42± 0.21 (1.40)	11.87 ± 0.48 (3.16)	14.57 ± 0.65 (11.92)	17.40 ± 1.21 (14.86)	18.26 ± 1.95 (19.35)
KCh	1.24 ± 0.26	9.17 ± 0.63 (8.12)	9.41 ± 1.32 (14.61)	11.24 ± 1.46* (26.32)	11.63 ± 1.22 * (37.34)	10.05± 1.43* (52.33)	1.43 ± 0.16	10.20 ± 1.05 (3.47)	11.21 ± 1.43 (8.57)	12.34 ± 1.75* (20.59)	13.18 ± 1.15* (35.50)	11.81 ± 1.46* (47.87)
KJ	1.27 ± 0.25	8.96 ± 0.87 (10.25)	9.12± 1.04 (17.28)	10.72 ± 0.81* (29.72)	10.71 ± 1.05* (42.33)	9.17 ± 1.24* (56.54)	1.44 ± 0.15	9.89± 0.64 (6.39)	10.86± 1.17 (11.02)	12.26 ± 1.24* (25.90)	12.42± 1.02* (39.24)	10.49± 0.56* (53.67)
BHT	1.29 ± 0.23	8.75 ± 0.82 (12.34)	8.89 ± 0.32 (19.40)	10.28 ± 0.54* (32.62)	9.96 ± 0.76* (40.98)	9.39 ± 1.64* (55.46)	1.43 ± 0.15	9.83± 0.21 (6.97)	10.53 ± 0.62 (14.08)	11.98 ± 0.78* (27.57)	12.71± 0.81* (37.83)	10.23± 0.65* (54.82)

Results are Mean ±S.D. of triplicate experiments. *P < 0.05 with respect to their control values.
KC-1: *Kufri Chipsona* -1; KC-3: *Kufri Chipsona* -3; KC-4: *Kufri Chipsona* – 4; KCh: *Kufri Chandramukhi*; KJ: *Kufri Jyoti*; BHT: Butylated hydroxytoluene. Values in parentheses indicate percent inhibition with respect to control.

Table 5.2 Effects of potato peel extracts on secondary oxidation (*p*-anisidine value; *p*-AV) in omega-3 fatty acids enriched bulk oil and oil-in- water emulsion model systems

Treatment	Bulk oil (meq/kg oil)						Oil-in-water emulsion (meq/kg oil)					
	Days						Days					
	0	3	6	9	12	15	0	3	6	9	12	15
Control	1.14 ± 0.06	11.56 ± 1.15	17.07 ± 1.10	25.89 ± 2.75	29.77 ± 2.59	32.25 ±2.75	1.78 ± 0.38	16.25 ±2.66	19.59 ± 2.58	28.92 ± 2.98	34.09 ± 3.58	40.78 ± 3.25
KC-1	1.12 ± 0.08	11.03 ± 0.67 (4.60)	15.83 ± 1.16 (7.29)	22.61 ± 2.02 (12.67)	24.59 ± 2.90 (17.43)	26.36 ± 2.47 (18.26)	1.79 ± 0.36	14.84 ± 0.61 (2.58)	18.65 ± 0.89 (4.82)	25.98 ± 2.63 (10.18)	28.51± 2.15 (16.37)	33.98 ± 3.63 (16.67)
KC-3	1.17 ± 0.07	10.31 ± 0.19 (10.84)	13.72 ± 0.45 (19.63)	17.43 ± 1.05* (32.71)	15.73± 1.69* (47.18)	13.79 ± 2.06* (57.27)	1.76 ± 0.35	11.98 ± 2.52 (8.72)	4.07 ± 0.89 (18.44)	12.64 ± 1.25* (30.57)	16.83 ± 2.10* (41.22)	23.32 ± 2.66* (53.76)
KC-4	1.14 ± 0.05	11.24 ± 0.78 (2.73)	16.22 ± 1.66 (4.93)	23.49 ± 2.10 (9.27)	26.17 ±1.25 (12.09)	26.33 ± 1.91 (18.35)	1.75 ± 0.36	15.76 ± 0.55 (3.07)	19.11 ± 1.01 (4.03)	26.13 ± 1.21 (9.65)	29.11 ± 2.25 (14.63)	34.55 ± 3.48 (15.27)
KCh	1.16 ± 0.07	10.86 ± 0.92 (6.14)	14.84 ± 1.06 (13.07)	19.52 ± 2.14* (24.61)	18.32 ± 1.62* (38.47)	16.31 ± 2.64* (49.43)	1.71 ± 0.37	15.40 ± 0.93 (5.31)	17.55 ± 1.46 (10.44)	22.74 ± 2.17* (21.37)	21.56 ± 2.61* (36.78)	22.87 ± 2.84* (43.92)
KJ	1.15 ± 0.08	10.58 ± 1.52 (8.56)	14.29 ± 1.05 (16.34)	18.59 ± 1.27* (28.21)	17.41 ± 1.36* (41.53)	15.08 ± 1.52* (53.27)	1.77 ± 0.35	15.25 ± 2.07 (6.17)	17.01 ± 1.14 (13.17)	21.90 ± 1.35* (24.28)	20.68 ± 2.43* (39.35)	20.62 ± 2.83* (48.72)
BHT	1.12 ± 0.05	10.56 ± 2.27 (8.67)	14.03 ± 1.24 (17.82)	18.22 ± 1.66* (29.63)	12.53 ± 1.57* (43.25)	15.01 ± 1.89* (53.47)	1.75 ± 0.39	15.12 ± 2.89 (6.97)	18.32 ± 2.63 (15.32)	21.02 ± 1.72* (27.23)	27.56 ± 1.92* (38.34)	19.04 ± 2.78* (51.75)

Results are Mean ±S.D. of triplicate experiments. *P < 0.05 with respect to their control values.
KC-1: *Kufri Chipsona* -1; KC-3: *Kufri Chipsona* -3; KC-4: *Kufri Chipsona* – 4; KCh: *Kufri Chandramukhi*; KJ: *Kufri Jyoti*; BHT: Butylated hydroxytoluene. Values in parentheses indicate percent inhibition with respect to control

- **Results on total oxidation**

Figure 5.1 and Figure 5.2 show the results of total oxidation of omega-3 fatty acids in bulk oil and oil-in-water emulsion model systems respectively before and after treatment of peel extract of selected varieties of potato at their individual effect. It was observed that peel extracts of *Kufri Chandramukhi, Kufri Chipsona-3* and *Kufri Jyoti* potato varieties reduced the total oxidation significantly ($p < 0.05$) both in bulk oil (Figure5.1) and oil-in-water emulsion (Figure 5.2) model systems whereas peel extracts of *Kufri Chipsona-1* and *Kufri Chipsona-4* potato varieties was found to be less effective against total oxidation of omega-3 fatty acids enriched food supplement in both the test lipid model systems (Figure 5.1 and Figure 5.2) compared to other tested potato peel extracts and BHT. Peel extract of *Kufri Chipsona-3* potato variety showed highest anti-lipid peroxidation potential against oxidation of omega-3 fatty acids enriched oil in both the test lipid model systems followed by peel extracts of *Kufri Jyoti* and *Kufri Chandramukhi* potato varieties.

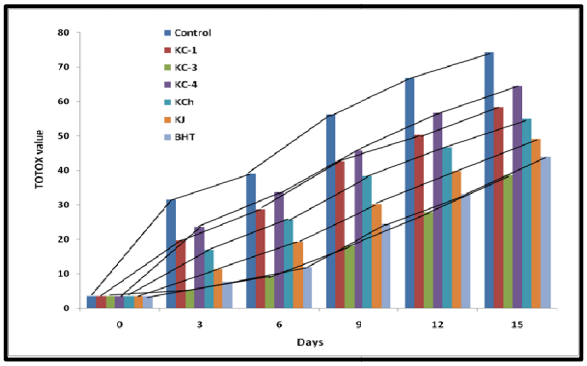


Figure 5.1 TOTOX values of potato peel extracts in bulk oil
KC-1 : *Kufri Chipsona*-1; KC-3 : *Kufri Chipsona* -3; KC-4 : *Kufri Chipsona* – 4; KCh :*Kufri Chandramukhi*; KJ : *Kufri Jyoti*; BHT : Butylated hydroxytoluene

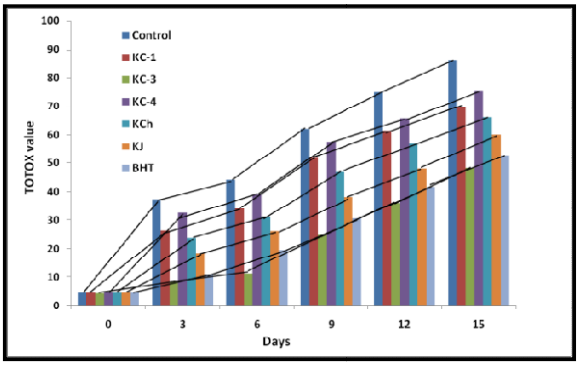


Figure 5.2 TOTOX values of potato peel extracts in oil-in-water emulsion
KC-1 :*Kufri Chipsona*-1; KC-3 : *Kufri Chipsona* -3; KC-4 : *Kufri Chipsona* – 4;
KCh :*Kufri Chandramukhi*; KJ : *Kufri Jyoti*; BHT : Butylated hydroxytoluene

(B) Results on individual antioxidant potential of peel extracts in omega-3 fatty acids fortified functional foods (whole milk, paneer and yoghurt)

(Results on primary oxidation and secondary oxidation)

The results on antioxidant potential of test potato peel extracts against primary and secondary oxidation of omega-3 fatty acids fortified various functional food (whole milk, paneer and yoghurt model systems) are shown in Table 5.3, Table 5.4 and Table 5.5 respectively. It was observed that among the peel extracts of potato varieties tested, only peel extracts of *Kufri Chandramukhi*, *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties exhibited strong antioxidant efficacy against both primary and secondary oxidation of omega-3 fatty acids fortified functional foods. Peel extract of *Kufri Chipsona-3* showed highest antioxidant potential in all the test food model systems followed by peel extracts of *Kufri Jyoti* and *Kufri Chandramukhi*. They were found to be more effective in paneer (Table 5.4) and yoghurt (Table 5.5) food models than whole milk model (Table 5.3). The peel extracts of other two tested potato varieties i.e. *Kufri Chipsona*-1 and *Kufri Chipsona*-4 were found to be less effective against both primary and secondary oxidation of omega-3 fatty acids fortified functional foods (Tables 5.3, 5.4 and 5.5)

Table 5.3 Antioxidant efficacy of peel extracts of selected varieties of potato against primary and secondary oxidation of omega-3 fatty acids fortified whole milk model system

Treatment	Primary oxidation (Peroxide value; PV) (meq/kg fat)						Secondary oxidation (*p*-anisidine value ; *p*-AV) (meq/kg fat)					
	Incubation period (Days)						Incubation period (Days)					
	0	3	6	9	12	15	0	3	6	9	12	15
Control	3.26 ± 0.25	12.98 ± 2.69	17.02m± 1.10	21.25 ± 1.26	28.46± 1.06	35.08 ± 2.98	2.42 ± 0.14	11.56 ± 2.54	23.25 ± 1.66	26.54 ± 1.25	33.23 ± 1.02	38.83 ± 1.43
KC-1	3.28 ± 0.27	10.34 ±1.42	16.56 ± 1.79	20.12± 1.23	26.63 ± 2.82	33.21± 2.18	2.40 ± 0.12	10.32 ± 1.89	21.59± 2.13	24.19 ± 2.62	31.50 ± 2.34	36.14 ± 2.54
KC-3	3.20 ± 0.27	7.92 ± 0.35	9.23 ± 1.27	12.73 ± 2.21*	19.42 ± 1.42*	25.22 ± 1.05*	2.35 ± 0.15	9.54 ± 2.24	11.08± 1.86	15.24 ± 1.40*	20.02 ± 1.74*	25.57± 1.73*
KC-4	3.25 ± 0.24	10.77 ± 1.78	17.61± 0.25	21.21 ±1.22	25.35± 2.65	35.02 ± 1.62	2.41 ± 0.14	11.42± 1.11	22.17 ± 2.28	25.57 ± 1.65	32.40 ± 1.01	37.16 ± 2.55
KCh	3.24 ± 0.26	9.17 ± 1.63	12.41 ± 1.02	17.24 ± 1.46*	22.03 ± 1.22 *	29.65± 2.43*	2.38 ± 0.16	10.20 ± 2.15	17.31 ± 2.43	19.76 ± 1.05*	25.18 ± 2.18*	31.72 ± 1.26*
KJ	3.23 ± 0.25	8.96 ± 1.87	11.12± 1.04	16.98 ± 1.81*	21.75 ± 1.32*	27.47 ± 2.24*	2.37 ± 0.15	9.89± 1.24	15.26± 1.07	17.42 ± 1.62*	23.29± 2.63*	29.49± 2.52*
BHT	3.21 ± 0.23	8.45 ± 0.42	10.89 ± 1.37	14.38 ± 2.54*	20.96 ± 2.76*	28.69 ± 1.24*	2.39 ± 0.15	9.83± 1.71	13.13 ± 1.22	14.98 ± 2.28*	22.24± 1.25*	27.21± 1.65*

Results are Mean ±S.D. of triplicate experiments. *P < 0.05 with respect to their control values.
KC-1: *Kufri Chipsona* -1; KC-3: *Kufri Chipsona* -3; KC-4: *Kufri Chipsona* – 4; KCh: *Kufri Chandramukhi*; KJ: *Kufri Jyoti*; BHT: Butylated hydroxytoluene. Values in parentheses indicate percent inhibition with respect to control

Table 5.4 Antioxidant efficacy of peel extracts of selected varieties of potato against primary and secondary oxidation of omega-3 fatty acids fortified paneer food model system

Treatment	Primary oxidation (peroxide value; PV) (meq/kg fat)						Secondary oxidation (*p*-anisidine value ; *p*-AV) (meq/kg fat)					
	Incubation period (Days)						Incubation period (Days)					
	0	3	6	9	12	15	0	3	6	9	12	15
Control	4.26 ± 0.21	15.98 ± 1.23	22.42± 1.32	34.14 ± 2.08	39.43± 1.24	45.37 ± 2.12	3.46 ± 0.24	13.50 ± 2.74	25.29 ± 1.86	35.48 ± 1.47	43.13 ± 1.22	48.83 ± 1.29
KC-1	4.24 ± 0.27	13.39 ±2.12	20.06 ± 1.87	32.21± 2.33	37.24 ± 2.22	41.11± 2.08	3.40 ± 0.82	11.36± 1.49	21.23± 2.16	32.36 ± 2.22	40.87 ± 2.24	44.19 ± 2.25
KC-3	4.16 ± 0.57	9.72 ± 1.25	11.29 ± 1.41	15.03 ± 2.01*	22.32 ± 1.56*	27.12 ± 1.65*	3.33 ± 0.49	8.33 ± 2.24	11.43± 1.53	15.84 ± 1.78*	20.62 ± 1.24*	25.98± 1.49*
KC-4	4.25 ± 0.34	14.67 ± 1.48	21.61± 1.57	33.27 ±1.75	38.96± 2.75	44.02 ± 1.62	3.45 ± 0.15	12.52± 1.91	23.16 ± 2.51	33.69 ± 2.34	42.39 ± 1.85	46.19 ± 1.53
KCh	4.21 ± 0.22	12.15 ± 1.29	15.77 ± 1.22	21.86 ± 1.11*	28.73 ± 1.92 *	34.25± 2.23*	3.37 ± 0.56	11.27 ± 2.78	15.54± 2.35	21.98 ± 1.63*	27.98 ± 1.57*	32.13 ± 1.45*
KJ	4.22 ± 0.45	11.36 ± 1.02	13.19± 1.54	19.98 ± 1.09*	25.68 ± 1.42*	31.23 ± 2.14*	3.36 ± 0.15	10.49± 1.64	13.23± 1.64	19.87 ± 2.53*	24.26± 2.35*	30.26± 2.89*
BHT	4.20 ± 0.73	10.23 ± 1.22	12.39 ± 1.83	16.33 ± 2.24*	24.77 ± 2.22*	29.44 ± 1.04*	3.34 ± 0.24	9.83± 1.21	13.25 ± 1.62	16.43 ± 2.73*	23.25± 1.93*	28.41± 1.36*

Results are Mean ±S.D. of triplicate experiments. *P < 0.05 with respect to their control values. KC-1: *Kufri Chipsona* -1; KC-3: *Kufri Chipsona* -3; KC-4: *Kufri Chipsona* – 4; KCh: *Kufri Chandramukhi*; KJ: *Kufri Jyoti*; BHT: Butylated hydroxytoluene. Values in parentheses indicate percent inhibition with respect to control

Table 5.5 Antioxidant efficacy of peel extracts of selected varieties of potato against primary and secondary oxidation of omega-3 fatty acids fortified yoghurt food model system

Treatment	Primary oxidation (peroxide value; PV) (meq/kg fat)						Secondary oxidation (*p*-anisidine value ; *p*-AV) (meq/kg fat)					
	Incubation period (Days)						Incubation period (Days)					
	0	3	6	9	12	15	0	3	6	9	12	15
Control	2.24 ± 0.29	10.08 ± 1.12	13.29± 2.21	19.13 ± 1.48	24.89± 1.98	32.73 ± 1.28	2.42 ± 0.77	11.56 ± 1.34	14.38 ± 2.07	18.19 ± 1.05	25.12 ± 2.22	34.98 ± 1.38
KC-1	2.26 ± 1.34	9.78 ±1.57	12.38 ± 1.15	17.58± 2.01	21.88 ± 0.98	28.18± 1.77	2.39 ± 1.39	11.29 ± 2.19	13.79± 1.16	17.78 ± 1.39	23.19 ± 0.99	31.39 ± 2.06
KC-3	2.23 ± 0.75	6.88 ± 1.45	7.19 ± 0.91	10.56 ± 1.39*	12.73 ± 1.08*	14.37 ± 1.78*	2.37 ± 1.09	7.34 ± 2.33	9.08± 1.35	11.52± 2.93*	14.85 ± 1.28*	17.54± 1.37*
KC-4	2.28 ± 1.35	8.89 ± 2.35	12.83± 1.36	18.77 ±0.63	22.31± 1.25	30.18 ± 2.19	2.40 ± 0.31	11.29± 2.15	13.39 ± 1.89	16.28 ± 1.07	24.87 ± 0.75	33.23 ± 1.32
KCh	2.22 ± 1.07	9.19 ± 1.86	11.37 ± 2.59	15.86 ± 1.79*	20.87 ± 1.31 *	23.76± 2.79*	2.31 ± 1.30	9.71 ± 1.85	10.19 ± 2.01	14.19 ± 0.87*	16.73 ± 1.78*	19.33 ± 2.22*
KJ	2.25 ± 2.19	8.18 ± 1.35	10.58± 0.75	13.45 ± 1.66*	17.59 ± 1.02*	20.11 ± 1.33*	2.30 ± 2.22	8.21± 1.39	8.98± 1.09	12.68 ± 2.45*	15.47± 1.11*	18.39± 2.68*
BHT	2.23 ± 1.11	8.89 ± 2.18	10.31 ± 0.89	13.39 ± 1.38*	18.19 ± 1.82*	19.58 ± 2.16*	2.35 ± 2.29	8.04± 1.54	8.34 ± 3.19	12.08 ± 1.79*	14.35± 2.37*	18.29± 1.65*

Results are Mean ±S.D. of triplicate experiments. *P < 0.05 with respect to their control values. KC-1: *Kufri Chipsona* -1; KC-3: *Kufri Chipsona* -3; KC-4: *Kufri Chipsona* – 4; KCh: *Kufri Chandramukhi*; KJ: *Kufri Jyoti*; BHT: Butylated hydroxytoluene. Values in parentheses indicate percent inhibition with respect to control

- **Results on total oxidation**

The results on the effects of peel extracts of selected varieties of potato on total oxidation of omega-3 fatty acids fortified whole milk, paneer and yoghurt food models are shown in Figure 5.3, Figure 5.4 and Figure 5.6 respectively. It was observed that the peel extracts of *Kufri Chandramukhi*, *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties exhibited varying degrees of time dependent antioxidant efficacy against total oxidation of omega-3 fatty acids fortified whole milk (Figure 5.3), paneer (Figure 5.4) and yoghurt (Figure 5.5) food model systems. The antioxidant potential of peel extract of *Kufri Chipsona-3* was higher than reference standard antioxidant BHT in all the food model systems tested. Peel extract of *Kufri Jyoti* was found to almost equipotent to BHT followed by peel extract of *Kufri Chandramukhi*. But, peels extracts of *Kufri Chipsona-1* and *Kufri Chipsona-4* were found to be ineffective against total oxidation of omega-3 fatty acids fortified various functional foods (Figure 5.3, Figure 5.4 and Figure 5.5).

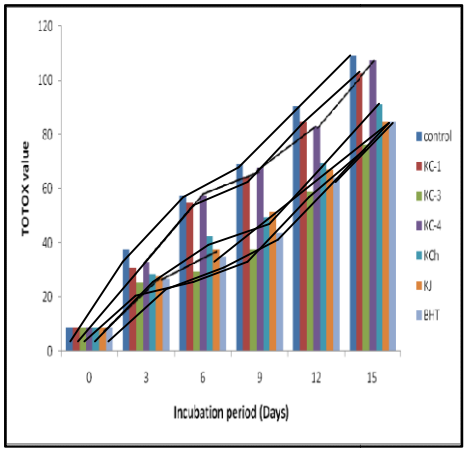


Fig 5.3 Effect of potato peel extracts on total oxidation (TOTOX) of omega-3 fatty acids fortified whole milk

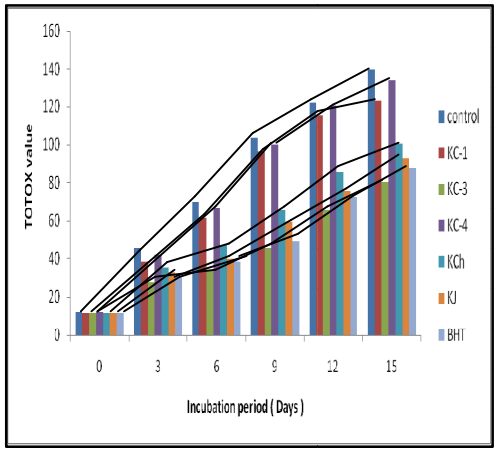


Figure 5.4 Effect of potato peel extracts on total oxidation (TOTOX) of omega-3 fatty acids fortified paneer

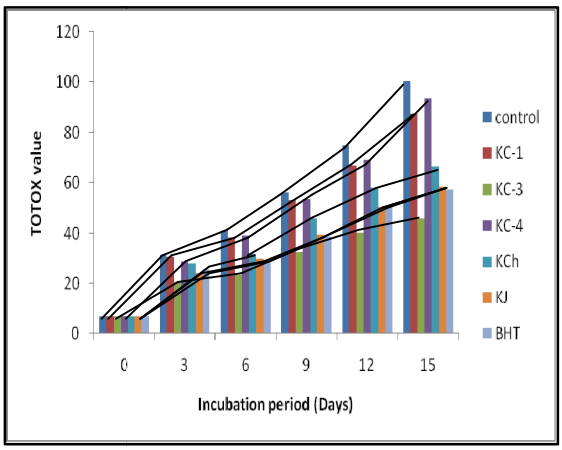


Figure 5.5 Effect of potato peel extracts on total oxidation (TOTOX) of omega-3 fatty acids fortified yoghurt

Discussion

After evaluating the antioxidant efficacy of phenolic extract of peels of selected varieties of potato at their individual effect in various *in vitro* models (DPPH radical scavenging, β-carotene-linoleic acid bleaching and Fe^{2+} ion chelating) (**Chapter 4**; Objective 1), their antioxidant efficacy in omega-3 fatty acids enriched food supplement (marine fish oil) and omega-3 fatty acids fortified various functional foods (whole milk, paneer and yoghurt) at their individual effects was also evaluated with a view to gather knowledge about their antioxidant efficacy in food lipids. To fulfill this objective, at first antioxidant potential of peel extracts of selected varieties of potato on primary and secondary oxidation of omega-3 fatty acids enriched lipid substrate (marine fish oil) and in omega-3 fatty acids fortified various functional foods both in bulk oil and oil-in-water emulsion model systems was evaluated. The results obtained from this study revealed that among the peel extracts tested, only peel extracts of *Kufri Chipsona-3*, *Kufri Jyoti* and *Kufri Chandramukhi* were effective antioxidant against oxidation of lipids both in omega-3 fatty acids enriched food supplement (Table 5.1 and Table 5.2) and in omega-3 fatty acids fortified functional foods (Table 5.2, 5.3, 5.4). Besides, peel extract of these three potato varieties were found to be more effective in bulk oil than oil-in-water emulsion model system. This difference in activity in oil-in-water emulsion model system compared to bulk oil may be due to their interfacial partitioning into the water phase of emulsion, thus becoming less protective than in bulk oil system (Kumar et al., 2016).

The third important oxidative indicator of lipid oxidation is the total oxidation (TOTOX) which is considered to be the most important evaluation required to determine oil's rancidity because combination of both primary oxidation (PV) and secondary oxidation (*p*-AV) give a good indication of the overall rancidity or quality of oil. The lower the TOTOX value, the better the quality of oil. Consequently after evaluation of primary and secondary antioxidant efficacy of test potato peel extracts at their individual effect, their effect on total oxidation both in bulk oil and oil-in-water emulsion model systems in omega-3 fatty acids enriched food supplement and omega-3 fortified functional foods was also determined. It was observed that peel extract of *Kufri Chipsona-3* showed highest antioxidant activity against total oxidation of omega-3 fatty acids enriched food supplement and omega-3 fatty acids fortified various functional foods both in bulk oil and oil-in-water emulsion model systems, followed by peel extracts of *Kufri Jyoti and Kufri Chandramukhi.* Peel extracts of *Kufri*

Chipsona-1 and *Kufri Chisona-4* were found to have insignificant antioxidant effect against total oxidation of lipids both in omega-3 fatty acids enriched food supplement and omega-3 fortified functional foods. These findings are in consistent with the observed findings of other workers in their study on anti-lipid peroxidation potential of peel extract of "Diamond" and "Agria" potato varieties in soybean oil (Mohdaly et al., 2010; Amado et al., 2014), "Diamond" potato variety in sunflower oil (Franco et al., 2016) and "Sava" and "Bintje" potato varieties in fish–rapeseed oil mixture (Habeebullah et al., 2010). Many studies have shown a strong positive correlation between total phenolic contents of fruits and vegetables and their antioxidant as well as antimicrobial efficacy (Reddy et al., 2010, Someya et al., 2002).

Therefore, in the present study an attempt has been made to evaluate possible correlation between total phenolic content and antioxidant as well as antimicrobial potential of active extracts of potato peels (*Kufri chpsona-3, Kufri Jyoti and Kufri Chandramukhi)*.The total phenolic content of *Kufri Chipsona-3* potato peel extract was found to be highest (51.47± 0.02 mg GAE/g of dry extract) followed by peel extracts of *Kufri Jyoti* (42.83 ± 0.03 mg GAE/g of dry extract) and *Kufri Chandramukhi* (34.30 ± 0.03 mg GAE/g of dry extract) and a strong positive correlation between total phenolic content of active extracts of potato peels and their antioxidant (R^2: 0.967) (Figure 5.6a) as well as antimicrobial [*L. monocytogenes (*R^2: 0.987), *S. typhimurium* (R^2:0.993) and *A. niger (*R^2:0.986) ; (Figure 5.6(b)] efficacy was observed. These findings suggest that among the peel extracts of potato varieties tested only three extracts of potato peels (*Kufri Chipsona-3, Kufri Jyoti* and *Kufri Chandramukhi*) have significant role against oxidation of lipids both in omega-3 fatty acids enriched food supplement as well as in omega-3 fatty acids fortified various functional foods. The phenolic compounds present in active extracts of potato peels may be responsible for their antioxidant and antimicrobial efficacy.

From our previous findings **(Chapter 4)** , it was observed that based on threshold values of antioxidant efficacy (IC_{50} < 50 µg /ml) and antimicrobial susceptibility screening (IZD ≥ 11 mm ; MIC or MFC ≤ 100 µg /ml) , among the peel extract treated, only peel extracts of *Kufri Chipsona-3* , *Kufri Jyoti* and *Kufri Chandramukhi* potato varieties were found to be active antioxidant and antimicrobial agents at their individual effect.

These three extracts of peels of selected varieties of potato (*Kufri Chipsona-3* , *Kufri Jyoti* and *Kufri Chandramukhi*) were also found to be effective antioxidant against oxidation

of omega-3 fatty acids enriched food supplement and omega-3 fatty acids fortified various functional foods among the peel extracts of potato varieties treated (**Chapter 5**).

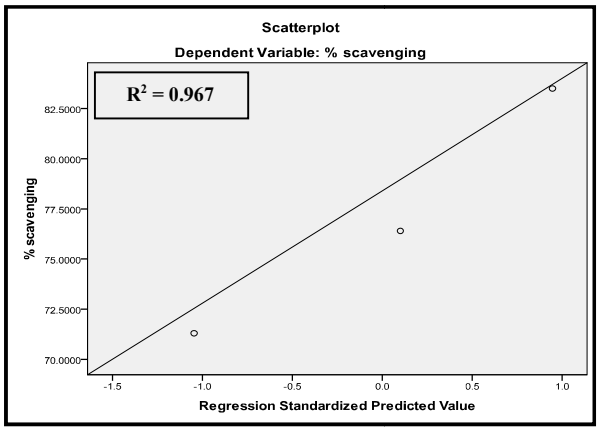


Figure 5.6(a) Correlation between DPPH radical scavenging activity and total phenolic contents of active peel extracts of potato peels (*Kufri Chipsona-3*, *Kufri Jyoti* and *Kufri Chandramukhi*)

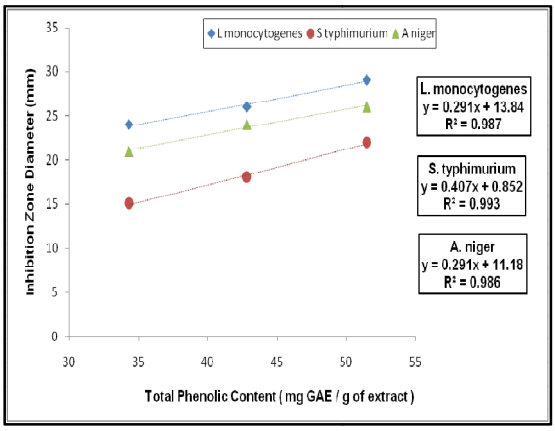


Figure 5.6(b) Correlation between total phenolic content and inhibition zone diameter (IZD) of peel extracts of *Kufri Chandramuhi*, *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties against foodborne microbes

Therefore, these three active extracts of potato peels (*Kufri Chipsona-3*, *Kufri Jyoti* and *Kufri Chandramukhi*) were subjected to further studies for **(i) evaluating their combined antioxidant and antimicrobial potential (Chapter 6;** objective 3**), (ii) chemical characterization of bioactive compounds responsible for antioxidant and antimicrobial efficacy along with cytotoxicity study (Chapter 7 ;** objective 4**) and (iii) elucidation of possible modes of antioxidant and antimicrobial action (Chapter 8 ;**objective 5 **)**

CHAPTER 6

OBJECTIVE 3

TO EVALUATE COMBINED ANTIOXIDANT AND ANTIMICROBIAL POTENTIAL OF ACTIVE PHENOLIC EXTRACTS OF PEELS OF SELECTED VARIETIES OF POTATO IN VARIOUS *IN VITRO* MODELS

INTRODUCTION

RESULTS

DISCUSSION

Introduction

Combined activity of crude plant extract or bioactive compounds is one of most important and intriguing aspect of their antioxidant as well as antimicrobial potential. The combination activity can be broadly classified into three categories i.e. (i) additive, (ii) synergistic and (iii) antagonistic. Among these three types of combined interactions, synergistic interactions have proven to be an essential feature of antioxidant and antimicrobial treatment because synergistic interactions have many beneficial effects viz. (i) they increase the spectrum of activity of plant extracts/ purified compounds having synergistic effect; (ii) they are able to thwart drug resistance; (iii) they decrease the amount of required doses, which in turn promotes the reduction of both cost and adverse/toxic side effects; (iv) they exhibit multimodal action. Therefore, it is a field of immense opportunity to protect food both from oxidative deterioration as well as microbial contamination at sufficiently low concentrations with higher efficacy and multimodal action (Hyldgaard et al., 2012; Nguefack et al., 2012). Previous study revealed that among the peel extracts of five selected varieties of potato tested, peel extracts of only three varieties (*Kufri Chandramukhi*, *Kufri Chisona-3* and *Kufri Jyoti*) exhibited strong antioxidant as well as antimicrobial efficacy at their individual effect **(Chapter 4** and **Chapter 5)**. Keeping this in mind, in the present work an attempt has been made to evaluate combined antioxidant and antimicrobial efficacy of these three active extracts of potato peels (*Kufri Chandramukhi*, *Kufri Chisona-3* and *Kufri Jyoti*) in various *in vitro* models taking the advantages of their possible synergistic interactions to fulfil objective 3. Combined antioxidant efficacy was evaluated using DPPH radical scavenging method followed by isobologram analysis and Briggs-Rauscher oscillation reaction method and combined antimicrobial potential was evaluated by checkerboard titration method followed by kill-kinetics assay.

Results

- **Results of combined antioxidant potential**

- **DPPH radical scavenging method**

Table 6.1 shows the results of combined antioxidant potential of active extract of potato peels in DPPH radical scavenging method. It was observed from Table 6.1 that among the possible combinations tested, the IC_{50} value of only one combination between peel extracts of *Kufri*

Chipsona-3 and *Kufri Jyoti* potato varieties was significantly lower ($p < 0.05$) compared to the IC_{50} value of their most active single component. On the basis of antioxidant combination index (CI), peel extracts of *Kufri Chipsona-3/Kufri Jyoti* blend exhibited synergistic antioxidant activity (CI: 0.82) whereas other tested possible combinations showed additive antioxidant effect (CI = 1) in DPPH radical scavenging assay. The findings suggest that free radical scavenging potential of peel extracts of *KufriChipsona-3/Kufri Jyoti* blend was significantly higher compared to their most active single components effect as well as other tested possible combinations of active extracts of potato peels.

Table 6.1 Combined antioxidant potential of active extracts of potato peels in DPPH free radical scavenging method

Potato peel extract	IC_{50} (µg/mL)	$CI_1 = (D)_1 / (Dx)_1$	$CI_2 = (D)_2 / (Dx)_2$	$CI = CI_1 + CI_2$	Remarks
KCh	96± 4	-	-	-	-
KC-3	38± 3	-	-	-	-
KJ	60± 4	-	-	-	-
KCh + KC-3	27± 3*	0.28	0.71	0.99 ≈1.0	ADD
KCh + KJ	37± 3*	0.38	0.62	1.0	ADD
KC-3 + KJ	19± 2*	0.50	0.32	0.82	S

Results are mean ± SD of triplicate experiments. ADD: additive (CI = 1); S: Synergistic (CI <1); KC-3, *Kufri Chipsona-3*; KCh, *Kufri Chandramukhi*; KJ, *Kufri Jyoti*; Synergistic.*$p < 0.05$ compared to IC_{50} value of most active single component in combination

- **Briggs-Rauscher Oscillation method**

The results of combined antioxidant potential of active extracts of potato peels in Briggs–Rauscher (BR) Oscillation reaction method are shown in Table 6.2. On the basis of inhibition time ($t_{inhibition}$), the antioxidant potential of potato peel extracts at their individual effect was found to be in the following decreasing order: *Kufri Chipsona-3* > *Kufri Jyoti* > *Kufri Chandramukh*i. In combination, peel extracts of *Kufri Chipsona-3/Kufri Jyoti* blend showed synergistic antioxidant potential ($t_{inhibition}$ of combination was significantly ($p < 0.05$) increased compared to average $t_{inhibition}$ of most single component in combination), whereas other tested combinations showed additive antioxidant effect ($t_{inhibition}$ of combination was not significantly ($p \geq .05$) increased or decreased compared to average $t_{inhibition}$ of individual component in combination) (Table 6.2).

Table 6.2 Combined antioxidant potential of active extracts of potato peels in Briggs-Rauscher oscillation reaction

Potato peel extract	$t_{inhibition}$ (sec)	Remarks
KCh	465±7	_
KC-3	497±8	_
KJ	472±5	_
KCh + KC-3	484±7	ADD
KCh + KJ	477±6	ADD
KC-3 + KJ	585±7*	S

Results are mean ± SD of triplicate experiments.
ADD: Additive (No significant increase or decrease in $i_{nhibition}$ of the combination compared to the average $t_{inhibition}$ of individual components); S: Synergistic (Significant increase ($P < 0.05$) in $i_{nhibition}$ of the combination compared to the average
$t_{inhibition}$ of individual components).KC-3, *Kufri Chipsona-3;* KCh, *Kufri Chandramukhi*; KJ, *Kufri Jyoti*

➢ Results of combined antimicrobial potential

• MIC and MFC values of active peel extracts against studied microbes

Table 6.3 shows the results on MIC and MFC values of active extracts of potato peels against the studied bacterial and fungal pathogens. On the basis of MIC and MFC values, the antimicrobial potency of potato peel extracts at their individual effect against the studied microbes were found to be in the following decreasing order: *Kufri Chipsona-3>Kufri Jyoti>Kufri Chandramukhi*. Potato peel extracts at their individual effects were found to be most effective against *L. monocytogenes* followed by *A. niger* and *S. typhimurium*. From Table 6.3, it was also observed that in combination, MIC and MFC values of peel extracts of *Kufri Chipsona-3/Kufri Jyoti* blend against both the studied bacterial and fungal pathogens were significantly ($p < 0.05$) lower compared to the MIC and MFC values of their most active single component whereas other tested possible combinations did not show any significant ($p \geq .05$) difference in MIC and MFC values against all the studied microbes compared to the MIC and MFC values of their most active single component.

Table 6.3 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of potato peel extracts alone and in combination against studied foodborne microbes

Potato peel extract	Foodborne Bacteria		Foodborne Fungus
	L.monocytogenes(MTCC 657)	*S. typhimurium* (MTCC 3224)	*A. niger (ATCC 16404)*
KCh	79.21±4.37	98.32±5.67	87.75 ± 3.71
KC-3	44.38±4.77	63.54±3.12	57.64 ± 2.43
KJ	55.34±3.80	74.87±4.53	62.27 ±2.16
KCh + KC-3	70.61±3.58 (KCh)	110.47±4.87 (KCh)	79.37 ± 3.67 (KCh)
	67.43±3.44 (KC-3)	107.72±3.44 (KC-3)	77.74 ± 2.27(KC-3)
KCh+ KJ	71.92±4.36 (KCh)	90.53±3.74 (KCh)	76.82 ± 2.61 (KCh)
	67.63±3.83 (KJ)	87.84±2.76 (KJ)	75.76 ± 2.41 (KJ)
KC-3 + KJ	14.21±2.38*(KC-3)	28.17±2.17* (KC-3)	18.33±1.57* (KC-3)
	11.74±2.40* (KJ)	22.29±2.73* (KJ)	15.10 ± 1.33* (KJ)

Values are mean ±SD of triplicate experiments. KCh: *Kufri Chandramukhi*; KC-3: *Kufri Chipsona*-3; KJ: *Kufri Jyoti*;

*P < 0.05 compared to MIC or MFC of individual components

- **Fractional Inhibitory Concentration Indices (FICI) values of active peel extracts against studied microbes**

Table 6.4 shows the results of combined antimicrobial potential of active extracts of potato peels in checkerboard titration method. Based on FICI values, the types of antimicrobial interactions (synergistic, additive or antagonistic) between active extracts of potato peels in combination against the studied microbes were interpreted. On the basis of FICI values, peel extracts of *Kufri Chipsona-3/Kufri Jyoti* combination was found to have synergistic antibacterial activity against bacterial pathogens *L. monocytogenes* (FICI: 0.42) and *S. typhimurium* (FICI: 0.50) and fungal pathogen *A. niger* (FICI: 0.45) whereas other tested possible combinations showed additive antimicrobial potential against all the studied microbes with FICI ranged from 1.83 to 2.21.

Table 6.4 Fractional inhibitory concentration indices (FICI) values of potato peel extracts in combination against studied bacterial and fungal pathogens

Microorganisms	Potato peel extracts in combination (1:1)								
	KCh + KC-3			KCh + KJ			KC-3 + KJ		
	FIC	FICI	Remarks	FIC	FICI	Remarks	FIC	FICI	Remarks
L. monocytogenes (MTCC 657)	0.98 (KCh) / 0.93 (KC-3)	1.91	ADD	0.80 (KCh) / 1.03 (KJ)	1.83	ADD	0.20 (KC-3) / 0.22 (KJ)	0.42	S
S. typhimurium (MTCC 3224)	1.14(KCh) / 1.07 (KC-3)	2.21	ADD	0.88 (KCh) / 1.19 (KJ)	2.07	ADD	0.24 (KC-3) / 0.26 (KJ)	0.50	S
A. niger (ATCC 16404)	0.89 (KCh) / 1.04 (KC-3)	1.93	ADD	0.87 (KCh) / 1.12 (KJ)	1.99	ADD	0.24 (KC-3) / 0.21 (KJ)	0.45	S

Results are mean ± SD of triplicate experiments.
ADD: additive (0.5>FICI ≤4)); S: Synergistic (FICI<0.5) KC-3: *Kufri Chipsona-3;* KCh: *Kufri Chandramukhi*; KJ*: Kufri Jyoti*

- **Results on confirmation of synergistic antimicrobial interaction by kill-kinetics assay**

In kill-kinetics assay, a reduction in colony count by >2Log$_{10}$ CFU/ml of studied bacterial pathogen *L. monocytogenes* (Figure 6.1), *S. typhimurium* (Figure 6.2) and fungal pathogen *A. niger* (Figure 6.3) by peel extract of *Kufri Chipsona-3/Kufri Jyoti* combination at 24 h was observed compared to the reduction in colony count by their most active single component confirming synergistic antimicrobial efficacy of *Kufri Chipsona-3/Kufri Jyoti* peel extract combination against the studied microbes.

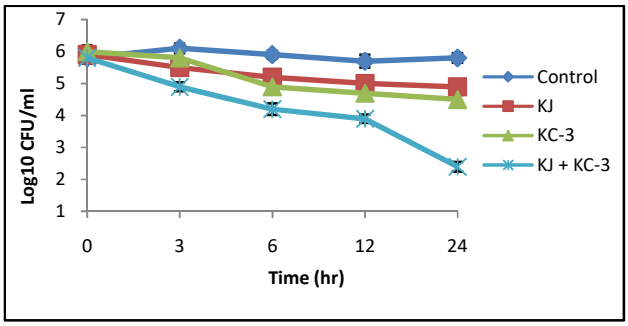


Figure 6.1 Kill kinetics curves of peel extracts of *Kufri Chipsona-3(KC-3)* and *Kufri Jyoti* (KJ) alone and in combination against *L. monocytogenes* (MTCC 657)

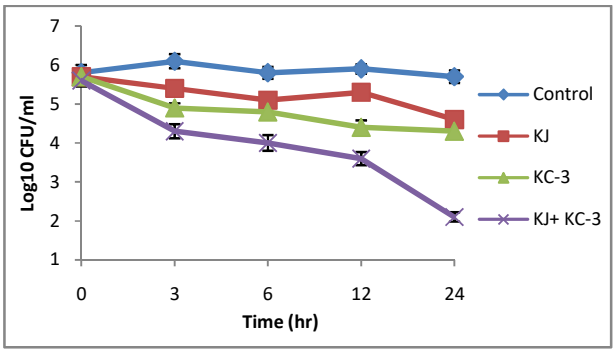


Figure 6.2 Kill kinetics curves of peel extracts of *Kufri Chipsona-3(KC-3)* and *Kufri Jyoti* (KJ) alone and in combination against *S. typhimurium*(MTCC 3224)

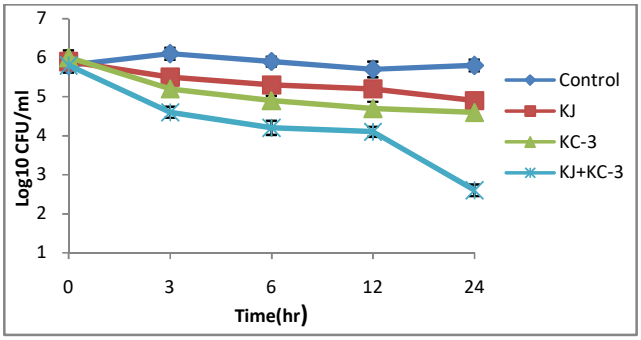


Figure 6.3 Kill kinetics curves of peel extracts of *Kufri Chipsona-3*(KC-3) and *Kufri Jyoti* (KJ) alone and in combination against *A. niger* (ATCC 16404)

Discussion

After evaluating antioxidant efficacy of peel extracts of selected varieties of potato at their individual effect in various *in vitro* models (**Chapter 4**) and in lipid substrate (**Chapter 5**), their antioxidant potential in combination was evaluated. This was done because oxidative rancidity is a major cause of food quality deterioration, which subsequently leads to the formation of undesirable off-flavors, off-odors as well as unhealthful compounds. Olive oil, walnuts, and marine fish are a heart-healthy choice, but concern is rising that, if not stored and used properly, these foods actually could be harmful for our health due to high degree of unsaturation in the molecular structure of essential fatty acids present in them (El-Ghorab & El-Massry, 2003). It is therefore important to seek natural antioxidant agents that have strong antioxidant efficacy against oxidation of polyunsaturated fatty acids bearing foods at sufficiently low concentration and this can be achieved by possible synergistic interaction of antioxidants in combination.

Therefore , in this investigation, an attempt has been made to evaluate combined antioxidant potential of active extracts of potato peels against oxidation of omega-3 fatty acids enriched food supplement (marine fish oil) and omega-3 fatty acids fortified various functional foods (whole milk , paneer, yoghurt) taking the advantages of their possible synergistic interactions. To evaluate combined antioxidant efficacy of active peel extracts, DPPH radical scavenging method followed by isobologram analysis and Briggs- Rauscher oscillation reaction method were used.

In DPPH radical scavenging method, combined antioxidant potential was evaluated using isobologram analysis based on their median effective dose (IC_{50}). It was observed that among the possible combinations tested between active extracts of peels of selected potato varieties, only one combination between peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* exhibited synergistic antioxidant efficacy (CI : 0.82) whereas other tested possible combinations showed additive antioxidant effect (CI : 1) (Table 6.1) suggesting that peel extract of *Kufri Chipsona-3/Kufri Jyoti* blend exhibited strong radical scavenging activity which may be mediated by their strong hydrogen donating ability compared to the other tested combinations.

The combined antioxidant potential of active extracts of potato peels of selected varieties of potato was also evaluated by Briggs-Rauscher (BR) Oscillation reaction method. This method was used because the pH of BR reaction is about 2.0, which is similar to that of the fluids of the main digestive process (human stomach). This can give us *in vitro*

information on antioxidant activity which takes place during "real digestion conditions" and can help in assessment of nutrition for maintenance of health and prevention of diseases (Cervellati, Furrow, Grecoa, Honer, & Venturi, 2010; Cervellati, Honer, Furrow, Needens, & Costa, 2001; Honar & Cervellati, 2002). In B-R reaction, the antioxidant efficacy of potato peel extract is expressed as the length of inhibition time ($t_{inhibition}$) in seconds before oscillation restart. An antioxidant having a higher concentration of it leads to a longer inhibition time ($t_{inhibition}$) (Briggs & Rauscher, 1973). Therefore, useful *in vitro* information on the antioxidant efficacy of a substance at acidic pH (≈2) can be determined by the BR reaction which is the prime advantage of this method. This is why, in this study, an attempt has been made to evaluate antioxidant potential of active potato peel extracts alone and in combination at low pH with a view to gather information about their possible effectiveness in real digestion system. It was observed that among the active peel extracts tested, the peel extract of *Kufri Chipsona-3/Kufri Jyoti* blend exhibited strong antioxidant activity in BR reaction compared to other possible combination tested (Table 6.2). These important findings suggest that combination of this active potato peel extracts may serve as a potent antioxidant in real digestion system.

It is well documented that foodborne disease caused by consumption of spoiled and contaminated food is a major public health problem worldwide. A safe food supply (free from microbial spoilage and contamination as well as from other toxic materials) is important because of significant disease burden as well as economic burden to the society and Nation (Fung et al., 2018). Therefore, after evaluating combined antioxidant efficacy of active extracts of potato peels in combination, their combined antimicrobial efficacy against foodborne bacterial and fungal pathogens was also evaluated taking the advantages of their possible synergistic antimicrobial action. To achieve the goal, fractional inhibitory concentration (FICI) of active potato peel extracts in combination against foodborne Gram-positive (*L. monocytogenes*) and Gram-negative (*S. typhimurium*) bacterial pathogens as well fungal pathogen (*A. niger*) was determined by checkerboard titration method followed by kill-kinetics assay. The results revealed that the peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* in combination exhibited synergistic antimicrobial efficacy against the studied microbes (Table 6.3) which was confirmed by kill kinetics assay (Figures 6.1, 6.2 and 6.3).

From the obtained findings on combined antioxidant and antimicrobial efficacy study, it can be concluded that peel extract of *Kufri Chipsona-3/Kufri Jyoti* blend may be used both as an antioxidant as well as antimicrobial agents in food preservation system at sufficiently low concentration with higher activity due to their synergistic interactions.

CHAPTER 7

OBJECTIVE 4

TO ISOLATE, IDENTIFY AND CHARACTERIZE THE BIOACTIVE ANTIOXIDANT AND ANTIMICROBIAL COMPOUNDS FROM ACTIVE PHENOLIC EXTRACTS OF PEELS OF SELECTED VARIETIES OF POTATO AND EVALUATION OF THEIR CYTOTOXIC POTENTIAL, IF ANY

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- INTRODUCTION
- RESULTS
- DISCUSSION

Introduction

In the process of natural products development, bioassay-guided isolation is a rapid and validated method for isolation of compounds with bioactivities. In an effort to discover new lead compounds, among the natural sources, many research groups screen plant extracts to detect secondary metabolites with relevant biological activities, because plant secondary metabolites are rich sources of bioactive compounds and have many beneficial effects on human health (Kandlakunta et al. 2015; Tungmunnithum et al. 2018; Swallah et al. 2020). Various bioassay-guided isolation techniques are used by several workers for isolation of plant secondary metabolites from complex plant extracts, of which the TLC bioautography technique is gaining much importance in recent years due to a number of important considerations, viz., (i) this assay allows a direct and rapid localization of bioactive compounds in plant extracts; (ii) it is a fast, cheap, and simple method for isolation of compounds from complex plant extracts; (iii) it facilitates accurate identification of chemical compounds in plant extracts; (iv) it is particularly important to avoid the time-consuming isolation of inactive compounds; and (v) this assay combines chromatographic separation and in situ activity determination facilitating the localization and identification of bioactive compounds. It was observed from previous experiments that active peel extract of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties exhibited synergistic antioxidant and antimicrobial interactions in combination **(Chapter 6).** In this study, an attempt has therefore been made to seek bioactive compounds from peel extracts of two potato varieties (*Kufri Chipsona-3* and *Kufri Jyoti*) that are responsible for synergistic interactions in combination by TLC-bioautography guided isolation followed by isobologram analysis, checkerboard titration and spectrometric (UV/Vis, FT-IR, HR-LCMS/MS) analysis to fulfil objective 4.

Results

- **Separation and detection of antioxidant components by analytical TLC and TLC bioautography analysis**

Figure 7.1a shows the R_f values of separated components of peel extract of *Kufri Chipsona-3* separated by analytical TLC and Figure 7.1b shows the R_f values of antioxidant components of peel extract of *Kufri Chipsona-3* in TLC- bioautography. The analytical TLC of peel extract of *Kufri Chipsona-3* revealed the presence of four components (R_f: 0.57, R_f: 0.52, R_f: 0.47 and R_f: 0.33) (Figure 7.1a). Among these four separated components, only three

components with R_f : 0.57, 0.52, and 0.33 were found have antioxidant activity in TLC bioautography detected as yellow spots against purple background (Figure 7.1b).

Figure 7.2 (a, b) represent the photographs of analytical TLC and TLC bioautography analysis of peel extract of *Kufri Jyoti* potato variety respectively. It was observed from Figure 7.2a that the peel extract of *Kufri Jyoti* potato variety contains five components (R_f : 0.73, R_f: 0.61,R_f : 0.36 , R_f : 0.22 and R_f :0.14) of which only four component (R_f : 0.61, R_f: 0.36, R_f : 0.22 and R_f : 0.14) demonstrated antioxidant activity in TLC bioautography analysis (Figure 7.2b).

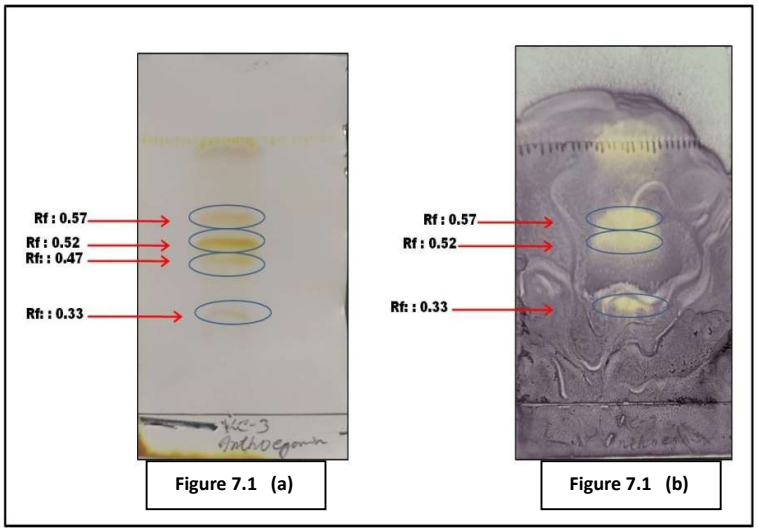


Figure 7.1 (a): R_f values of separated components of *Kufri Chipsona-3* by analytical TLC

Figure 7.1 (b): R_f values of antioxidant components of *Kufri Chipsona-3* by TLC -bioautography

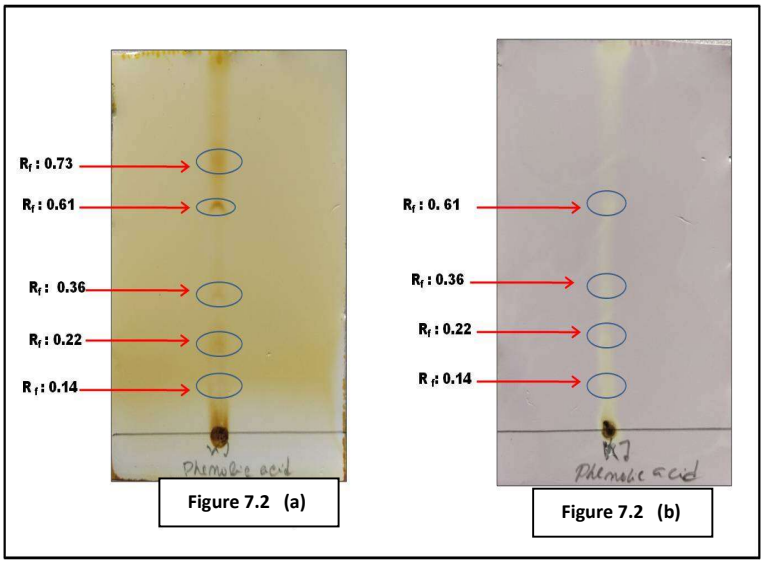


Figure 7.2 (a): R_f values of separated components of *Kufri Jyoti* by analytical TLC

Figure 7.2 (b): R_f values of antioxidant components of *Kufri Jyoti* by TLC -bioautography

Table 7.1 a shows the R_f values of antioxidant components of active peel extract of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties obtained by TLC-bioautography analysis. Peel extract of *Kufri Chipsona-3* contains three antioxidant components with R_f: 0.52, 0.57 and 0.33, whereas peel extract of *Kufri Jyoti* contains four antioxidant components with R_f: 0.61, 0.36, 0.22 and 0.14.

Table 7.1 b shows the results of IC_{50} values of all the isolated antioxidant components from phenolic fraction of peels of *Kufri Chipsona -3* and *Kufri Jyoti* potato varieties alone and in combination. It was observed from Table 7.1 that on the basis of IC_{50} values, the isolated component with R_f: 0.57 from peel extract of *Kufri Chipsona-3* showed highest *in vitro* antioxidant potential in DPPH radical scavenging method at its individual effect compared to other two isolated antioxidant components (R_f : 0.52 and R_f : 0.47) of *Kufri Chipsona-3* peel extract. In case of *Kufri Jyoti* potato peel extract, the highest *in vitro* antioxidant efficacy was shown by the isolated component having R_f : 0.61 over the other three isolated antioxidant components (R_f : 0.36, 0.22 and 0.14) of *Kufri Jyoti* peel extract. In combination, among these seven isolated antioxidant components (three from *Kufri Chipsona-3* and four from *Kufri Jyoti*), only one component with R_f 0.57 from peel extract of *Kufri Chipsona-3* and one component with R_f 0.61 from peel extract of *Kufri Jyoti* showed synergistic antioxidant

efficacy (CI = 0.78), whereas other tested possible combinations showed additive antioxidant effect (Table 7.1b).

Table 7.1 a R_f values of antioxidant components of peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties obtained by TLC-bioautography

Peel extracts	Separated components by analytical TLC	R_f value of separated components	R_f value of active antioxidant components in TLC –bioautography
Kufri Chipsona-3	Component 1	0.33	0.33
	Component 2	0.47	_
	Component 3	0.52	0.52
	Component 4	0.57	0.57
Kufri Jyoti	Component 1	0.14	0.14
	Component 2	0.22	0.22
	Component 3	0.36	0.36
	Component 4	0.61	0.61
	Component 5	0.73	_

Table 7 .1 b Combined antioxidant efficacy (CI) of active components from peel extract of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties

Test potato peel	R_f value of active components	IC_{50} (µg/ml)	$CI_1 = (D)_1/(D_X)_1$	$CI_2 = (D)_2/(D_X)_2$	$CI=CI_1+CI_2$	Remarks
Kufri Chipsona-3	0.33	71.67 ± 1.23	_	_	_	_
Kufri Chipsona-3	0.52	64.34 ± 1.05	_	_	_	_
Kufri Chipsona-3	0.57	34.56 ± 1.02	_	_	_	_
Kufri Jyoti	0.14	75.78 ± 2.01	_	_	_	_
Kufri Jyoti	0.22	59.43 ± 1.34	_	_	_	_
Kufri Jyoti	0.36	66.37 ± 1.54	_	_	_	_
Kufri Jyoti	0.61	42.64 ± 2.23	_	_	_	_
Kufri Chipsona-3 + *Kufri Jyoti*	0.33 + 0.14	37.54 ± 1.43	0.52	0.49	1.01 ≈ 1	Additive
Kufri Chipsona-3 + *Kufri Jyoti*	0.33 + 0.22	33.32 ± 1.11	0.46	0.56	1.02 ≈ 1	Additive
Kufri Chipsona-3 + *Kufri Jyoti*	0.33 + 0.36	41.35 ± 1.21	0.57	0.62	1.19	Antagonistic
Kufri Chipsona-3 + *Kufri Jyoti*	0.33 + 0.61	26.98 ± 2.13	0.37	0.63	1	Additive

Test potato peel	R_f value of active components	IC_{50} (µg/ml)	$CI_1 = (D)_1/(D_X)_1$	$CI_2 = (D)_2/(D_X)_2$	$CI=CI_1+CI_2$	Remarks
Kufri Chipsona-3 + *Kufri Jyoti*	0.52 + 0.14	35.65 ± 1.57	0.55	0.47	1.02	Additive
Kufri Chipsona-3 + *Kufri Jyoti*	0.52 + 0.22	42.18 ± 2.07	0.65	0.70	1.35	Antagonistic
Kufri Chipsona-3 + *Kufri Jyoti*	0.52 + 0.36	32.98 ± 1.78	0.51	0.49	1	Additive
Kufri Chipsona-3 + *Kufri Jyoti*	0.52 + 0.61	25.91 ± 1.28	0.40	0.60	1	Additive
Kufri Chipsona-3 + *Kufri Jyoti*	0.57 + 0.14	29.63 ± 1.11	0.85	0.39	1.23	Antagonistic
Kufri Chipsona-3 + *Kufri Jyoti*	0.57 + 0.22	22.12 ± 2.43	0.64	0.37	1.01 ≈ 1	Additive
Kufri Chipsona-3 + *Kufri Jyoti*	0.57 + 0.36	24.67 ± 1.09	0.71	0.37	1.08 ≈ 1	Additive
Kufri Chipsona-3 + *Kufri Jyoti*	0.57 + 0.61	16.94 ± 1.15*	0.49	0.39	0.88	Synergistic

Results are mean ± SD of triplicate experiments

KC-3: *Kufri Chipsona* -; KJ: *Kufri Jyoti*

*P < 0.05 with respect to the average of their individual components

Synergistic (CI<1); Additive (CI=1); Antagonistic (CI > 1) [Ref: Chou et al (1994)]

- **Results on combined antimicrobial efficacy study**

These seven isolated antioxidant components three from *Kufri Chipsona-3* (R_f: 0.57, 0.52, 0.33) and four from *Kufri Jyoti* (R_f: 0.61, 0.36, 0.22, 0.14) (Table 7.1 a) potato peel extracts] alone and in combination were also evaluated for their antimicrobial potential in combination against foodborne Gram-positive [*L. monocytogenes* (MTCC 657)] and Gram-negative [*S. typhimurium* (MTCC 3224)] bacterial pathogens. Table 7.2 shows the result of MIC values of these seven components alone and in combination against the studied bacterial pathogens. Based on individual and combined MIC values of seven active components, FICI values against the studied bacterial pathogens were calculated to determine type of antimicrobial interactions. From FICI values it was observed that among the possible combinations of isolated components tested, only one components from *Kufri Chipsona-3* potato peel extract (R_f : 0.57) and one component from *Kufri Jyoti* potato peel extract (R_f : 0.61) showed synergistic antimicrobial interaction against the studied foodborne bacterial pathogens (FICI < 0.50) whereas other tested possible combinations showed additive antimicrobial effect (Table 7.3).

Table7.2 Minimum inhibitory concentration (MIC) values of active components of phenolic fraction of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties alone and in combination against studied foodborne bacterial pathogens

Test potato peel extracts	R_f value of active components	MIC (µg/ml)	
		Foodborne Bacteria	
		L. monocytogenes (MTCC 657)	*S. typhimurium* (MTCC 3224)
	Individual effect of potato peel extract components		
Kufri Chipsona-3	0.33	86.45 ± 3.71	125.87 ± 2.21
Kufri Chipsona-3	0.52	71.27 ±2.16	112.63 ±3.44
Kufri Chipsona-3	0.57	39.64 ± 2.43	55.37 ± 2.18
Kufri Jyoti	0.14	75.37 ± 3.67	115.19 ± 2.78
Kufri Jyoti	0.22	79.74 ± 1.27	114.80 ± 2.12
Kufri Jyoti	0.36	73.82 ± 2.61	108.74 ± 2.24
Kufri Jyoti	0.61	43.76 ± 2.41	61.78 ± 2.40
Kufri Chipsona-3 + *Kufri Jyoti*	Combined effect of potato peel extract components		
	0.33 + 0.14	71.33 ± 1.57 (R_f : 0.33)	89.35 ± 2.06 (R_f :0.33)
		78.45 ± 2.17 (R_f : 0.14)	97.54 ± 1.13(R_f : 0.14)
Kufri Chipsona-3 + *Kufri Jyoti*	0.33 + 0.22	76.67 ± 1.09 (R_f : 0.33)	102.89 ±1.98(R_f: 0.33)
		81.02 ± 1.42 (R_f :0.22)	112.23±1.08(R_f :0.22)
Kufri Chipsona-3 + *Kufri Jyoti*	0.33 + 0.36	67.90 ± 2.13 (R_f: 0.33)	109.72±2.27 (R_f: 0.33)
		79.34 ± 2.89 (R_f: 0.36)	118.12 ±1.23(R_f: 0.36)
Kufri Chipsona-3 + *Kufri Jyoti*	0.33+ 0.61	74.63 ± 3.18 (R_f: 0.33)	91.34 ± 2.88 (R_f: 0.33)
		84.87 ± 1.65 (R_f: 0.61)	99.45 ± 2.06 (R_f: 0.61)
Kufri Chipsona-3 + *Kufri Jyoti*	0.52 + 0.14	68.45 ± 3.91 (R_f: 0.52)	94.87 ± 2.29 (R_f: 0.52)
		78.31 ± 2.95 (R_f: 0.14)	114.13 ± 1.34 (R_f: 0.14)

Contd…..

Kufri Chipsona-3 + *Kufri Jyoti*	0.52 +0.22	58.27 ± 2.19 (R_f: 0.52)	116.13 ±3.81 (R_f: 0.52)
		69.56 ± 1.05(R_f: 0.22)	126.33 ± 3.21 (R_f: 0.22)
Kufri Chipsona-3 + *Kufri Jyoti*	0.52 + 0.36	60.64 ± 2.03 (R_f: 0.52)	71.37 ± 2.96 (R_f: 0.52)
		74.87 ± 2.85 (R_f: 0.36)	83.98± 1.12 (R_f: 0.36)
Kufri Chipsona-3 + *Kufri Jyoti*	0.52 + 0.61	73.26 ± 1.92 (R_f: 0.52)	110.12 ± 3.28 (R_f: 0.52)
		85.22 ± 2.33 (R_f: 0.61)	125.66 ± 1.34 (R_f: 0.61)
Kufri Chipsona-3 + *Kufri Jyoti*	0.57 + 0.14	64.52 ± 2.24 (R_f: 0.57)	117.92 ± 1.72 (R_f: 0.57)
		75.89 ± 3.08 (R_f: 0.14)	129.31 ± 2.65 (R_f: 0.14)
Kufri Chipsona-3 + *Kufri Jyoti*	0.57 + 0.22	72.19 ± 2.61 (R_f: 0.57)	78.83 ± 1.57 (R_f: 0.57)
		87.09 ± 1.76 (R_f: 0.22)	86.13 ± 1.99 (R_f: 0.22)
Kufri Chipsona-3 + *Kufri Jyoti*	0.57 + 0.36	55.76 ± 2.41 (R_f: 0.57)	105.32 ± 1.39 (R_f: 0.57)
		68.09 ± 1.63 (R_f: 0.36)	112.65 ± 2.38 (R_f: 0.36)
Kufri Chipsona-3 + *Kufri Jyoti*	0.57 + 0.61	8.24±1.31* (R_f: 0.57)	12.62 ± 2.26 * (R_f: 0.57)
		10.78 ± 1.21* (R_f: 0.61)	15.19 ± 2.87 * (R_f: 0.61)

Table 7.3 Fractional inhibitory concentration indices (FICI) values of bioactive compounds potato peel extracts in combination against studied foodborne bacterial pathogens

Test potato peel extracts	R_f value of active components	Foodborne Bacteria					
		L. monocytogenes (MTCC 657)			*S. typhimurium* (MTCC 3224)		
		FIC	FICI	Remarks	FIC	FICI	Remarks
Kufri Chipsona-3+*Kufri Jyoti*	0.33 + 0.14	0.82 (R_f0.33) 0.96 (R_f0.14)	1.78	ADD	0.69 (R_f0.33) 0.84 (R_f0.14)	1.53	ADD
Kufri Chipsona-3+*Kufri Jyoti*	0.33 + 0.22	0.88 (R_f0.33) 1.02 (R_f0.22)	1.90	ADD	0.79 (R_f0.33) 1.02 (R_f0.22)	1.81	ADD
Kufri Chipsona-3+*Kufri Jyoti*	0.33 + 0.36	0.78 (R_f0.33) 1.07 (R_f0.36)	1.85	ADD	0.87 (R_f0.33) 0.92 (R_f0.36)	1.79	ADD
Kufri Chipsona-3+*Kufri Jyoti*	0.33 + 0.61	0.86(R_f0.33) 1.93(R_f0.61)	2.79	ADD	0.72 (R_f0.33) 1.60 (R_f0.61)	2.32	ADD
Kufri Chipsona-3+*Kufri Jyoti*	0.52 + 0.14	0.96 (R_f0.52) 1.03 (R_f0.14)	1.99	ADD	0.82 (R_f0.52) 0.99 (R_f0.14)	1.81	ADD
Kufri Chipsona-3+*Kufri Jyoti*	0.52 + 0.22	0.81 (R_f0.52) 0.87 (R_f0.22)	1.68	ADD	1.03 (R_f0.52) 1.10 (R_f0.22)	2.13	ADD
Kufri Chipsona-3+*Kufri Jyoti*	0.52 + 0.36	0.85 (R_f0.52) 1.01 (R_f0.36)	1.86	ADD	0.63 (R_f0.52) 0.77 (R_f0.36)	1.40	ADD

Contd…..

Kufri Chipsona-3+*Kufri Jyoti*	0.52 + 0.61	1.02 (R_f0.52) 1.94 (R_f0.61)	2.96	ADD	0.97 (R_f0.52) 0.48 (R_f0.61)	1.42	ADD
Kufri Chipsona-3+*Kufri Jyoti*	0.57 + 0.14	1.62 (R_f0.57) 1.00 (R_f0.14)	2.62	ADD	2.12 (R_f0.57) 1.12 (R_f0.14)	3.24	ADD
Kufri Chipsona-3+*Kufri Jyoti*	0.57 + 0.22	1.82 (R_f0.57) 1.09 (R_f0.22)	2.91	ADD	1.42 (R_f0.57) 0.75 (R_f0.22)	2.17	ADD
Kufri Chipsona-3+*Kufri Jyoti*	0.57 + 0.36	1.40 (R_f0.57) 0.92 (R_f0.36)	2.32	ADD	1.91 (R_f0.57) 1.03 (R_f0.36)	2.94	ADD
Kufri Chipsona-3+*Kufri Jyoti*	0.57 + 0.61	0.20(R_f0.57) 0.24 (R_f0.61)	0.44	S	0.22 (R_f0.57) 0.24 (R_f0.61)	0.46	S

Results are Mean ±S.D. of triplicate experiments; KC-3: *Kufri Chipsona -3*;

KJ: Kufri *Jyoti* *P < 0.05 with respect to the average of their individual components

Results are mean ± SD of triplicate experiments. ADD = 1; S < 1. ADD, additive; S, Synergistic.

From Table 7.1(b) and Table 7.3, it was also observed that among the possible combinations on antioxidant and antimicrobial potential tested between three isolated components of peel extracts of *Kufri Chipsona-3* (R_f : 0.57, 0.52, 0.33) and four isolated components from peel extract of *Kufri Jyoti* (R_f : 0.61, 0.36, 0.22, 0.14) potato varieties, only one combination between component of *Kufri Cipsona-3* peel extract (R_f: 0.57) and one component of *Kufri Jyoti* peel extract (R_f : 0.61) exhibited both synergistic antioxidant and antimicrobial efficacy whereas other tested possible combinations showed additive antioxidant and additive or antagonistic antimicrobial effect. Therefore, these two peel extract components, one from peel extract of *Kufri Chipsona-3* (R_f: 0.57) and the other from peel extract of *Kufri Jyoti* (R_f: 0.61) that showed both synergistic antioxidant and antimicrobial interactions in combination

were subjected to spectrometric analysis for chemical characterization of bioactive compounds.

Figure 7.3 represents the UV/Vis spectra of *Kufri Chipsona -3* peel extract component (R_f: 0.57). It was observed that the maximum absorbance ($UV_{\lambda max}$) of *Kufri Chipsona-3* peel extract component (R_f: 0.57) was 278 nm, which corresponds with the absorbance maxima of proanthocyanidin compounds (Ku and Mun, 2007).

Figure 7.4 represents the UV/Vis absorption spectra of *Kufri Jyoti*) peel extract component (R_f: 0.61). It was observed that the maximum absorbance ($UV_{\lambda max}$) of *Kufri Jyoti* peel extract component (R_f: 0.61) was at 350 nm and 260 nm, which corresponds with the absorbance maxima of flavones (Jurasekova et al., 2006).

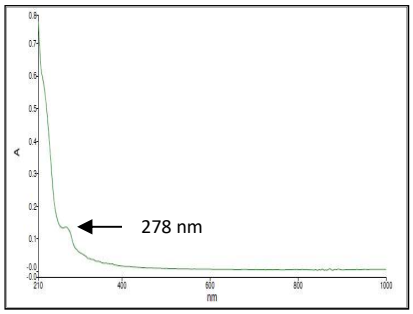


Figure 7.3 UV-Vis absorption spectra of *Kufri Chipsona-3* peel extract component (R_f : 0.57)

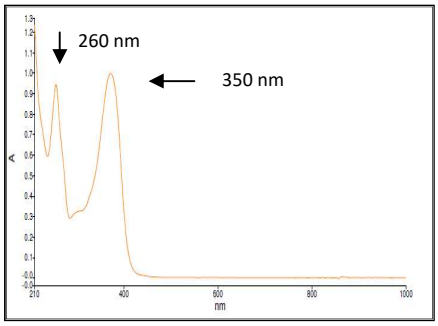


Figure 7.4 UV/Vis absorption spectra of *Kufri Jyoti* peel extract component (R_f : 0.61)

Figure 7.5 represents the FT-IR spectrum of *Kufri Chipsona -3* peel extract component (R_f: 0.57). From FT-IR curves (Figure 7.5) the major peaks of *Kufri Chipsona-3* peel extract component (Rf : 0.57) were found at 3429.61 (stretching vibrations of O-H groups); 2906.48 and 2851.53 (stretching vibration of C-H (CH_2, CH_3);1575.36 (aromatic ring vibrations); 1417.19 (related to CH_2, CH_3, flavonoids and aromatic rings where the vibrations would be bending (δ) vibrations of C-H and stretching vibration of aromatics); 1120.80-1020.44 (stretching vibration of C-O-C). These bands represent structure of proanthocyanidins (Ku and Mun, 2007)

Figure 7.6 represents the FT-IR spectrum of *Kufri Jyoti* peel extract component (R_f: 0.61). Major peaks at FT-IR spectra of *Kufri Jyoti* peel extract component (R_f: 0.61) were found at 3442.28 (stretching vibration of O-H group); 2927.83-2821.82 (stretching vibration of C-H); 1594.57 (aromatic ring vibration); 1353.23 (stretching vibration of aromatic C=C); 1075.60

(C-O stretching). These bands represent the molecular structure of flavonoids (Oliveira et al. 2016).

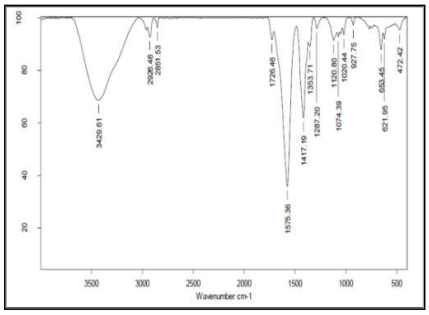


Figure7.5 FTIR spectra of *Kufri Chipsona-3* peel extract component (R_f : 0.57)

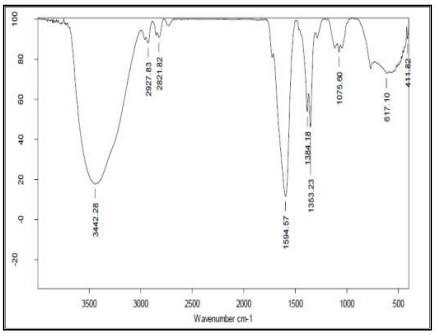


Figure 7.6 FTIR spectra of *Kufri Jyoti* peel extract component (R_f : 0.61)

Figure 7.7 represents the HR-LCMS/MS analysis of *Kufri Chipsona -3* peel extract component (R_f : 0.57).The retention time of this component was found to be 7.89 min and its molecular mass (*m/z)* was found to be 897.0.

Figure 7.8 represents the HR-LCMS/MS analysis of *Kufri Jyoti* peel extract component (R_f: 0.61). From HR-LCMS/MS analysis curves, the retention time of *Kufri Jyoti* peel extract component (R_f : 0.61) was found to be 10.04 min and its molecular mass (*m/z*) was 327.5.

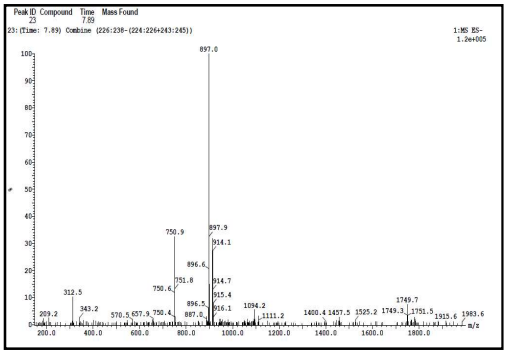


Figure7.7.HR –LCMS/MS spectra of *Kufri Chipsona-3* peel extract component (R_f : 0.57)

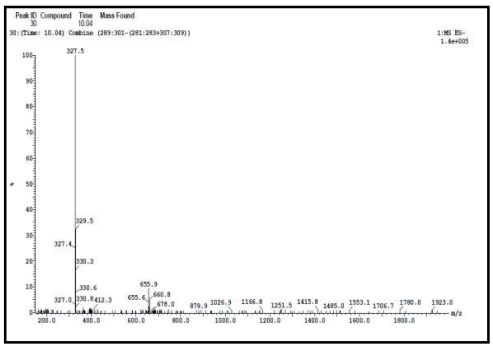
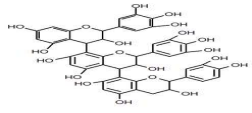
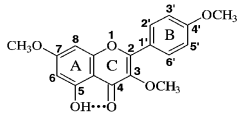


Figure7.8.HR –LCMS/MS spectra of *Kufri Jyoti* peel extract component (R_f : 0.61)

Table 7.4 Collective spectrometric data of active compounds from peel extract of *Kufri Chipsona-3* (R_f : 0.57) and *Kufri Jyoti* (R_f : 0.61) potato varieties

Spectrometric parameters	Active components from peel extract of *Kufri Chipsona-3* potato (R_f : 0.57)	Active components from peel extract of *Kufri Jyoti* potato (R_f : 0.61)
$UV_{\lambda max}$ (MeOH)	Only one band at 278 nm	Two bands. Band A at 350 nm and Band B at 260 nm
HR-LCMS/MS (m/z) **$[M-H]^-$**	897 (calcd. molecular formula : $C_{45}H_{38}O_{20}$; 898.8)	327.5 (calcd. molecular formula : $C_{18}H_{16}O_6$; 328.3)
Retention Time (min)	7.89	10.04
FT-IR $_{KBr}$ (cm^{-1})	Major peaks are at 3429.61 (stretching vibrations of O-H groups); 2906.48 and 2851.53 (stretching vibration of C-H (CH2, CH3);1575.36 (could be related to aromatic ring vibrations); 1417.19 (related to CH2, CH3, flavonoids and aromatic rings where the vibrations would be bending (δ) vibrations of C-H and stretching vibration of aromatics); 1120.80-1020.44 (stretching vibration of C-O-C) .	Major peaks are at 3442.28 (stretching vibration of O-H group); 2927.83-2821.82 (stretching vibration of C-H); 1594.57 (aromatic ring vibration); 1353.23 (stretching vibration of aromatic C=C); 1075.60 (C-O stretching).
Proposed compound	Prodelphinidin trimer	5-Hydroxy-3',4',7-trimethoxy flavone
Molecular structure		

The collective spectrometric analyses data of two potato peel extract components one from *Kufri Chipsona-3* (R_f: 0.57) and the other from *Kufri Jyoti* (R_f: 0.61) that showed synergistic antioxidant and antimicrobial interactions in combination are shown in Table 7.4.Comparing these data with available literature data (Qa'Dan 2003; Macedo et al.,2019) and MS library data, the bioactive compound from *Kufri Chipsdona-3* (R_f : 0.57) was identified as prodelphinidin trimer and the compound isolated from peel extract of *Kufri Jyoti* was identified as 5-Hydroxy-3',4',7-trimethoxy flavone (Table 7.4).

Discussion

For isolation, identification and chemical characterization of bioactive compounds responsible for synergistic antioxidant and antimicrobial interactions of peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties, at first TLC bioautography guided detection and

isolation of antioxidant components was performed. TLC bioautography analysis revealed the presence of three antioxidant components in peel extract of *Kufri Chipsona-3* having R_f values 0.57, 0.52, and 0.33 (Figure 7.1b) and four antioxidant components with R_f : 0.61, 0.36, 0.22 and 0.14 in peel extract of *Kufri Jyoti* potato varieties (Figure 7.2 b). These seven antioxidant components of different R_f values from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties were subjected to combined antioxidant efficacy study in DPPH radical scavenging method followed by isobologram analysis and also for combined antimicrobial efficacy study against foodborne bacterial pathogens using checkerboard titration method followed by Kill-kinetics assay. Results obtained from combined antioxidant and antimicrobial efficacy study revealed that among the possible combinations tested for antioxidant and antimicrobial efficacy study, only one combination between *Kufri Chipsona-3* peel extract component (Rf: 0.57) and *Kufri Jyoti* peel extract component (Rf: 0.61) exhibited both synergistic antioxidant (Table 7.1b) and synergistic antimicrobial (Table 7.3) interactions.

Now, these two bioactive components from peel extract of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties were subjected to spectrometric (UV/Vis, FT-IR, HR-LCMS/MS) analysis for chemical characterization of bioactive compounds responsible for synergistic antioxidant and antimicrobial interactions. Obtained spectrometric analysis data (Table 7.4) of these two bioactive components of peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties were compared with MS library data as well as relevant references (Qa'Dan 2003; Macedo et al.,2019). Comparing the obtained spectral data of bioactive components from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties with MS library data as well as relevant literature data, the bioactive component from peel extract of *Kufri Chipsona-3* with R_f: 0.57 and m/z: 327.5 was found to be prodelphinidin trimer and the bioactive compound from peel extract of *Kufri Jyoti* (R_f: 0.61; m/z 897) was found to be 5-Hydroxy-3',4',7-trimethoxyflavone. Thus, bioactive compounds responsible for synergistic antioxidant and antimicrobial efficacy in combination from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties were prodelphinidin trimer and 5-Hydroxy-3',4',7-trimethoxyflavone respectively.

(B) Evaluation of cytotoxic potential of active extracts of potato peels and their bioactive compounds in various *in vitro* models.

Introduction

Cytotoxicity studies are a useful step in determining the potential toxicity of a test substance including plant extracts and biologically active natural and synthetic compounds. Minimal to nontoxicity is essential for the successful development of a pharmaceutic or cosmetic preparation and in this regard, cellular toxicity studies play a crucial role. To evaluate the potential of bioactive compounds or active plant extracts on various biological activities without toxicity, the selectivity index (SI) was introduced. The SI is an important measure to identify substances with promising biological activity and negligible cytotoxicity (McGaw et al., 2014). SI is the ratio between cytotoxic potential (IC_{50}, EC_{50}) and biological activity (ED50). Low SI (SI < 1) of plant extract or bioactive compound indicates that the biological activity is probably due to cytotoxicity rather than other mechanism of action. A substance with $1 \geq SI < 10$ can be considered as moderately toxic. In contrast, high SI ($SI \geq 10$) offers a potential safer therapy (Valdes et al., 2010). Various bioassays and a number of different cell lines have been used to assess the cytotoxicity of crude plant extracts or purified bioactive compounds. In this work, among the peel extracts of selected varieties of potato tested for their possible antioxidant and antimicrobial efficacy alone and in combination, only peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* combination (1:1) showed synergistic antioxidant and antimicrobial interaction **(Chapter 6)**. Chemical analysis revealed that bioactive compounds prodelphinidin trimer from peel extract of *Kufri Chipsona-3* and 5-Hydroxy-3',4',7-trimethoxyflavone from peel extract of *Kufri Jyoti* potato varieties were responsible for both synergistic antioxidant as well as antimicrobial interactions in combination **(Chapter 7)**. It is, therefore, necessary to evaluate the cytotoxic potential of active extracts of peels of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties as well as their bioactive compounds prodelphinidin trimer and 5-Hydroxy-3',4',7-trimethoxyflavone in combination using various *in vitro* methods with a view to assess their cytotoxic potential if any, for the successful development of safe and effective novel natural antioxidant and antimicrobial agents for preserving foods. Considering this, the present work has been designed accordingly to evaluate the cytotoxic potential of these active peel extracts and their bioactive compounds in combination in various *in vitro* models [*Allium cepa* root growth

inhibition assay and WST-1 based colorimetric assay using human keratinocyte cell line (HaCaT cells)].

Results

- *Allium cepa* root growth inhibition assay

Figure 7.9 shows the cytotoxic results of active peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties in combination in *Allium cepa* root growth inhibition method. It was observed that the active peel extracts of *Kufri Chipsona-3/Kufri Jyoti* blend upto 1000 µg/ml concentration did not show any significant effect in root growth inhibition compared to control. The IC_{50} of peel extracts of *Kufri Chipsona-3/ Kufri Jyoti* combination was found to be > 1000 µg /ml.

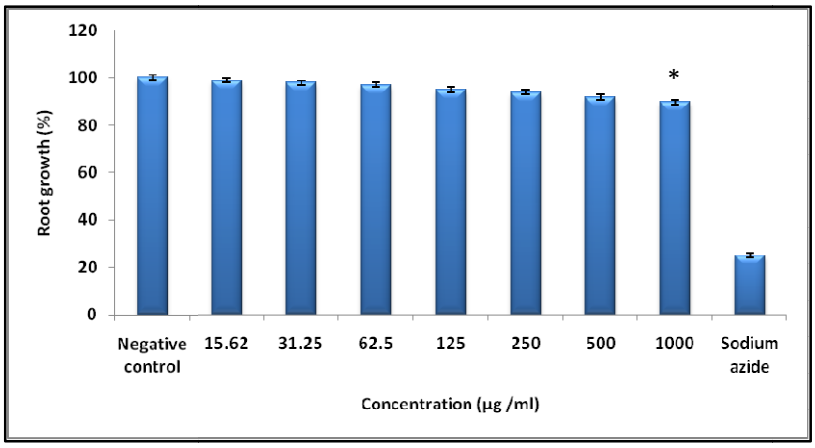


Figure 7.9Showing the results of cytotoxic potential of *Kufri Chipsona-3/Kufri Jyoti* potato peel extract combination in *Allium cepa* root growth inhibition assay

Figure 7.10 Photographs of evaluation of cytotoxic potential of *Kufri Chipsona-3/Kufri Jyoti* potato peel extract in combination in *Allium cepa* root growth inhibition assay

- **WST-1 based colorimetric assay**

Next, cytotoxic potential of bioactive compounds prodelphinidin trimer and 5-Hydroxy-3',4',7- trimethoxyflavone isolated from active peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties respectively responsible for synergistic antimicrobial and antioxidant interactions in combination was evaluated by WST-1 based colorimetric assay using human normal keratinocyte cell line and the results are shown in Figure 7.11. It was observed that there was no significant difference in cell viability up to 100 µg/ ml concentration of bioactive compounds tested in combination compared to negative control but was found to be significantly ($p < 0.05$) different when compared with positive control (Triton-X 100) and IC_{50} of test bioactive compounds in combination was found to be > 100µg/ml (Figure 7.11).

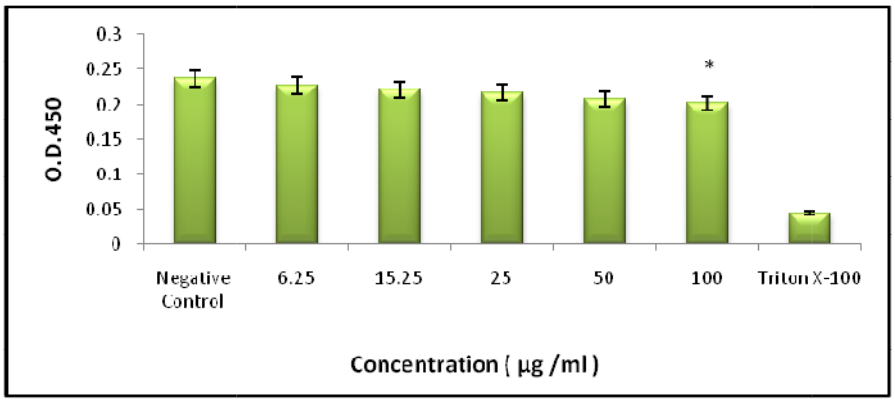


Figure 7.11 Showing the results of cytotoxic potential of bioactive compounds in combination isolated from *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties in WST-1 based colorimetric assay

Discussion

Cytotoxic potential of test active extracts of peels of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties in combination was evaluated using *Allium cepa* root growth inhibition assay. *Allium cepa* test was used because this *in vitro* test is very useful as a first-tier analysis of cytotoxicity and genotoxicity due to its simplicity, low relative cost, versatility and minimum laboratory facilities required for its performance (Khora et al., 1997). It has been shown to have a correlation with tests in other living systems and serve as an indicator of toxicity of the tested material (Fiskesjo, 1985). Inhibition of root growth appears to be the most sensitive parameter because root growth inhibition is manifestation of an arrest of a cell division (Webster and MacLeod 1996). According to Meyer et al., (1982), crude plant extracts with

IC_{50} > 1000 µg/ml are considered non-toxic. In our study, we observed that IC_{50} of *Kufri Chipsona-3/Kufri Jyoti* combination was > 1000 µg/ml (Figure 7.2). Besides, no visible morphological changes in root consistency and colour as well as the presence of swelling, hooks or twists in the roots were observed. Therefore, active peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties in combination can generally be considered as non-toxic substances.

Now, the bioactive purified compounds responsible for synergistic antioxidant and antimicrobial interactions of peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* i.e. prodelphinidin trimer and 5-Hydroxy-3',4',7-trimethoxyflavone respectively in combination were subjected to cytotoxicity evaluation by WST-1 assay using human keratinocyte cell line. In this study, IC_{50} of test bioactive compounds of active potato peel extracts in combination against keratinocyte cell line was found to be >100 µg/ml. According to the American National Cancer Institute (NCI) plant screening programme, a crude plant extract is generally considered to have an *in vitro* cytotoxic activity with an IC_{50} value ≤ 30 µg/ml. However, a crude extract with IC_{50}< 20 µg/ml is considered to be highly cytotoxic. The IC_{50} value of cytotoxic purified compounds will be lower than that (Vijarathna and Sasidharan, 2012; Sriwiryajan et al., 2014). In our study, it was observed that IC_{50} of test bioactive compounds in combination was > 1000 µg / ml and therefore can generally be considered as safe.

CHAPTER 8

OBJECTIVE 5

TO ELUCIDATE POSSIBLE MODE OF ANTIOXIDANT AND ANTIMICROBIAL ACTION OF BIOACTIVE COMPOUNDS ISOLATED FROM ACTIVE EXTRACTS OF POTATO PEELS ALONE AND IN COMBINATION

INTRODUCTION

RESULTS

DISCUSSION

(A) Elucidation of possible modes of antioxidant action of bioactive compounds alone and in combination

Introduction

Free radicals are produced in the form of reactive oxygen species (ROS) as a normal part of metabolism. Antioxidants maintain a delicate balance between the production and neutralization of ROS, but if this balance is disturbed, the cells start to suffer the consequences of oxidative stress (Wiernsparge, 2003).This leads to the development of several chronic diseases viz. cardiovascular diseases, diabetes mellitus, atherosclerosis and stroke, neurological disorders, renal disorders, rheumatoid arthritis, Alzheimer's disease and even cancer (Lu et al., 2010; Lobo et al, 2010). Free radical mediated damage to lipids, proteins, and DNA is mainly responsible for these negative effects (Ranjbar et al., 2006). Although the human body produces antioxidant enzymes to neutralize free radicals, exogenous antioxidants are required to assist the human body to protect itself (Poljak et al., 2014).

Different antioxidants follow different mechanisms or pathways under various stress conditions in order to show their antioxidative response. The main mechanisms involve: inhibiting free radical oxidation reactions (preventive oxidants), interruption of propagation of the autoxidation chain reaction (chain breaking antioxidants) inhibiting formation of free lipid radicals; quenching single oxygen species; reducing hydro peroxides converting them into stable compounds; inhibiting prooxidative enzymes; chelating metals and through synergism with other antioxidants (Kancheva, 2009; Heim et al, 2002).

Since the oxidation process follows a complicated set of mechanism, mixture of compounds (phytochemicals or synthetic) would have greater bioactivity than a single compound because a mixture of bioactive compounds has the ability to affect multiple targets (Schmidt et al., 2008) and also the activity of one antioxidant is dependent on the other. Antioxidants have a wide range of biological and pharmacological activities and are considered to be of great benefit in nutrition and health (Newman et al., 2000). These can also prevent or delay the oxidation of food and thus are used in food preservation (Vagi et al., 2005) because these natural antioxidants avoid undesired health problems that may arise from the use of synthetic antioxidants. The interactions among different antioxidant components can be synergistic, additive or antagonistic. It may be successful to use antioxidant combinations in which the antioxidants produce a synergistic effect.

Considering this, in the present study an attempt has been made to evaluate the effect of purified bioactive compounds prodelphinidin trimer and 5 –Hydroxy -3',4',7-trimethoxy flavone at their individual effect in various *in vitro* methods (DPPH radical scavenging; ABTS•+radical cation decolourization; Trolox equivalent antioxidant capacity (TEAC); Fe^{2+} ion chelating; Ferric reducing antioxidant power (FRAP) and Lipoxygenase inhibition) and in combination against oxidation of lipids (omega-3 fatty acids enriched oil) and in Briggs-Rauscher Oscillation reaction method with a view to elucidate their possible modes of antioxidant action alone and in combination.

Results

➢ **Antioxidant efficacy at their individual effect**

- ***DPPH radical scavenging activity***

Figure 8.1 shows the results of antioxidant potential of isolated bioactive compounds from active peel extracts of *Kufri Chisona-3* and *Kufri Jyoti* potato varieties against DPPH radical scavenging method at their individual effect. It was observed that both the test compounds exhibited varying degrees of concentration dependent free radical scavenging activity in DPPH radical scavenging method. The bioactive compound prodelphinidin trimer (compound 1) isolated from peel extract of *Kufri Chipsona-3* showed higher antioxidant potential compared to the bioactive compound 5–Hydroxy -3',4',7-trimethoxy flavones isolated from *Kufri Jyoti* potato peel extract (compound 2).

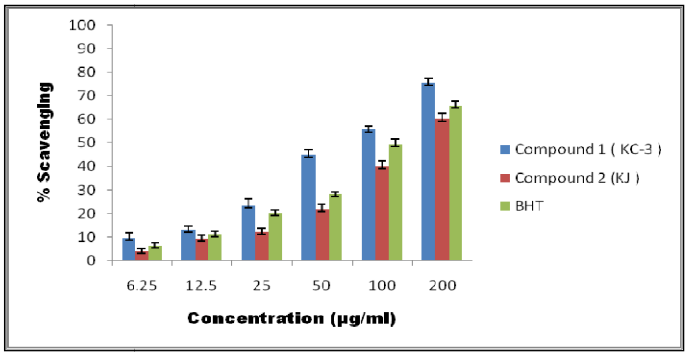


Figure 8.1: DPPH radical scavenging activity of bioactive compounds isolated from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* at their individual effect

Compound 1: Prodelphinidin trimer (from *Kufri Chipsona-3*); Compound 2 : 5 –Hydroxy -3',4',7-trimethoxyflavones (from *Kufri Jyoti*), Values are mean ± SD of triplicate experiments

- ***ABTS •+ radical cation decolourization activity***

The ABTS •+ radical cation decolourization activity results of isolated bioactive compounds prodelphinidin trimer and 5 –Hydroxy -3',4',7-trimethoxyflavone at their individual effect are shown in Table 8.1. It was observed that IC_{50} value of prodelphinidin trimer (compound 1) was much lower than 5-Hydroxy -3',4',7-trimethoxy flavones (compound 2) as well as reference standard antioxidant BHT suggesting higher inhibitory activity of prodelphinidin trimer isolated from peel extract of *Kufri Chipsona-3* potato variety against ABTS •+ radical cation decolourization compared to both 5 –Hydroxy -3',4',7-trimethoxyflavone isolated from peel extract of *Kufri Jyoti* potato variety as well as reference standard antioxidant BHT.

Table 8.1 $ABTS^{\cdot+}$ decolourization activity of active compounds isolated from *Kufri Chipsona-3* and *Kufri Jyoti* potato peel extracts at their individual effect

Active components of potato peel extract	**Concentration (µg/ml)**						**IC_{50} (µg/ml)**
	6.25	12.5	25	50	100	200	
	Scavenging (%)						
Compound 1 (KC-3)	10.43 ± 2.1	18.78± 1.5	27.32 ± 2.4	45.47 ± 2.5	63.58 ±3.2	85.53 ± 2.9	51.41
Compound 2 (KJ)	5.89 ± 1.76	10.62 ± 2.1	18.89 ± 1.8	22.47 ± 1.7	35.32 ± 2.7	52.51 ± 3.5	184.56
BHT	8.12± 1.3	14.67± 1.86	24.73 ± 1.4	38.6 ± 1.2	59.41 ± 2.8	75.87 ± 3.4	60.88

Compound 1: Prodelphinidin trimer (*Kufri Chipsona-3*); Compound 2: 5 –Hydroxy -3',4',7-trimethoxyflavone (*Kufri Jyoti*)

Values are mean ± SD of triplicate experiments

- ***Trolox equivalent antioxidant capacity (TEAC)***

Table 8.2 shows the results of Trolox equivalent antioxidant capacity value (TEAC) of bioactive compounds isolated from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties in ABTS•+ radical cation decolourization assay. Here also it was observed that trolox equivalent antioxidant capacity of bioactive compound prodelphinidin trimer from *Kufri Chipsona-3* exhibited higher antioxidant potential compared to bioactive compound 5 – Hydroxy -3',4',7-trimethoxyflavone isolated from peel extract of *Kufri Jyoti* potato variety.

Table 8.2 Trolox equivalent antioxidant capacity (TEAC) value of active compounds isolated from *Kufri Chipsona-3* and *Kufri Jyoti* potato peel extracts at their individual effects in $ABTS^{\cdot+}$ decolourization activity assay

Active components of potato peel extract	IC_{50} (µg /ml)	TEAC value (µg /ml)
Compound 1 (KC-3)	51.41	5.56
Compound 2 (KJ)	184.56	4.81

Compound 1: Prodelphinidin trimer (*Kufri Chipsona-3*)

Compound 2: 5 –Hydroxy -3',4',7-trimethoxyflavone (*Kufri Jyoti*)

- ***Fe^{2+} ion chelating activity***

Results of Ferrous (Fe^{2+}) ion chelating activity of isolated bioactive compounds Prodelphinidin trimer from peel extract of *Kufri Chipsona-3* and 5-Hydroxy -3',4',7-trimethoxy flavone from peel extract of *Kufri Jyoti* potato varieties are shown in Figure 8.2. Here, it was observed that EDTA, the reference standard chelating agent showed higher ferrous ion chelating activity followed by 5 –Hydroxy -3',4',7-trimethoxyflavone isolated from peel extract of *Kufri Jyoti* and prodelphinidin trimer isolated from peel extract of *Kufri Chipsona-3* potato varieties.

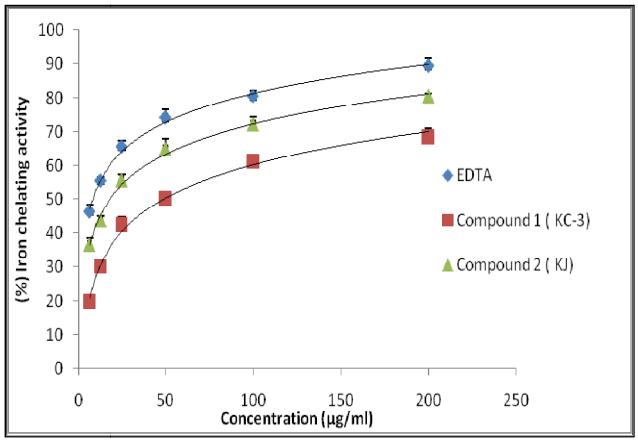


Figure 8..2 Iron chelating activity of active compounds isolated from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* at their individual effect

Compound 1 : Prodelphinidin trimer (*Kufri Chipsona-3*)

Compound 2: 5 –Hydroxy -3',4',7-trimethoxyflavone (*Kufri Jyoti*)

Values are mean ± SD of triplicate experiments

- ***Ferric reducing antioxidant power (FRAP)***

Table 8.3 shows the results of ferric reducing antioxidant power (FRAP) of test bioactive compounds prodelphinidin trimer form *Kufri Chipsona-3* and 5–Hydroxy -3',4',7-trimethoxy flavone from *Kufri Jyoti* potato peel extracts at their individual effect. Both the test compounds exhibited concentration dependent varying degrees of ferric reducing antioxidant power at their individual effect. Based on their IC_{50} values, the compound prodelphinidin trimer showed higher antioxidant potential followed by BHT, a reference standard antioxidant and bioactive compound 5 –Hydroxy -3',4',7-trimethoxy flavone isolated from *Kufri Jyoti* potato variety.

Table 8.3 Ferric reducing antioxidant power (FRAP) of active compounds isolated from *Kufri Chipsona-3* and *Kufri Jyoti* potato peel extracts at their individual effect

Active components of potato peel extract	Concentration (µg/ml)						IC_{50} (µg/ml)
	6.25	12.5	25	50	100	200	
	Activity (%)						
Compound 1 (KC-3)	18.27 ± 1.1	21..39 ± 1.27	43.9 ± 2.2	56.43 ± 1.02	74.5 ± 2.16	81.32 ± 1.23	45.87
Compound 2 (KJ)	8.19 ± 1.26	16.13 ± 1.89	24.52± 2.31	33.24 ± 1.07	43.7 ± 2.9	58.5 ± 1.5	170.71
BHT	9.02 ± 1.45	19.59 ± 2.11	38.42± 1.98	49.32 ± 1.52	63.24 ± 2.2	75.67 ± 1.5	53.54

Compound 1: Prodelphinidin trimer (*Kufri Chipsona-3*)

Compound 2: 5 –Hydroxy -3',4',7-trimethoxyflavone (*Kufri Jyoti*)

Values are mean ± SD of triplicate experiments

- ***Lypoxygenase (LOX) inhibition activity***

Table 8.4 shows the results of lipooxygenase inhibitory activity of bioactive compounds prodelphinidin trimer from peel extract of *Kufri Chipsona-3* and 5-Hydroxy-3',4',7-trimethoxy flavone isolated from *Kufri Jyoti* peel extract at their individual effect. On the basis of IC_{50} values, the bioactive compound 5 –Hydroxy -3',4',7-trimethoxyflavone isolated from *Kufri Jyoti* potato peel extract exhibited higher lipooxygenase inhibitory effect followed by indomethacin, a reference standard lipooxygenase inhibitory agent and the bioactive compound prodelphinidin trimer isolated from peel extract of *Kufri Chipsona-3* potato variety.

Table 8.4 Lipoxygenase inhibition activity of active compounds isolated from *Kufri Chipsona-3* and *Kufri Jyoti* potato peel extracts at their individual effect

Active components of potato peel extract	Concentration of test components (µg/ml)						IC_{50} (µg/ml)
	6.25	12	25	50	100	200	
	Inhibition activity (%)						
Compound 1 (KC-3)	8.3 ± 2.1	12.12± 1.54	20.23 ± 1.63	39.26 ± 1.82	49.29± 2.5	60.61 ± 1.3	108.85
Compound 2 (KJ)	15.13 ± 2.16	22.5 7± 2.2	34.29 ± 1.06	45.28 ± 1.62	59.92 ± 1.33	76.55 ± 2.12	57.39
Indomethacin	10.19± 1.74	16.51± 1.63	29.87 ± 1.09	38.45± 2.12	55.43 ± 2.36	66.76 ± 1.32	79.83

Compound 1: Prodelphinidin trimer (*Kufri Chipsona-3*)

Compound 2: 5 –Hydroxy -3',4',7-trimethoxy flavone (*Kufri Jyoti*)

Values are mean ± SD of triplicate experiments

- **Antioxidant efficacy at their alone and combined effect**

- **Oxidation of lipids**

Table 8.5 shows the results of antilipid peroxidation potential of bioactive compounds prodelphinidin trimer and 5 – hydroxy -3',4',7 – trimethoxyflavone at their individual and combined effects against oxidation of omega-3 fatty acids enriched bulk oil (marine fish oil). From Table 8.5 it was observed that prodelphinidin trimer was significantly ($p < 0.05$) effective against both primary and secondary oxidation of lipids whereas 5-hydroxy-3',4',7-trimethoxyflavone was significantly ($p < 0.05$) effective only against primary oxidation of lipids at their individual effect. In combination the antioxidant efficacy against both primary as well as secondary oxidation of omega-3fatty acids enriched lipid substrate was found to be significantly greater ($p < 0.05$) compared to their individual effect.

Table 8.5 Effects of bioactive compounds isolated from *Kufri Chipsona-3* and *Kufri Jyoti* alone and in combination on primary and secondary oxidation products of lipids in bulk oil

Active components of potato peel extract	Peroxide value (meq/kg oil) (Primary oxidation product)						*p*-anisidine value (p-AV) (meq/kg oil) (Secondary oxidation product)					
	Incubation period (days)						Incubation period (days)					
	0	**3**	**6**	**9**	**12**	**15**	**0**	**3**	**6**	**9**	**12**	**15**
Control	1.26 ± 0.25	9.98 ± 0.69	11.02 ± 1.10	15.25 ± 1.27	20.56 ± 1.66	29.08 ± 2.98	1.34 ± 0.06	11.56 ± 1.15	17.07 ± 1.10	25.89 ± 2.75	29.77 ± 2.59	32.25 ±2.75
Compound 1 (KC-3) (200µg/ml)	1.23 ± 0.75	2.18 ± 1.95	4.58 ± 1.71	6.45 ± 1.30	7.73 ± 1.08*	10.37 ± 1.78	1.32 ± 1.09	6.34 ± 2.01	8.28± 1.08	15.85± 2.13*	18.32 ± 1.19*	20.35± 1.27*
Compound 2 (KJ) (200µg/ml)	1.25 ± 1.19	5.78 ± 1.35	6.58± 1.75	9.45 ± 1.66	11.59 ± 1.02 *	16.11 ± 1.33*	1.30 ± 2.22	10.21± 1.39	16.28± 1.29	20.25 ± 2.19*	28.18± 1.23	32.19± 1.08
Combination (1:1) (100µg/ml each)	1.23 ± 1.11	1.89 ± 2.18	3.31 ± 0.89	4.39 ± 1.18*	6.9 ± 1.89 *	8.58 ± 2.06 *	1.35 ± 2.29	3.84± 1.54	5.39 ± 1.49	7.28 ± 1.56*	9.35± 2.17*	11.09± 1.32*
BHT (200µg/ml)	1.29 ± 0.23	2.95 ± 0.27	6.06 ± 0.32	8.92 ± 0.54	9.84 ± 0.76*	15.17 ± 1.64*	1.33 ± 0.05	10.56 ± 2.27	14.03 ± 1.24	18.22 ± 1.66*	20.53 ± 1.57*	22.01 ± 1.89*

Compound 1: Prodelphinidin trimer (*Kufri Chipsona-3*); Compound 2: 5 –Hydroxy -3',4',7-trimethoxy flavone (*Kufri Jyoti*)

Values are Mean ±SD of triplicate experiments. *P < 0.05; with respect to their control values

Figure 8.3 represents the total oxidation (TOTOX) value of isolated bioactive compounds from phenolic extracts of peels of *Kufri Chipsona-3* (prodelphinidin trimer) and *Kufri Jyoti* (5 –Hydroxy -3',4',7-trimethoxy flavones) at their individual and combined effects. It was observed from Figure 8.3 that at their individual effect they exhibited varying degrees of time dependent antioxidant activity against total oxidation of lipids in bulk oil model system. Prodelphinidin trimer showed higher antioxidant efficacy than 5 –Hydroxy -3',4',7-trimethoxyflavone at its individual effect. In combination, the inhibitory effect on total

oxidation was increased significantly ($P < 0.05$) from day 6 onward compared to their individual effects.

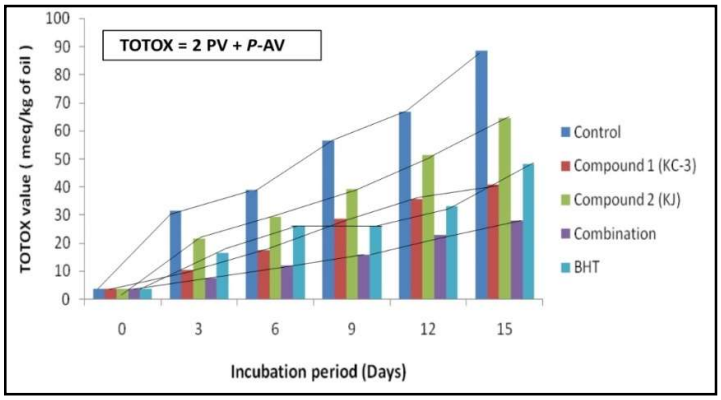


Figure 8.3 TOTOX value of active compounds isolated from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato peel extract alone and in combination

Compound 1 : Prodelphinidin trimer (*Kufri Chipsona-3*) ; : Compound 2: 5 –Hydroxy -3',4',7-trimethoxyflavone (*Kufri Jyoti*) Values are Mean ±SD of triplicate experiments

- ***In Briggs-Rauscher Oscillation reaction***

Table 8.6 shows the results of antioxidant efficacy of isolated bioactive compounds from phenolic extracts of peels of *Kufri Chipsona-3* (prodelphinidin trimer) and *Kufri Jyoti* (5 – Hydroxy -3',4',7-trimethoxyflavone) potato varieties at their individual and combined effect in Briggs- Rauscher oscillation reaction method. It was observed that both of the compounds tested showed varying degrees of antioxidant efficacy, and the bioactive compound prodelphinidin trimer from *Kufri Chipsona-3* showed significantly higher antioxidant potential ($P < 0.05$) than 5-Hydroxy -3',4',7-trimethoxyflavone isolated from peel extract of *Kufri Jyoti* at their individual effect. In combination, however both of the compound showed significant increase in antioxidant efficacy which is evident from the significant decrease ($P < 0.05$) in inhibition time ($t_{inhibition}$) as compared to the average $t_{inhibition}$ of their individual effect.

Table 8.6 Antioxidant potential of active compounds isolated from potato peel extracts of *Kufri Chipsona-3* and *Kufri* Jyoti alone and in combination in B-R oscillation reaction method

Treatment	$t_{inhibition}$ *(seconds)*	Remarks
Compound 1 (KC-3) (200µg/ml)	97 ± 7#	–
Compound 2 (KJ) (200µg/ml)	72 ± 6	–
Combination (1:1) (100µg/ml each)	194 ± 5*	S

Compound 1 : Prodelphinidin trimer (*Kufri Chipsona-3*); Compound 2: 5 –Hydroxy -3',4',7-trimethoxyflavone (*Kufri Jyoti*)

Values are Mean ±S.D. of triplicate experiments ; # Significant : ($p < 0.05$) where compound 2 (5 –Hydroxy -3',4',7-trimethoxyflavone)

*Synergistic (S) : If $t_{inhibition}$ in combination increased significantly ($P < 0.05$) with respect to the average $t_{inhibition}$ of their individual components.

Discussion

Several hypotheses have been proposed to explain the mechanisms of antioxidant synergism and antagonism. It has been reported by several workers that some antioxidants in combination act in a regenerating manner, with either the stronger regenerating the weaker (antagonistic effect) or the weaker regenerating the stronger (synergistic effect) (Peyrat et al., 2003). Synergistic combinations may also involved in the interaction of compounds in combination, which include reaction rates of antioxidants, the polarity of the interacting molecules and the effective concentration of the antioxidants at the site of oxidation (Cuvelier

et al., 2000; Frankel et al, 1994; Koga and Terao, 1995). Decker (2002) reported that following three mechanisms are generally involved in synergism among antioxidants: (i) a combination of two or more different free radical scavengers in which one antioxidant (primary antioxidant) is regenerated by others (secondary antioxidant or synergist), (ii) sacrificial oxidation of an antioxidant to protect another antioxidant, and (iii) a combination of two or more antioxidants whose antioxidant mechanisms are different. It has also been reported that regeneration of a more effective free radical scavenger by a less effective free radical scavenger (coantioxidant, synergist) occurs mostly when one free radical scavenger has a higher redox potential (primary antioxidant) than the other (secondary antioxidant). Regeneration of primary antioxidants contributes to a higher net interactive antioxidant effect than the simple sum of individual effects leading to the formation of synergistic interactions (Decker, 2002).Antioxidants can be classified as primary or secondary antioxidants according to their antioxidant mechanisms. The primary antioxidants are able to react directly with free radicals by transforming them to more stable, non-radical products. Hence, primary antioxidants play an important role in lipid oxidation. The secondary antioxidants, on the other hand, work indirectly on limiting lipid oxidation (Decker, 2002).Besides, primary antioxidants function as chain breaking antioxidants and interfere with the initiation, propagation and peroxide decomposition of oxidation reactions by donating hydrogen atom (through HAT mechanism) or single electron (through SET mechanism). Some secondary antioxidants can work synergistically by regenerating primary antioxidants and thereby restore the antioxidant activity of primary antioxidants to ensure their continuous antioxidant activity (Haahr and Jacobsen 2008).

Considering this, to elucidate possible mode of antioxidant action of bioactive compounds, their individual antioxidant potential using some *in vitro* models (DPPH radical scavenging; $ABTS^{\cdot+}$radical cation decolourization activity; Trolox equivalent antioxidant capacity (TEAC); Fe^{2+} ion chelating; Ferric reducing antioxidant power (FRAP) and Lipoxygenase inhibition) and combined antioxidant potential against oxidation of omega-3 fatty acids enriched bulk oil and in Briggs-Rauscher Oscillation reaction methods were evaluated. At their individual effect, it was observed that the bioactive compound prodelphinidin trimer from *Kufri Chipsona-3* potato peel extract showed higher antioxidant potential in DPPH radical scavenging (Figure 8.1) ; ABTS •+ radical cation decolourization (Table 8.1) ; Trolox equivalent antioxidant capacity (TEAC) (Table 8.2) and Ferric reducing antioxidant power (FRAP) activity (Table 8.3) compared to 5-Hydroxy-3',4',7-

trimethoxyflavone isolated from peel extract of *Kufri Jyoti.* But 5-Hydroxy-3',4',7-trimethoxy flavone was found to be more effective in Fe2+ ion chelating (Figure 8.2) and lipoxygenase inhibition assays (Table 8.4) than prodelphinidin trimer. Besides, in lipid oxidation, prodelphinidin trimer was found to be significantly ($P < 0.05$) effective against both primary and secondary oxidation of lipid substrate (omega-3 fatty acids enriched oil) at its individual effect, but 5-Hydroxy-3',4',7-trimethoxyflavone at its individual effect showed its significant antioxidant efficacy ($p < 0.05$) against primary oxidation of lipid substrate and failed to do so against secondary oxidation (Table 8.5). These findings suggest that at their individual effect, the mode of antioxidant action of bioactive compounds prodelphinidin trimer and 5-Hydroxy-3',4',7-trimethoxyflavone are different. Prodelphinidin trimer functions as primary oxidant and 5-Hydroxy-3',4',7-trimethoxyflavone as secondary antioxidant or synergist.

In combination, both in lipid oxidation (Table 8.5) and in Briggs-Rauscher Oscillation reaction (Table 8.6), the antioxidant efficacy was found to be significantly greater ($P < 0.05$) compared to the simple sum of their individual effects suggesting their synergistic antioxidant interactions in combination. The possible reason behind this synergistic antioxidant interaction is not clear right now. From the observed findings, it can be inferred that synergistic antioxidant interactions may be due to combined antioxidant effect of both primary antioxidant prodelphinidin trimer and secondary antioxidant (co-antioxidant or synergist) 5-Hydroxy-3',4',7-trimethoxyflavone where 5-Hydroxy-3',4',7-trimethoxyflavone (secondary antioxidant) as a synergist increase the activity of primary antioxidant prodelphinidin trimer by regenerating it in oxidative chain reaction and this regeneration of primary antioxidant prodelphinidin trimer contributes to a higher net interactive effect than the simple sum of the individual effects leading to the formation of synergistic interactions. Our findings corroborate with the findings of other workers where tocopherols as primary antioxidant and ascorbic acid as secondary antioxidant in combination showed synergistic antioxidant efficacy following the same mechanism of action **(**Liebler, 1993; Buettner, 1993).

(B) Elucidation of possible mode of antimicrobial action of bioactive compounds alone and in combination

Introduction

It is well documented that plant phenolics exhibit antimicrobial potential against a large number of Gram-negative and Gram-positive bacteria and their mode of antimicrobial action is mainly based on their ability to disrupt cell wall and cytoplasmic membrane, leading to lysis and leakage of intracellular compounds (Burt, 2004; Rota et al.,2004). It has also been reported by several workers that plant phenolics primarily destabilize the cellular architecture, leading to the breakdown of membrane integrity and increased permeability, which disrupts many cellular activities (Cox et al., 2000; Carson et al., 2002) and this antimicrobial activity of phenolic compounds are related to their composition, configuration, concentration and their possible synergistic interactions **(**Lis-Balchin et al., 1998). Besides, biofilm formation is one of the mechanisms, used by foodborne microbes especially *L. monocytogenes* and *S. typhimurium* for developing drug resistance (Fuqua and Greenberg, 1998). The biofilm formation is controlled by cell-to-cell communication of bacteria, which is widely known as quorum sensing. The inhibition of quorum sensing is one of the methods among the different strategies developed to control biofilm forming bacterial pathogens without causing drug resistance (Singh et al, 2013). In recent years, several antiquorum sensing compounds are reported in plants and microbes that may have beneficial effects on human health from protecting foodborne diseases caused by biofilm forming bacterial pathogens (Choo et al., 2006; Adonizio et al., 2008; Ni et al., 2009; Kalia and Purohit, 2011; Kalia et al., 2012). Therefore, in this study an attempt has been made to evaluate the effects of bioactive compounds prodelphinidin trimer and 5-Hydroxy -3,4',7 trimethoxyflavone alone and in combination on cell viability, cell membrane integrity, cell membrane permeability, antiquorum sensing ability and atomic force microscopy (AFM) analysis against studied foodborne bacterial pathogens *L.monocytogenes* (MTCC 657) and *S. typhimurium* (MTCC 3224) with a view to elucidate their possible mode of synergistic antimicrobial interactions.

Results

- ***Effects on cell viability***

The results on the effects of bioactive compounds alone and in combination on cell viability against the studied Gram-positive (*L. monocytogenes;* MTCC 657) and Gram-negative (*S. typhimurium;* MTCC 3224) foodborne bacterial pathogens are shown in Figure 8.4(a) and Figure 8.4(b) respectively. It was observed from these two Figures (Figure 8.4a and Figure 8.4b), that bioactive compound prodelphinidin trimer from peel extract of *Kufri Chipsona-3* at 0.5× MIC concentration showed a significant reduction ($p < 0.05$) in cell viability of both the studied bacterial pathogens at its individual effect from 3h onward. At 1×MIC concentration of prodelphinidin trimer, the reduction in viable cells was further increased from 3h onward against both the studied bacterial pathogens. On the other hand, bioactive compound 5 –Hydroxy -3',4',7-trimethoxy flavone from peel extract of *Kufri Jyoti* at its individual effect at 0.5× MIC concentration failed to significantly reduce the viable cell count of studied bacterial pathogens throughout the experimental period. However, at its higher concentration (1×MIC), the reduction in cell viability against *L. monocytogenes* was found to be significant ($p < 0.05$) at 6h and against *S. typhimurium* for 12h onward. In combination (1:1) of bioactive compounds at their 0.5×MIC concentrations, a significant reduction ($p < 0.05$) in colony count of viable cells was observed from 3h onward against *L. monocytogenes* and 6h onward against *S. typhimurium* compared to their individual component's effect at 0.5 × MIC concentration. A complete reduction in cell viability by test bioactive compounds in combination was observed at 12h against *L. monocytogenes* and at 24h against *S. typhimurium* (Figure 8.4 (a) and Figure 8.4(b)).

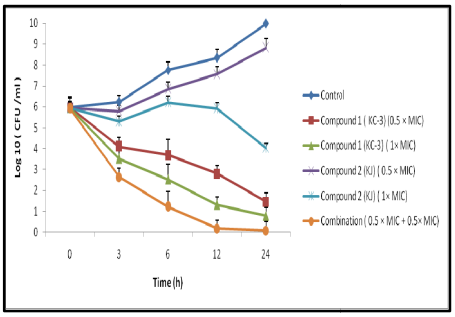


Figure 8.4 (a) Effect of bioactive compounds isolated from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato peel extracts alone and in combination on cell viability of *Listeria monocytogenes* (MTCC 657)

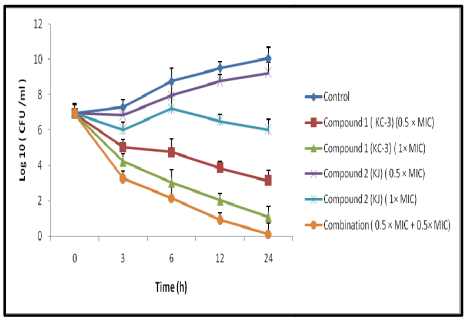


Figure 8.4 (b) Effect of bioactive compounds isolated from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato peel extracts alone and in combination on cell viability of *Salmonella typhimurium* (MTCC 3224)

Compound 1: Prodelphinidin trimer (*Kufri Chipsona-3*) ; Compound 2: 5 –Hydroxy -3',4',7-trimethoxyflavone (Kufri Jyoti)

Values are mean ± SD of triplicate experiments

- **Effects on cell membrane integrity**

- ***Release of 260-nm absorbing materials***

Figure 8.5 shows the results on the release of 260-nm absorbing materials by bioactive compounds prodelphinidin trimer and 5–Hydroxy-3',4',7-trimethoxy flavone alone and in combination against the studied bacterial pathogens *L. monocytogenes* (MTCC 657) and *S. typhimurium* (MTCC 3224). From this Figure, it was observed that 5–Hydroxy -3',4',7-trimethoxyflavone at 0.5×MIC concentration did not show any significant effect on release of 260-nm absorbing materials against both the studied bacterial pathogens but, at 1×MIC concentration of it, the release of 260-nm absorbing material was significantly increased ($p < 0.05$) against both the studied bacterial pathogens compared to control values. On the other hand, prodelphinidin trimer at 0.5×MIC concentration at its individual effect showed a significant release ($p < 0.05$) in 260-nm absorbing materials against both the studied bacterial pathogens. At 1×MIC concentration of it, the release of 260-nm absorbing materials was found to be greater compared to its 0.5 × MIC concentration. In combination (1:1), at 0.5×MIC concentrations of bioactive compounds, the release of 260-nm absorbing materials was also found to be significantly ($p < 0.05$) increased against both the studied bacterial pathogens compared to the average of the sum of their individual component's effect (Figure 8.5).

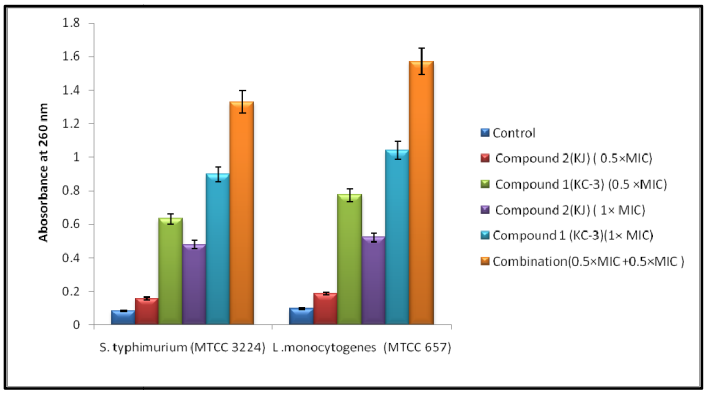


Figure 8.5 Effect of bioactive compounds isolated from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* alone and in combination on release of 260-nm absorbing materials of foodborne bacterial pathogens *Listeria monocytogenes* (MTCC 657) and *Salmonella typhimurium*(MTCC 3224)

Compound 1 : Prodelphinidin trimer (*Kufri Chipsona-3*)

Compound 2: 5 –Hydroxy -3',4',7-trimethoxyflavone (*Kufri Jyoti*)

Values are mean ± SD of triplicate experiments

- ***Effects on release of cellular proteins***

Figure 8.6 shows the results on the effect of test bioactive compounds prodelphinidin trimer and 5 –Hydroxy -3',4',7-trimethoxyflavone alone and in combination on release of cellular proteins of studied bacterial pathogens. It was observed from this Figure 8.6 that release of cellular proteins of both the studied bacterial pathogens was increased significantly ($P < 0.05$) by the bioactive compound prodelphinidin trimer at its individual effect both at 0.5×MIC and 1×MIC concentrations whereas in case of 5–Hydroxy -3',4',7-trimethoxyflavone, this effect was observed only at its 1×MIC concentration. In combination (1:1) of bioactive compounds at their 0.5×MIC concentrations, a significant increase ($p < 0.05$) in release in cellular proteins against both the studied bacterial pathogens was observed compared to the average of the sum of their individual effects at 0.5 ×MIC concentrations.

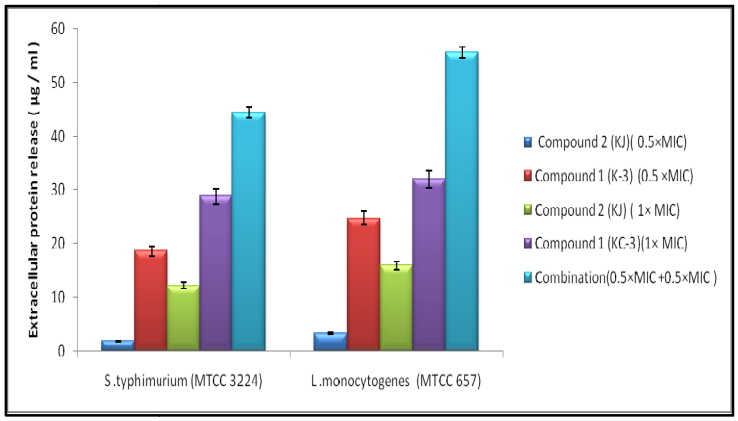


Figure 8.6 Effect of bioactive compounds isolated from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* alone and in combination on release of extracellular protein of test foodborne bacterial pathogens *Listeria monocytogenes* (MTCC 657) and *Salmonella typhimurium*(MTCC 3224)

Compound 1 : Prodelphinidin trimer (*Kufri Chipsona-3*)

Compound 2: 5 –Hydroxy -3',4',7-trimethoxyflavone (*Kufri Jyoti*)

Values are mean ± SD of triplicate experiments

- **Effect on cell membrane permeability**

- ***Effects on β-galactosidase enzymes***

Figure 8.7 (a) and Figure 8.7 (b) show the results on the effect of test bioactive compounds alone and in combination on release of β-galactosidase enzymes of both the studied bacterial pathogens *L. monocytogenes* (MTCC 657) and *S. typhimurium* (MTCC 3224) respectively. It was observed from these two Figures (8.8(a) and 8.8 (b)) that the bioactive compound 5-Hydroxy-3',4',7-trimethoxyflavone at its individual effect upto 1×MIC concentration did not show any significant effect of release of β-galactosidase enzymes against both the studied bacteria. On the other hand, the bioactive compound prodelphinidin trimer at 0.5×MIC concentration showed a significant release ($P < 0.05$) in β-galactosidase enzyme against both the studied bacterial pathogens. In combination, the release of β-galactosidase enzymes of both the studied bacteria was significant higher ($P< 0.05$) compared to the 1×MIC concentration of most effective bioactive compound prodelphinidin trimer.

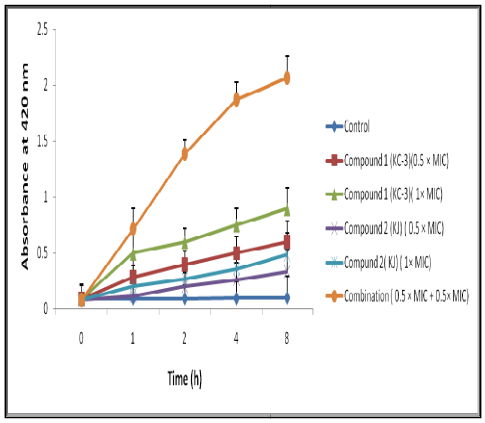


Figure 8.7(a) Effect of active compounds isolated from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato peel extracts alone and in combination on membrane permeability of *Listeria monocytogenes*(MTCC 657)

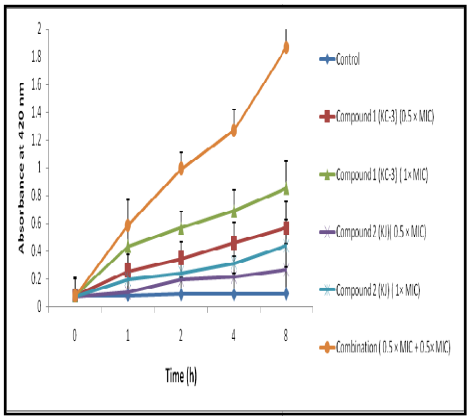


Figure 8.7(b) Effect of active compounds isolated from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato peel extracts alone and in combination on membrane permeability of *Salmonella typhimurium* (MTCC 3224

Compound: Prodelphinidin trimer (*Kufri Chipsona-3)* ; Compound 2: 5 –Hydroxy -3',4',7-trimethoxyflavone (*Kufri Jyoti)*

Values are mean ± SD of triplicate experiments

- ***Atomic Force Microscopy (AFM) analysis***

Figures 8.8 (a-d) and Figures 8.9 (a-d) show the atomic force microscopy (AFM) images of control and bioactive compounds treated cellular morphology of studied bacterial pathogens *L. monocytogenes* (MTCC 657) and *S. typhimurium* (MTCC 3224) respectively. From these AFM images of *L.monocytogenes* and *S. typhimurium*, it was observed that the cell surfaces of control cells of both the studied bacterial pathogens [*L. monocytogenes* (Figure 8.8a) and [*S. typhimurium* (Figure 8.9a)] are smooth and well-structured. Changes in cellular morphology by prodelphinidin trimer at its individual effect at 1×MIC concentration against both *L. monocytogenes* (Figure 8.8b) and *S. typhymurium* (Figure 8.9b) was found to be greater compared to the effect of 5 –Hydroxy 3'4',7-trimethoxy flavone at its 1×MIC concentration against cellular morphology of *L. monocytogenes* (Figure 8.8c) and *S. typhimurium* (Figure 8.9c). In combination, at their 0.5×MIC concentrations, a severe changes in cell surface morphology of the treated cells of both the studied bacterial pathogens *L. monocytogenes* (Figure 8.8d) and *S. typhimurium* (Figure 8.9 d) compared to their individual effect at 1×MIC concentration against *L. monocytogenes* (Figure 8.8 b and Figure 8.9 b) and *S. typhimurium* (Figure 8.8 c and Figure 8.9 c) was observed.

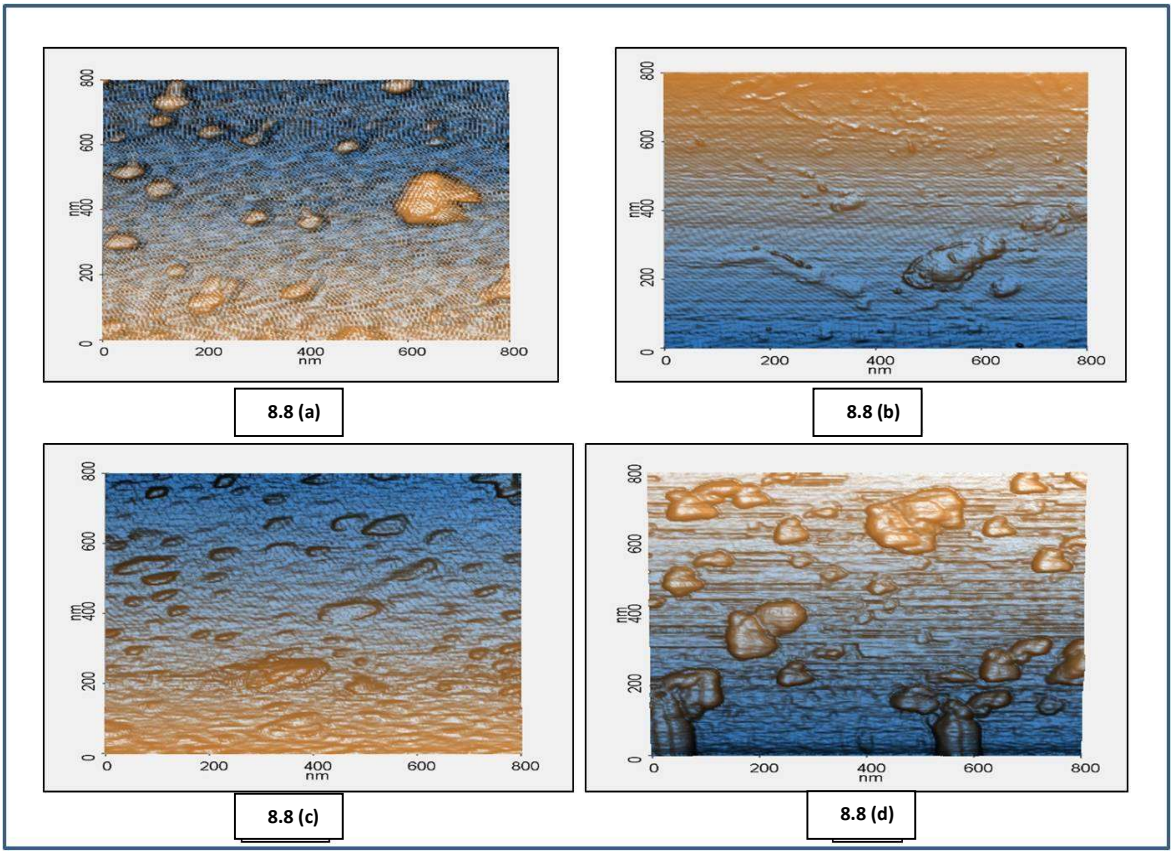


Figure.8.8 (a) AFM image of *Listeria monocytogenes* (control)

Figure 8.8 (b) AFM image *of Listeria monocytogenes* treated with prodelphinidin trimer (1 × MIC)

Figure 8.8 (c) AFM image of *Listeria monocytogenes* treated with 5- Hydroxy -3',4', 7 trimethoxyflavone (1 × MIC)

Figure 8.8 (d) AFM image of *Listeria monocytogenes* treated with both of the compounds in combination (0.5 × MIC +0.5 × MIC)

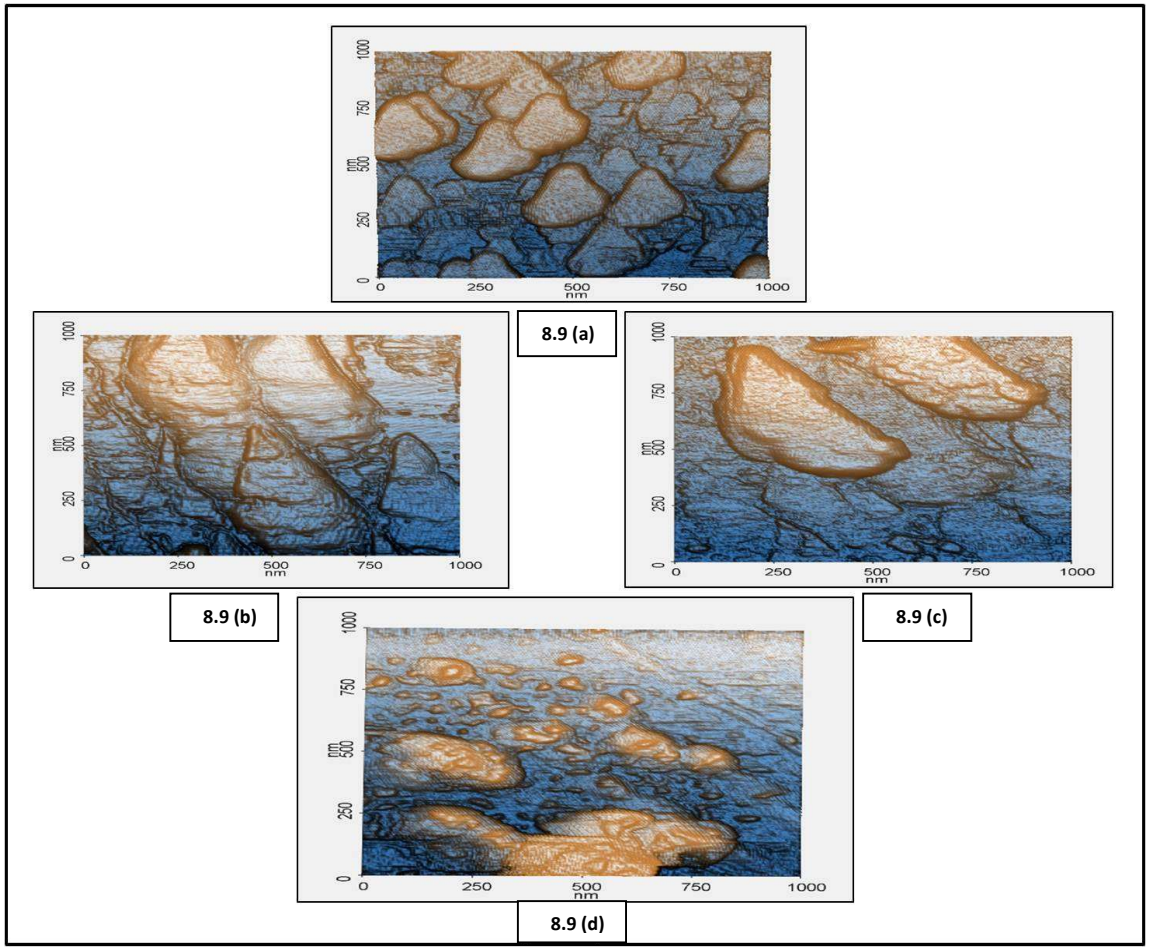


Figure 8.9 (a) AFM image of *Salmonella typhimurium* (control)

Figure 8.9 (b) AFM image of *Salmonella typhimurium* treated with prodelphinidin trimer (1 × MIC)

Figure 8.9 (c) AFM image of *Salmonella typhimurium* treated with 5- Hydroxy -3',4', 7 trimethoxyflavone (1 × MIC)

Figure 8.9 (d) AFM image of *Salmonella typhimurium* treated with both of the compounds in combination (0.5 × MIC + 0.5 × MIC)

- ***Anti-quorum sensing (anti-biofilm formation) activity***

Figure 8.10 shows the effect of isolated bioactive compounds from phenolic extracts of peels of *Kufri Chipsona-3* (prodelphinidin trimer) and *Kufri Jyoti* (5 –Hydroxy -3',4',7-trimethoxy flavone) at their individual and combined effect on antiquorum sensing efficacy of both of the studied foodborne pathogens *L. monocytogenes* (MTCC 657) and *S. typhimurium* (MTCC 3224) measured in terms of anti-biofilm formation efficacy. It was observed that the compound 5- Hydroxy -3',4',7-trimethoxyflavone from *Kufri Jyoti* potato peel extract at its

individual effect at 0.5×MIC concentration did not show any significant anti-biofilm formation efficacy (reduction ≤ 50%) against both the studied foodborne bacterial pathogens. At its 1×MIC concentration, a significant inhibition in biofilm biomass of both the studied bacterial pathogens was observed. On the other hand, prodelphinidin trimer from *Kufri Chipsona-3* potato peel extract at 0.5×MIC concentration showed a significant anti-biofilm formation efficacy against both the studied bacterial pathogens. At its 1×MIC concentration, this inhibition was found to be much more greater compared to its 0.5×MIC concentration as well as 1×MIC concentration of 5- Hydroxy -3',4',7-trimethoxyflavone. In combination at their 0.5×MIC concentrations, the reduction in biofilm formation of both the studied bacterial pathogens was found to be significantly greater ($P < 0.05$) compared to the 1×MIC concentration of their most effective single component (prodelphinidin trimer) against both the studied Gram-positive and Gram-negative bacterial pathogens.

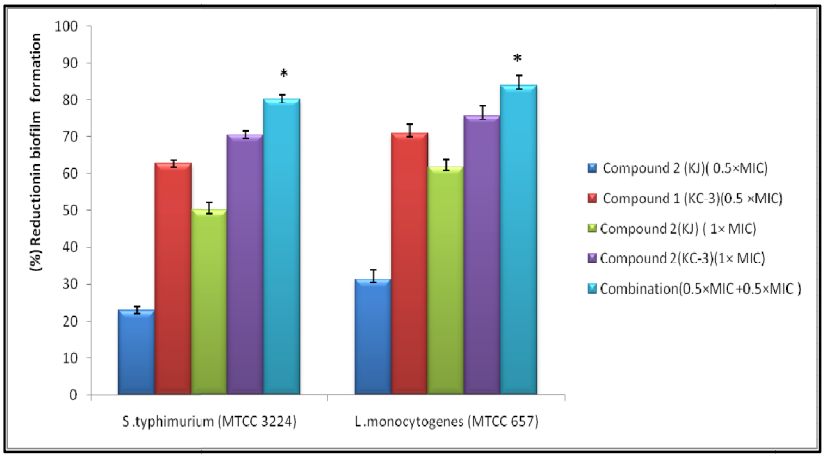


Figure 8.10 Antiquorum sensnsing (anti-biofim formation efficacy) efficacy of bioactive compounds isolated from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* alone and in combination against test foodborne bacterial pathogens *Listeria monocytogenes* (MTCC 657) and *Salmonella typhimurium*(MTCC3224)

Compound 1: Prodelphinidin trimer (*Kufri Chipsona-3*)

Compound 2: 5-Hydroxy-3',4',7-trimethoxyflavone (*Kufri Jyoti*)

Values are mean ± SD of triplicate experiments

Discussion

To elucidate the possible mode of synergistic antimicrobial action of bioactive compounds prodelphinidin trimer from *Kufri Chipsona-3* and 5 –Hydroxy -3',4',7-trimethoxyflavone from *Kufri Jyoti* potato varieties alone and in combination against the studied Gram-positive (*L. monocytogenes*) and Gram-negative (*S. typhimurium*) bacterial pathogens, their effects on cell viability, cell membrane integrity, cell membrane permeability, atomic force microscopy analysis and anti-quorum sensing activity were evaluated.

In cell viability study (Figure 8.4a and 8.4b), it was observed that the test bioactive compounds at their individual effect exhibited concentration dependent varying degrees of growth inhibitory activity against the studied bacterial pathogens. The bioactive compound prodelphinidin trimer showed higher growth inhibitory activity against the studied microbes than 5-Hydroxy-3',4',7-trimethoxyflavone and was strongly effective against both the studied bacteria at low (0.5×MIC) concentration. In combination, at low concentration, a significant reduction ($P < 0.05$) in cell viability against the studied bacterial pathogens compared to the average of sum of their individual effects (Figure 8.4 (a) and Figure 8.4 (b)) was observed. These findings suggest that growth inhibitory activity was concentration dependent and prodelphinidin trimer exhibited higher growth inhibitory activity than 5-Hydroxy-3',4',7-trimethoxyflavone against the studied foodborne bacterial pathogens.

In bacterial cell membrane integrity study, release of 260-nm absorbing materials (Figure 8.5) and release of extracellular proteins (Figure 8.6) by the test bioactive compounds at their individual effect against the studied microbes, it was observed that at low concentration (0.5×MIC), prodelphinidin trimer was able to release 260-nm absorbing materials significantly ($P < 0.05$) whereas 5-Hydroxy-3',4',7-trimethoxyflavone failed to do it. At 1×MIC concentration, 5-Hydroxy-3',4',7-trimethhoxyflavone decreased significantly 260-nm absorbing materials and extra-cellular proteins of both the studied bacteria. In combination, the release of both 260-nm absorbing materials and extra cellular proteins was increased significantly ($P < 0.05$) at their 0.5 × MIC concentration compared to their most effective single components effect (Figure 8.5 and Figure 8.6). These findings indicate that prodelphinidin trimer exhibited bacterial cell membrane disintegrating activity at low concentration at its individual effect whereas 5-Hydroxy-3',4',7-trimethoxyflavone failed to do it at low concentration. Only at high concentration (1×MIC), it can destabilize cell membrane integrity.

In bacterial membrane permeability study, the bioactive compound prodelphinidin trimer showed a significant effect on release of β-galactosidase enzymes at low concentration (0.5×MIC) at its individual effect against the studied microbes but 5-Hydroxy-3',4',7-trimethoxyflavone did not show any significant effect on it at its low concentration [Figure 8.7(a,b)] suggesting that at low concentration prodelphinidin trimer has the ability to increase the membrane permeability of studied bacterial pathogens but 5-Hydroxy-3',4',7-trimethoxyflavone did not have any such effect at low concentration. Only at high concentration, it can destabilize the cell membrane of studied bacterial pathogens.

Atomic Force Microscopy analysis revealed that the cellular morphology of studied bacterial pathogens at low concentration of prodelphinidin trimer and 5-Hydroxy-3',4',7-trimethoxy flavone at their individual effect was not significantly disturbed compared to the cellular morphology of their controls although prodelphinidin showed its higher activity against the studied microbes than 5-Hydroxy-3',4',7-trimethoxyflavone. In combination, a significant disturbance in cellular morphology of both the studied bacterial pathogens compared to their control images was observed (Figure 8.8 (a-d) & 8.9 (a-d)). These findings confirmed the results of our previous findings obtained in cell viability, membrane integrity and cell membrane permeability studies.

In antiquorom sensing activity study, at their individual effect, the bioactive compound prodelphinidin trimer showed moderate activity against formation of biofilms of studied bacterial pathogens but the bioactive compound 5-Hydroxy-3',4',7-trimethoxyflavone at its individual effect showed weak activity against formation of biofilms of studied bacterial pathogens. In combination, at low concentration a significant inhibition in the formation of biofilm of studied bacterial pathogens was observed (Figure 8.10). These findings suggest that the test bioactive compounds also exhibited anti-quorum (antibiofilm) activity against the studied bacterial pathogens.

Collectively, the obtained findings suggest that the mode of antimicrobial action of two bioactive compounds prodelphinidin trimer and 5-Hydroxy-3',4',7-trimethoxyflavone at their individual effect against the studied bacterial pathogens are different and concentration dependent. Prodelphinidin trimer at its individual effect, at low concentration acted upon bacterial cell envelope leading to membrane damage and cytoplasm leakage. On the other hand, 5-Hydroxy-3',4',7-trimethoxyflavone at low concentration at its individual effect, did not show any promising activity on cell membrane integrity and cell membrane permeability.

Only at high concentration it can destabilize membrane function. In combination, at low concentration prodelphinidin trimer at low concentration increased membrane permeability of both the studied bacterial pathogens and allows 5 -Hydroxy -3',4',7-trimethoxyflavone to enter inside the bacterial cells at its low concentration with ease as evidenced from permeability study. Once they entered inside the cell, the release of intracellular materials was increased significantly ($P < 0.05$) as evident from membrane integrity study. At the end, the loss of membrane integrity caused a significant change in cellular morphology which subsequently led to cell lysis and cell death as evident from atomic force microscopy and antiquorom sensing activity study.

CHAPTER 9

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

- **Summary**

Omega-3 fatty acids have immense health benefits. In recent years to increase the nutritional value of foods and reduce the risk of chronic diseases, omega-3 fatty acids enriched oils are added to foods. But developing omega-3 fatty acids fortified stable functional food is challenging because two principal omega-3 fatty acid components eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are highly susceptible to oxidation due to high degree of unsaturation in their molecular structures. Besides, microbial spoilage and contamination of various food products including lipid bearing foods is another great concern. Although synthetic antioxidants and antimicrobials are generally used separately as food preservatives in food industry to overcome these problems, they have accumulated evidence that they could be toxic and carcinogenic. Therefore, innovation should continue to seek safe and effective, novel, natural food preservatives having dual preservative properties i.e. both antioxidant and antimicrobial efficacy at sufficiently low concentration from other sources especially from plant origin for extending the shelf life of food products especially omega-3 fatty acids fortified functional foods. The use of a combination of preservation factors has been suggested as a viable alternative to synthetic preservatives to preserve foods for extended periods of time because a single molecule of plant origin having both antioxidant and antimicrobial properties is considered to be more effective than synthetic preservatives. Potato peels are rich in phenolic compounds and may serve as a potential source of such compounds due to their both strong antioxidant as well as antimicrobial potential. But detailed knowledge about their use both as an antioxidant and antimicrobial agents in food preservation system is lacking. This work dealt with a detailed and systematic study on antioxidant and antimicrobial potential of phenolic extracts of peels of five selected varieties of potato (*Kufri Chipsona-1*, *Kufri Chipsona- 3*, *Kufri Chipsona-4*, *Kufri Chandramukhi* and *Kufri Jyoti*) and their bioactive compounds along with cytotoxicity evaluation and elucidation of possible modes of antioxidant and antimicrobial action with a view to seek a more potent, safe and effective novel, natural preservatives from waste potato peels having both antioxidant and antimicrobial properties which may serve as a promising alternative to synthetic food preservatives for preserving foods especially omega-3 fatty acids fortified functional foods. To achieve the goal (i) individual effect on antioxidant potential of phenolic extract of peels of five selected varieties of potato in various *in vitro* models, omega-3 fatty acids enriched food supplement, and in omega-3 fatty acids fortified various functional foods along with antimicrobial potential against foodborne microbes were

evaluated, (ii) their combined antioxidant and antimicrobial efficacy in various *in vitro* models were evaluated taking the advantages of their possible synergistic interactions, (iii) bioactive compounds responsible for synergistic antioxidant and antimicrobial efficacy were isolated, identified and chemically characterized, (iv) cytotoxic potential of active extracts of potato peels as well as their bioactive compounds in combination, if any, was evaluated and (v) possible modes behind synergistic antioxidant and antimicrobial action of bioactive compounds were elucidated using standard methods. The important findings obtained from this study are:

- Among the peel extracts of five selected varieties of potato tested to evaluate their individual antioxidant and antimicrobial efficacy in various *in vitro* models, only peel extracts of three potato varieties (*Kufri Chandramukhi*, *Kufri Chipsona-3* and *Kufri Jyoti*) showed both strong antioxidant and antimicrobial efficacy at their individual effect.
- Among the possible combinations tested between these three active extracts of potato peels (*Kufri Chandramukhi*, *Kufri Chipsona-3* and *Kufri Jyoti*) for their possible synergistic antioxidant interactions, only one combination between peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties showed synergistic antioxidant efficacy whereas other tested possible combinations showed additive antioxidant effect.
- TLC bioautography analysis revealed that three components with R_f: 0.33, 0.52 and 0.57 from peel extract of *Kufri Chipsona-3* and four components with R_f : 0.14, 0.22,0.36 and 0.61 from peel extract of *Kufri Jyoti* potato varieties exhibited antioxidant efficacy at their individual effect.
- These seven antioxidant components (three from peel extract of *Kufri Chipsona-3* and four from peel extract of *Kufri Jyoti*) were subjected to combined antioxidant and antimicrobial efficacy study and only one combination between component with R_f: 0.57 from peel extract of *Kufri Chipsona-3* and one component with R_f: 0.61from peel extract of *Kufri Jyoti* showed both synergistic antioxidant and antimicrobial interactions in combination.
- Spectrometric analysis revealed that these two bioactive compounds that are responsible for synergistic antioxidant and antimicrobial interactions in combination are prodelphinidin trimer from peel extract of *Kufri Chipsona-3* (R_f: 0.57) and 5 -

Hydroxy -3',4',7-trimethoxyflavone from peel extract of *Kufri Jyoti* (R_f: 0.61) potato varieties.

- Results of cytotoxicity study revealed that *Kufri Chipsona-3/Kufri Jyoti* potato peel extracts as well as their bioactive compounds prodelphinidin trimer and 5 -Hydroxy -3',4',7-trimethoxyflavones that showed synergistic antioxidant and antimicrobial interactions in combination did not show any cytotoxic effect at recommended dosage level and can generally be considered as safe substances.
- Results on possible modes of synergistic antioxidant action of prodelphinidin trimer from peel extract of *Kufri Chipsona-3* and 5 -Hydroxy -3',4',7-trimethoxyflavone from peel extract of *Kufri Jyoti* potato varieties revealed that at their individual effect, prodelphinidin trimer acts as a primary antioxidant whereas5 -Hydroxy -3',4',7-trimethoxyflavone as a secondary antioxidant (synergist).

  In combination, secondary antioxidant 5- Hydroxy-3',4'.7- trimethoxyflavone works by regenerating primary antioxidant prodelphinidin trimer and thereby restore the antioxidant activity of prodelphinidin trimer in oxidative chain reaction to ensure their antioxidant effect. This regeneration of primary antioxidants contributes to a higher net interactive antioxidant effect than the simple sum of their individual effect leading to a formation of synergistic interactions.
- Results on possible modes of antimicrobial action of prodelphinidin trimer isolated from peel extract of *Kufri Chipsona-3* and 5 -Hydroxy -3',4',7-trimethoxyflavone isolated from peel extract of *Kufri Jyoti* at their individual effect revealed that modes of antimicrobial action of these two bioactive compounds against the studied bacterial pathogens at their individual effect are different. Prodelphinidin trimer at its individual effect, at low concentration acted upon bacterial cell envelope leading to membrane damage and cytoplasm leakage. On the other hand, 5-Hydroxy-3',4',7-trimethoxyflavone at low concentration at its individual effect, did not show any promising activity on cell membrane integrity and cell membrane permeability. Only at high concentration it can destabilize membrane function.

  In combination, prodelphinidin trimer at low concentration increased membrane permeability of both the studied bacterial pathogens and allows 5-Hydroxy -3',4',7-trimethoxyflavones to enter inside the bacterial cells at its low concentration with ease. Once they entered inside the cell, both the bioactive compounds simultaneously induced a significant disturbance in the cytoplasm of bacterial cells leading to

significant release of cytoplasmic materials. At the end, the loss of membrane integrity caused a significant change in cellular morphology which subsequently led to cell lysis and cell death.

Collectively, the important findings obtained from this study revealed that the bioactive compounds prodelphinidin trimer and 5 -Hydroxy -3',4',7-trimethoxyflavone isolated from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties respectively in combination at sufficiently low concentration not only exhibited strong antioxidant efficacy against omega-3 fatty acids enriched food supplement and omega-3 fatty acids fortified various functional foods but also have strong antimicrobial efficacy against foodborne microbes with multimodal action without any cytotoxic potential and this blend of potato peel extract components may serve as a promising alternative to synthetic food preservatives especially for developing omega-3 fatty acids fortified stable functional foods.

- **Conclusion**

Thus, both synergistic antioxidant and antimicrobial efficacy of bioactive compounds prodelphinidin trimer isolated from peel extract of *Kufri Chipsona-3* and 5 -Hydroxy -3',4',7-trimethoxyflavone isolated from peel extract of *Kufri Jyoti* potato varieties in combination with multimodal antioxidant and antimicrobial action and without any cytotoxic potential may help in developing a more potent, safe and effective, novel natural alternative to synthetic food preservatives for preserving foods especially omega-3 fatty acids fortified functional foods for extended periods of time in food and pharmaceutical industries. These findings may also be helpful to convert potato peels from waste by-product to value added products.

- **Recommendations for future research**

1. The way of application of isolated bioactive compounds from potato peels in food products is an interesting and growing area for researchers whose results could end up having a great use for food industries. Therefore, the way in which isolated bioactive compounds from potato peel extracts in combination is to be applied in foods (i.e. directly into the food matrix or as a packaging ingredients or as an edible coated films) that has to be investigated.
2. Attention has to be given on low-cost extraction method and further investigation should also be done on innovative products from similar by-products.

3. Potato peel as a by-product of food processing industry possesses to be totally inexpensive, valuable and affordable starting material for the production of economically important substances. Therefore, research focus should gear to use potato peel waste as eco-friendly industrial products.
4. In order to prevent lipid oxidation in foods, the mechanism of lipid oxidation should be comprehensively understood and in particular, the control of free radical formation is very important because free radical is able to initiate oxidative chain reaction.

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List of publications

1. Bhattacharya, A., Purkait, S., Bag, A., & Chattopadhyay, R.R.(2020). Chemical profiling, cytotoxicity study and assessment of antioxidant potential of hydro ethanol extract of peels of some selected varieties of potato in various in vitro models and in lipid substrate enriched with Omega-3 fatty acids. *European Journal of Food Research and Technology*, **246** :1469–1482. Springer. Impact Factor : 3.498 (2021)

2. Bhattacharya, A., Purkait, S., Bag, A. & Chattopadhyay, R.R.(2021). Evaluation of antimicrobial and antioxidant efficacy of hydro ethanol extract of peels of *Kufri Chandramukhi*, *Kufri Chipsona-3*, and *Kufri Jyoti* potato varieties alone and in combination. *Journal of food safety,* **41(4)** , e12901. Impact Factor : 2.449 (2021)

3. Bhattacharya, A., Mitra, S., Goswami, A., Chattopadhyay, R.R. (2022). Possible modes of synergistic antimicrobial action of prodelphinidin trimer and 5-Hydroxy-3',4',7-trimethoxyflavone isolated from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties in combination against foodborne bacterial pathogens *Listeria monocytogenes* and *Salmonella typhimurium* (under preparation).

4. Bhattacharya ,A., Chattopadhyay, R.R. (2022). Possible modes of synergistic antioxidant action of prodelphinidin trimer and 5-Hydroxy-3',4',7-trimethoxyflavone isolated from peel extracts of *Kufri Chipsona-3* and *Kufri Jyoti* potato varieties against oxidation of omega-3 fatty acids enriched food supplement. (Under preparation).