Microbial Decontamination and Shelf Life Extension of Leafy Vegetables by Ionizing Radiation

By

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

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Publications from this thesis in Refereed Journals

1. "Application of ionizing radiation (Gamma and Electron Beam) for inactivation of coliform and shelf life extension of fresh leafy vegetables (spinach, coriander and mint) in combination with water washing and sanitizer treatment" **Khade H. D.**, Hajare Sachin N., Sarma K. S. S. and Gautam Satyendra, "Indian Journal of Experimental Biology". Accepted (2019)

2. "Radiation treatment enhanced the free form of phenolic acid and flavonoids in leafy greens and therefore bioactivity in terms of antimutagenicity**" Khade H. D.**, Hajare Sachin N., and Gautam Satyendra, "International Journal of Pharmacy and Biological Sciences", 2019; 9(4): 74-86.

Abstracts in Conferences/Symposia

1."Radiation processing of leafy vegetables to ensure microbial safety" **Khade H. D.**, Jain M. P. and Gautam S. DAE- BRNS Life Science Symposium, BARC, Mumbai, 3-5 Feb, 2015.

2."Hygienization and shelf life extension of leafy vegetables by combination treatment including radiation processing" Khade H D, Jain M P, Gautam S., "International conference on "Innovative Trends in Chemical, Physical and Biosciences-2016", Univ. of Pune, Feb. 9-10, 2016.

3. Radiation Processing of Fresh Leafy Vegetables by Electron Beam Treatment. Khade H D, Jain M P, and Gautam S**.** Conference on "Climate Change Biodiversity and Bioresource Management" Jawaharlal Neharu Rajkeeya Mahavidyalaya, Port Blair, February 1-3, 2017

4. "Screening of pathogens in leafy vegetables sold in retail outlets in Mumbai". Khade H D, Hajare**S. N., and** Gautam S. "International Conference on Antimicrobial Resistance - 2018" 19th -20th January, 2018, at SASTRA University, Tirumalaisamudram, Thanjavur, Tamil Nadu.

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TO

MY PARENTS.....

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CONTENT

SUMMARY

India is the second largest producer of vegetables, next to China. Fresh agri produce like *Spinacia oleracea* (Spinach), *Coriandrum sativum* (Coriander), and *Mentha arvensis* (Mint), which are consumed in raw form, are heavily contaminated with high microbial load more than the permissible limit. Vegetables get easily contaminated with bacteria at any step from harvesting in field to consumption. Surface sanitization with water as well as treatment with chemicals including, sanitizer (up to permissible limit) and other treatments are being used on processed fresh leafy agri produce mainly to reduce microbial load, decay and browning.

In the present study combination treatments including sanitizer, radiation (gamma or electron beam) with compatible packaging and low temperature storage have been evaluated to ensure microbial safety and extend shelf life of fresh leafy vegetables. Also, organoleptic and physiochemical and functional properties of treated produce have been analysed during the storage. Freshly harvested prominent variety of spinach (cv. Semi-Savoy), coriander (cv. Co-2) and mint (cv. Japanese mint / menthol mint) from Nashik region were selected for the study.

These commodities were found to be heavily contaminated with microbes including presumptive coliforms and *Salmonella*. To hygienize these leafy vegetables, a CP (Combination Process) including potable water washing followed by washing with sanitizer (NaOCl at 200 ppm for 5 min) and irradiation (2 kGy gamma or electron beam) was attempted. The combination including gamma radiation was termed as CP1 and that with electron beam as CP2. These treatments significantly reduced the microbial count (by 5-6 log cfu/g) and yeast and mould count (by 4-5 log cfu/g). The presumptive coliform count was decreased below detectable level after the combination treatment. Such treated leafy vegetables could be stored up to 15 days at 4-6 ºC. During storage, there was increase of 1-2 log cfu/g in total microbial count as well as yeast and mould count. However, there was no

increase in the presumptive coliform count. In case of presumptive coliform and presumptive *Salmonella* isolates, confirmation was done by carrying out PCR analysis of pathogen specific genes. In case of presumptive coliform, the isolates were confirmed to be *E. coli* (amplification of *usp A* gene). However, none of the isolates were pathogenic (*E. coli* O157:H7) as confirmed by serological methods. None of the presumptive *Salmonella* isolates were pathogenic one (as confirmed by amplification of *Inv A* gene).

The flavonoids extracted from coriander showed about 46% reduction in mutagenicity. Gamma irradiation and electron beam irradiated samples showed about 57 and 54 % reduction in the mutagenicity compared to control. The combination treatments did not have any negative effects on physical, biochemical, nutritional and functional attributes. In spinach, coriander and mint the content of kaempferol, quercetin and rosmarinic acid, respectively showed significant increase upon CP1 (Combination Process 1) / CP2 (Combination Process 2) treatments. It was also observed that, in case of CP2 enhancement was more as compared to CP1. Thus, these treatments can ensure safety of leafy greens and extend their shelf life up to 15 days at 4 to 6 ºC with enhanced flavonoids and phenolic content which have functional relevance. Use of electron beam treatment can make the process very much cost effective due to its high through put.

CHAPTER – 1

INTRODUCTION

AND

LITERATURE REVIEW

1.1. Production and global status

From the old age (10,000 BC to 7,000 BC) vegetables were mostly collected in forest. One of the major vital components of human diet is vegetables and is consumed since archaic time. It provides nutrition and can be eaten in the form of raw or cooked, it is a source of low fat & carbohydrates and high in vitamins and minerals. They also contain fibres. The healthiest choice is to consume five or more portions of fruits and vegetables per day to prevent illness.

India is leading agriculture producing country next to China. Now a day's production of leafy green is produced on industrial scale with controlled production and packaging (WHO) (Table 1.1 and 1.2 (Harper Douglas, wiki). Spinach production has doubled in developing countries in the last 1decade (FAO statistical database; FAO, STAT, 2007).

As per the FAO, STAT data, the difference of productions between developed and developing countries varies. Developed countries produced over nine million tonnes of lettuce and chicory in year 2006, while 14 million tonnes (WHO) of these two types of vegetable were produced in developing countries (FAO, STAT, 2007). Out of total global production around 70-75% leafy vegetables are sold as fresh vegetables therefore to avoid post-harvest losses in these commodities, minimal processing of leafy vegetables is viable option as popularity of canned vegetables have decreased globally.

Due to easy market access, countries like Mexico, Spain and Netherland has dominating the market of North America and EU trade. Considering the fact, the world wide area under production of coriander may be approximate at 550,000 ha per annum (Diederichsen, *et al.,* 1996). The Russia, Ukraine, Argentina, Mexico, Morocco, Romania and India are the major producers of coriander seed. Many countries produce coriander at various locations of the world. Categories are Middle East region: (Kasachstan, Bhutan, Kirgysia and Tadjikistan, Pakistan); Near East region: (Israel, Iran, Kuwait, Lebanon, Turkey and Syria); Far East

region: Burma, China and Thailan; Americas: Argentina, Chile, Costa Rica, Canada and Paraguay; Africa, Europe: Bulgaria, Czechoslovakia Hungary, England, Italy, France, Poland, (Nadeem, Muhammad et al., 2013).

Table 1.1 Global vegetable production (2017-18)			
Leading Countries	Area in '000' Ha	Production '000'MT	Productivity MT/Ha
China	24561	573935	23.4
India	10259	184387	17.6
USA	1105	35948	32.5
Turkey	1112	27819	25.0
Islamic Republic	877	23486	26.8
World	58971	1159179	19.7

Source: Horticultural Statistics at Glance, 2018, FAO/WHO, NHB and APEDA, wikipedia, List of world production # Vegetable

Table 1.2 Some common vegetables cultivated in the world					
Image	**cultivars**	**Origin**	**Parts used**	**Types (common name)**	**Global production ($\times 10^6$ tons, 2012)**
	*Brassica oleracea*	Europe	Flower heads, Leaves, axillary buds, stems,	cabbage, Brussels sprouts, cauliflower, broccoli, kale, kohlrabi, red cabbage, Savoy cabbage, Chinese broccoli, collard greens (cole crops)	70.1
	*Brassica rapa*	Asia	root,	Chinese cabbage, napa cabbage, turnip, bok choy	-
	*Raphanus sativus*	South east Asia	root, leaves, seed oil, sprouting, seed pods,	seedpod varieties, daikon, radish,	-
	*Daucus carota*	Persia	root, leaves, stems	carrot	36.9
	*Pastinaca sativa*	Eurasia	Root	parsnip	-
	*Beta vulgaris*	Near East and Europe	Leaves, root	sugar beet, Swiss chard, beetroot, sea beet, ,	-
	*Lactuca sativa*	Egypt	stems, leaves, seed oil	celtuce, lettuce,	24.9

	*Phaseolus vulgaris*, *Phaseolus coccineus*, *Phaseolus lunatus*	South and central America	pods, seeds	French bean, rgreen bean, unner bean, Lima bean, haricot bean,	44.6
	*Viciafaba*	Mediterranean and Middle East	pods, seeds	broad bean	-
	*Pisum sativum*	Mediterranean and Middle East	pods, seeds, sprouts	pea, snow pea, split pea, snap pea,	28.9
	*Solanum tuberosum*	South America	Tubers	potato	365.4
	*Solanum melongena*	South and East Asia	Fruits	eggplant (aubergine)	48.4
	*Solanum lycopersicum*	South America	Fruits	tomato	161.8
	*Cucumis sativus*	Southern Asia	Fruits	cucumber	65.1
	*Cucurbita spp.*	Mesoamerica	fruits, flowers	squash, marrow, zucchini (courgette), pumpk in, ,gourd	24.6

	*Allium cepa*	Asia	bulbs, leaves	spring onion, scallion, onion, shallot, list of onion cultivars	87.2
	*Allium sativum*	Asia	Bulbs	garlic	24.8
	*Allium ampeloprasum*	Middle East and Europe	leaf sheaths	elephant garlic, leek	21.7
	*Capsicum annuum*	North and South America	Fruits	pepper, sweet pepperbell, pepper,	34.5
	*Spinacia oleracea*	south western Asia and Central and	Leaves	spinach	21.7
	*Dioscorea spp.*	Tropical Africa	Tubers	yam	59.5
	*Ipomoea batatas*	South America and Central America	tubers, leaves, shoots	sweet potato, list of sweet potato cultivars	108.0
	*Manihot esculenta*	South America	Tubers	cassava	269.1

Source: *Harper, Douglas.* 'Vegetable'. Dictionary Online Etymology ., Swedenborg, manual (2003). Swedenborg Concordance, 1888. Kessinger Publishing, p. 502. https://en.wikipedia.org/wiki/Vegetable

1.1.1. Indian production status of leafy vegetables

India ranks second largest in production of the vegetables followed by the Brazil and USA (APEDA, 2015). There are around thousand species of tropical edible leafy plants with annual and perennial habit in India but at present only ten percent of it is being commercially grown in India (Table 1.3). Some of these include spinach, coriander, mint, fenugreek and others.

India increases the production of total horticulture produces 184 million metric tons in 2017-18. This data was published by India's National Horticulture Board. The vegetables are cultivated under area 10259 thousand hectares. India has tremendous potential for export of vegetables. During 2017-18, India earned revenue of Rs. 9,410.81 crores by exporting fruits and vegetables out of which fresh vegetables shared revenue of Rs. 1848 crores. Vegetables like okra, chillies, potato, bitter gourd, and onion are mainly exported. In 2017-18, India exported fresh onions of Rs 12.7 crores to the United Kingdom and about 30 -50 tonnes of vegetables are exported to London routinely (Economictimes.indiatimes.com/news, 15 March 2019).

Table 1.3 Area and Cultivation of Crops: India					
Year	Production, horticulture crop Qty (Million MT)	Area under cultivation horticulture crop (in '000' ha)	Export of other fresh vegetables (Million MT)	Revenue generated by exporting other fresh vegetables Rs. (crore)	Major export destinations
2010-11	146.5	8495	0.45	804	Pakistan Bangladesh Malaysia Sri Lanka Nepal UAE UK and others
2011-12	156.3	8989	0.66	1152	
2012-13	162.1	9205	0.72	1368	
2013-14	162.8	99396	0.88	2027	
2014-15	169.4	9542	0.79	2158	
2015-16	169.1	10,106	0.70	2008	
2016-17	178.1	10,238	0.98	2589	
2017-18	184.3	10,259	0.73	1848	

Source NHB Horticultural Statistics at a Glance 2018 and APEDA, Govt. of India, MOSP, Govt. of India statistical-year-book; Agri-exchange (APEDA)

1.1.2. Post - harvest losses

The production depends on many factors such as climate conditions, variety of land, season and location. Harvesting of vegetables followed by process like grading, storing, processing prior to marketing. Various varieties of vegetables can grow in India because of the diverse climatic conditions.

India cultivates variety of vegetables according to region. However, the major are potato, tomato, onion, chilli and leafy produces. In agriculture, soil erosion causes through wind, water and through farming activities, like tillage and it affects the cultivation efficiency. Cultivation of leafy vegetables can generate employment as well as act as cash crop for farmers. India has many opportunities in sector of agriculture for production, export and processing of agri produce.

India has many challenges with respect to quality, uniformity to sustain in the global market. To contribute in the world's value chains it is very important to support export with high profit agro products, processed foods and sustainable brands in the market (Dept. of commerce, Govt of India). Also, it is crucial to understand the global market needs and to focus on the demands.

India lacks adequate storage infrastructure, transportation and maintaining demand supply ratio. Fresh vegetables required timely and appropriate shipping which plays important role. Supply chain management plays significant role in case of fresh leafy vegetable. Farm to fork maintenance of produce quality is main task in case of perishable fresh agri-produce.

New segments are growing in the market such as dairy, horticulture, inland aquaculture and poultry. No wonder that India is capturing the leading agricultural market globally, as India has most diverse background from centuries in area of food and non-food agro base products

(Agriculture Export Policy, Department of Commerce, Ministry of Commerce & Industry, Government of India).

1.2. Microbiological status

World Health Organization (WHO, 2006) and Food and Agriculture Organization (FAO) like agencies are monitoring the concerns for food borne infections and poisoning outbreaks is reported by Ijabadeniyi (2010). However, many countries were not ranked the outbreaks issues. Limited data often cause a concern with disease control surveillance system (WHO).

Water contamination is the major issue due to animal and human wastes poses into water and it leads to major health risks like *Salmonella* and *Listeria* spp. (Combarro *et al.,* 1997; Johnson *et al.,* 1997).

The water contamination is critical factor as it affects the pre and post-harvest activities (Suslow *et al.,* (2003). Also, another issue addressed by Ibenyassine *et al.,* (2006) that irrigation water and surface water contaminates the fruits and vegetables. The data (European Commission, 2002) suggests that foods contaminating major pathogens are Shigella spp., *E. coli* (STEC), *Campylobacter* spp., *Salmonella* spp., Shiga toxigenic, *Listeria monocytogenes.* Food infections and outbreaks are raising issues and majorly associated with ready to eat vegetables (Pezzoli, 2008).

Vegetables can often be contaminated in the field during irrigation with water contaminated with pathogens. Hence, the water quality in terms of purification and microbiological safety is important in fresh and minimally processed vegetables. (Solomon, *et al.,* 2002).

Majorly used type of irrigation systems are sprinkler and the drip irrigation. Data suggests (Keraita *et al.,* 2007) that vegetables are more prone to contamination in overhead irrigation than the drip irrigation. Although the irrigation practice and how it affects the transfer of pathogens are not examined yet in full depth. Hence the need of research to understand

various irrigation techniques and affect on human pathogens in cultivation of crops are raising concerns (Matthews *et al.,* 2014).

Changing dietary habits leads to maximize the consumption of fruits and vegetables and minimally processed fresh produce (Ijabadeniyi 2010). Garg *et al.,* (2010)

Many factors affect the food borne illness such as changes in the harvesting, methods of processing, patterns of consumption (Beuchat and Ryu, 1997). So, the safety and quality of fresh vegetables are major concerns in the global market.

It was blamed for its widespread presence in feces of livestock and animals (Cooley *et al.,* 2013). Another case associated with lettuce found in Taco Bell restaurants in northern USA (CDC, 2015) where an *E. coli* O157:H7 outbreak linked. Other information linked on fresh vegetables in Ethiopia reported by investigators (Ashenaf, 1989; Aberra *et al.,* 1991; Guchi and Ashenaf, 2010).

Also, due to lack of clean water availability pathogen contamination in vegetables leads as water is the primary source for harvesting to cleaning in every step. Burden of the diseases and outbreaks is major concern in most of the developing countries (WHO). Though the many cases were reported there was no specific pattern observed to take future steps. The leafy greens, lettuce and Spinach product recalls were associated due to *Escherichia coli* O157:H7 in 2006 and 2007.

1.2.1. Common pathogens associated with leafy greens

Fresh vegetables were generally considered that free from bacteria and nutrios however, it may cause adverse health effects on human body. Many factors are taking into consideration for the contamination like use of contaminated water, environmental factors, fertilizer, processing and handling methods etc. food borne illnesses affects to children, elderly, and low immunity concern people. General awareness and taking precautions while eating raw salads are important practices. While Good manufacturing practices (GMP) are implementing

in large scale all across the manufacturing units. Outbreaks issue also occurred in Europe in local produced as well as imported foods. The fresh produce market needs to ensure the safety and preventive actions against outbreaks.

Report from the United States, Centers for Disease Control and Prevention (CDC) shows the data of increasing outbreaks related to fresh produce consumption in past decades (1995 - 2005) (Johnston *et al.,* 2005). USA reported around 200 illness and 03 deaths in 2006 due to contamination by *E. coli* O157:H7 *in* spinach (Smith DeWaal and Bhuiya, 2008). There is evidence of fresh tomatoes outbreak of *Salmonella* Typhimurium. *E. coli O157*:H7 at restaurants in the USA. Major illness is not concerned with fruits than the consumption of vegetables as it caused by various bacteria and viruses. Pre –packed spinach was recalled by FDA due to *E. coli* outbreak and it was found in in California, USA Ijabadeniyi (2010).

Fresh leafy agro products are contaminated by pathogens of enterotoxigenic *E. coli and reason of causing* diarrhoea (Beuchat, 1996). Meat and poultry consumption cause food borne illnesses (Albrecht *et al.,* 1995). The study of Beuchat (1998) shows *E. coli*, *Listeria, Campylobacterand Salmonella were present* less than 8, 3.1, 25 and 22 % respectively in fresh produce. In England, Norway and Sweden an outbreak of *Salmonella* spp was recorded in 2004 and was associated with rucola lettuce which was imported from Italy (Nygard *et al.,* 2008). In 2005, bagged salad containing romaine lettuce was linked to an outbreak of *E. coli* O157:H7 food borne illness (Smith DeWaaland Bhuiya, 2008) in the United States. Small scale (less than 50 cases) outbreaks related to *E. coli* O157:H7 contaminated lettuce was also been documented in the USA.

Tambekar and Mundhada (2006) were reported that the food borne organisms generally presents in vegetables were Salmonella spp, *E. coli*, *S. aureus*. Aycicek *et al.,* (2004) also stated about faecal coli form. If plate count of aerobic mesophilic microorganisms presents in food; it confirms the microbiological contamination.

The study of Johnston *et al.* (2005) resulted the total plate count (TPC), total colifrom and *Escherichia coli were* (~4.5 - 6.2), (~1.0 - 3.4) and (~1.0 - 1.5) log cfu/g respectively for vegetables and herbs. All the samples were collected from the region of southern USA. Fifteen varieties of (5.7%) of *Salmonella* spp. with high prevalence rate was found in vegetables at Mexico region. The principally isolate was S*almonella* Typhimurium. This study was also reported *Salmonella* Typhimurium from parsley (Quiroz-Santiago *et al.,* 2009). Outbreaks of food borne illnesses often associated with fresh produces because consumption habits of eating ready to eat salads, raw foods or minimally processed foods (Ilic *et al.,* 2012). The surveillance study related to microbial quality of fresh produce was conducted in Singapore and *E. coli* O 157:H7 or *Salmonella* spp. were found. Also, in bean, sprouts and fresh cut salad high level of coliforms were detected. Amongst that almost 50 % of samples were found more than 5 log cfu/g (Seow *et al.,* 2012). Lettuce contamination was reported in Spain by *Salmonella* Typhimurium var. Copenhagen (DT104B) and it leads to outbreaks. Eleven cases of HUS were identified due to contaminated lettuce. *E. coli* O157:H7 contamination was resulted.

In various studies total aerobic plate count in vegetables at various stages of farm to market reported approx 8 log cfu/g (Mukherjee *et al.,* 2007; Mukherjee *et al.,* 2004). In Israel Salmonella outbreak associated with fresh basil caused illness in 4 countries, while one reported death (WHO). The implicated hazards are linked to severity of the disease. For an example, enterohaemorrhagic *Escherichia coli* are responsible for acute illnesses and even deaths in some cases (World Health Organization). Many outbreaks were reported in developing countries south-west Pacific, North America and in Europe; it has significant reflection of illness monitoring in particular these reg ions. The main identified concerns were related to the group of pathogens like *Escherichia coli* O157:H7, Salmonella or Norovirus and all were associated with consumption of leafy greens. While some countries

reported primary concern with sprouted seeds and melons. One case was found for *Yersinia pseudotuberculosis* in carrots.

1.2.2. Trade status

India's contribution in export increased, in the global market from 1 to 2.2 % since 2016 (Department of commerce, Govt. of India). However, India has major challenges as its agro products are not fully processed, less market value and mostly sold in wholesale. India's contribution in global market is not more than 15 % while USA and China are contributing high as 25% and 49% respectively. India produces 70 types of vegetables amounting to about 184 million MT of which is ~ 20 % of global vegetable production (Table 1.3). There is vast deference between today's agriculture and the time during Green revolution. India's GDP gradually increased from 1970's to late 1990's.

The data shows GDP expansion from US$25 billion to > US$100 billion. Initially the growth was stagnant and it was majorly focused on the cereals, rice and wheat. From early 2000 to 2014, agricultural cultivation has grasped the level US$101 to US$367 billion. The upmost market to sell fresh produce for India are countries like Saudi Arabia and Qatar, Sri Lanka, Bangladesh, UAE, United Kingdom, Nepal, Malaysia and Netherland. As per WTO trade data 2016 India is ranking tenth amongst the major exporters worldwide.

Improvement towards cultivation and harvests management is necessary to improve production yield and value-added products. Many studies were carried out on vegetables and it focused on major losses of post-harvest processing's from 8 - 18 % on account of lack of or inadequate post-harvest management. Agro processing and agricultural exports are key sectors require immediate attention.

India’s role is nearly 2.2% in the international market. However, there is increasing market for horticulture products and this positive impact occurred due to developments of cold chain and quality assurance measures in the industry. APEDA's assisting to public sectors as well hence the market is increasing for private as well as public organizations. They created centers for taking care of post harvesting processes and creating awareness in farmers in terms of export process.

1.2.3. Impact of contaminations on trade

The Rapid Alert System for Food and Feed (RASFF) sent alerts in Europe to highlight the risk of pathogens on fresh produce among European countries or outside the European Union (WHO). The data is not available to check the effect on trade and economics. There is no significant statistical summary available about outbreaks and its impact on international trades. Though, the most recognized impacts are related to microbiological hazards of fresh agro produce (WHO).

Mexican states are taking extensive effort to implement Good Agricultural Practice (GAP) program me to aware the growers and distributors for the certification. The good result was visible as only 1 state in 2001 to 22 states in 2005; the growth of GAP (Good Agricultural Practices) programme implementation expanded and was up to 32 by 2007. Mexico reported outbreaks to the Canada or USA, or both; for cantaloupes with *Salmonella* serovar Poona in green onions with hepatitis A virus also in with cyclospora in basil (WHO). Outbreaks caused deaths and other health issues due to consumption of contaminated vegetables and it also leads to the economic impact. The United States Department of Agriculture (USDA) and the Economic Research Service (ERS) was recorded the Spinach outbreaks. The demand of Spinach was still maintained by over 40%, while contaminated commodity consumption caused long term health and economic consequences.

The incident of product recall of Spinach from Canada in trade with USA in 2006 was reported due to large spinach *E. coli* O157:H7 outbreak and it impacted the trade and resulted into Canada restricted on USA grown spinach for several months (WHO). The Commodity Specific Food Safety Guidelines for the Production and Harvest of Lettuce and Leafy Greens compliance and certification was implemented by California growers to ensure the food safety.

1.2.4. Quality factors and trade

Even within the same country, the crops variety, how it grows and marketed are completely different. GAPs compliance and verification by third-party auditors are mandatory for fresh produce distribution and export. Some countries did not require GAP application however growers and distributors doing a voluntary GAP program for safety and standardize export of fresh produce. As a result of voluntary steps, many countries noticed a significant difference in the quality and safety of fresh produce and it impacted on economic and reduces the health concerns.

Differences in crops are intended for domestic as well as export market. The quality of leafy vegetables is determined by various factors. Many countries are increasing the production of fresh produce. However, many new crops are being introduced along with the traditional crops. Many raw eaten vegetables are newly introduced in market of China and Ghana.

Post-harvest practices vary throughout the distribution chain. Post-harvest losses in developing countries often results of untrained workers and not access or very limited access to cold chains.

Many variables can contribute to microbial contamination of vegetables (WHO). But the land and the location of harvesting is one of major factor. The demand of fresh produces increasing due to population and lack of space leads to chances of harvesting vegetables near livestock production and near to urban areas and this is also a factor responsible for

microbiological contamination. Developed countries may have solution for this but many developing countries do not have infrastructure to focus on such concerns. Environmental factors like no rainfall or extensive rainfall, extreme geographical locations cause problems and livestock production also remains the problem. Local wildlife and ecology and their significant role in the environment are also another aspect of contamination issues.

Due to increasing water demands, the search for alternative water sources is one of important challenge in water shortages countries. Seventy percent of fresh water uses the agriculture industry. Many countries facing the water shortages issues and almost 40% of their renewable resources are exploiting (WHO). The total surface area of raw or partially diluted wastewater is estimated at 20 million hectares in fifty countries. It is 10% of the total land (WHO). It is difficult to estimate the proportion of water used in fresh produce production. However, supply of wastewater for irrigation is increasing in many areas. One of the reasons of increasing demand is increasing competition for limited water resources and multiple schemes for irrigation and with attractive return's farmers can earn from producing fruits and vegetables. The wastewater supplies increased due to population growth in big cities and villages throughout the world. Another issue is the volume of wastewater has increased multiple times than the ability to build and operate treatment facilities.

Wastewater re-use is still in new trends and varies with treatment levels, water involved and other information related to public concerns. The factors can improve the water management are tradable permits, taxes and effluent standards, it also helpful for household water management and in industries wastewater treatment plants. Policies are taking care of many things like water rights, pricing, full-cost energy pricing, and restrictions on groundwater pumping. Also, framers can get incentives for investment in water saving implementation of irrigation methods.

Synthetic fertilizers and some other options like animal and poultry manure are used without composting and that leads to the microbial hazards. Organic waste is economic and re-uses treatment often used in agriculture land. However, these methods still lead to potential contaminants into the environment and hence not necessarily be used for horticulture (WHO). The role of workers causing the contamination in fresh produce has been evaluated and studied shows it's linked to the outbreaks. Worker hygiene is important and the availability and accessibility of wash and comfort stations are necessary on the farms. If the workers or children are sick and they involved in the processing, packing sections which directly increases the chance of pathogens. Hence, personal hygiene of workers and multidisciplinary approach requires addressing the issues (WHO). Cross-contamination and contamination may lead due to equipment's and tools used in horticulture. For example, the knives used in harvesting root vegetables and lettuce heads, spring onions.

Consumption patterns in the both developed and developing countries, in recent years the consumption of fresh produce has been increased. Traditionally, people cooked food but now the consumers are eating more raw foods. Eating habits differs among regions with respect to consumption practices and preparation techniques. For example, baby corn is eaten raw in western countries but in Thailand eaten as cooked form. Another example is baby spinach. It is very popular salad and eaten as raw salad in North America. However, many other countries consumed eat in cooked form (WHO).

1.3. Importance of Green Leafy Vegetables in diet

Green leafy vegetables are high in demand due to health benefits and moreover it is easy to cook, prepare and eat. Vegetables plays vital role in addressing issues like malnutrition hence to focus on safety of leafy green produce is very important.

Green leafy vegetables provide important nutrition's like vitamins, dietary fibers, minerals, micro nutrients. Especially in rural diet; vegetables contribute majorly as a nutritious source.

(Asaolu *et al.,* 2012) (Table 1. 4). Green Leafy Vegetables are the vital source of human nutrition. Young generation generally ignores the power of simple nutritious source of vegetables due to lack of awareness (Odhav *et al.,* 2006). Vegetables also help in weight loss and maintaining healthy weight as It has law calorific value (Nwanekezie and Obiakor, 2014). It consists of pectin substances, hemicelluloses and cellulose. It provides firmness and texture (Mohammed and Sharif, 2011). It also protects against diseases and helps to build the immunity (Mohammed and Sharif, 2011).

	Table 1.4 Nutritive value (per 100 g) of some of the commonly consumed leafy greens					
Nutrients	**Drumstick Leaves**	**Amaranth**	**Spinach**	**Roselle Leaves (Gogu)**	**Coriander Leaves**	**Mint**
Calories	92	45	26	56	44	48
Protein (g)	6.7	4.0	2.0	1.7	3.3	4.8
Calcium (mg)	440	397	73	1720	184	200
Iron (mg)	7.0	25.5	10.9	2.28	18.5	15.6
Carotene (µg)	6780	5520	5580	2898	6918	1620
Thiamine (mg)	0.06	0.03	0.03	0.07	0.05	0.05
Riboflavin (mg)	0.06	0.30	0.26	0.39	0.06	0.26
Vitamin C (mg)	220	99	28	20.2	135	27.0

Source: National Institute of Nutrition / http://vikaspedia.in/health/nutrition/nutritive-value-of-foods/the-goodness-of-greens

1.3.1. Health Benefits of Green Leafy Vegetables

World Health Organization (WHO) and Food and Drug Administration (FDA) are contributing vital role in awareness among people and ensuring safety of products. As the recent data suggested 5 to 9 servings of fruits and vegetables gives healthy life among 2.7 million lives per year (Johnston *et al.,* 2006). Leafy vegetables which are rich in iron content can lead to treat anaemia. Consumption of spinach enriched with iron which can effectively maintaining iron level. As per nutritionist 3-4 serving of spinach per week can recover deficiency of iron**.**

Magnesium content present in green leafy vegetables play important role against scalp infection. In addition to this some level of antioxidant also found in spinach, kale and cabbage vegetables. Free radical which deteriorates the body silently can be prohibited by consuming these antioxidants containing leafy vegetables.

Studies have concluded that a single serving of leafy vegetables can provide multiple nutrients like calcium, dietary fiber, iron, minerals, vitamin A, B and C. Due to fibre content it improves the digestive health. To maintain proper digestion nutritionist, recommend daily consumption of leafy vegetables. Daily vegetables consumption can maintain nutrients like potassium, magnesium and calcium level in body. Night blindness can be cured or prevented by consuming vegetables containing high vitamin A. Source of potassium from fresh green leafy vegetables can ably maintain eyesight properly. Cholesterol level increased due to consumption of junk foods containing high trans-fat, sugars and cholesterol, can be overcome by consuming leafy vegetables. The evidence of health benefits of leafy vegetables is reported (Lerici *et al.,* 2000). It plays important role in providing healthy life and preventing illnesses like cancer, diabetes and heart disease hence regular consumption of leafy produces important (Ijabadeniyi *et al.,* 2010).

Consumption of leafy vegetables gives very less fat and calories. Green leafy vegetables can help to reduce cardio vascular diseases, eyesight and weight loss. Consumption of leafy vegetables can balance the cholesterol. The minerals and vitamins in the leafy vegetables can limit the deposition of cholesterol in arteries. Cholesterol which deposit in the arteries can lead to heart attack and stroke diseases. Nutrients present in the leafy vegetables can nourish the skin by providing antioxidants. Skin aging can be reverted by consuming leafy greens.

1.4. Nutritional status and health protecting properties of selected leafy greens

Green vegetables have much nutrition and have capacity to fulfil the ever-increasing human population. Due to lack of awareness many vegetables remained underutilized and needs to focus on its benefits in the society. The consumption of fruit and vegetables have many health benefits including improving human health, reducing risk of chronic diseases like cardiovascular disease, diabetes and many types of cancer treatments. (Fuhrman and Rutherford, 2010).

Herbs plays important role as a source of medicines. Herbs are rich in metabolites and are act as main ingredient in preparation of drugs and essential oils. Medicinal plants are mainly advantageous for its therapeutic uses. They are easily available, economical and effective. (Gupta and Prakash, 2005).

1.4.1. Spinach

Spinach word came from Spanish word Hispania. The family of spinach is *Amaranthaceae*. Spinach is known as edible flowering plant. Spinach also called as *Spinacia oleracea.* In different languages it has different names. In *Sanskrit* called Chhurika. In language Hindi, Gujarati and Marathi spinach is known as Palak. Also, in Kashmiri it is identified by name Palakh. In Bengali and Tamil, it is known as Palang and Pasalai respectively and in Telugu it is called Mathubucchali. (Kirtikar and Basu, 2005).

It is believed that the first cultivation of spinach was by Arabians. About 2000 years ago, the Persians were cultivated spinach too (Bakhru, 2001). In the eleventh century it was introduced into Europe and then spread all over the world. India cultivated large amount of spinach. It is incorporated in many foods in India and favourite vegetables available in winter. (Guha and Das, 2008). Spinach requires moist climate and cool environment to grow.

Spinach provides minerals like calcium, magnesium, phosphorus, manganese and copper, zinc, potassium and Iron. (Anonymous, 2004; Tindall, 1983). Spinach is not only source of nutrition but also provides antioxidant hepatoprotective (Gupta and Singh, 2006) and helps in different biological activities like antiviral, anthelmentic (Patil *et al.,* 2009), and anticancer (Longnecker *et al.,* 1997).

Spinach is well known for its high nutritional value. It contains high level of antioxidants. Also, it contains carotenoids like neoxanthin, ß-carotene and violaxanthin. Spinach is rich source of Vitamins. It contains vitamins A, C, E, and K and it also contains oxalic acid, folic acid. It also has source of variety of minerals (Table 1.5) (Guha and Das, 2008).

It is one of the richest sources of vitamins than other leafy greens, an excellent source of vitamin A. It is superior to yellow vegetables as the carotene of green leafy vegetables is better absorbed than that of yellow vegetables. The carotene absorption of spinach is from 45 to 58 percent. It is relatively rich in iron so it is employed as a food medicine in anaemia (Mukherjee, 1983). Spinach is consumed in many ways. As salads, with addition of other vegetables like cucumbers, onions and tomatoes it makes a perfect dish. Also, Lemon juice can be incorporated not only for taste but also vitamin C in lemon juice helps to absorb iron (Bakhru, 2001). In the south-western Asia, *Spinacia oleracea* Linn is famous for its medicinal properties.

Table 1.5 Proximate composition of fresh spinach Vegetables as per USDA data		
Nutrient	Unit	Value per 100 g spinach
Water	g	91.40
Energy	kcal	23
Protein	g	2.86
Total lipid (fat)	g	0.39
Carbohydrate, by difference	g	3.63
Fiber, total dietary	g	2.2
Sugars, total	g	0.42
Minerals		
Calcium, Ca	mg	99
Iron, Fe	mg	2.71
Magnesium, Mg	mg	79
Phosphorus, P	mg	49
Potassium, K	mg	558
Sodium, Na	mg	79
Zinc, Zn	mg	0.53
Vitamins		
Vitamin C, total ascorbic acid	mg	28.1
Thiamine	mg	0.078
Riboflavin	mg	0.189
Niacin	mg	0.724
Vitamin B-6	mg	0.195
Folate	µg	194
Vitamin B-12	µg	0.00
Vitamin A	µg	469
Lipids		
Fatty acids, total saturated	g	0.063
Fatty acids, total monounsaturated	g	0.01
Fatty acids, total polyunsaturated	g	0.165
Fatty acids, total trans	g	0
Cholesterol	mg	0

U S Department of Agriculture., Agricultural Research Service, National Nutrient Database, Standard Reference Legacy Release.

Spinach has been found valuable for constipation (Kumar *et. al.,* 2013), dyspepsia (chronic indigestion), anaemia (Bassey & Khan, 2015). Also, spinach has health benefits in arthritis, obesity, nerve exhaustion, high blood pressure, tumours and bronchitis. The study also showed the positive effects on patients recovering from ailments of the kidney, liver and bladder (Joseph, 1975). Especially in arteriosclerosis, the compound present in spinach inositol and choline are helps to prevent the hardening of the arteries. (Kar & Borthakur, 2008). Spinach has also a source of adequate nutrition of Iron. (Hanif *et al.,* 2006) and also contains Vitamin K (Hanif *et. al.,* 2006). This provides the protection in blood clotting (Kadans *et al.,* 1975; Robinson, *et al.,* 1990).

Spinach plays important role in preventing from aging process and gives protection to cells (Cruess, 1958). This makes a Spinach one of best anti-aging foods (McKeowyn-Eyssen *et. al.,* 1994; Miller, 1994). Spinach is vital source of lipophilic compounds and various carotenoids like zeaxanthin, neoxanthin, lutein and chlorophyll (Lomnitski *et. al.,* 2003). It is also a good source of flavonoids. It acts as strong antioxidant (Pemberton *et. al.,* 1991) and helped in preventing and fighting against radical damage in the body (Miller, 1994).

The similar antioxidants are also available in vegetables such as broccoli, kale, carrots (Hugo *et. al*., 2005). Sufficient intake of spinach and its effects were studied. It is very beneficial for various types of cancer like breast, colon, prostatic, ovarian and lung (Lomnitski *et al.,* 2003). Carotenoids also helps in decreasing the risk of heart disease (Miller, 1994; Gaikwad et.al., 2010; Diet and Health, 2014) diabetes (BMJ 2010), it also helps in preventing neurodegenerative diseases (Miller, 1994).

Food Value (chemical constitutes)

The spinach is highly esteemed for its high mineral and vitamin content (Table 1.6). Its other values are not so significant.

Table: 1.6 Food Value of spinach			
Moisture	92.1%	Fibre	0.6%
Proteins	2.0%	Carbohydrates	2.9%
Fats	0.7%		
Mineral	1.7%		

Source: Foods that heal Natural way to good health, By Author; H K Bakhru, published by orient paper backs

1.4.2. Coriander

Around 5000 BC, it is mentioned in Sanskrit prose and also in Eber Papyrus in Greek around 1550 BC. (Uhl., 2000). The reference showed the name Coriander called as “kusthumbari” or as “dhanayaka” in the Sanskrit (Prakash, 1990). Coriander has medicinal uses and it was mentioned by Hippocrates in Greek medicines (460-377 BC). Also, in Egyptians this herb is well-known as spice of happiness.

Coriander named as *Coriandrum sativum.* The genus *Coriandrum embrace* comes from plant *Coriandrum sativum.* Coriander is well known with various names in different languages. In English it is called coriander. In Urdu and Hindi, it is Dhania. Arabic and Chinese names are Kuzbara and Yuan sui respectively. The “coriander” word originated from Greek word for “bed-bug” and it means smell with spanking as herbs.

Coriander is spice as well as herb. It has many medicinal properties well known from thousands of years. It has antifungal properties (Basılico and Basılico, 1999). It is also a source of antioxidant (Chithra and Leelamma, 1999) and helps in hypolipidemic conditions as well (Chithra and Leelamma, 2000). Corianders have antimicrobial benefits (Singh *et al.,*

2002) and have anticonvulsant and hypocholesterolemic contains (Chithra and Leelamma, 1997). Compounds present in essential oils are geraniol (1.9%), camphor (3.0%); pinene (10.5%); γ-terpinene (9.0%); and linalool (67.70%); (Nadeem *et al.,* 2013). From thousands of year coriander has been applied as medicine (Mathias, *et al.,* 1994). Seed and leaves having diuretic, antimutagenic, anticough, antioxidant, anthelmintic properties (Rajeshwari, Andallu, 2011., and Pathak *et al.,* 2011). From ancient times coriander is used as anti-inflammatory to prevent joint pain and rheumatism (Wichtl, *et al.,* 1994).

Phenolic content and antioxidant activity are directly correlated. The positive correlation between phenolic content indicates the presence of antioxidant activity (Wangensteen *et al.,* 2004; El-Ghorab *et al.,* 2006). Many studies evaluate the antioxidant activity in herbs like coriander, peppermint, laurel, basil, marjoram, thyme, cumin and clove (Baratta *et al.,* 1998), also other ingredients like fennel, nutmeg, dill and black pepper (Lagouri and Boskou, 1995). Many essential oil ingredients such as carvone can obtained from caraway and linalool be formed from coriander, thymol from thyme can be obtained and possess the high antioxidant activity.

Fruits are act as flavouring agent as a carminative, spasmolytic and stomachic. Coriander oil has ability to retain flavour than any other oils of its group (Purseglove *et al.,* 1981). It has numerous benefits for sub-acid gastritis, diarrhoea and digestive stimulation (Patel and Srinivasan, 2004). Coriander has very powerful lipolytic activity (Leung and Foster, 1996). However, taking cautions are necessary as study suggested allergic reactions from furanocoumarins compound (Brinker, *et al.,* 1998). There is Strong antioxidant activity in Coriander leaves than its seeds; however, the antioxidant contribution during plants growing is small. (Dorman *et al.,* 2008; Cozzi *et al.,* 1997).

Coriander to flavor wine was used by The Romans and Greeks as a medication (Livarda and van der Veen, 2008). The etymology of coriander consists of a Greek word korannon and combination of koris and aroma (Smell) (Uchibayashi, 2001). The naturalist from Rome was first used the name Coriandrum with reference to describe fragrances and its immature fruit (Blumenthal, 2000).

Pharmaceutical Industries uses coriander oil as a flavouring agent (Leung and Foster, 1996). It has many other applications in cocoa, liquor and chocolate industries. The flavouring compounds in essential oils rustics the use of antioxidants in herbs (Madsen and Bertelsen, 1995). Coriander is helpful in correcting taste and other medicinal values in oils. Aroma therapy of coriander is one of its applications (Cooksley, 2003). Coriander seeds also used as other volatile oils and as spices like curry powder and other combination of spices.

Coriander has many functional properties which contribute to the health nutrition and medicinal benefits. Coriander oil has natural antimicrobial properties which help to fight against *Campylobacter jejuni* (Rattanachaikunsopon and Phumkhachorn, 2010). Major functional activities involve antioxidant activity and nutritional aspect (Table 1.6).

Hydro-alcoholic extracts of the seeds of coriander were studied on the egg and adult nematode parasite Haemonchuscontortus by in vitro anthelmintic activities of crude aqueous. It was also investigated for in vivo anti helminitic activity in sheep to control the infection of *H. contortus*. Both results were shown the concentration was less than 0.5 mg/ml found effective (Debella *et al.,* 2007).

Table 1.7 Proximate composition of fresh coriander Vegetables as per USDA data		
Nutrient	Unit	Value per 100g coriander
Water	g	92.21
Energy	kcal	23
Protein	g	2.13
Total lipid (fat)	g	0.52
Carbohydrate, by difference	g	3.67
Fiber, total dietary	g	2.8
Sugars, total	g	0.87
Minerals		
Calcium, Ca	mg	67
Iron, Fe	mg	1.77
Magnesium, Mg	mg	26
Phosphorus, P	mg	48
Potassium, K	mg	521
Sodium, Na	mg	46
Zinc, Zn	mg	0.5
Vitamins		
Vitamin C, total ascorbic acid	mg	27
Thiamine	mg	0.067
Riboflavin	mg	0.162
Niacin	mg	1.114
Vitamin B-6	mg	0.149
Folate, DFE	µg	62
Vitamin B-12	µg	0
Vitamin A,	µg	337
Vitamin A, IU	IU	6748
Vitamin E (alpha-tocopherol)	mg	2.5
Vitamin D (D2 + D3)	µg	0
Vitamin D	IU	0
Vitamin K (phylloquinone)	µg	310
Lipids		
Fatty acids, total saturated	g	0.014
Fatty acids, total monounsaturated	g	0.275
Fatty acids, total polyunsaturated	g	0.04
Fatty acids, total trans	g	0
Cholesterol	mg	0

U S Department of Agriculture, Agricultural Research Service.
National Nutrient Database, Standard Reference Legacy Release.

1.4.3. Mint

Mentha spicata L is the commonly known for Labiatae (Laminaceae Family). Mint is widely grown in almost every region of the world and it is famous for herbal properties. Mint is herbaceous rhizome plants and mostly emits the quadrangular green or purple steams (Dattatreya *et al.,* 2010).

The family of Mint (*Mentha arvensis L.) is Lamiaceae.* It is cultivated in Hot and medium temperature regions. Mint is an important crop which produces fresh or dry leaves, and essential oil. Fresh and dry leaves are used for herbal teas. The essential oil from herbs used in cosmetics, food applications, nutraceutical and pharmaceutical products (Arzani *et al.,* 2007; Kumar and Patra, 2012).

Mint is popular herb. It has many medicinal benefits such as remedy for vomiting in pregnancy, fever, bronchitis, hysteria and indigestion (Majumdar *et al.,* 2012). Mint also acts as carminative, stimulant and antispasmodic. Mint Leaves are beneficial for human health as they contain significant amount of micronutrients, vitamins, antioxidants, photochemical and fibre content that may help protect against degenerative diseases and micronutrient malnutrition (Table 1.7). (Kodanda Ramreddy and Kavita, 2013). Peppermint provides relive in anaesthetic conditions. Mint provides coolness against pain and itching. Menthol helps small blood vessels to dilate and helps increasing the blood flow by making skin feel warm.

Table 1.8 Proximate composition of fresh mint vegetables as per USDA data		
Nutrient	Unit	Value per 100 g mint
Water	g	85.55
Energy	Kcal	44
Protein	g	3.29
Total lipid (fat)	g	0.73
Carbohydrate, by difference	g	8.41
Fiber, total dietary	g	6.8
Minerals		
Calcium, Ca	mg	199
Iron, Fe	mg	11.87
Magnesium, Mg	mg	63
Phosphorus, P	mg	60
Potassium, K	mg	458
Sodium, Na	mg	30
Zinc, Zn	mg	1.09
Vitamins		
Vitamin C, total ascorbic acid	mg	13.3
Thiamine	mg	0.078
Riboflavin	mg	0.175
Niacin	mg	0.948
Vitamin B-6	mg	0.158
Folate, DFE	mg	105
Vitamin B-12	mg	0
Vitamin A,	mg	203
Vitamin A, IU	IU	4054
Vitamin D (D2 + D3)	mg	0
Vitamin D	IU	0
Lipids		
Fatty acids, total saturated	g	0.191
Fatty acids, total monounsaturated	g	0.025
Fatty acids, total polyunsaturated	g	0.394
Fatty acids, total trans	g	0
Cholesterol	mg	0

Source: U S Department of Agriculture, Agricultural Research Service
National Nutrient Database. Standard Reference Legacy Release

As mentioned in herbalpedia since antediluvian times, mint was a remedy for treatments of cough, fever and cold. It has references in Ayurveda. Medieval German named abbess/herbalist Hildegard recommended that mint is good for digestion and gout. Culpeper studied the mint effects as cough remedy, digestive aid and treatments for cold. Peppermint acts as a breath freshener. Mint tea is very popular for curing lifts depression and eases colic. Mint oil has beneficial in relieving ear aches. Also, when few drops of mint oil added in water, it helps to treat against itching and burning, sunburn. For curing hives allergy methanol used as a sensitizer.

1.5. Processing parameters

Microbiological contamination is big concern and specially affects the fresh, pre cuts and raw vegetables. The treatments for reducing the load of contamination are to include washing vegetables with antimicrobial compounds. Treatments are available for reduction of the contamination but complete elimination of microorganisms very difficult. Prevention plays an important role between chain of farm and consumer. The need of alternative treatment is rising to make sure the quality and safety of vegetables (IAEA).

Washing with sanitizer: Submerged treatment or sprinkling chlorinated water has been routinely used which leads to reduce surface microbial contamination (Figure 1.1). Sanitizer like sodium hypochlorite has been recommended for use up to 200 ppm. Produce should be maintained at low temperature and cross contamination through carriers or other contaminated leafy vegetables should be avoided. After pre-cooling of produce it must be stored in temperature preferably 3 - 5°C.

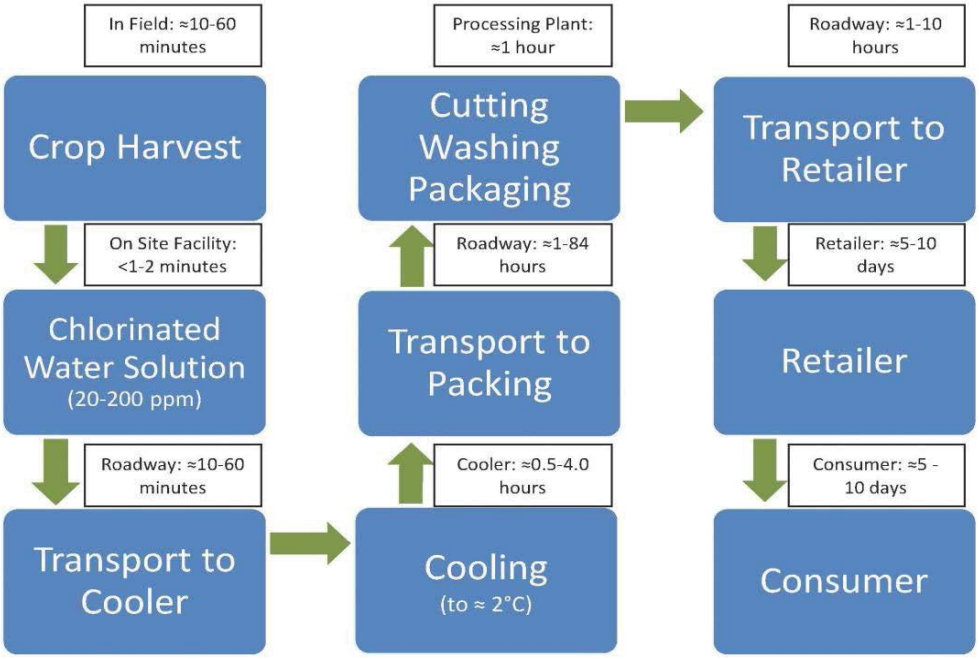


Figure 1.1 Flow sheet of Processing of fresh green leafy produce

Source: Ohio state university, fact sheet.

1.6. Radiation treatment (Nutritional safety and beneficial aspects)

Radiation also occurs due to natural environment. Various types of radiations are present such as infrared light, radio frequency, microwave, visible light and ultraviolet light. Others are ionizing radiations, more energetic types of radiation like electron beams, gamma ray and X-ray. These types of radiation produce electronically charged atoms or ions or molecules. Radiation treatment can be defined as when food is exposed and comes in contact with a definite dose of ionizing radiation (IR) or controlled application of radiation for specific purpose the process called Irradiation treatment. Ionizing radiation operates on the same mechanisms with respect to how it affects the food and reduces the microorganisms.

These techniques include gamma rays, X-rays, high-energy ultraviolet (UV) radiation and secondary radiation (Table 1.8, 1.9). The unit of measurement radiation is Gy. One gray equals to ionizing energy and is measured in Joule and it is absorbed 1Kg irradiated material. IR represents the regulatory limits and the doses are expressed in (Table 1.10 and 1.11).

Radiation uses mainly in food industry for preservation. Ultraviolet-C (UV-C) helps to kill bacteria and induces the effect between neighbouring pyrimidine bases in DNA. This technique also used ad surface disinfectant (Kang *et al.,* 2013, Qiulian Kong et al., 2014). However, (UV-C) has poor penetrating capacity. So, it restricts applications in food processing. A nuclear source (cobalt-60) produces Gamma rays. It has high penetration capability and applicable in treatment of commercial packages (Qiulian Kong et al., 2014).

Electrically generated radiations are E-beam and X-rays. The choice is varying according to density of food and mass anticipated. If product has greater density it multiplies by its thickness > 20 g/cm$^{2.}$ It is useable for high penetrating X-rays.

E-beams are more superior to X-rays. E-beams possesses high dose rate while both X-rays and E-beams have switch –on and off capacities (Castell-Perez and Moreira, 2011). E-beam have more advantages like control methods, thermal and high pressure, chemical washes and fumigants.

Other advantages of E-beams are Nature friendly, more rapidly processing,) Shorter exposure time and No pre-treatment needed. It also beneficial because of No aeration process needed after sterilization, no chemical residues and Reduction of microorganisms without using liquids. This technology helps to reduce pathogens and decrease the chances of contamination from food (Qiulian, Kong et al., 2014; Espinosa *et al.,* 2012; Grasso *et al.,* 2011; Cabeza *et al.,* 2010; Neal *et al.,* 2008).

Radiation technology also has limitations and many other concerns linked to the uses. Major challenges include concerns associated with decontamination technology and lack of awareness in people. General awareness in very important about irradiated foods (Qiulian Kong et al., 2014). Numerous studies demonstrated about dosimeter and dose range various with respect to products. Hence, requirement of irradiation process parameters is important. The basic use of this technology is to maximize the effect and reduces the dose of radiation. High dosage is costly while low dosage can cause a safety concern as well (Kim *et al.,* 2011). Many studies are available on the irradiation processing of fresh produces and its impact on reduction of microorganism (Grasso *et al.,* 2011; Mintier and Foley, 2006; Sanglay *et al.,* 2011; Schmidt *et al.,* 2006).

Table 1.9 Technologies for Food Irradiation

Type of radiation	X-Ray	Electron beam	Gamma ray
Source	Induced by impingement of electron beam onto a metal plate. Conversion efficiency 5–10%	Accelerated electrons, typically, 5–10 MeV	Radioactive decay of Co-60 (2.5 MeV) Or Cs-137 (0.66 MeV)
Penetration	30–40 cm, suitable for all products	6–8 cm, suitable for relatively thin or low-density products	30–40 cm, suitable for all products
Shielding for operator	>2 m concrete or ~0.7 m lead	>2 m concrete or 0.7 m lead	>5 m water or > 2 m concrete or 0.7 m lead

Source “Irradiation food” by Niemira

https://naldc.nal.usda.gov/download/37365/PDF

Table 1.10 Food Irradiation Application (Regulatory limits)

Type of dose	Products	Dose kGy	Benefits
Low dose (up to 1 kGy)	Potatoes, onions, garlic, root ginger, yam, etc.	0.05–0.15	Inhibition of sprouting
	Cereals and pulses, fresh and dried fruits, dried fish and meat, fresh pork, etc.	0.15–0.5	Insect disinfestations and parasite disinfection
	Fresh fruits and vegetables	0.25–1.0	Delay of physiological processes (e.g., ripening)
Medium dose (1–10 kGy)	Fresh fish, strawberries, mushrooms, etc.	1.0–3.0	Extension of shelf life
	Fresh and frozen seafood, raw or frozen poultry and meat, etc.	1.0–7.0	Elimination of spoilage and pathogenic microorganisms
	Grapes (increasing juice yield), dehydrated vegetables (reduced cooking time), etc.	2.0–7.0	Effect on food properties
High dose (10–50 kGy)	Meat, poultry, seafood, prepared foods, sterilized hospital diets	30–50	Industrial sterilization
	Spices, enzyme preparations, natural gum, etc.	10–50	Decontamination of certain food additives

Source: A series of Fact Sheets from the International Consultative Group on Food Irradiation, ICGFI is an international group of experts designated by Governments to evaluate and advise on global activities of food irradiation

Source: https://moreira.tamu.edu/BAEN625/TOC_files/foodirradiation.pdf

Nutritional safety upon radiation treatment: There are safety concerns with respect to radiation treatment and its nutritional safety. The Nutritional loss varies on many factors like food composition, radiation dose, the presence or absence of oxygen and temperature of irradiation. The loss of micronutrients and vitamins are very minute which is occurred due to food processing like cooking, heating.

The facilities of radiation treatment plants must carry out routine quantitative dosimetry to ensure proper dose delivery during operation, which is suggested by as per the Atomic Energy (Radiation Processing of Food and Allied Products) Rules, 2012 (Table 1.11 and 1.12).

Beneficial aspects of radiation treatment: It reduces the efficacy of chemicals and other traditional treatment options. (Niemira and Fan, 2005). This is suitable approach for environment protection. This technique is similar to any other processing techniques in terms of factors affecting processing technology, type of method, duration of processes, Handling and other safety protocols. The validation of the processes is required for treatments.

New studies have evidence that irradiation is effective technique to kill bacterial pathogens on fresh produce (Smith and Pillai, 2004; Niemira and Fan, 2005). 1–3 kGy irradiation doses require reducing pathogenic viruses and fungi as they are more resistant to radiation (Niemira and Fan, 2005). High dosage required to get maximum results against fungi and viruses. 67% of food borne illnesses is caused by viruses while 60% and 72% illnesses caused by bacterial pathogens in hospitalizations and deaths (Mead *et al.,* 1999). Especially for elimination safety concerns in fruits and vegetables irradiation is the most significant technique. The safety is the major concern. The antimicrobial efficacy also varies with many factors including the type of fresh produce treated, pathogen, and condition of the fruit or vegetable like whole, cut or any other packaging material included and the commodity factors (Niemira and Fan,

2005). The protocols are different with different commodities and pathogen types. For *E. coli* O157:H7 elimination from leafy greens has different safety guidelines than the elimination of *Salmonella* from tomatoes. Some research observed a determined the ability of irradiation to bio film-associated, to kill internalized and protected pathogens (guidelines, post-harvest center, Univ. of California).

1.7. Regulatory aspects

The Food and Drug Administration (FDA) plays significant regulatory role in food irradiation. In 1958, the Food Additives Amendment to the Federal Food, Drug, and Cosmetic Act in that legislation included the material and sources of irradiations under the food additives. The regulation related to packaging and marketing within the country or internationally prescribes the safety and applications for consumers. Before the FDA amendments about food additive regulations, the FD & C Act describes the supporting data and the information required for FDA approvals. The Food and Drug Administration (FDA) allows the use of irradiated foods as it has impact on extending shelf life and safety of vegetables, fruits, spices, eggs, raw poultry and red meats. The first approval from FDA was issued in 1963 for wheat and flour which is suitable to kill pests (FDA, 2011).

A little more than a decade, BFlFC (Bureau of Foods Irradiated Food Committee) was established by FDA to focus on toxicological testing requirements and ensuring the safety of food irradiation. The goal is to take in to account the characteristics and doses of radioactive materials and analyze the human exposure and sensitivity. They referred many new recommendations and guidelines to the FDA committee. They helps to exempt toxicological testing for food irradiation which is less than 1 kilogray (kGy) or a very little impact on diet and nutrition. For other irradiated foods, the Committee recommended testing consisting of short mutagenicity tests which conducted under conditions that maximize the concentration

of radiolysis products. The effect was studied on rodents and on non-rodent. After 90 days feeding of two tests subject the need of further studies required to clarify the any inconclusive findings in the basic battery of tests.

BFIFC reviewed and published the animal feeding and mutagenicity studies. The committee evaluated the toxicological effects of food irradiation and recommended that toxic effects would not be considered below doses l kGy from foods irradiated and such a food do not require following the toxicological testing. The task group evaluated many studies. However, some of them are inadequately designed and hence the more data required for evaluating the safety of irradiation impact on foods exposed to more than 1 kGy. Also, the task group shows the support to evaluate and consider requests for authorization for food irradiations on case to case basis.

A joint Committee with FAO/IAEA/WHO Experts decided to review food Irradiation extensive data in 1980. The wholesomeness of irradiated foods confirms the irradiation findings and time at when the dose of 10 kGy presents. Also, findings suggested that no toxicological hazards and nutritional or microbiological problems were observed. The Codex Alimentarius Commission in 1993 approved use of radiation in food processing, while in 2003 the Commissions made revisions to the General standards for Irradiated Foods and categories includes for doses higher than 10 kGy.

Under the World Trade Organization (WTO) agreements done in between Technical Barriers to Trade (TBT) and Sanitary and Phytosanitary (SPS) and the combined efforts to adopt the irradiation in in international trade with safety measures and significant equivalence. India implemented commercial radiation processing of food with the regulation by Atomic Energy Rules, 1996 (Control of Irradiation of Food).

Food irradiation is approved and accepted in over sixty countries in the world. Since the announcement of National Standards for Food Irradiation in 1997, Chinese regulatory supports the use of food irradiation.

Apart from above lists the Food Safety and Standards Authority of India (FSSAI) also has regulations for irradiated foods and its processing, safety and measures (Atomic Energy (Radiation Processing of Food & Allied Products) Rules 2012). As per new amendments of regulations, product generic approval process required for treatments and the list of food products (Table 1.11 and 1.12).

Table 1.11 Classes of food products and dose limits

Class	Food	Dose Limit (kGy) kilo Gray Minimum	Maximum	Purpose
Class 1	Stem and bulb, root, tubers and rhizomes	0.02	0.2	Inhibit sprouting
Class 2	Fresh (fruits & vegetables (other than Class - 1)	0.2	1.0	Ripening delay
		0.2	1.0	Insect disinfestations
		1.0	2.5	Shelf -life extension
		1.5	5.0	Reduction of microbial load
Class 3	Cereals, pulses and their milled products, oilseed, nuts, dried fruits and dried fruit products	0.25	1.0	Insect disinfestations
		1.5	5.0	Microbial load reduction
Class 4	Seafood, Fish, aquaculture, & seafood products (fresh or frozen) & crustaceans	1.0	7.0	Elimination of the Pathogenic microorganisms
		1.0	3.0	Extension of Shelf -life
		0,3	2.0	Control of human parasites
Class 5	Meat and meat products including poultry (fresh and frozen) and eggs	1.0	7.0	Elimination of pathogenic microorganisms
		1.0	3.0	Shelf -life extension
		0.3	1.0	Control of human parasites
Class 6	Dry vegetables, seasonings, spices, condiments, dry herbs and their products, tea, coffee, cocoa and plant products	6.0	14.0	Microbial de-contamination
		0.3	1.0	Insect disinfestation
Class 7	Dried foods of animal origin and their products	0.3	1.0	Insect disinfestation
		1.0	3.0	Control of moulds
		2.0	7.0	Elimination of pathogenic microorganisms
Class 8	Ethnic foods, military rations, space foods, ready-to-eat, ready-to-cook/ minimally processed foods	0.23	1.0	Quarantine application
		2.0	10.0	Reduction of microbial load
		5.0	25.0	Sterilisation

Source: Atomic Energy (Radiation Processing of Food & Allied Products) Rules 2012

Table 1.12 **Radiation processing of allied products (Dose Limits)**				
Sr. No.	Allied Product	Purpose	Purpose Dose Limit (kGy) (kilo Gray)	
			Minimum	Maximum
1.	Packaging materials used for food and allied product	Microbial decontamination Sterilisation	5.0	10.0
			10.0	25.0
2.	Food additives	Insect disinfestation	0.25	1.0
		Microbial decontamination	5.0	10.0
		Sterilisation	10.0	25.0
3.	Dietary supplements, Health foods, and Nutraceuticals	Insect disinfestation	0.25	1.0
		Microbial decontamination	5.0	10.0
		Sterilisation	10.0	25.0

Atomic Energy, (Rules 2012), Radiation Processing of Food & Allied Products

SCOPE OF THE THESIS

AND

AIMS AND OBJECTIVES

Background

According to the Centres for Disease Control and Prevention (CDC), 12% of reported food borne illness outbreaks was linked to fresh leafy agro-produce. Over the last several years, there has been an increased concern about the microbiological safety of the world food supply. As a reflection of this concern, new research and testing programs have been initiated both nationally and internationally. UN body such as the World Health Organization has also declared food safety a top priority.

Over 95% of India's fresh agro produce is handled by the unorganized sector. The goods typically change at least 5 hands before reaching end customer. India's flora comprises of 6000 species of plants used for consumption, 1/3rd of which are fresh leafy agro-produce. Fresh leafy agro-produce is a very good source of minerals and vitamins and when consumed regularly they can substantially improve micronutrient status of the population. These vegetables are herbaceous, shrub, or tree origin where leaf is the edible part. The leaf contains vitamin A, C, folic acid, riboflavin, thiamine, B carotene, minerals like iron and calcium, significant level of fibre and antioxidants. The nutritive value of vegetables varies with the season, type of chemical used and the variety used for cultivation.

Short shelf life and microbial contamination are the major problems associated with green leafy vegetables. Much research efforts are directed to improve the microbiological quality of these vegetables along with increasing the shelf life. The rationale of the present research work was to develop a combination treatment for treatment of three leafy vegetables *viz*. *Coriandrum sativum* (Coriander), *Spinacia oleracea* (spinach), *Mentha arvensis* (Mint) which can reduce the microbial load significantly while eliminating the presumptive pathogens. Further, it was also proposed to study the effect of this treatment on the keeping quality, sensory, nutritional as well as biochemical properties of these leafy vegetables. The results of the study will be helpful in developing SOPs for treatment of the leafy vegetables

on the commercial scale. This research is focused on the application of irradiation technology targeted to preservation of leafy vegetables, instead of use of chemical preservatives. Application of irradiation is a relatively an innovative technology to enhance food safety, quality and trade of food products.

Preamble:

Food security is essential for life of human being and uplifting economic welfare and reassurance of a nation. Amount of agricultural produce lost due to infestation, microbial contamination and physical damages during post-harvest packing, transport and storage is very high. The gap between production and demand losses can be minimized by preventing post-harvest losses. The control mechanism used for post-harvest losses like chemical fumigants are being phased out because of their residual effect on human health as well as environment. Globally radiation processing by high energy ionizing radiation is used to minimize post-harvest losses. It is a cold non exothermic and physical process in which food is exposed to radiation under controlled application of energy which achieves microbial decontamination, disinfestations and sprout inhibition in case of onion and potatoes. Speciality of this technology lies in its use to pre-packaged foods. Radiation brings its effects indirectly through radiolysis of water as well as directly through deposition of energy on bio molecules. More pronounced effect of radiation is found in aqueous medium in which free radicals are generated which interact with other molecules. Now electron beam (machine based) is becoming a popular option compared to gamma and X-ray radiation. Ionizing radiation like gamma and electron beam has enough high energy which ionizes atoms and result in to breakage of bond. Food exposed to radiation up to prescribed dose limit doesn't undergo any undesirable physical or chemical changes. Commercial application of radiation processing in pharmaceutical sector is for radiation sterilization of medical devices etc. In

engineering sector, it has been widely applied for material modification. Gamma and electron beam radiations have their own unique features; gamma having high penetration power with requirement of radioisotopes and its continuous emission in nature is one of the limitations. On the other hand, electron beam irradiation can give high through put and the machine can be switched on/off as per the need.

Aims and objectives:

1. Microbiological analysis of selected leafy agro-produce for total microbial load and characterization of pathogenic organisms.
2. Standardization of process parameters for radiation hygienization of fresh agro-produce *Spinacia oleracea* (spinach), *Coriandrum sativum* (Coriander), *Mentha arvensis* (Mint).
3. Development of proper packaging and storage conditions for the shelf life extension.
4. Biochemical analysis of fresh as well as processed agro-produce for characterizing their nutritional and organoleptic properties.

CHAPTER 2

MATERIALS AND METHODS

2.1 Procurement of leafy vegetables

Freshly harvested popular varieties of coriander (cv. Co-2), mint (cv. Japanese mint / menthol mint) and spinach (cv. Semi-Savoy) were selected for the study. Farm fresh matured and dark greens leafy vegetable sample were collected from Nashik, Maharashtra and transported to laboratory within 4 - 6 hour of collection.

Minimal processing of sample: sample was washed with different type of waters (potable, filter and distilled) for separate set of experiment to evaluate the efficacy of water purity for microbial hygienization.

Microbial Analysis

2.2. Preparation of sample homogenate:

Vegetables samples (25 g) was weighed and mixed with 225 mL, peptone water (0.1%) in a blender jar of capacity 500 mL and were mixed for 2 minutes. Serial dilutions were prepared under aseptic conditions from each sample using 0.85% saline. Bacteriological Analysis Manual with some modification (Rev. Ed. 8, USFDA, BAM 2017)

2.2.1. TAPC (Total Aerobic Plate count)

TAPC was determined using Plate Count Agar (PCA) which is recommended by APHA (American public Health Association) and FDA. In plate count agar tryptone is used as a source of nitrogen and yeast extract provides vitamin B requirement. Using pipettes, dilution of 10^{-1} to 10^{-8} was prepared as in duplicate mentioned above. An aliquot of (0.1 mL) was aseptically spread on plate count agar plates. An aliquot of 1 mL was used for pour plate technique. Plates were incubated for 48 h at 35 $^{\circ}$C. Colony counts were taken and calculated with respective dilution and expressed in log CFU/g.

2.2.2. YMC (Yeast Mold Count):

YMC was analysed by using Potato Dextrose Agar for enumeration of Yeast and Mold from food samples. On the basis of pigments formation dermatophytes can be screened using PDA having pH 3.5 which inhibits the growth of other bacteria.

Sample (25 g) was diluted in 225 mL of saline (0.85 %) water and vigorously mixed in stomacher. Appropriate dilutions were prepared and used for analysis. Yeast and Mold count was carried out by pour plate method on PDA. Serially diluted 1.0 mL samples were transferred aseptically in sterile Petri plates, overplayed with molten PDA agar maintained at 45 °C temperature and mixed thoroughly by rotating plate. Further plates were incubated for 5 days at 25 °C. Colony counts were taken and expressed in terms of log CFU/g. Each of analysis was performed in duplicate.

2.2.3. Enumeration of presumptive coliform bacteria

The sample suspension prepared was diluted serially. One mL of each one dilution was transferred aseptically into separate, Petri plates in duplicate. 12-15 ml of molten (cooled to 45 °C) Violet Red Bile Agar (VRBA) (Himedia make) was added to each plate. The plates were swirled gently and the agar overlaid with 5 mL VRBA allowed to solidify. Colonies with pink to red colour having diameter 2 - 3 mm after incubation for 18 to 24 h at 37 °C were counted.

2.3. Screening of pathogens

2.3.1. E. coli:

Fresh 25 g raw vegetable sample was mixed in stomacher with 225 mL of the Brain Heart Infusion broth and homogenized. Further this homogenate was incubated at 42 °C for 3 h for pre enrichment. After incubation whole pre enriched content was added in to the 2X TP (Tryptone Phosphate broth) and incubated at 37 °C for 24 h for further selective enrichment.

Then 100 µl of enriched broth was spread plated onto MacConkey's agar (Hi Media Lab Pvt. Ltd.) plates as well as VRBA plates (Hi Media Lab. Pvt. Ltd.). Plates were then incubated for 16-18 h at 37 °C. Pink coloured colonies were observed on MacConkey's agar and colonies with pink to red colour were observed on Violet Red Bile Agar. These isolates were used for further studies. (screening of pathogens carried out with some modification as per Revised edit. 8th, US FDA, BAM), 2017).

Biochemical Confirmation of *E. coli* by IMVIC test:

Indole Test:

Some bacterial spp. produces Indole from tryptophan by action of enzyme typtophanase. Indole production in broth was detected by adding Kovac's reagent. Indole present in broth reacts with aldehyde in presence of reagent and gives red colour at the top of broth.

$$\text{Tryptone} \xrightarrow{\text{Tryptophanase}} \text{Indole} + NH_3 + H_20$$

Tryptone broth (TB) was inoculated with isolates and incubated for 18-24 h at 35 °C. Indole production in broth was tested by adding of Kovacs' reagent (0.3 mL). Presence of red colour in upper layer was observed for positive Indole test.

Methyl red (MR) Voges - Proskauer (VP) Test

Methyl red and Voges - Proskauer broth contains sugar glucose, peptone and phosphate buffer. Some organisms produce acids which lead to lowering of the pH. Methyl red was used as pH indicator as it is red in colour if pH is less than 4.4 and yellow if pH is more than 6. Those organisms which are MR negative they further metabolize initially produced fermentation products by decarboxylation and produce acetoin which decrease acidity of medium and pH of the medium increases towards neutrality (~ 6 or above). In presence of alkali and atmospheric oxygen the end product and acetoin oxidized to diacetyl which further react with creatine and produce red colour.

Inoculated tube of MR-VP broth was inoculated with the cells and incubated at 35 °C for 48 h. 1 mL of broth was transferred in the test tube and mixed using vortex after adding ±-naphthol solution (0.5 mL) and potassium hydroxide (0.2 mL 40 %) and vortexed. Few crystals of creatine were added and shaken for some time and allowed the solution to stand for 2 h. The positive result shows presence of pink colour in top of broth (Harley and Prescott, 1990).

MR-VP test:

Principle

Glucose $\longrightarrow$ Acetoin

Acetoin $\xrightarrow[\text{Atomospheric O2}]{\text{KOH}}$ Di - acetyl + water

Diacetyl + guanidine $\longrightarrow$ Alpha-naphthol Pink color
component of peptone (figure 2.2)

Citrate Utilization test:

In this test microorganisms use citrate as sole source of carbon. Sodium citrate is used as carbon source and bromo thymol blue is used as pH indicator dye. pH of medium becomes alkaline and pH sensitive bomothymol blue changes colour of medium from green to blue.

Citrate $\longrightarrow$ Oxaloacetate+ Acetate

Oxaloacetate $\longrightarrow$ Pyruvate+CO_2

CO_2+Na+H_2O $\longrightarrow$ Na_2CO_3

Isolated organisms were inoculated into Koser's Citrate medium slants and incubated for 48 h at 37 $^{\circ}$C. Upon citrate utilization turbidity increases and colour of media slant becomes blue from green (Brown, 2001).

2.3.2. Screening and isolation of *Salmonella* Typhimurium:

Vegetable samples (25 g) was weighed and added into a 225 mL Lactose broth mixed and incubated for 24 h at 37°C. The Pre enrichment was carried out by incubating at 35 ºC in Tetrathionate broth (TT) for 24 h. Further enrichment was carried out by inoculating 1 mL enriched broth TT (Tetrathionate) Broth and 0.1 mL in RV (Rappaport-Vassiliadis) broth. Further it was kept in water bath at 42 °C for overnight. After incubation the cells were streaked on XLDA (Xylose-Lysine Deoxycholate Agar) medium. Positive isolates give red colour colonies with black center because it contains sodium thiosulfate, and phenol red. On HEA (Hektoen Enteric Agar) agar isolates give blue-green colour with or without a black centre. On BSA agar Olive-black to black colonies with metallic sheen are seen if Salmonella spps are present. Biochemical confirmation is carried out by LIA (Lysine Iron Agar) test on which Salmonella positive culture produce H_2S in LIA media. Salmonella having typical ability to produces alkaline, turbid (purple) reaction in the butt of tube. Triple sugar iron test was carried out where growth of organisms showed media colour red (Alkaline slant and yellow acidic butt, without / with production of H_2S which leads blackening of agar. IMVIC test was also performed as mentioned above for *E. coli*. (USFDA, BAM)

2.3.3. Screening and isolation of *Staphylococcus aureus*

25 g sample was mixed with 225 mL Tryptic soya (TS) broth and incubated at 35 °C for 24 h, further sub cultured on BHI Broth and Tryptic Soy broth. Enriched media were streaked on Baird Parker with Agar and Egg Yolk Tellurite and incubated at 35 °C for 48 h. Staphylococcus spp. reduces tellurite to telluride. Presumptive *S. aureus* have ability to demonstrate lecithinase activity, which appear as black coloured colonies bordered by opaque zone, encircled by a zone of clearance that indicate due to lipase activity. Biochemical

confirmation was carried out by catalase test by emulsifying a bit of the growth on 2 % hydrogen peroxide. Bubbling and frothing is observed for confirmation of the isolates.

2.3.4. Screening and isolation of Shigella spp.

Enrichment was carried out by taking of 25 g sample in Trypticase soy-yeast extract (TSYE) *Shigella* broth (225 mL) containing novobiocin (0.5 µg/mL) was added. The suspension was held for 10 minutes at ambient temperature and shaken periodically. Adjusted the pH 7 and incubated at 42 °C for 20 h. Isolation was carried out by streaking enriched sample on MacConkey's agar. Observed colony's having colour slight pink and translucent, colonies without or with rough edges. On Hektoen enteric agar bluish-green colonies for *Shigella* spp. were expected. Further biochemical confirmation is carried out by inoculating presumptive colonies into the TSI agar slant, Glucose broth, , lysine decarboxylase and tryptone broth incubating at 35 °C for 48 h and examining after 20 h.

2.3.5. Screening and isolation of Listeria spp

Primary enrichment was carried out by adding sample (25 g) in to 225 mL *Listeria* Enrichment Medium (having nalidixic acid and acriflavin) and Incubated for at 35 °C for 48 h, aliquot (0.2 mL) of enriched broth was spread plated on Listeria Selective Agar plate. Further secondary enrichment carried out by simultaneously transferring 0.1 mL of enriched broth to 10 mL of fresh Listeria enrichment medium base with (nalidixic acid and acriflavin). Isolation was carried out by spreading 0.2 mL of enriched broth on Selective Agar and incubated at 35 °C for 24 h. PALCAM agar was used as selective medium and observed for grey green colonies on PALCAM agar. Presumptive isolates show brown red coloured colonies on Listeria selective agar. Further confirmation was carried out by ttransferring some colonies on Tryptone Soya Yeast Extract Agar- (TSYEA) and Incubating TSYE agar plates at 30 °C for 24 - 48 h. *Listeria* colony colour appear blue-gray to blue.

2.3.6. Serology of coliforms with LK 13 Himedia kit for *E. coli* O157: H7

Isolation of *E. Coli.*

Brilliant Green Bile Broth 2 % (BGB) tubes were inoculated with pink colonies from VRBA plates and incubated at 44.5 ± 0.5 °C. The tubes which produced acid and gas in Durham tube were examined by streaking loopful on VRBA and incubating at temperature 37 °C for 18-24 hours. Colonies were biochemically tested by inoculating TSI (Triple Sugar Iron) (tubes, Simmon's citrate, urea and Indole broths.

Isolation of *E. coli* O157:H7

MUG (4 methyl-umbelliferyl-^{2} -D-glucuronide) was added to the VRBA (Violet Red Bile Agar) with concentration of 100 mg/l before medium was prepared. Sample 25g was homogenized in 225 mL of peptone water diluents. Tenfold serial dilutions of the homogenate in peptone water diluents were further prepared. An aliquot of 0.1 mL each of dilution was transferred, in duplicate, onto the surface of VRBA agar with MUG (4-methylumbilliferyl-ß-gluconide) and spreaded uniformly across each of the plate. Plates were incubated at 35 ºC and colonies were counted after period of 18 h. After incubation period plates were exposed to UV light, colonies having colour purple red and surrounded by the zone of bile acids precipitation were examined.

Principle of the Test

Antibodies are raised against the lipopolysaccharide and latex particles are coated with O157 antigen of *E. coli* O157:H7. Upon mixing of latex particles sensitized with the suspension contains *E. coli* O157 antigens, a immunochemical reaction takes place and latex particles (fine, dispersed) agglutinated in to aggregates which easily become able to be seen to eyes.

Contents of Kit (Figure 2.1).

Test Latex Reagent *E. coli*: Latex particles which coated with rabbit antibodies which is reactive to *E. coli*

Control Latex Reagent *E. coli*: Latex particles which coated with rabbit antibodies which is non-reactive to the *E. coli* O157. Sample diluents (0.85 % Saline)

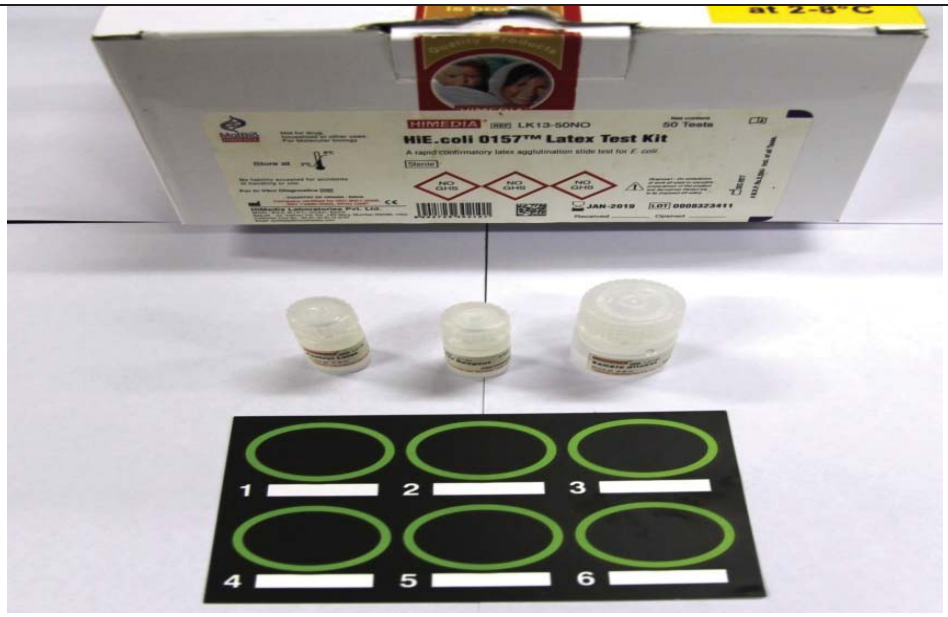


Fig. 2.1 Latex Kit LK13 Himedia Make used for serology

Source: from web site of Hi-media http://himedialabs.com/TD/LK13.pdf,

2.3.7. Screening of pathogens in leafy vegetables cultivated and sold in Mumbai

Sample collection:

Leafy vegetable samples were purchased from local markets in Mumbai namely *Coriandrum sativum* (coriander), *Mentha arvensis* (mint), and *Spinacia oleracea* (spinach). These three samples were obtained for each vegetable type from retail outlet as well as cultivated along the railway line. The samples were collected aseptically and refrigerated until analyzed within 24 h.

Details from where sample collected from Mumbai for screening of pathogens detailed in Table 2.1.

Table 2.1. Sample collection data table		
Location No. / Place code	**Type of sample**	**No. of replicates**
1	Spinach, Mint	3
2	Spinach, Coriander, Mint	3
3	Coriander, Mint	3
4	Mint	3
5#	Spinach	3
6#	Coriander	3
7*	Spinach	3
8*	Coriander	3
9*	Mint	5
10	Mint	4
11	Coriander	10
12	Coriander, spinach	5
13	Coriander, spinach, Mint	4
# Retail outlet hospital periphery, *Wholesale market		

2.4. Screening and confirmation of pathogens

2.4.1. The screening confirmation of coliforms (Standard method in BAM (Bacteriological Analysis Manual)

Upon arrival of sample in laboratory analysed within 72 h for the purpose of screening of coliforms. 25 g samples of vegetable leaves from the outer and internal side of were used. Further mixing carried out with the stomacher vigorously for the 2 minutes in the 225 mL of BHI broths. Whole content was incubated at 37 °C for 3 h. Upon enrichment the whole contents were added in of Tryptone Phosphate (2X) 250 mL broth. Further broth was incubated at 37 °C for 24 h. A loopful of suspension enriched broth was streaked on EMB plates and further incubated for 18 - 24 h at 37 °C. Colonies having dark centre and flat, without / with metallic sheen were used. From each plate five numbers of colonies were transferred to EMB plate. Further storage was done in Plate Count Agar for further use. Also, colonies on EMB and MacConkey's agars were streaked which was further incubated at 37 °C for 18 - 24 h. On basis of colony morphology putative isolates were categorized.

2.4.2. Screening and confirmation of *Salmonella* spp

25 g of vegetable sample was kept in stomacher along with 225 mL of Lactose broth (Hi Media make) and further incubated at 37 °C for 24 h. After pre-enriched some content was then transferred in to tubes containing 9 mL of the TT / tetrathionate (Hi Media make) and RV Rappaport Vassiliadis (Hi Media Laboratories Pvt. Ltd. Mumbai) both the broths were incubated in water bath at 42.5 °C. After incubation loopful suspension was streaked on the XLDA (xylose lysine deoxycholate) (Hi media Laboratories Pvt. Ltd., Mumbai) and BSA (bismuth sulphite) (Hi media Make) plates. Isolates were categorised presumptive *Salmonella*, on basis of colony morphology. Further screening as well as confirmation of the *Salmonella* spps was carried out by methods as described in to the FDA, (BAM).

2.4.3. Identification of pathogen specific gene by PCR in putative isolates of presumptive coliform and Salmonella isolates

A colony PCR was performed for amplification of specific genes for *Escherichia coli* and *Salmonella* Typhimurium. For presumptive coliforms, Universal Stress protein – A *(Usp A)* gene of *E. coli* was selected. Presumptive *Salmonella* screening Invasion-A *(Inv A)* gene of *Salmonella* was selected.

Usp A	F 5'CCGATACGCTGCCAATCAGT3' and R 5'ACG CAG ACC GTA GGC CAG AT 3',
Inv A	F 5'GTG AAA TTA TCG CCA CGT TCG GGC AA3' and R 5'TCA TCG CAC CGT CAA AGG AAC C3',

Coliform isolates which showed amplification of 16S rRNA gene were further used for amplification of pathogen specific *Usp-A* gene of *E. coli* and *Inv-A* (Invasion) gene of *Salmonella* Typhimurium.

Colony PCR conditions for *E. coli* was Initiation - 5 min, 94 °C, Denaturation - 94 °C, 45 Seconds, Annealing-55.2 °C for 10 seconds and Extension- 72 °C for 1 Min, 35 cycles, Product size 884 bp were detected on 1 % Agarose gel. (Godambe. *et al.,* 2017). Colony PCR for *Salmonella* Typhimurium was carried out by keeping following conditions: Initiation: 94 °C, 60 sec, Denaturation: 94 °C, 60 Seconds, Annealing: 64 °C for 30 Sec, Extension- 72 °C for 7 Min, 35 cycles, product size: 284 bp (Karmi *et al.,* 2013).

2.4.4. Electrophoresis (Agarose gel)

The DNA (amplified products) from the PCR was analyzed on agarose (1 %) gel and stained by ethidium bromide. 10 µL of each DNA amplified product was mixed with 2 µL of dye and then loaded onto the gel. 1 Kb step-up ladder was used as a marker; a current of 120 volts for 30 Min was passed through the electrophoresis tank.

2.5. Effect of combination treatment on pathogen isolated from leafy vegetables

Presumptive isolates of *E. coli* and *Salmonella* were analysed in this study. Single colony of the presumptive pathogen was inoculated in 20 mL of MacConkey's and LB broth and grown at 37 °C for overnight. Next day, one mL culture was centrifuged and washed with 0.85 % NaCl. Subsequently, about 4 log CFU/mL cells were treated with single treatment of NaOCl (200 ppm) or gamma (2 kGy) or combination treatment of NaOCl (200ppm) + gamma (2 kGy). After the treatment, the cells were serially diluted and dilutions plated on Luria agar plates, incubated at 37 °C for overnight. Next day, the count was taken and expressed as log CFU/g.

2.6. Process Optimization

Vegetables samples were minimally processed by removing soil and slightly cutting the damaged leaves and roots. Plant shoots were cleaned physically and washed initially with potable water for 5 minute and treatment designated or named as T1. Further these vegetable samples were treated with sodium hypochlorite solution having concentration 200 ppm for 5 minutes and treatment named or designated as T2. All these samples were dried in air for 30 min in open area and were packed in circular tray. For EB processing it was ensured that, the samples did not have total thickness more than 6 cm.

Sample treated with gamma radiation 2 kGy or electron beam radiation 2 kGy were named as T3 and T4, respectively. Cellulose based material soaked in water was used to maintain the moisture and whole tray along with vegetable and wet cellulose material at base was wrapped in PVC film.

2.6.1. **Irradiation of samples**

2.6.1.1. Gamma irradiation

Gamma radiation was delivered to fresh vegetables at the institute in a ^{60}Co based food package irradiator. The dose rate was 30 Gy/min and dose uniformity ratio (DUR) was observed to be 1.4 (D_{max} / D_{min}). ASTM, 2017, standard dosimetry was performed by ceric-cerous dosimetry.

2.6.1.2. Electron Beam (EB) irradiation

The EB (Electron Beam) processing of packaged vegetable samples was carried out at the e-beam facility at institute. Radiation dose (D_{min}= 2 kGy) was delivered to the samples. Dose delivery was pre-optimized using film dosimeters (Miller A. *et al.,* 1988)

2.6.2. Combination treatment with water at different purity level (Potable, Filter and Distilled) water

Combination treatments was developed which includes combination of washings and radiation processing. In the first combination, the vegetable samples were washed with water (T1) for 5 min and then were washed with sodium hypochlorite (200 ppm) for 5 min (T2). Later the samples were dried in air for 30 min, packed in Styrofoam trays with wet bed and irradiated at 2 kGy using gamma irradiation (T3). This combination treatment (T1 + T2 + T3) was designated as CP1. Similarly, in second combination treatment, the same process was followed except that the samples were irradiated at 2 kGy using electron beam (T4) instead of gamma. This combination treatment (T1 + T2 + T4) was designated as CP2.

2.6.3. To study effect of radiation on packaging material (polystyrene tray and cling film)

Approximately 1 g of each packaging material was accurately weighed and put into 250 mL capacity flask and to it 100 mL of MQ water was added. The flasks were sealed using aluminum foil. The small pieces of plastic films in the water were then irradiated with gamma radiation doses of 1 and 2 kGy. The water was then concentrated to the final volume of 1 mL. 20 µl of the concentrate was loaded on commercially prepared TLC (Thin Layer Chromatography) plate and the TLC plate was developed in a solvent system Butanol: Acetic acid: Water (6:2:1). Simultaneously, preparative Thin Layer Chromatography was used to separate the compounds migrated from packaging material to the water and the separated compounds were analyzed by GC / MS.

2.7. Physical analysis of processed and stored leafy vegetable samples.

2.7.1. Moisture content during storage

For measurement of water loss by dry wet basis carried out. Results were expressed as a percentage of weight loss (Chien *et al.*, 2007).

2.7.2. Colorimetry

Colour of the vegetables was measured by a colorimeter. Nine leaves of spinach were selected at random from each one packaged tray at different storage period for 15 days. Colour of the samples was measured by 'Minolta spectrophotometer' (Model: CM – 3600 d manufactured by: Konica- Minolta, Sensing Inc, Japan). Calibration of instrument were done with a black and white standard supplied by Konica Minolta and then used to find out the colour using the three coordinates, L – Lightness; a − green, + red; b − blue, + yellow. Reflectance was measured at three different positions of the leaf in the spectrum 360-780 nm

at the wavelength of 10 nm. Illuminant, observer, was used D65 lamp and 10° viewing angle were used as respectively (Pathare P. *et al.,* 2013)

2.7.3. Sensory analysis using 9-point hedonic scale

Leafy vegetable samples were evaluated by the panellist in various sessions during the storage period in a taste panel laboratory at the institute. Coded vegetable samples were served in trays (white) to the panel. Sensory analysis of all tests samples was carried out on 9-point hedonic test employed by sensory panel of 15 members. Panellists selected were having earlier experience of carrying out sensory analysis of food. On 9-point hedonic score 1 (dislike extremely) and score 9 indicate (like extremely) (Lopez - Rubira *et al.,* 2005). Parameters analysed were colour, taste, texture, aroma, and overall acceptability (Onyeoziri IO, *et al.,* 2018). To decide the visual acceptance of the samples at various storage points, packaging and irradiation processing treatment all the parameters evaluated were compared with freshly procured control samples on occasion of each day. The scores of each sample for each attribute were noted in tabulated form. For all attributes mean value was calculated. Panellist's opinion about the sensory analysis of the food and the significant difference were calculated by ANOVA (Analysis of Variance).

2.8. Biochemical Analysis

2.8.1. Determination of total soluble phenolics

Folin–Ciocalteu procedure was used for phenolic content estimation. The absorbance was measured at 725 nm in the Ultra violet visible spectrometer. Methanolic extract was diluted appropriately and added with 0.2 N Folin - Ciocalteu reagent and kept at ambient temperature at 32 °C for 5 minutes. Sodium bicarbonate solution (6 %) of 0.75 mL used for neutralization and incubated at ambient temperature 32 °C for 90 minutes. Absorbance was measured using a spectrophotometer at wavelength 725 nm. Calibration curve was plotted by using standard

Gallic acid. The results were expressed as GAE (mg Gallic Acid Equivalent / g). Distilled water was used as a blank, with a quartz cell (Nunzia C. *et al.,* 2009).

2.8.2. Chlorophyll content by Arnon method

Arnon (1949) method was used for chlorophyll content estimation. 1 g of sample was homogenized in mortar and pestle by using 80 % acetone. During the grinding, added pinch of calcium carbonate. Homogenate was centrifuged at 3000 rpm for 15 minutes and final solution was made up to 25 mL in acetone (80 %). Supernatant was transferred to a tube of colorimeter and the OD (Optical Density) was measured at 663 and 645 nm. 80% acetone as used as blank. Determined chlorophyll 'a' and 'b' by using Arnon equation given below:

Chlorophyll a (µ/g/ mL) = (12.7 x O.D. at 663 nm) – (2.69 x O.D. at 645 nm)

Chlorophyll b (µ/g/ mL) = (22.9 x O.D. at 645 nm) - (4.08 x O.D. at 663 nm)

Total chlorophyll (µ/g/ mL) = (20.2 x O.D. at 645 nm) + (8.02 x O.D. at 663 nm)

Chlorophyll content was expressed as mg chlorophyll / g of fresh weight of the sample.

2.8.3. Ash content estimation by method AOAC (19th Edition 930.05, 2012)

About 5 - 10g of the sample of each cultivar of spinach, coriander and mint was weighed precisely in tared platinum crucible. At 600 ºC temperature crucible was placed and heated in muffle furnace. Then cooled in desiccators and weighed. This process was repeated till the dilution between two consecutive weights was less than 1 %.

$$\text{Percentage of Ash vegetable sample (\%)} = \frac{\text{Weight of dry ash residues (g)}}{\text{Weight of vegetable sample (g)}}$$

2.8.4. Fat content estimation by (method IS: 12711 – 1989)

Procedure: Weighed accurately about 10 to 30 g of the material sufficient to give about 1.0 g of fat in a suitable thimble and dried for hours at 100 ± 2 °C. Placed the thimble in the Soxhlet extraction apparatus and extracted with the solvent for about 16 hours. The extract contained in the Soxhlet flask was dried the empty mass of the flask has been previously determined by taring at 100 °C for an hour. Cooled gradually in the desiccator and weighed. Continued the alternate drying and weighing at 30 min intervals until the loss in mass between two successive weighing is not more than 1 - 2 mg. Recorded the lowest mass.

$$\text{Fat, \% by mass} = \frac{100\,(M1 - M2)}{M} \times 100$$

M1 == weight in gram of soxhlet flask with extracted fat

M2 = weight in gram of the empty soxhlet flask clean and dry

M = weight in gram of sample taken for test

2.8.5. Carbohydrate estimation by method By Difference

The carbohydrate content in the sample was computed by determining difference among mass of total sample (100 g) and the summation of the values of protein, moisture, fat, ash and crude fibre (value per100 g of each) in three leafy vegetables spinach coriander and mint.

Calculations

Carbohydrate in gms/100g) = 100 g - (protein + moisture + fat + ash + crude fibre) g

2.8.6. Total Sugars estimation by method AOAC 19th Edition 923.09, 2012 and FSSAI

In the 250 mL conical flask 10 mL of Fehling's solution pipette and from the burette the whole solution of the standard dextrose added which required for the reduction of whole

copper. By adding dextrose solution ensured that, for completion of titration more than 1 mL solution will be required. Further this mixture was heated and 2 minute prior to boiling 1 mL methylene blue indicator solution added without interfering the boiling. When content in the flask begin to boil added standard dextrose solution from the burette till blue colour disappears. Dextrose factor was obtained by multiplying titration value to anhydrous dextrose (mg/mL) present in the standard dextrose solution.

Filtrate of 25 mL which containing reducing sugar in range of 50 to 200 mg was titrated with solution of fehling A & B for reducing sugar. For inversion sugar in 100 mL capacity volumetric flask added 50 mL deleaded solution and 10 mL HCl and kept at room temperature for 24 hours. Further by using concentrated NaOH Solution was neutralised and phenolphthalein was used as indicator. Further this solution was diluted up to 100 mL. Fehling A and B solution ued for titration and total sugar was determined. For calculation of an invert sugar calculated added sugar by subtracting reducing sugars from total sugars. Formula for reducing sugar and total sugar as follows:

$$\text{Total reducing sugar (\%)} = \frac{\text{mg. of invert sugar x final vol. made up x original volume x 100}}{\text{TR x Wt. of sample x aliquot taken for inversion x1000}}$$

$$\text{Reducing sugar (\%)} = \frac{\text{mg. of invert sugar x vol. made up x 100}}{\text{TR x Wt. of sample x 1000}}$$

Total sugar (sucrose) % = (Total reducing sugar – Reducing sugar) x 0.95 + Reducing sugar

2.8.7. Energy (by Calculation method)

Total energy was calculated as the protein value multiplied by 4.0, fat value multiplied by 9.0 and total carbohydrate value multiplied by 4.0. Summing up of the values carried out. This values was expressed or reported KCal /100g on dry weight basis

2.8.8. Protein estimation by method IS: 7219-1973 (Reaffirmed 2010)

Micro kjeldhal method was used for protein estimation by using the factor 6.25 which is used for conversion of nitrogen content to protein. Protein results were expressed in decimal points.

In the block digestion apparatus the portion of the sample was digested with a mixture containing potassium sulphate, concentrated sulphuric acid, sulfate (copper (II)) which act as catalyst. This digestion converts organic nitrogen existed in the sample to ammonium sulfate. Further sodium hydroxide was added to the cooled digest for liberation of ammonia. By semiautomatic steam distillation unit steam distillation of ammonia was carried out from the digest and excess ammonia trapped in the boric acid solution. Further titration of boric acid carried out with hydrochloric acid.

In the digestion tube added K_2SO_4, 12 g, copper sulfate solution 1.0 mL, sample 5 g, and added 20 mL of concentrated H_2SO_4. Mixed the contents and digestion block was set at low temperature (180 to 230 °C) for 30 min. Further temperature (410 to 430 °C) was increased, till digest is clear. By setting distillation unit ensured that it will dispense 55 mL of sodium hydroxide solution. Boric acid solution was used for the titration with standard 0.1 hydrochloric acid solution to trace pink colour. Accurate burette reading was taken nearest to 0.05 mL.

Formula for nitrogen content (percentage by mass) calculation

$$Wn = \frac{1.4007 \times (Vs - VB) \times N}{W}$$

Wn = Nitrogen content (% by mass) in sample
VS = volume of standard HCl in mL (used for sample)
VB = volume of standard HCl in mL (used for blank test)
N = Normality of HCl (four decimal places)
W = weight of test portion (g), nearby 0.1 mg.

2.8.9. Niacin estimation by method IS 5400-1969 (Reaffirmed 2010)

Principle – Reaction of cyanogen bromide and Nicotinic acid gives a pyridinium compound. This pyridinium undergoes further rearrangement and yields derivatives which couple with aromatic amines (analine) and which form coloured compounds. This coloured compound was estimated photometrically.

Procedure: 1 g of sample was grounded in 30 mL of 4N H_2SO_4, further in this mixture steam was passed for 30 min. Transferred to a 50 mL flask containing distilled water. Then the solution was filtered and added 5 mL of lead acetate (60 %) solution. By usigsodium hydroxide pH was adjusted to 9.5. Centrifugation was carried out; supernatant was collected and added 2.0 mL of concentrated H_2SO_4. The solution was kept stable up to one hour, at room temperature. After that centrifugation was carried out precipitate of lead sulphate was removed and 5 mL of zinc sulphate (40 %) was added. pH 8.4 was adjusted with 10 N NaOH. Repeatedly once again, supernatant was collected and pH was adjusted to neutral, that solution turned colour just green.

2.8.10. Vitamin C estimation by method IS 5838-1970 (Reaffirmed 2005)

5 gram sample was grounded in the mortar (acid wash, sand by TCA reagent) further transferred in the 100 mL flask. Then the mixture was thoroughly mixed and final volume 100 mL made with TCA reagent. With the help of filter paper solution was filtered.

Determination of Vitamin C

Filtrate (10 mL) was titrated immediately with the indophenol solution. Blank determination was carried by using 11 mL of the reagent by using water to make volume of the 15 mL and the indophenol's solution volume necessary for direct titration.

Vitamin C calculation formula:

$$\text{Vitamin C mg per 100 g} = \frac{A \times B \times 1000}{W}$$

A = volume indophenols solution in mL used for the titration

B = ascorbic acid weight in mg equivalent to 1 mL solution of the indo phenol

W = weight in g (solution used for test)

Formula Source: Bureau of Indian standards

2.8.11. Vitamin A estimation by method IS 5886-1970 (Reaffirmed 2010)

Principle: The vitamin A is extracted from the assay sample, purified by saponification or chromatography and dissolved in optically pure isopropanol, hexane or cyclo hexane. This method is based on OD measurement of light absorption of the vitamin solution. This light absorption is directly proportional to concentration of the vitamin at a. wavelength of 325 nm where maximum absorption occurs.

Weighed accurately a quantity of the material containing 30 to 50 IU of vitamin A and transferred the material quantitatively to the saponification flask (150 mL) and added 30 mL of ethanol (95 %) and five mL solution of potassium hydroxide.

Electrically water bath heated for 30 to 40 min. Cooled and further added 30 mL of water. Transferred the content quantitatively to a separating funnel and washed the flask twice with 10 mL of the water and added the washings to separating funnel.

Extracted the saponified solution thrice, respectively with 50 mL, 20 mL and 20 mL ether; combine the ether extracted in another separating funnel and washed this with 50 mL ice cold water containing a little sodium acid sulphate or potassium acid phosphate.

Transferred the ethereal extract to a stoppered measuring cylinder (100 mL) rinsed the separating funnel with a little ether and then added this to the cylinder. Further ether was added to make the volume to 100 mL mark. Then added 15 g of anhydrous sodium sulphate; mildly shaken and then kept it to settle in a dark cool place.

Residue was taken in the 5 mL of petroleum ether. Placed a wad of fat-free cotton in the lower stem of the chrormatographic tube and set it up in a stand. Poured in petroleum ether up to the middle of the 10 mm section then added sufficient alumina absorbent, with continuous tapping, to a height of 100 mm in the column; then added anhydrous sodium sulphate to make a layer of 1 cm. Attached the separator funnel at the top and apply pressure to drained off excess solvent, but taking particular care that at no time, the level of solvent falls below the alumina surface in the column. Released the pressure, removed the funnel and transferred the petroleum ether extract of the unsaponifiable matter quantitatively to the column, using more petroleum ether. This washing would contain carotene fraction, which may be collected and estimated. Replaced the receiver and gradually added 30 mL of the petroleum ether-diethyl ether elution mixture of appropriate composition, determined by a pilot test. Eluted carefully and preserved for estimation.

Evaporated the ethereal extract of the unsaponifiable residue or the eluted from the chromatographic procedure, using moderate heat and in a dark place away from light in the stream of the nitrogen. Taken up residue in sufficient isopropanol or hexane to given the concentration, expected to yield absorbance reading of 0.2 to 0.5 at 325 nm. Absorbance was determined as (E) of this solution at 334, 325, 310 and nm in the spectrophotometer.

a. Determination of the corrected absorbance at 325 mμ per 100 g of the sample

$$= \frac{E_{325}\ (\text{corrected}) = 6.815E_{325}-2.555E_{310}-4.260E_{334}}{W}$$

b. Vitamin A content in I.U. per 100 g of sample

$$= \frac{E_{325}\ (\text{corrected0} \times 1830 \times 100}{L \times C}$$

L= length of light path in absorption cell in cm

C = amount of assay sample in g per 100 ml of isopropyl alcohol solution

Formula source: Bureau of Indian Standards, archive

2.8.12. Riboflavin estimation by method IS 5399-1969

Riboflavin phosphate and flavin adenine dinucleotide reveal the same feature as colour yellow and fluorescence yellow green as riboflavin. Upon the expose to the light (440 to 500 nm), riboflavin gives fluoresces. Concentration of riboflavin is proportional to the intensity of the fluorescence in diluted solutions. The amount of riboflavin is estimated by calculating difference between the fluorescence (before and after) due to chemical reduction. His chemical reduction carried out by hydrosulphite. This hydrosulphite will reduce the riboflavin and their co-enzymes towards colour less compounds. These colourless compounds do not fluoresce.

Procedure: Taken two reaction vessels and filled with sample solution of 10 mL in each tube. One of the tube, filled with one mL of the standard riboflavin solution. Another tube filled with one millilitre of water and thoroughly mixed at vortex. Further both of the tubes filled with 1 mL of acetic acid and mixed thoroughly. Further 0.5 mL of potassium permanganate solution was added and mixed. Solution kept stable at room temperature for the 2 minutes.

Both the tubes filled with O.5 mL of the hydrogen peroxide solution and mixed. Permanganate colour formed which gets disappear within ten seconds. By using the fluorometer, fluorescence was measured. The tube of sample solution which containing, one millilitre of the standard riboflavin called as reading A. Another tube containing one mL of water called as reading B. Added to in the same tube by mixing, 20 mg of sodium hydrosulphite. Within the 5 sec fluorescence was measured and called as reading C.

Milli gram of riboflavin per ml of the final sample solution =

$$= \frac{B - C}{A - B} \times \frac{1}{10} \times \frac{1}{1000}$$

(Value of the $\frac{B - C}{A - B}$ should not be d 0.66 and e 1.5).

Result was expressed as riboflavin per 100 g.

(Bureau of Indian standards, https://archive.org/details/gov.in.is.5399.1969/page/n3

2.8.13. Analysis of flavonoids extracted from coriander and spinach and phenolic acid from mint.

2.8.13.1. Extraction of sample for flavanoids (quercetin and kaempferol)

40 g of spinach and coriander leaves were finely chopped and grounded in liquid nitrogen. The extraction was carried out three times in 80% (100 mL) methanol. After centrifugation at10000g for 10 Minutes supernatant was collected and concentrated in a flash evaporator. The process flash evaporator continued till all the traces of methanol were removed. Further this aqueous portion was extracted three times with hexane (100 mL). Finally extraction was done twice with ethyl acetate three times. Ethyl acetate phases were pooled together and it washed thrice with water. The obtained aqueous phase was treated with 1 % HCL for

hydrolysis. Hydrolysis of glycoside bound flavonoids was carried out by incubating sample in water bath for 1h at 70 °C. Flavonoids were extracted in ethyl acetate and further concentrated in methanol. 1 % solution of flavanoid was used for identification and quantification.

2.8.13.2. TLC of extracted sample

Spotting was done on commercially available TLC plates (Merck make) in sequentially as A (Un-hydrolysed 10 µL), B (Hydrolysed): spot 1 (10 µL), spot 2 (30 µL), and spot 3 (Standard quercetin 10 µL). Samples (10 µL) were also spotted on to the TLC plate (20 cm X 20 cm). Air drying of all spots was carried out. Solvent system consisting of toluene: ethylformate: formic acid in the ratio of 5:4:1 respectively. When TLC plates were exposed to iodine fumes in closed chamber, spots were visualized. The standard quercetin (2 µg) and kaempferol (2 µg) were spotted on same plate along with sample for comparison and identification of flavanoids.

2.8.13.3. Preparation of standards

Standard Stock solutions were prepared by weighing 10 mg of kaempferol, quercetin and gallic acid standards into separate volumetric flasks. Reference standards were dissolved in methanol by sonication. Working standard solutions 10 to 100 µg/mL was prepared using dilution from the stock standard solutions in methanol. All solutions were stored at 4 °C.

2.8.13.4. Quercetin and kaempferol analysis by HPLC

HPLC (DIONEX make) equipped with C18 analytical column was used. A doble beam wavelength Ultra Violet detector was set at 350 nm. Initially the HPLC column was pre-treated with methanol (HPLC grade). For the mobile phase Solvent A: Orthophosphoric acid (1.5%); Solvent B: Acetonitrile 20%, Glacial acetic acid 24%, O-phosphoric acid 1.5%, water 54.5%. Gradient conditions were set as reported earlier (Hussain *et al.,* 2013). The flow rate was 1.2 mL/min. The gradient flow used was 0-5 min: 100% A; 5-10-min: 100 % A to 80 %

A; 10-40 min: 80 % A 62 % A; 40- 45 min: 62% A to 0 % A for 45 minutes. Standard curves were constructed using quercetin (0.01 to 0.05 mg/mL) and kaempferol (0.001 to 0.003 mg/mL). The concentration of quercetin and kaempferol in vegetable samples was calculated using the standard curve.

2.8. 13.5. Extraction Procedure for rosmarinic acid: About 0.5 g of sample was finely ground using liquid nitrogen and extracted in 80 % methanol for 10 minutes, later, the homogenate was centrifuged at 10000 RPM. Supernatant was collected and used for HPLC analysis.

2.8.13.6. Rosmarinic acid analysis by HPLC

Rosmarinic acid was analysed by HPLC at 330 nm. Prior the sample run, the HPLC column was pre-treated with methanol (HPLC grade). The analysis was carried out by using an elution at the flow rate of 1.0 mL/ min. The mobile phase used was solvent A: Orthophosphoric acid (0.1 %) in water (*v/v*); Solvent B: Orthophosphoric acid (0.1 %) in the methanol water (*v/v*). Flow of gradient was used 0-10 min, linear gradient was from 40 to 50 % of B; linear gradient from 50 – 60 % of B for 10-15 min; maintained at 60 % B, for 25 min. one mL/min flow rate was maintained. The chromatographic peak of rosmarinic acid was confirmed by comparing their RT (Retention Time) of 8 min in the solvent system as reported by Wang *et al.,* 2004. A standard curve was plotted using the gallic acid concentration in the range (0.005 to 0.020 mg/mL) and its content as expressed equivalent to gallic acid.

2.9. Functional analysis

Measurement of antimutagenic potential Antimutagenicity rif R

Antimutagenicity assay was carried out by using *E. coli* (MG1655) culture. The culture was diluted in 25 mL Luria broth (LB) and incubated at 37 °C at 150 rpm for 3h (Mid log phase).

Further, the enriched culture was kept on ice for 15 minutes and centrifuged (7000X g for 15 min). Further pellet was washed twice with 10 mL Luria broth and cells were re-suspended in 25 mL of the Luria broth. The cells were then incubated separately with 1% extract of spinach and coriander for 15 min. An aliquot of cell suspension (2 mL) was incubated at 37 °C on a rotary shaker (150 rpm) for 45 min with EMS having concentration 133 mM. On basis of earlier studies concentration of EMS has been used as an effective concentration for the assay. Sterile distilled water was used as blank instead of vegetable extract. After mutagen (EMS) treatment cells were centrifuged and washed twice with Luria broth (2 mL) and suspended in 2 mL of Luria broth. From this 50 µL cells were inoculated in fresh Luria broth and were incubated for the overnight at 37 °C on rotary shaker at 150 rpm. After incubation next day proper dilution of culture was plated by spread plate technique on Luria agar plates containing rifampicin (100 µg/mL) and also plain Luria agar plates and incubated at 37 °C for 24 hours. The total viable count was counted from Luria agar plates while Rif^R mutants were estimated by taking the count from Luria agar plates with rifampicin. Frequency of mutation was depicted by the ratio of the total number of Rifampicin resistant mutants per millilitre to the total viable cells per millilitre of culture. Cell suspension which incubated without mutagen gave spontaneous mutation frequency (Hajare S. N. *et al.,* 2014; Cupples, C. G., *et al.,* 1989).

Statistical analysis

Means as well as standard deviations were calculated for each set of experimets. For each set of experiment at least three replicates were used. Representative sets of data have been presented in the thesis. The Origin 6.1 version v6.1052 (B232) of Origin Lab Corp., Northampton, Mass., U.S.A.) was used for data analysis. One-way analysis of variance (ANOVA) at 0.05 level of significance was used for establishing statistical significance.

CHAPTER - 3

RESULTS AND DISCUSSION

CHAPTER - 3.1

MICROBIOLOGICAL PROFILE

OF

LEAFY GREENS COLLECTED

FROM

FARMS AND URBAN SOURCES

Green leafy vegetables have been designated as Natures wonders because they are rich sources of phytochemicals which provide advantageous health protecting properties. They are good sources of various vitamins and minerals which provide a range of micronutrients in a single served bowl. Green leafy vegetables are also a rich source of bioactive compounds like caroteniods, flavonoids and other non-flavonoid phenolic compounds (Gupta and Prakash, 2009). Green leafy vegetables have short shelf life due to their higher moisture content. Attention has been devoted to the commonly available green leafy vegetables, which though underexploited in most cases, possess a tremendous potential to help people overcome the life-threatening diseases (Subhasree et al., 2009).

The selected plants for this study are being used for consumption by the general population in India, since ancient time namely Spinach (*Spinacia oleracea*), Coriander (*Coriandrum sativum*) and mint (*Mentha arvensis*). The study aims to microbiological analysis of leafy agri-produce consumed raw or after partial cooking such of leafy vegetables, screening and characterization of pathogenic organisms, e.g. *E. coli, Salmonella, Shigella, Listeria, and Staphylococcus* spp in these produces.

Study also focuses on the standardization of process parameters for radiation (Gamma and EB) treatment of these agri-produce and also development of appropriate packaging and storage conditions for their shelf life extension. Analysis of biochemical, nutritional and organoleptic properties of control and processed agri-produce was also carried out. A functional property like antimutagenicity has also been evaluated for these leafy vegetables.

3.1.1. Fresh leafy vegetables selected for study found heavily burdened with microorganisms

Vegetables were procured yearly in between month of July to September from Nashik region of Maharashtra since 2012. Total 07 lots of vegetables were procured from farm in different years. One lot of vegetable weighing ~ 05 kg was transported on same day from farm to

laboratory and kept at low temperature till analysis. Sample of 25 g in triplicate was used for analysis. Initial study was carried out for microbial analysis of freshly procured leafy vegetables. Microbial profile data of one representative lot of fresh vegetables has been shown in Table 3.1.

Microbial load in fresh Spinach (*Spinacia oleracea*)

Total aerobic plate plate (TAPC) was observed to be 7.51 log CFU/g. Yeast and mould count (YMC), and presumptive coliforms (PC) counts were 5.69 log CFU/g, and 5.78 log CFU/g, respectively. Surprisingly all the batches of spinach were found to have PC (Table 3.1). Anaerobic bacteria and spore counts were found to be nil. TAPC, YMC and PC counts ranged between 6 to 8 log CFU/g, 5 to 6 log CFU/g and 4 to 6 log CFU/g, respectively.

Microbial load in fresh Coriander (*Coriandrum sativum*)

Total aerobic plate count in the fresh coriander was observed to be 7.59 log CFU/g. Yeast and mould count was 5.50 log CFU/g and presumptive coliforms was 4.70 log CFU/g. Like spinach here also PC was found in all the batches (Table - 3.1). Anaerobic bacteria and spore counts were found to be nil. The range of TAPC, YMC and PC in different batches was like spinach.

Microbial load in fresh Mint (*Mentha arvensis*)

Total aerobic plate count in the fresh mint was observed to be 7.15 log CFU/g. Yeast and mould count in fresh mint was 5.39 log CFU/g and presumptive coliform was 5.62 log CFU/g. Like spinach and coriander here also PC was found in all the batches (Table 3.1). Anaerobic bacteria and spore counts were found to be nil. The range of TAPC, YMC and PC in different batches was like other two aforesaid leafy greens.

Table No. 3.1. Microbial profile of fresh spinach, coriander and mint			
Vegetable	**TAPC (log CFU/g)**	**YMC (log CFU/g)**	**PC (log CFU/g)**
Spinach	7.51 ± 0.45^{a} (Range 6 to 8)	5.69 ± 0.88^{x} (5 to 6)	5.78 ± 0.43^{l} (4 to 6)
Coriander	7.59 ± 0.54^{a} (6 to 8)	5.50 ± 0.54^{x} (5 to 6)	4.70 ± 0.43^{m} (4 to 6)
Mint	7.15 ± 0.92^{a}	5.39 ± 0.49^{x}	5.62 ± 0.19^{n}

Different superscript letters across columns indicate significant difference among mean (p d0.05).

The mean faecal coliform values of all the three vegetable samples exceed the ICMSF (International Commission on Microbiological Specification for Foods) recommended level of 10 fecal coliform per gram of fresh weights. This may be due to quality of water/ manure used in field for cultivation of these vegetables.

Study carried out by Amoah *et al.,* (2005) had also shown that, sources of fecal coliform contamination of lettuce may include overhead irrigation with already contaminated water, planting in contaminated soils and frequent application of poultry manure which was not well composted. Food safety is a major public health concern worldwide. During the last decades, the increasing concern on food safety has initiated research regarding the risks associated with consumption of food stuffs contaminated with pathogenic microorganism. Several studies have revealed that contamination of vegetables with pathogens poses a threat for consumers (D'Mello, et al., 2003; Zandstra and De Kryger, et al., 2007; Heaton and Jones, 2008).

Generally, variation in the TAPC, YMC and PC values of the present study and previous works may be either due to differences in the geographical location of the cultivation area, or due to the differences in the contamination load at different sections of the drainage canal and different pre-harvest handling practices. As the presence of coliforms is indicator of fecal contamination. Most of the researchers focus on pathogenic bacteria in leafy vegetables. Leff JW, Fierer N (2013) have shown overall diversity of bacterial communities which are produce specific. Having comprehensive data on these bacterial communities in leafy vegetables give fair idea about potential risk of exposure of consumer to various bacterial communities. Leff, J. W., Fierer N. (2013) addressed that 16 S rRNA sequencing of various samples (spinach, lettuce and sprout) which were brought from various locations have shown contaminations with organisms from family Enterobacteriaceae.

Minimally processed vegetables too are vulnerable to microorganism such as saprophytic, pectinolytic and lactic acid bacteria and every stage of processing there is chances of contamination (Christophe Nguyen *et al.,* 2009). Compared to fruits, bacteria in vegetables were found to be more frequent because they are soil growing as fruits are tree borne. Different varieties of bacteria occur in different crops; however, bacteria appeared heavily burdened in one field may rare in others. Study carried out by Serkan Kemal Buyukunal *et al.,* (2015) dealing with screening of 261 supermarket samples in Turkey shows Aerobic Mesophillic Count in the range of 3 - 4 log 10 CFU/g and Aerobic Psychrotrophic count in the range of 0 - 3 log CFU/g. The highest counts of coliforms were found in carrot, spinach, green leaf lettuce, lettuce and iceberg lettuce.

3.1.2. **Occurrence of pathogens in farm fresh leafy vegetables is not very common**

All the above said seven numbers of lots of fresh leafy vegetables (spinach, coriander and mint) was also screened for the presence of pathogens like *Escherichi coli*, *Salmonella* Typhimurium, Shigella spps, Staphylococcus spps and Listeria Spp.

In initial screening presumptive coliforms spps were observed in the all these lots and presumptive *Salmonella* were found only in the two lots. However, *Shigella*, *Staphylococcus aureus* and *Listeria* spps were not found in any of the farm fresh sample lots procured from Nashik region (Table 3.2, figure 3.2). Presumptive pthogens were subjected for confirmation.

Presumptive isolates of salmonella and coliform spp. Frm farm fresh samples was not confirmed by biochemical analysis

For biochemical confirmation IMVIC, TSI and LIA test was carried out. All the presumptive isolates of coliform and Salmonella were not shown IMVIC test positive for *E. coli*. and *Salmonella* Typhimurium (Table 3.3). Presumptive isolates of *Salmonella* spp. also showed

negative results for TSI and LIA test. Thus presumptive isolates from farm fresh vegetable samples was not confirmed to be *E. coli*. and *Salmonella* Typhimurium.

Table 3.2 Farm fresh vegetable samples from Nashik region with presumptive coliforms and presumptive Salmonella species								
Presumptive Pathogen	Lot'	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7
Presumptive Coliform spps.	Spinach	+	+	+	+	+	+	+
	Coriander	+	+	+	+	+	+	+
	Mint	+	+	+	+	+	+	+
Presumptive Salmonella Spps.	Spinach	-	+	-	-	+	-	-
	Coriander	-	-	-	-	-	-	-
	Mint	-	-	-	-	-	-	-

Table – 3.3 IMVIC results for Presumptive isolate from farm fresh vegetable samples

Test' Presumptive isolate "	Indole	MR	VP	Citrate utilization
Presumptive coliform 1	-	-	-	+
Presumptive coliform 2	-	-	-	+
Presumptive coliform 3	-	-	-	+
Presumptive coliform 4	-	-	-	+
Presumptive coliform 5	-	-	-	+
Presumptive coliform 6	-	-	-	+
Presumptive coliform 7	-	-	-	+
Presumptive Salmonella 1	-	-	+	-
Presumptive Salmonella 2	-	-	+	-

As per BAM (Bacteriological Analysis Manual) expected positive results are: *E. coli.* (+ + - -) and *Salmonella* Typhimurium (- + - +)

3.1.3. Leafy greens from urban sources indicated random existence of pathogens

Presumptive *Salmonella* and presumptive coliforms were occurred in all the lots of vegetable samples (Table 3.4). Out of all these presumptive isolates only five isolates (three from spinach and two from coriander) were confirmed as *E. coli.* Confirmation was done by amplifying *Usp-A* gene specific to *E. coli* (Table 3.5). However, presumptive S*almonella* isolates does not shown amplification of *Inv-A* gene. Thus among all these lots from urban sources none of the leafy vegetable samples showed presence of *Salmonella* species.

***E. coli* specific *uspA* gene amplification**: Amongst of all these isolates only three isolates from spinach samples which was collected from locality having code No. 5) and two samples of coriander collected from locality having code No. 6 (Table 3.4) showed amplification of *usp-A* gene (884 bp) which were confirmed the isolates to be *E. coli* (Fig. 3.3).

Further these *E. coli* isolates were tested serologically by using LK13 *E. coli* O157™ Test kit (Hi-media). None of the 5 isolates show agglutination reaction indicating absence of *E.coli* O157 (Table 3.6 and 3.7). Thus *E. coli* isolates screened from vegetables shown amplification to *E. coli* specific *uspA* gene indicating faecal contamination could have occurred. Thus, this is an issue of safety concern.

Fig. 3.1 Cultivation of green vegetables in urban

Fig 3.2 Farm view from Nashik region

Table 3.4 Sample collection data table		
Location No. / Place code	**Type of sample**	**No. of replicates**
1	Spinach, Mint	3
2	Spinach, Coriander, Mint	3
3	Coriander, Mint	3
4	Mint	3
5#	Spinach	3
6#	Coriander	3
7*	Spinach	3
8*	Coriander	3
9*	Mint	5
10	Mint	4
11	Coriander	10
12	Coriander, spinach	5
13	Coriander, spinach, Mint	4
# Retail outlet hospital periphery *Wholesale market		

Table 3.5. Primer details for the genes used for identification of the putative bacterial pathogenic isolates				
Gene	**Primer name**	**Direction**	**Sequence**	**Annealing Temp. (°C)**
Inv-A (S. Typhi.)	S139	Forward	5’ GTG AAA TTA TCG CCA CGT TCG GGC AA 3’	**65**
	S141	Reverse	5’ TCA TCG CAC CGT CAA AGG AAC C 3’	**63**
Usp-A (E. coli)	uspAF	Forward	5’ CCG ATA CGC TGC CAAT CAG T 3’	**62**
	uspAR	Reverse	5’ ACG CAG ACC GTA GGC CAG AT 3’	**65**

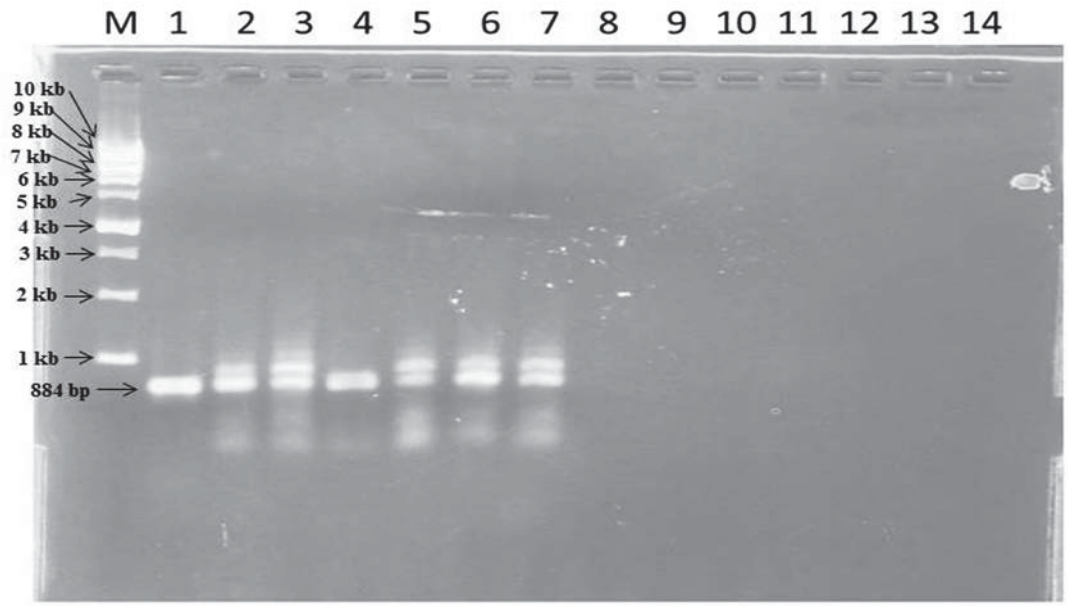


Fig. 3.3 Amplification of specific gene *usp A* in presumptive *E. coli* isolates in leafy vegetable samples:

PCR amplification (884 bp) of usp A gene in presumptive *E. coli* isolates in leafy vegetable samples. M- 1 kb step up ladder Marker; 1 & 2- Positive controls; 3-5 Spinach, from location No. 5; 6- 7 Coriander from location No. 6; 8-10 Spinach from location No. 7; 11- 13 Coriander from location No. 8; 14- Mint from location No. 9.

Table 3.6 Observation protocol for serology:

Test latex	**Control latex**	**Interpretation**
+	-	*E. coli* 0157 is present
-	-	*E. coli* 0157 is absent
-	+	Non-specific agglutination
+	+	Inconclusive result

Source: http://himedialabs.com/TD/LK13.pdf

Table 3.7 Identification of *E. coli* O157			
Samples No.	Test latex	Control latex	Interpretation
1	-	-	*E. coli* O157 is absent
2	-	-	*E. coli* O157 is absent
3	-	-	*E. coli* O157 is absent
4	-	-	*E. coli* O157 is absent
5	-	-	*E. coli* O157 is absent

The similar results were reported by Leonal *et al.,* (2018) where he screened 60 samples of coriander leaves for salmonella detection by PCR technique. Out of these 04 isolates of biofilm producing *Salmonella* spps was confirmed. Isolated salmonella spps tested for antibiotic resistance shown 92 % sensitivity. Nelly Denis *et al.,* 2016 carried out the survey of wide range of produce available in the market. Most of the samples shown >100 CFU/g for coliform but pathogen remain below detection level. Salmonella spps and *Listeria monocytogens* were found in the leafy herbs. Soderqvist K *et al.,* (2019) used Quantitative microbial risk assessment approach to detect risk of shiga toxin producing *E. coli* per serving of leafy vegetables. They found that 87 % lower probability of infection when consuming rocket compared to spinach in autumn season. They also concluded that there are less chances of STEC infection before harvest of leafy vegetables, however once harvested and processing done chances of contamination were more.

Heredia N, *et al.,* (2016) collected fresh produce samples (636 Numbers) from Northern Mexico during 2011 and 2012 shown that presence of microbial contamination in produce is farm and production step specific which gives information for design approach for prevent contamination. During the study they found only pathogenic salmonella with lowest detection limits: 0.0033CFU/mL, 0.0013CFU/mL and 0.04 CFU/g in fresh produce, water and soil respectively. Study carried out by Johnston L M, *et al.,* 2005 showed leafy greens mean indicator ranged between 4.5 to 6.2, 1 to 4.3 and 1 to 1.5 log CFU/g in case of the aerobic plate count, coliforms as well as Enterococcus and *E. coli* respectively. Study demonstrated that all steps of production to consumption may affect microbial load of produce. Rodriguez, *et al.,* (2006) in his study on prevalence of *salmonella* in farm samples shown that, samples collected from, North Carolina, Alabama, Tennessee, Washington and California when analyzed by USFDA using BAM method resulted 4.7 % salmonella serovar on all these samples, and majority of isolates (57%) was from the swine farm. Salmonella is carried by

both domesticated and wild animals and can contaminate freshwater by direct or indirect contact. In some cases, direct contact of produce or seeds with contaminated manure or animal wastes can lead to contaminated crops. (Christophe *et al.,* 2016).

Our results are similar to the above results and the study supported the presence of bacterial pathogens in the leafy vegetables, which could be because of use of contaminated water during irrigation and washing of produce. These possibly may be associated in a large number of outbreaks. Thus, the fresh produce (processed fruit and vegetables) continues to be the main source of food borne illness outbreaks implicating pathogens such as *Escherichia coli* O157:H7, Salmonella, *Listeria monocytogenes* and human parasites (e.g. hepatitis A, Cyclospora). Previously, outbreaks were primarily limited to leafy greens, tomatoes, and cantaloupes, but more recently there has been a trend of more diverse produce types (e.g. cucumbers and papayas) being implicated (Larry, 1996).

CHAPTER - 3.2

OPTIMIZATION OF COMBINATION PROCESS

FOR

HYGIENIZATION & SHELF LIFE EXTENSION

OF

LEAFY GREENS

3.2.1. Water wash of fresh leafy greens improved their microbial hygiene

Further studies were performed to optimize a combination process to reduce microbial load within the permissible limit and presumptive coliforms to below detectable level. Minimal processing of spinach, coriander and mint samples was carried out by trimming and removing the partial root portion. First step of combination was decided to be washing with water. For this water at three purity level (potable, filtered and distilled) were used.

Effect of water wash on spinach, coriander and mint improve the microbial quality of produce

In spinach samples washed with potable water TAPC, YMC and PC were reduced from 7.51 to 6.72, 5.69 to 4.60, and 5.78 to 4.76 log CFU/g, respectively. Findings indicated that, around one log count reduction in the microbial load was achieved after washing the fresh produce with potable water (Table 3.9).

In coriander samples washed with potable water TAPC, YMC and PC were reduced from 7.59 to 6.76, 5.50 to 4.77, and 4.70 to 3.71 log CFU/g, respectively. Findings indicated that, around one log count reduction in the microbial load was achieved after washing the fresh produce with potable water.

In mint samples washed with potable water TAPC, YMC and PC were reduced from 7.15 to 6.90, 5.39 to 4.27, and 5.62 to 4.60 log CFU/g, respectively. Findings indicated that, around one log count reduction in the microbial load was achieved after washing the fresh produce with potable water.

Almost similar results were obtained with filtered and distilled water too. Thus, the study carried out by using three different type of waters in combination process resulted that potable water is can be used in combination treatment as alternate to distilled and filtered water to make the process cost effective. Therefore, hence forth discussed data deals with

potable water washed samples only and the treatment was termed as T1.

Seong gi Hong *et al.,* (2010) designed a system that ensures leafy vegetables packed in boxes were continuously passed through the washing and dehydration stages. After washing and dehydration showed that optimum time using air bubble for treatment of lettuce and perila was five minutes and in case of Chinese cabbage was 10 minutes. However, by applying air bubble treatment percentage of bacteria removed after washing were 76, 98 and 94 for Chinese cabbage, perilla and lettuce respectively (Siddig *et al.,* 2013; Luchesi *et al.,* 2016; Popova et al., 2018; Beltrán *et al.,* 2005; Suan, et al., 2016; Seong *et al.,* 2010; Luchesi *et al.,* 2016). Gil et al., 2005 reported that the use of potable water instead of water containing chemical disinfection agents for washing fresh-cut vegetables is being advocated in some European countries. However, the problems of using an inadequate sanitizer or even none are an issue.

Contamination level impacts the effectiveness of water quality and helps to reduce pathogen concentrations. It was studied and observed that the cross-contamination of fresh-cut escarole with *E. coli* occurs and it affects the overall product contamination. Also, it indicates the necessity of using wash water sanitizers to eliminate pathogens (Allende 2008). The water quality influences the microbial load and sensory quality of fresh produce.

Thery used in study three different types of wash water such as diluted recirculated water, potable water and recirculated water. All were having different microbial counts and organic loads. This phenomenon was studied in *Escherichia coli* cross-contamination between inoculated and uninoculated products after washing. In this study found that, the microbial load ($P > \text{or} = 0.02$) and sensory quality ($P > 0.625$) of the product were not affected due to the water wash and storage (Allende A et al., 2008).

The *E. coli* slightly increased and not up to a statistically significant level when the

uninoculated product was washed with recirculated water ($P > 0.035$). When fresh-cut escarole was contaminated at a low inoculums level (3.2 log CFU/g) it was observed that the wash water quality not influence the level of cross-contamination.

When fresh produce was contaminated at a high inoculums level (5.1 log CFU/g) then the wash water changes the level of cross contamination. The *E. coli highest* load ($P < 0.001$) was to be found in uninoculated fresh produce Escarole after washing with recirculated water. After washing, the significant transmission of *E. coli* cells ($P < 0.001$) was observed due to cross-contamination between inoculated and uninoculated products (Allende A et al., 2008).

3.2.2. Effect of sodium hypochlorite wash on the microbial quality of water washed samples (T2)

The efficacy of sanitizers in killing human pathogenic microorganisms on a wide range of whole and fresh-cut fruits and vegetables has been studied extensively (Beuchat L.R. et al., 2001). Combination of washing with potable water followed by dip treatment in sodium hypochlorite (200 ppm) an approved sanitizer solution for 5 min (termed as T2) resulted in further reduction in TAPC and YMC.

In spinach samples TAPC reduced to 5.69 log CFU/g whereas YMC reduced to 3.82 log CFU/g. The Presumptive Coliforms reduced to 3.77 log CFU/g (Table 3.9).

In coriander samples TAPC reduced 5.37 log CFU/g whereas YMC reduced to 3.46 log CFU/g, and the PC reduced to 2.66 log CFU/g.

In mint samples TAPC reduced to 5.60 log CFU/g whereas YMC reduced to 3.30 log CFU/g and the PC reduced to 3.32 log CFU/g.

Siddig *et al.,* 2013 carried out study for purpose of to determine suitable treatment for lettuce

to avoid contamination. For study purpose he collected 150 samples from Saudi Arabia. They found that washing with tap water and 5 % acetic acid reduced microbial contamination level which make safe for human consumption. Further they found in their study presence of *E. coli* in 5 numbers of samples but salmonella not detected in any sample. Soaking in vinegar in 25 - 100 % reduced contamination by 3 log cycles. Luchesi *et al.,* (2016) in their study concluded that washing of leafy vegetables with running water and with chlorine was not efficient for reducing microbial load. However they obtained nematodes, coliforms and free living protozoa after water wash. In their study they used technique like, Hoffman's spontaneous sedimentation technique for parasite analysis and MPN for *E. coli* detection. Abadias M, *et al.,* (2008), in one of the studies, shown efficacy of neutral electrolyzed water for microbial decontamination from leafy vegetables. They show neutral electrolyzed water is the best alternative for sodium hypochlorite and they tested neutral electrolyzed water against listeria, salmonella and *E. coli* O157:H7 and *Erwinia carotovora* in lettuce.

Diluted Neutral electrolysed water containing 50 ppm of free chlorine, at pH 8.60 similar to that chlorinated water of 120 ppm of free chlorine with reduction 2 log unit against salmonella, *E. coli* O157:H7, and *E. carotovora* on lettuce. Popova, Teodora & E. Petrova, Toshka, (2018) shown that, ionized aqueous solution has been used for decontamination of lettuce; ionized aqueous solution was prepared by anolytes and catholytes which was prepared without addition of salt solution but by adding 0.8% NaCl (0.4% NaCl and 0.4 % Na2CO3). In their study they shown catholyte which obtained from activation with NaCl also shown high antimicrobial activity.

Beltran D, 2005 *et al.,* shown for the purpose of shelf life extension ozonated water was used as sanitizer in fresh cut lettuce which also resulted in effect on polyphenols and vitamin C content. In their study they also shown ozonated water does not stimulate respiration. They

used ozonated water of three different types for lettuce washing and were compared with water and chlorine rinses which resulted reduction of mesophilic count by 1.6 & 1.2 log by ozonated water and chlorine respectively. They also found that, most effective treatment is ozone activated with UV-C.

3.2.3. Both Gamma and Electron Beam radiation displayed similar hygienization efficacy

After minimal processing of spinach, coriander and mint, followed by washing with potable water (5 min), drying (air), and optimized packaging (as detailed later), the produce was subjected to radiation doses of 1, 2, and 3 kGy. However dose of 1 kGy was not found to be very effective in reduction of coliform count; however 2 kGy doses of Gamma and electron beam radiations reduce the coliform count to below detectable limit. Dose of 3 kGy was found equally effective in terms of reduction of coliform count but was not found be retain sensory quality. So on the basis of these observation 2 kGy dose of Gamma and Electron Beam was selected and this termed as T3 and T4, respectively (Table 3.9).

3.2.3.1 Effect of gamma radiation (dose 2 kGy) treatment on microbial quality of spinach (T3)

Upon the gamma radiation 2 kGy, TAPC and YMC reduced to 4.52 log CFU/g and 1.70 log CFU/g respectively, in these samples whereas PC reduced below detection level. Thus the findings indicate the use of these treatments eliminated PC and reduced TAPC and YMC significantly (Table 3.9).

3,2,3,2, Effect of gamma radiation (dose 2 kGy) treatment on microbial quality of coriander (T3)

Gamma radiation treatment effectively reduced TAPC and YMC to 4.59 log CFU/g and 2.43 log CFU/g respectively, in these samples whereas PC reduced below detection level. Thus, the findings indicate the use of these treatments eliminated PC and reduced TAPC and YMC significantly (Table 3.9).

3.2.3.3. Effect of gamma radiation (dose 2 kGy) treatment on microbial quality of mint (T3)

TAPC and YMC reduced to 4.44 log CFU/g and 2.62 log CFU/g respectively, in these samples whereas PC reduced below detection level. Thus, the findings indicate the use of these treatments eliminated PC and reduced TAPC and YMC significantly (Table 3.9).

3.2.3.4. Effect electron beam (dose 2 kGy) treatment on microbial quality of spinach (T4)

After the treatment of electron beam, the TAPC and YMC reduced to 4.93 log CFU/g and 1.65 log CFU/g respectively, PC reduced to below detection level. Thus findings indicate that, treatment eliminated PC and reduced TAPC and YMC significantly.

3.2.3.5. Effect electron beam (dose 2 kGy) treatment on microbial quality of coriander (T4)

After the treatment of electron beam, TAPC and YMC reduced 4.98 log CFU/g and 2.93 log CFU/g respectively, PC reduced to below detection level. Thus findings indicate that, treatment eliminated PC and reduced TAPC and YMC significantly.

3.2.3.6. Effect electron beam (dose 2 kGy) treatment on microbial quality of mint (T4)

After the treatment of electron beam, TAPC and YMC reduced to 4.73 log CFU/g and 2.91 log CFU/g respectively, PC reduced to below detection level. Thus findings indicate that, treatment eliminated PC and reduced TAPC and YMC significantly (Table 3.9).

Reduction of microbial load in coriander (*Coriandrum sativum* L.) and mint (*Mentha arvensis* L.) was observed similarly upon CP1 and CP2 treatments (Table 14). Study carried out by Lee, Jo, & Shin (2006) shown similar decrease of count (4 log cycles) was observed upon irradiation (2 kGy) of spinach inoculated with *Salmonella* Typhimurium. Mahmoud (2010) observed upon irradiation (2 kGy X- ray) more than 5 log cycles reduction of *E. coli* and other pathogens found in spiked shredded iceberg lettuce. Neal, Cabrera-Diaz, & Marquez-Gonzalez (2008) also observed that, there was reduction of more than 6 log cycles in *E. coli* when irradiated (e 1 kGy electron beam) in baby spinach. Study carried out by Lu, Yu, & Gao (2005) resulted that showed that upon cut celery leaves was irradiated (1 kGy gamma) the count of *E. coli* cells reduced to less than 30 cfu/g while maintaining the organoleptic quality till 9 days at 4 °C storage. Similarly, Prakash, Inthajak, & Huibregtse (2000) compared conventional treatment such as blanching, acidification, and chlorination in combination with gamma radiation or over the gamma irradiation on and sensory characteristics and microbial load of diced celery. It was concluded that radiation dose of 1 kGy could eliminate *E. coli* while maintaining the organoleptic quality.

Foley, Dufour, & Rodriguez (2002) showed that iceburg lettuce packed in film bags and irradiated (0.55 kGy) in combination with chlorination, reduced the count by 5.4 log in *E. coli*. This dose was not affected the textural and other qualities of lettuce. Similarly, study carried out by Lopez, Avendano, & Romero (2005) resulted that, irradiation of celery at 1 kGy reduced the total plate count by 4.7 log cfu/g and enterobacterial count by 3.8 logs cfu/g. Results also conclude that, there was no significant change in the organoleptic properties of fresh control and irradiated celery samples when stored at 5 °C for 7 days.

Hsu, Simonne, Jitareerat (2010) showed that a radiation processing (2 kGy) eliminated the *E. coli* completely, without affecting visual quality. These treated samples were stored up to 9 days only at 4 °C and found no significant change in the visual quality. Our present study

resulted minimum shelf life of the vegetables is 15±2 days which is considerably more than earlier studies.

3.2.3.7. Above treatments were found effective on isolates in saline suspension

Putative isolates of *E coli* and presumptive salmonella was used for study of the survival of pathogen upon individual and combination treatment. Purpose of study to see the effect of individual treatment on pathogens isolated from leafy vegetables. After the treatments as detailed in the Table 3.8 efficacy was found. Presumptive pathogen count was reduced to below detectable level by either single or combination treatment of NaOCl (200 ppm), 5 min and gamma irradiation (2.0 kGy). Thus we ensure that in aqueous medium (as vegetables also contain ~ 90 % moisture) all the treatments are effective against pathogens isolated from leafy vegetables.

Table 3.8 Profile of presumptive pathogens in liquid culture after individual or combination treatment			
Presumptive Pathogen	**Log CFU /mL**	**Treatment**	**Log CFU/mL**
coliform	4.3 ±0.4	NaOCl (200 ppm), 5 min.	<detection level
Coliform	4.4 ±0.4	Gamma (2 kGy)	<detection level
Coliform	4.4 ±0.3	NaOCl (200 ppm)+ Gamma (2kGy)	<detection Level
Salmonella spp	4.3 ±0.3	NaOCl (200 ppm), 5 min.	<detection level
Salmonella spp	4.2 ±0.3	Gamma (2 kGy)	<detection level
Salmonella spp	4.1±0.4	NaOCl (200 ppm)+ Gamma (2kGy)	<detection Level

3.2.4. Development of combination processes:

For optimization of combination process microbial profile, screening of pathogens study was carried out. Further study of dose (Gamma and EB) optimization for hygienization of leafy vegetables were also carried out which resulted 2 kGy is sufficient for reduce TAPC, YMC and PC below detection level. Effect of individual treatment on organoleptic and effect of radiation 2 kGy on moisture content of fresh leafy vegetables were also carried out. It indicates that, 2 kGy does not affect the organoleptic quality of fresh leafy vegetables. Based upon above data, it was decided to optimize the packaging material and storage temperature to extend the shelf life of fresh leafy vegetables.

For radiation dose optimization Food irradiation rules notified by "FSSAI" Government of India and United States (code of regulation, 21 CFR 179.26). 2 kGy dose of radiation was selected on basis of preliminary trials carried out by delivering range of dose from 1 to 3 kGy, for purpose to achieve desired hygienization level and extension of shelf life. A set criterion for optimization of radiation dose was reduction of coliform count to below detection level and retention of organoleptic attributes. On basis of the observations 2 kGy dose was selected for study. Apart from radiation treatment other combinations like, minimal processing, washing, and dip with approved sanitizer (sodium hypochlorite) were also combined in the combination process. Combination process ensured the hygienization as well as shelf life extension up to 15 days. Samples those processed with < 2 kGy of radiation dose were found presence of coliforms and were remain detectable during storage.

Samples treated with more than 2 kGy radiation did not detected coliforms but lost the organoleptc quality. Vegetables were minimally processed, treated with potable water (5min), then dip treatment with sodium hypochlorite (200 ppm), air dried (30 min) and Radiation treated at 2 kGy. Approved sanitizer sodium hypochlorite (200 ppm) was used because no

bacterial resistance develop to it. Further this sanitizer is easily available in market (Adams, Hartley & Cox, 1989). To retain the freshness, moisture content during storage (4 to 6 °C), moist cellulose-based material was used in tray. Further to prevent moisture loss trays covered with oxygen permeable pvc film. Produce treated with such combination process were found acceptable upon storage at 4 to 6 °C for 15 days. Other hand samples neither processed nor stored at low temperature were resulted very shelf life of one to two days only. Thus, optimized combination process 1 (CP1) included T1+T2+T3 and combination process 2 (CP2) included T1+ T2+ T4.

3.2.4.1. Effect of combination treatment (CP1) including, potable water wash (T1), sodium hypochlorite wash (T2) followed by gamma (2 kGy) treatment (T3) on microbial count

Spinach: Combination process (CP1) including potable water (5min) washing, sodium hypochlorite (200 ppm) dip treatment 5 min, drying, and gamma radiation treatment showed significant efficacy in hygienization of the spinach samples. Upon combination treatment microbial count (TAPC) were 1.46 log CFU/g, YMC were 1.25 log CFU/g and presumptive coliforms reduced to below detection level (Table 3.9).

Coriander: CP1 treatment showed their efficacy in hygienization of the coriander samples and reduced overall microbial count. TAPC were 1.25 log CFU/g, and YMC count was 1.30 log CFU/g and presumptive coliforms reduced to below detection level.

Mint: CP1 treatment showed their efficacy in hygienization of the mint samples and reduced overall microbial count. TAPC reduced to 1.1 log CFU/g, and YMC count reduced to 1.25 log CFU (Table 3.9).

3.2.4.2. Effect of combination treatment (CP2) including, potable water wash (T1), sodium hypochlorite wash (T2) followed by electron beam (2 kGy) treatment (T4) on microbial count

Spinach: Combination process (CP2) including potable water washing (5min), sodium hypochlorite (200 ppm) dip treatment 5 min, drying, and electron beam treatment showed their significant efficacy in hygienization of these samples. Microbial count in spinach samples reduced singinficantly: TAPC were 1.59 log CFU, YMC were 1.63 log CFU/g (Table 3.9) and presumptive coliforms reduced to below detection level (Table 3.9).

Coriander: CP2 treatment showed their efficacy in hygienization of the coriander samples and reduced overall microbial count: TAPC reduced to 1.61 log CFU/g, and YMC count were 1.88 log CFU/g and presumptive coliforms reduced to below detection level.

Mint: CP2 treatment showed their efficacy in hygienization of the mint samples and reduced overall microbial count: TAPC reduced to 1.9 log CFU/g and YMC were reduced 1.82 log and presumptive coliforms reduced to below detection level.

2 kGy dose of gamma irradiation dose was selected as per the US federal regulation (21 CFR 179.26) code and new FSSAI rules notified by Government of India for radiation processing of fresh leafy vegetables. In previous studies reported, the electron beam irradiation has been considered as effective method to improve the microbial quality and increase the shelf life of many food products include wild chamomile (Nemtanu et al., 2008), Spirulina (Brasoveanu et al., 2005), soy beans (Wilson et al., 2007) and Mucuna pruriens seeds (Bhat and Sridhar, 2008). Electron beam irradiation has a short processing time, does not produce radioactive waste and it destroys the major pathogenic food borne bacteria (Rodriguez et al., 2006). Living cells are inactivated when exposed to ionizing irradiation that substantially changes their cellular structure and physiological functions. The damages include DNA strand

breakage, cell membrane rupture and mechanical damage to cell walls (Lado and Yousef, 2002). Therefore, using irradiation microorganisms, insects and plant meristems are prevented from reproduction (Farkas, 2006).

For a gamma plant source strength and geometry, product conveyor geometry and mode of transport, and in a similar manner for electron accelerator plants, the beam characteristics, power and geometry, and product transport parameters are key aparmeters. A study performed at Korean laboratory with dried laver products showed higher microbial contamination. Effects of approved dose (7 kGy recommended in the Korean Food Code for algal food of electron beam) of E-beam irradiation on microbiological, physicochemical and luminescence properties of dried laver products indicated dose-dependent reductions in microbial load. For example, a 4 kGy irradiation dose reduced coliform (<2.5 log CFU/g) to undetectable levels (<10 CFU/g), while a 7 kGy irradiation dose reduced total aerobic bacteria count (6.6 log CFU/g) by approximately 2 log cycles. Physicochemical attributes of dried lavers were not significantly ($p > 0.05$) affected before and after 7 kGy irradiation, but carotenoid content was significantly ($p < 0.05$) reduced at 10 kGy irradiation. Irradiated samples (>4 kGy) could be detected from non-irradiated ones by luminescence techniques. Overall, results indicated that <7 kGy irradiation is recommended along with other heat treatment for improving microbiological contamination by at most 4 log CFU/g in dried lavers (Lee *et al.*, 2017).

Table 3.9 Microbial profile of fresh spinach, coriander and mint treated with various combination processes including Gamma / Electron Beam radiation.

	Spinach			Coriander			Mint		
Combination processes	TAPC (log CFU/g)	YMC (log CFU/g)	Pr.coliform (log CFU/g)	TAPC (log CFU/g)	YMC (log CFU/g)	Pr.coliform (log CFU/g)	TAPC (log CFU/g)	YMC (log CFU/g)	Pr. Coliform (log CFU/g)
Non-treated Control (C)	7.51 ± 0.45^{a}	5.69 ± 0.88^{p}	5.78 ± 0.43^{x}	7.59 ± 0.54^{a}	5.50 ± 0.54^{p}	4.70 ± 0.43^{x}	7.15 ± 0.92^{a}	5.39 ± 0.49^{p}	5.62 ± 0.50^{x}
Potable water wash (5min) T1(W)	6.72 ± 0.43^{b}	4.60 ± 0.48^{q}	4.76 ± 0.32^{y}	6.76 ± 0.71^{b}	4.77 ± 0.73^{q}	3.71 ± 0.30^{y}	6.90 ± 0.52^{b}	4.27 ± 0.51^{q}	4.60 ± 0.451^{y}
Sodium hypochlorite treatment, 200 ppm, (5min) T1+T2	5.69 ± 0.88^{c}	3.82 ± 0.45^{r}	3.77 ± 0.030^{z}	5.37 ± 0.47^{c}	3.46 ± 0.36^{r}	2.66 ± 0.81^{z}	5.60 ± 0.64^{c}	3.30 ± 0.29^{r}	$3.32\pm0.3.0^{z}$
Gamma irradiation (2 kGy) T3	4.52 ± 0.51^{d}	1.70 ± 0.15^{s}	<detection limit	4.59 ± 0.40^{d}	2.43 ± 0.16^{s}	<detection limit	4.44 ± 0.31^{d}	2.62 ± 0.142^{s}	<detection limit
EB irradiation (2 kGy) T4	4.93 ± 0.30^{e}	1.65 ± 0.17^{t}	<detection limit	4.98 ± 0.50^{e}	2.93 ± 0.30^{t}	<detection limit	4.73 ± 0.414^{e}	2.91 ± 0.089^{t}	<detection limit
CP1 (T1+T2+T3)	1.46 ± 0.15^{f}	1.25 ± 0.20^{u}	<detection limit	1.25 ± 0.24^{f}	1.30 ± 0.50^{u}	<detection limit	1.1 ± 0.173^{f}	1.25 ± 0.28^{u}	<detection limit
CP2 (T1+T2+T4)	1.59 ± 0.13^{g}	1.63 ± 0.16^{v}	<detection limit	1.61 ± 0.28^{g}	1.88 ± 0.17^{v}	<detection limit	1.95 ± 0.15^{g}	1.82 ± 0.130^{v}	<detection limit

TAPC: Total Aerobic Plate Count, YMC: Yeast and Mould count, PC: Presumptive Coliform, Different superscript letters in columns, used ($^{a\ to\ g}$ for TAPC) and ($^{p\ to\ v}$ for YMC) and ($^{x\ to\ z}$ for PC) indicate significant difference among (p d0.05)

3.2.5. Optimization of packaging material required for shelf life extension

Optimization of packaging was explored using pvc film, Styrofoam tray and cellulose based material. Initially only cling film is used to wrap vegetable (~150 g) in film with single layer. Wrapped vegetables was irradiated at dose of 2 kGy and kept at 4 °C , RH 85-90% which resulted shelf life of 3 days in case of spinach, coriander and mint samples over the control sample non treated and wrapped in cling film also resulted shelf life of 3 days.

Further combination of packaging material which includes styrofoam tray each containing vegetables (~ 150 g) wrapped in pvc film and irradiated at 2 kGy which resulted extended shelf life in range of 3 to 4 days when stored at 4°C. However, control sample also was resulted extended shelf life of 2-3 days.

After that combination was explored using cellulose based material having water holding capacity, which was placed at the base of porous tray. Around 150 g of vegetables was placed in each tray and wrapped with stretchable pvc film and irradiated at dose of 2 kGy and stored at 4°C which resulted extended shelf life of 15 days in case of test sample and 7 days in case of non irradiated packed control samples.

Thus packaging in vegetables in porous tray having cellulose based material at base for water holding and by keeping ~150 g of vegetable in tray with pvc wrap found most suitable for packaging (Figure 3.6).

Botondi *et al.,* 2015 also showed the effects of packaging material polyethylene terephthalate and polylactic acid, on fresh spinach (*Spinacia oleracea).* Samples were stored at low temperature at 4 °C for around 2 weeks, maintained physical water activity, color retention sensory. But problem was PLA packaging material showed condensation of water affecting

quality and shelf life. But in our packaged samples condensed water was not observed during storage period.

Suan et al., (2016) showed in his study comparative evaluation of two different storage methods by observing subjective or qualitative parameters like browning and off odour formation considered for comparison. In their study storage in active modified atmosphere for 12 days at 7 °C with head space between 0.2-3.2 kPa02 & 6-9 kPa CO2 found suitable for storage.

3.2.5.1. To study effect of radiation on packaging material (polystyrene tray and cling film)

As there is direct contact of packaging material with packaging material a preliminary study regarding release was carried out. Study resulted that, as seen from the TLC and GCMS no additives migrated / leached in water up to dose 2 kGy (Figure 3.4 and 3.5). Thus the packaging material was found tolerant to radiation up to 2 kGy (Table 3.10).

Table 3.10 Release study of packaging material

Material	Dose	Release	Compound
Polystyrene tray	2 kGy	Not detected	--
Cling film	2 kGy	Not detected	--

N : Non – Methylated control
M: Methylated

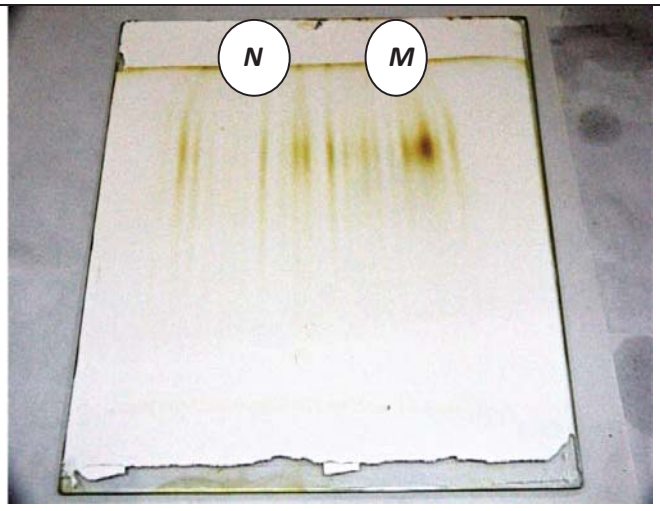
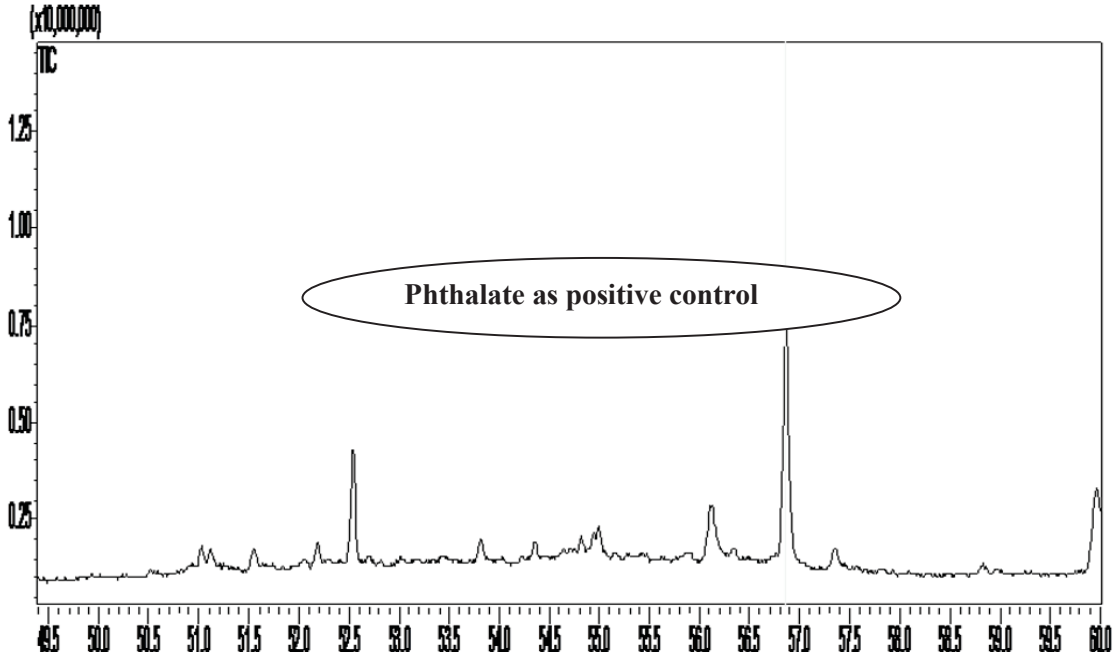


Figure 3.4 Methylated and non - methylated samples



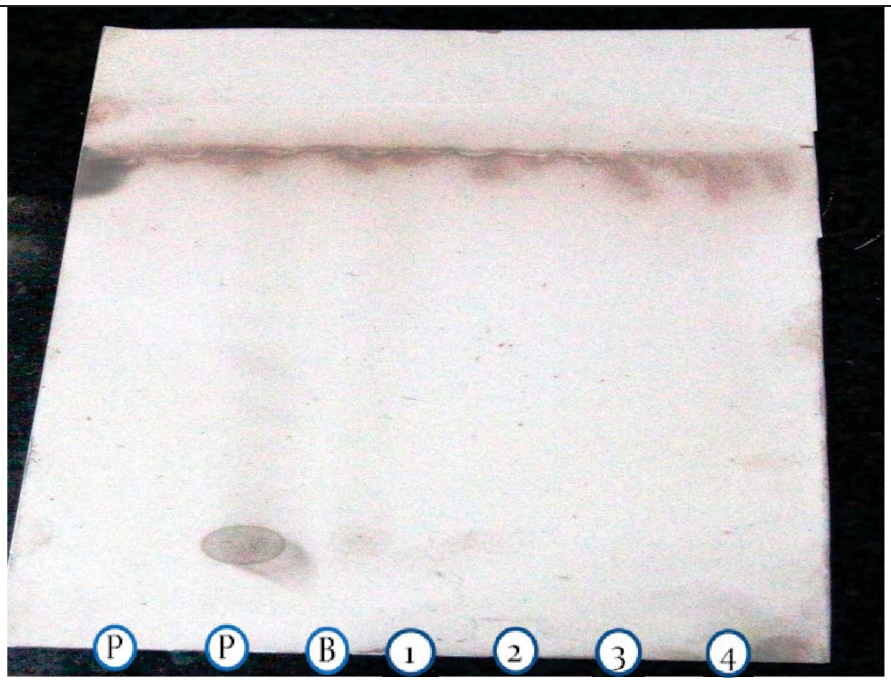


Figure 3.5 GCMS profile of positive control phthalate and TLC profile of migrated from packaging material to water P: Positive control bisphenol dissolved in water P: Positive control bisphenol dissolved in NaOH

B: blank, 1 kGy dose for polystyrene; 2 kGy dose for polystyrene; 1 kGy dose pvc film ;2 kGy dose for pvc film.

Figure 3.6 Front view and side view of packed sample for EB radiation.

3.2.6. Storage at 4 °C and RH (85 to 90 %) showed best quality retention during storage

In view of to enhance the shelf life of fresh leafy vegetables storage temperature is very crucial factor, so above packed sanples (in optimized packaging) were kept at 4°C, 10°C and ambient temperature (28±2 °C). This resulted in extended shelf life of 15 days in case of CP1 and CP2 treated samples (upon packaging) and 7 days in case of control (packaged but non irradiated samples) when stored at 4 °C and RH 85 to 90 %. However, CP1 and CP2 treated samples (upon packaging) have 5 to 7 days and 2 days shelf life when stored at 10 °C and ambient temperature (28 ±2 °C), respectively. Thus 4 °C and RH 85 to 90 % were found to be most suitable conditions for storage of processed and packed leafy greens.

3.2.7. Upon CP1 and CP2 treatment microbial hygiene of combination processed leafy greens retained during 15 days storage

Microbial analysis of fresh CP1 and CP2 processed leafy vegetables packed in optimized packaging (as detailed above) and kept at 4±2 ºC was carried out at different intervals till 15 days of storage period.

Spinach: The CP1 treatment resulted in reduction of TAPC to 1.2 log CFU/g, YMC to 1.4 log CFU/g and PC to below detection level on day 1. Similarly, CP2 treatment showed significant hygienization efficacy. TAPC reduced to 1.8 log CFU/ml, YMC to 1.9 log CFU/g and PC to below detection level (Table 3.11). Thus, combination process with irradiation (either gamma or electron beam) proved to be a better hygienization process. During storage there was increase of one log cycle in both TAPC and YMC till 15 days at 4±2 ºC and RH 85 to 90 %.. However, PC count was below detectable level even on 15 days of storage at 4±2 ºC and RH 85 to 90 %.

Coriander: Upon CP1 treatment TAPC count was 1.9 log CFU/g, while YMC was 1.1 log CFU/g (Table 3.11). PC was reduced to below detection level. CP2 also showed significant

reduction in microbial count and both TAPC and YMC was 1.9 log CFU/g. In this case also PC was below detectable level. During storage there was increase of one log cycle in both TAPC and YMC till 15 days of storage at 4 ±2ºC and RH 85 to 90 %.. However, PC count was below detectable level even after 15 days of storage at 4±2 ºC and RH 85 to 90 %.

Mint: In CP1 treated samples TAPC and YMC was 1.0 and 1.2 log cfu/g, respectively and PC count was below detectable level. CP2 treatment also showed significant reduction in overall microbial count of mint samples. TAPC reduced to 1.9 log CFU/g and YMC to 1.8 log CFU/g. PC was below detectable level (Table 3.11). Thus, in all the vegetable samples combination treatment was highly effective than single treatment. During storage there was increase of one log cycle in both TAPC and YMC till 15 days at 4±2 ºC and RH 85 to 90 %.. However, PC count was below detectable level even after 15 days of storage at 4±2 ºC and RH 85 to 90 %.

Even though the gamma irradiation is a very efficient technology, the profitability of gamma radiation plant is always depending upon the availability of food material for radiation processing. The reason behind this is whether radiation source is in use or not it will keep on depleting. However, in comparison to gamma radiation, electron beam treatment is economically more cost effective for two reasons. Electron - beam machine can be switched on or off as per the availability of food material for radiation processing and the other is its dose rate is very high compared to gamma radiation. So, if density of produce is not an issue then the electron beam is economically feasible due to high throughput.

Palekar *et al.,* 2006 studied the effect of Electron beam on sliced cantaloupe and analysed for total aerobic microbial counts, texture, color, and different sensorial parameters as a function of irradiation doses 0, 0.7, and 1.4 kGy and the wash treatments, 1 and 200 ppm chlorine applied to the melons before cutting. Irradiation resulted in a reduction in the total aerobic microbial counts with increasing doses. Across all doses of irradiation, counts were

consistently lower for cantaloupe pieces obtained from melons that had been subjected to chlorine rinse in comparison with those washed with water without chlorine. Melons washed with chlorine before cutting had total aerobic bacterial counts of 2.7, 0.7, and 0.5 log CFU/g on day 0 at irradiation doses of 0, 0.7, and 1.4 kGy, respectively. Thus decontamination of whole cantaloupes before cutting using chlorine wash may be combined with low dose irradiation for shelf life extension of sliced cantaloupe.

Similar study has been carried out by Jack *et al.,* 2010, He concluded that, irradiation dose did not affect the basic tastes, aromatics, or mouth feels of fresh spinach, however; hardness attributes decreased as irradiated dose increased and slimy attributes of fresh spinach were higher in control samples compared to irradiated samples.

Study carried out by Hagenmaier and Baker (1997) identified the increase in microbial populations on irradiated iceberg lettuce with respect to storage time and temperature. Therefore there is need to reduce the bacterial load on spinach during decontamination steps are important; however it helps to maintain the produce quality and improve the shelf life. Prakash and others (2000) observed that when cut romaine lettuce was irradiated with gamma radiation at 0.35 kGy, it showed the 1.5 log CFU/g reduction in the microbial load of aerobic microorganisms. Foley and others (2004) reported when cilantro irradiated with 0.5 kGy a 3.7 log reduction of yeasts and molds were observed. The variation between cilantro leaves and the results with spinach leaves emphasizes the importance of the food matrix in the effectiveness of irradiation. Gamma irradiation helps in reduction of APC count and shelf life extension of bagged spinach when treated with radiation dose of doses of 1.4 kGy. Gram positive microorganisms like lactic acid bacteria and yeasts and molds are more resistant to irradiation and causes more spoilage than the gram negative organisms (Monk and others 1995). Lactic acid bacteria can grow at low temperatures and therefore they cause spoilage during storage at low temperature, further these bacteria causes the fermentation of sugars

present in the spinach leaves. Therefore, it is essential to minimize the initial bacterial load to enhance the shelf life of bagged leafy greens. While, Babic and others (1996) study showed the lactic acid bacteria are present on spinach at lower levels than gram negative bacteria and they can grow at 10 °C. Hence, the reductions in APC counts were observed at initial level when spinach treated to irradiation. Otherwise the stressed bacterial cells may recover and begin to grow. Fermentation, lactic acid bacteria can reduce the pH level and it helps to contribute to benefits of load reduction over gram negative microorganisms. However these effects were not studied thoroughly. When refrigerated cut iceberg lettuce treated with irradiation at 0.2 kGy after the chlorine treatment causes major reductions in yeasts and molds counts while found that fungi recovered after some days.

TABLE 3.11 MICROBIAL PROFILE OF LEAFY VEGETABLES (STORAGE AT 4 to 6 °C) SUBJECTED TO COMBINATION PROCESS											
Treatments	Total Aerobic Plate Count (TAPC) (Log CFU/g)					Yeast Mould Count (YMC) (Log CFU/g)					Presumptive Coliform (PC) (Log CFU/g)
→	Storage period (days)										
	1st	3rd	7th	13th	15th	1st	3rd	7th	13th	15th	Day 1st to 15th
Spinach control	$7.5\pm1.4^{x,m}$	NA	NA	NA	NA	$5.6\pm2.1^{x,s}$	NA	NA	NA	NA	$5.7\pm2.2^{x,}$
T3	$4.6\pm1.2^{y,m}$	$4.7\pm1.6^{x,m}$	$4.9\pm2.1^{x,m}$	$4.9\pm2.5^{x,m}$	$5.5\pm2.1^{x,n}$	$1.9\pm0.8^{y,s}$	$2.5\pm1.1^{x,s}$	$2.6\pm1.1^{x,s}$	$2.9\pm1.7^{x,s}$	$3.6\pm1.2^{x,t}$	<detection level
CP1	$1.2\pm0.8^{z,m}$	$1.8\pm0.5^{y,m}$	$2.3\pm0.8^{y,n}$	$2.4\pm1.3^{y,o}$	$2.9\pm1.3^{y,o}$	$1.4\pm0.6^{z,s}$	$1.6\pm0.9^{y,s}$	$1.7\pm0.7^{y,s}$	$1.9\pm0.6^{y,s}$	$2.9\pm1.3^{y,t}$	<detection level
T4	$4.9\pm2.1^{a,m}$	$4.9\pm1.6^{z,m}$	$4.9\pm1.1^{z,m}$	$5.6\pm2.1^{z,n}$	$5.9\pm2.6^{z,n}$	$2.4\pm1.2^{a,s}$	$2.6\pm1.5^{z,s}$	$2.8\pm1.5^{z,s}$	$2.9\pm1.2^{z,s}$	$3.4\pm1.6^{z,t}$	<detection level
CP2	$1.8\pm0.3^{b,m}$	$1.8\pm0.9^{a,m}$	$1.9\pm0.8^{a,m}$	$2.6\pm0.8^{a,n}$	$2.9\pm1.7^{a,n}$	$1.9\pm0.7^{b,s}$	$1.9\pm0.7^{a,s}$	$1.5\pm0.8^{a,s}$	$2.2\pm1.4^{a,s}$	$2.8\pm1.1^{a,s}$	<detection level
Coriander control	$7.6\pm1.5^{c,m}$	NA	NA	NA	NA	$5.5\pm2.1^{c,s}$	A	NA	NA	NA	4.7 ± 0.4^{y}
T3	$4.6\pm1.4^{d,m}$	$4.6\pm1.3^{b,m}$	$4.8\pm1.7^{b,m}$	$4.9\pm1.1^{b,m}$	$5.4\pm2.4^{b,n}$	$3.4\pm1.3^{d,s}$	$3.6\pm1.4^{b,s}$	$3.8\pm1.2^{b,s}$	$4.2\pm1.7^{b,s}$	$4.8\pm2.2^{b,t}$	<detection level
CP1	$1.9\pm0.6^{e,m}$	$2.7\pm1.4^{c,n}$	$2.9\pm0.7^{c,n}$	$2.9\pm2.2^{c,n}$	$2.9\pm1.3^{c,n}$	$1.1\pm0.6^{e,s}$	$1.8\pm0.8^{c,s}$	$1.8\pm0.9^{c,s}$	$2.0\pm0.8^{c,s}$	$2.2\pm1.3^{c,s}$	<detection level
T4	$4.5\pm1.3^{f,m}$	$4.8\pm1.8^{d,n}$	$4.9\pm2.1^{d,o}$	$4.9\pm2.5^{d,p}$	$5.5\pm2.6^{d,q}$	$3.7\pm1.2^{f,s}$	$3.9\pm1.1^{d,s}$	$3.9\pm1.3^{d,s}$	$4.2\pm1.1^{d,s}$	$4.7\pm2.1^{d,s}$	<detection level
CP2	$1.9\pm0.7^{g,m}$	$2.8\pm0.1^{e,n}$	$2.9\pm0.9^{e,o}$	$2.9\pm0.9^{e,p}$	$2.9\pm1.4^{e,q}$	$1.9\pm0.7^{g,s}$	$2.0\pm1.2^{e,s}$	$2.1\pm1.3^{e,s}$	$2.8\pm1.3^{e,s}$	$2.9\pm0.9^{e,s}$	<detection level
Mint control	$7.1\pm1.9^{h,m}$	NA	NA	NA	NA	$5.3\pm2.2^{h,s}$	NA	NA	NA	NA	5.6 ± 0.1^{z}
T3	$4.3\pm1.5^{i,m}$	$4.6\pm1.4^{f,n}$	$4.8\pm2.5^{f,o}$	$5.2\pm2.3^{f,p}$	$5.6\pm2.8^{f,q}$	$2.5\pm1.7^{i,s}$	$2.8\pm1.5^{f,s}$	$2.9\pm1.1^{f,s}$	$3.3\pm1.2^{f,s}$	$3.7\pm1.2^{f,s}$	<detection level
CP1	1.0 ± 0.1^{jm}	$1.8\pm0.7^{g,n}$	$1.9\pm1.1^{g,o}$	$2.4\pm1.2^{g,p}$	$2.9\pm1.7^{g,q}$	$1.2\pm0.8^{j,s}$	$1.8\pm1.1^{g,s}$	$1.8\pm0.7^{g,s}$	$1.9\pm0.8^{g,s}$	$2.4\pm1.4^{g,s}$	<detection level
T4	$4.6\pm1.4^{k,m}$	$4.8\pm1.6^{h,n}$	$4.9\pm2.2^{h,o}$	$5.4\pm2.2^{h,p}$	$5.6\pm2.1^{h,q}$	$2.5\pm1.5^{k,s}$	$2.7\pm1.6^{h,s}$	$2.9\pm1.2^{h,s}$	$3.2\pm1.1^{h,s}$	$3.7\pm1.5^{h,s}$	<detection level
CP2	$1.9\pm0.8^{l,m}$	$1.9\pm0.8^{i,n}$	$1.9\pm0.9^{i,o}$	$2.8\pm1.1^{i,p}$	$2.9\pm1.4^{i,q}$	$1.8\pm1.1^{l,s}$	$1.8\pm0.8^{i,s}$	$1.8\pm1.6^{i,s}$	$1.9\pm0.9^{i,s}$	$2.4\pm1.1^{i,s}$	<detection level

Control- fresh samples non treated; T3 - Gamma irradiation (2 kGy); T4 - Electron beam irradiation (2 kGy); CP1- Potable water wash + Sodium hypochlorite treatment (200 ppm) + Gamma irradiation (2 kGy); CP2- Potable water wash + Sodium hypochlorite treatment (200 ppm) + Electron beam irradiation (2 kGy); NA –Not Available for analysis due to spoilage of sample (control); **x,y,z,a,b,c to j, k, l** Different letters across the column and **(m to q for TAPC) and (s to t for YMC)** different letters across the rows indicates significant difference (p d0.05) between the sample means as analysed by one way ANOVA. CFU/g- Colony Forming Unit per gram

3.3

QUALITY ATTRIBUTES

OF

PROCESSED LEAFY GREENS COMPARED

TO

FARM FRESH PRODUCE

3.3.1. Physico chemical analysis of fresh leafy vegetables

3.3.1.1. Organoleptic property of fresh vegetables retained after individual treatment

Sensory evaluation was performed by 20 panelists using 9-point hedonic scales (9-like extremely, 8-like strongly, 7-like very well, 6- like fairly well, 5-like moderately, 4- like slightly, 3- dislike slightly, 2- dislike moderately, 1- dislike externally) in an independent partitioned compartment in a sensory laboratory. Parameters evaluated were appearance, colour, flavour, texture, taste, after taste and overall acceptability. Samples in cooked form were used for taste and after taste parameters where as raw samples were used for color, flavour and texture analysis. Upon treatment with T3 (gamma radiation 2 kGy), T4 (electron beam radiation 2 kGy), CP1 and CP2 on day one sensory rating found in range of 7 to 7.5 which is well within acceptable limit (Table 3.12).

Sensory evaluation has recently utilized the methods developed by psychophysicists and psychometricians, who seek to represent data in terms of various scales and mathematical formulations. This covers the development of ratio scaling to develop relations between sensory and instrumental measures of food, the use of multivariate psychophysical procedures which relate a variety of physical variables to a single sensory response, and the use of multidimensional scaling to relate different sensory percept's to each other. Each of these approaches is nascent in applications to sensory evaluation, although the mathematics and formulations are very developed. Each approach gives the experimenter insights into subjective and objective correlations and the manner in which the panelist perceives relations among stimuli. The treatment of the reported literature for each approach follows the same course: necessary conditions for its application to sensory evaluation, experiences with model systems and real foods, and potential uses and limitations in sensory evaluation (Howard and Herbert, et al., 2009; Linda et al., 2013)

Table 3.12 Sensory analysis (over all acceptability)				
Treatments	Treatment code	Spinach	Coriander	Mint
Non-treated Control	C	ND	ND	ND
Potable water wash (5 min)	T1	ND	ND	ND
Sodium hypochlorite treatment (5 min)	T2	ND	ND	ND
Gamma irradiation (2 kGy)	T3	$7.6\pm.22^{x}$	7.5 ± 0.5^{y}	$7.5\pm.5^{z}$
Electron Beam treatment (2 kGy)	T4	7.5 ± 0.5^{x}	7.0 ± 0.5^{y}	7.0 ± 0.5^{z}
CP1 (T1+T2+T3)		7.0 ± 0.5^{x}	7.0 ± 0.5^{y}	$7.0\pm.5^{z}$
CP2 (T1+T2+T4)		7.0 ± 0.5^{x}	6.5 ± 0.5^{y}	7.0 ± 0.5^{z}

ND - Not Done, 9-point hedonic scale: 9 = like extremely, 8= like strongly, 7= like very well, 6= like fairly well, 5= like moderately, 4= like slightly, 3= dislike slightly, 2= dislike moderately, 1= dislike extremely. Different superscript letters columns, used ($^{x-x}$ for spinach), ($^{y-y}$ for coriander) and ($^{z-z}$ for mint) significant difference among (p d0.05), C –Control, T1 - Potable water wash, T2 - Sodium hypochlorite treatment, T3 -Gamma, radiation 2 kGy, T4 - Electron beam 2 kGy. ND- Not done control sample heavily burdened with microbes including pr. Coliform

3.3.1.2. Moisture Analysis (%) of fresh leafy vegetables:

Moisture content was analysed in fresh and radiation processed. Moisture content in fresh spinach, coriander and mint was in range of 92, 90 and 89 (%) respectively (Table 3.13). Fresh control spinach samples showed 92 % moisture at day 1, which reduced slightly but insignificantly to 91%. However, there was no change in moisture content of coriander and mint upon radiation treatment.

The results obtained in this study shows a close agreement with those found in literature (Oke, et al., 1986). From the experimental results, *Talinum triangulare* has the highest moisture (91.6%) which might be due to large number of cell saps they posses. The results fall within the range of results reported by Tindall (1988) (90-93%)

3.3.1.3. Colorimetry of fresh vegetable samples

Colorimetery parameters especially for greenness of spinach, coriander and mint were measured by Hunter Douglas Instrument as a* represents the redness/greenness (positive a* is red and negative a* is green)**;** a* value in control samples spinach; coriander and mint on day 1st were in range of -10 to -17, -11 to -17 and -9 to -18 respectively. The b* value represents the yellowness / blueness (positive b* is yellow and negative b* is blue). b* value in the case of fresh spinach, coriander and mint was in range of 12 to 28, 18 to 23 and 17 to 27 respectively (Table – 3.14).

Table No. 3.13 Moisture content of control / Fresh and radiation processed Test (2kGy) samples on day 1		
Name of vegetable	**Sample**	**Moisture (%) 0n Day 1st**
Spinach	Control	92 ± 0.571^{x}
	Test	91 ± 1.52^{x}
Coriander	Control	90 ± 0.57^{p}
	Test	90 ± 0.57^{p}
Mint	Control	89 ± 0.57^{a}
	Test	89 ± 1.0^{a}
Different superscript letters columns, treatments used ($^{x-x}$ for spinach) and ($^{p-p}$ for coriander) and ($^{a-a}$ for mint) indicate significant difference among (p d0.05)		

Table No. 3.14 Colorimetric analysis measured by Hunter Douglas make Instrument									
	L* value			a* value			b* value		
Treatment	**Spinach**	**Coriander**	**mint**	**spinach**	**Coriander**	**Mint**	**Spinach**	**coriander**	**Mint**
Non – treated control (C)	53 ± 5.0^{a}	52 ± 2.8^{p}	54 ± 7^{s}	-17 ± 2.06^{a}	-14 ± 1^{p}	-18 ± 3^{s}	20 ± 3^{a}	18 ± 3^{p}	11 ± 1^{s}
Potable water wash (5 min) (T1)	52 ± 6^{a}	62 ± 6^{q}	57 ± 5^{s}	-14 ± 2^{b}	-15 ± 3^{p}	-12 ± 1^{t}	14 ± 2^{b}	20 ± 2^{q}	19 ± 3^{t}
Sodium hypochlorite treatment (200 ppm) (T2)	49 ± 5^{a}	58 ± 5^{q}	55 ± 7^{s}	-16 ± 2^{c}	-13 ± 2^{q}	-14 ± 2^{u}	23 ± 3^{c}	21 ± 2^{q}	27 ± 2^{u}
Gamma irradiation (2 kGy) (T3)	53 ± 6^{a}	57 ± 4^{q}	43 ± 4^{t}	-10 ± 1^{d}	-17 ± 3^{r}	-11 ± 2^{v}	12 ± 2^{d}	11 ± 3^{r}	18 ± 3^{v}
EB treatment (2 kGy) (T4)	47 ± 5^{b}	55 ± 5^{q}	60 ± 7^{u}	-12 ± 3^{e}	-14 ± 2^{s}	-9 ± 2^{w}	18 ± 2^{e}	23 ± 3^{s}	17 ± 2^{v}
CP1 (T1+T2+T3)	61 ± 6^{c}	56 ± 5^{q}	56 ± 6^{u}	-14 ± 3^{e}	-11 ± 2^{t}	-14 ± 2^{x}	28 ± 3^{f}	22 ± 4^{s}	24 ± 3^{w}
CP2 (T1+T2+T4)	46 ± 6^{d}	57 ± 5^{q}	63 ± 2^{v}	-12 ± 3^{f}	-15 ± 2^{u}	-13 ± 2^{x}	18 ± 2^{g}	20 ± 3^{s}	18 ± 3^{x}

Colorimetric Analysis measured by Hunter Douglas Instrument as L* represents the Light / Dark (positive L* is Light and negative L* is dark). As a* represents the redness/greenness (positive a* is red and negative a* is green) and b*represents the yellowness / blueness (positive b* is yellow and negative b* is blue). Control; T1: Potable water wash; T2: sodium hypochlorite wash; T3: Gamma radiation treated; T4: EB Treated Different superscript letters column vegetables treatments used ($^{a-g}$ for spinach), ($^{p-u}$ for coriander) and ($^{s-x}$ for mint) significant difference among (p d0.05)

3.3.2. Physical quality attributes of processed leafy greens well retained during storage

3.3.2.1. Samples treated by combination process retained the freshness and visual characteristics

Spinach: As control samples of all the vegetables could not remain acceptable after 3 days, the colorimetric data for only first day was measured for all the control vegetable samples. As seen from L* values, the lightness of control spinach samples was 53, while that of combination treated samples (CP1) was significantly higher (61) (pd 0.05). Second combination treated samples (CP2) showed significantly lower (46) L* value as compared to control samples (Table 3.15A, B, C). However, the lightness of these samples remained unchanged during storage of 15 days. Greenness of the control spinach samples was -17, whereas the combination treated samples showed the greenness ranging from -10 to -14 (Table 3.15A, B, C). There was significant change in the greenness of the samples (p d 0.05) during the storage that can be attributed to the biological variation. Greenness of second combination treated samples (CP2) was in the range of -10 to -13. There was significant change (p d 0.05) in the greenness of these samples also. But it could be attributed to the biological variation. Yellowness (b*) of the control spinach samples was 20. Yellowness of combination treated samples (CP1) was in the range of 13 to 28. Over the period of 15 days, there was significant reduction in the yellowness of the samples. In case of second combination treated (CP2) the yellowness of the samples was in the range of 17 to 23. In this case, though there was significant variation in the yellowness of the samples, there was no storage dependent reduction till 15 days.

Coriander: In case of coriander, the lightness of control coriander samples (L* values) was 52, while that of combination treated samples (CP1) was 56 (Table 3.15A, B, C). Second combination treated (CP2) showed similar lightness (~57) and there was gradual but

insignificant decrease in lightness in both these samples during storage. In case of greenness, control coriander samples showed a* value as -14. Combination treated samples (CP1) showed initial greenness -11 while second combination treated (CP2) samples showed slightly more greenness (-15). CP2 treated samples showed significant reduction in greenness during storage till 13th day. Yellowness (b*) of the control samples was 18 while that of combination treated coriander samples (CP1) was 22. There was significant reduction in the yellowness of the combination treated samples during the storage of 15 days. Second combination treated (CP2) samples showed initial yellowness as 20 and there was no significant change in the yellowness of these samples during storage (Table 3.15A, B, C).

Mint: Initial lightness (L*) of control mint samples was 54 while that of combination treated mint samples (CP1) was 56. There was no significant change (p e 0.05) in the lightness of these samples during storage of 15 days. Similarly, initial lightness of second combination treated samples (CP2) was 63. In these samples also there was no significant change (p e 0.05) in the lightness of these samples during storage of 15 days. Greenness (a*) of control mint samples was -18 while that of combination treated mint samples (CP1) was -14. There was no significant change (p e 0.05) in the greenness of these samples during storage of 15 days. Similarly, initial greenness of second combination treated samples (CP2) was -14. In this case also there was no significant change (p e 0.05) in the greenness of these samples during storage of 15 days. Initial yellowness (b*) of control mint samples was 11, while that of combination treated mint samples was 24 (Table 3.15A, B, C). There was significant change (p d 0.05) in the yellowness of these samples during storage of 15 days. Similarly, initial yellowness of second combination treated samples (CP2) was 18. In this case also, there was significant change in the yellowness of these samples during storage of 15 days, though no specific trend was observed. Thus, overall in all the vegetable samples, there was no effect of radiation treatment (both gamma and electron beam) on the colour parameters

though there was some reduction in some of the colour parameters during storage of 15 days at 4 °C and RH 85 to 90 %.

Use of fresh-cut vegetables has increased and new technologies have been applied to preserve the quality during transportation and storage. According to Young and White (1985), a " E^*_{ab} value in the range of value above 12.0 indicates colour of different shade, 6.0–12.0 an extremely remarkable difference in colour, 3.0 – 6.0 a remarkable difference, 1.5 – 3.0 indicates a just noticeable difference, 0.5 – 1.5 indicates a slight difference and value 0 – 0.5 indicates an imperceptible difference in colour between the samples.

TABLE 3.15A. COLORIMETRIC ANALYSES OF LEAFY VEGETABLES TREATED WITH COMBINATION PROCESS AND STORED AT 4 to 6 °C.

Treatment	Index/Shade colour	Spinach				Coriander				Mint			
		Storage period (Days)											
		1st	7th	13th	15th	1st	7th	13th	15th	1st	7th	13th	15th
Control	L*	53±5^{m,a}	NA	NA	NA	52±2^{m,c}	NA	NA	NA	54±7^{m,c}	NA	NA	NA
CP1		61±6^{n,a}	59±6 ^{m,a}	48±5^{m,b}	48±4^{m,b}	56±5^{n,c}	53±5^{m,c}	51±3^{m,d}	51±5^{m,e}	56±6^{m,g}	61±6^{m,g}	50±5^{m,h}	50±5^{m,h}
CP2		56±6^{o,a}	53±5^{n,a}	55±6^{n,a}	50±6^{n,b}	57±5^{n,c}	54±4^{m,d}	52±5^{m,e}	50±5^{m,e}	58±2^{n,g}	55±6^{n,g}	58±6^{n,g}	53±7^{m,g}

Control samples: Non-treated; CP1: Potable water wash + Sodium hypochlorite treatment (200 ppm) + Gamma irradiation (2 kGy); CP2: Potable water wash + Sodium hypochlorite treatment (200 ppm) + Electron beam irradiation (2 kGy); NA –Not Available for analysis due to spoilage of sample (control), $^{m\ to\ n}$ different letters across the column and $^{a\ to\ b\ (spinach),\ c\ to\ e\ (coriander),\ g\ to\ h\ (mint)}$ different letters across the row indicates significant difference (p d0.05) between the sample means as analysed by one-way ANOVA.

TABLE 3.15B COLORIMETRIC ANALYSES OF LEAFY VEGETABLES TREATED WITH COMBINATION PROCESS AND STORED AT 4 to 6 °C.													
Treatment	Index/Shade/Colour	Spinach				Coriander				Mint			
		Storage period (Days)											
		1st	7th	13th	15th	1st	7th	13th	15th	1st	7th	13th	15th
Control	a*	-17±2^{p,x}	NA	NA	NA	-10±1^{p,l}	NA	NA	NA	-18±3^{p,d}	NA	NA	NA
CP1		-14±3^{p,x}	-14±2^{p,x}	-10±1^{p,y}	-10±2^{p,y}	-11±2^{p,l}	-10±1^{p,l}	-10±2^{p,l}	-10±2^{p,l}	-14±2^{p,d}	-10±2^{p,e}	-10±1^{p,e}	-10±4^{p,e}
CP2		-15±2^{p,x}	-13±2^{p,x}	-10±2^{p,y}	-10±1^{p,y}	-11±3^{p,l}	-11±2^{p,l}	-11±2^{p,l}	-11±1^{p,l}	-14±2^{p,d}	-14±2^{q,d}	-11±1^{p,e}	-11±2^{p,e}

Control samples: Non-treated; CP1: Potable water wash + Sodium hypochlorite treatment (200 ppm) + Gamma irradiation (2 kGy); CP2: Potable water wash + Sodium hypochlorite treatment (200 ppm) + Electron beam irradiation (2 kGy); NA –Not Available for analysis due to spoilage of sample (control), $^{p \text{ to } q}$ different letters across the column and $^{x \text{ to } y \text{ (spinach), } l \text{ to } l \text{ (coriander), } d \text{ to } e \text{ (mint)}}$ different letters across the row indicates significant difference (p d0.05) between the sample means as analysed by one-way ANOVA.

TABLE 3.15C. COLORIMETRIC ANALYSIS OF LEAFY VEGETABLES TREATED WITH COMBINATION PROCESS AND STORED AT 4 to 6 °C.

Treatment	Index/Shade colour	Spinach				Coriander				Mint			
		Storage period (Days)											
		1st	7th	13th	15th	1st	7th	13th	15th	1st	7th	13th	15th
Control	b*	20±3^{r,a}	NA	NA	NA	18±3^{r,x}	NA	NA	NA	19±1^{r,e}	NA	NA	NA
CP1		24±3^{s,a}	26±3^{r,a}	23±2^{r,a}	13±2^{r,b}	18±4^{r,x}	19±3^{r,x}	17±3^{r,x}	14±2^{r,y}	23±3^{s,e}	25±2^{r,e}	15±2^{r,f}	17±1^{,rf}
CP2		19±2^{t,a}	19±2^{s,a}	23±3^{r,b}	17±2^{s,a}	18±2^{r,x}	24±3^{s,y}	21±3^{s,z}	20±3^{s,z}	18±3^{t,e}	18±3^{s,e}	17±2^{r,e}	14±2^{s,f}

Control samples: Non-treated; CP1: Potable water wash + Sodium hypochlorite treatment (200 ppm) + Gamma irradiation (2 kGy); CP2: Potable water wash + Sodium hypochlorite treatment (200 ppm) + Electron beam irradiation (2 kGy); NA –Not Available for analysis due to spoilage of sample (control), $^{(r\ to\ t)}$ different letters across the column and $^{(a\ to\ b\ \ spinach),\ (x\ to\ z\ coriander)\ and\ (e\ to\ f\ mint)}$ different letters across the row indicates significant difference (p d0.05) between the sample means as analysed by one-way ANOVA.

3.3.2.2. Organoleptic attributes of combination processed leafy vegetables were well acceptable

During the storage at 4 to 6 °C for the period of the 15 days, sensory analysis (9 - point hedonic scale) was carried out for spinach, mint and coriander on day one of the treatment. The overall acceptability scoring was as per mentioned in Table 17. Due to the heavy microbial burden sensory evaluation fresh non treated control samples were not carried out. CP1 & CP2 processed samples of spinach rated over all acceptability sensory score of 7 (like very well). Sensory score was remains unchanged during the 15 days storage period. CP2 processed samples, shown the rating for overall acceptability lowered insignificantly up to 6.5 (p e 0.05) during the storage period of 15 days. (Table 3.16).

CP1 and CP2 processed samples of coriander scored overall acceptability 7.5 and in this case non-significant change were observed during the storage period. Mint samples treated or processed with CP1 and CP2 scored overall acceptability of 7.0 which also remained non significant throughout the storage of 15 days. CP2 processed mint samples were scored significantly less (p d 0.05) up to 6.0 during the period of 15 days storage (Table 3.16). Thus, it can be concluded that, mint CP2 processed samples, lowered in overall acceptability during storage period. This is may be due to higher dose rates of (EB) electron beam compared to gamma radiation. This may result in the some micro level changes in organoleptic attributes. However, remaining all the samples was remained acceptable limit at the end of the fifteen days storage period.

Earlier findings also shows no significant changes in sensory attributes of any other vegetables up to radiation doses in range of the (0.5 to 2.0 kGy) (Hsu et al., 2010; Gomes, Moreira, & Castell-Perez, 2008; Lopez et al., 2005; Lu et al., 2005). Thus, it shows sensory evaluation rating to the shelf life of CP1 and CP2 combination treated leafy vegetable

samples could be retained well thorough out the storage period of 15 days at 4 to 6 °C as opposed control samples which is untreated. The study of Trigo, Sousa, & Sapata (2009) also resulted that, gamma radiation treatment of fresh mint and coriander at doses of up to 1 kGy resulted in enhanced shelf life at 4 to 6 °C for 2 and 3 days. Study of Fatema, Mahfuza, & Afifa (2013) have resulted no significant changes in overall rating of fresh spinach upon radiation treatment (1.0 kGy) and storage up to 12 days at 12 °C.

TABLE 3.16 Sensory analysis (Overall Acceptability) of Leafy Vegetables treated with Combination process including Gamma and EB and Stored at 4 To 6 °C and RH 85 to 90 %

Treatment	Spinach				Coriander				Mint			
	Sensory Score- Overall Acceptability											
	Storage period in days											
	1st	7th	13th	15th	1st	7th	13th	15th	1st	7th	13th	15th
Control	ND	NA	NA	NA	ND	NA	NA	NA	ND	NA	NA	NA
CP1	$7.0\pm1.0^{x,a}$	$7.0\pm1.0^{x,a}$	$7.0\pm0.5^{x,a}$	$7.0\pm1.0^{x,a}$	$7.0\pm1.0^{x,a}$	$7.0\pm0.5^{x,a}$	$7.0\pm0.5^{x,a}$	$7.0\pm1.0^{x,a}$	$7.0\pm0.5^{x,a}$	$7.0\pm1.0^{x,a}$	7.0 ± 1.0^{xa}	$7.0\pm1.0^{x,a}$
CP2	7.0 ± 0.5^{xa}	$7.0\pm1.0^{x,a}$	$7.0\pm1.0^{x,a}$	$6.5\pm1.0^{y,a}$	$7.5\pm1.0^{y,a}$	$7.0\pm1.0^{x,a}$	$7.0\pm0.5^{x,a}$	$6.5\pm1.0^{y,a}$	$7.0\pm1.0^{x,a}$	$7.0\pm1.0^{x,a}$	$6.5\pm1.0^{y,b}$	$6.0\pm0.5^{y,b}$

9-point hedonic scale: 9 = like extremely, 8= like strongly, 7= like very well, 6= like fairly well, 5= like moderately, 4= like slightly, 3= dislike slightly, 2= dislike moderately, 1= dislike extremely. Control samples: Non-treated; CP1: Potable water wash + Sodium hypochlorite treatment (200 ppm) + Gamma irradiation (2 kGy);
CP2: Potable water wash + Sodium hypochlorite treatment (200 ppm) + Electron beam irradiation (2 kGy); NA –Not Available for analysis due to spoilage of sample (control), ND- Not done control sample heavily contaminated with microbial load including presumptive coliforms, $^{x-y}$ Different letters across the column and $^{a-b}$ different letters across the rows indicates significant difference (p d0.05) between the sample means as analyzed by one way ANOVA.

3.3.2.3. Moisture content of stored samples

Moisture content was analysed in fresh and radiation processed. Moisture content in fresh spinach, coriander and mint was in range of 92, 90 and 89 (%) respectively. Moisture content in fresh leafy greens was found to be in the normal range which did not change significantly in CP1 & CP2 treated samples during the course of storage up to 15 days at 4 to 6 °C. Similarly, the visual appeal of the combination processed leafy greens retained up to 15th days at low temperature storage.

Fresh control spinach samples showed 93% moisture at day 1 which reduced slightly but insignificantly to 91% at day 3 of storage at 4 °C and RH 85 to 90% (Table 3.17). This sample spoiled after 3 days. Moisture content of combination treated (CP1) samples was similar to control samples. However, it significantly reduced to 88% during storage till 15 days at 4 °C and RH 85 to 90 % (Table 3.17). Moisture content of second combination treated (CP2) samples was similar and showed similar trend.

Moisture content of control coriander samples was about 89 % at day 1. There was no reduction in moisture content till day 3 of storage. CP1 treated coriander samples showed initial moisture content of 90 % (Day 1) which did not significantly change (pe 0.05) during storage up to 10 days. However, at 15 days of storage, it reduced significantly (p d 0.05) to 87%. Similar was the trend of moisture in CP2 treated samples.

In case of mint, the moisture content of control samples was about 89 % which did not change significantly during storage of 3 days. The CP1 treated mint samples showed similar moisture content (89%) to the control samples which reduced significantly (p d 0.05) only at 15th day of storage (87%) (Table 3.17). Moisture content of second combination treated (CP2) mint samples was similar to the respective gamma irradiated samples.

Table 3.17 Moisture content (%) in control and treated samples during storage at 4 ºC					
Sample **Storage at** **(4 °C and RH 85 to 90 %)**		**Moisture content (%)**			
		Storage period (Days)			
		1	**3**	**10**	**15**
Spinach	**Control**	93±2.0^{a}	91±2.0^{a}	NA	NA
	CP1	93±2.0^{a}	91±3.0^{a}	91±2.0^{a}	88±3.0^{b}
	CP2	89±2.0^{a}	89±2.0^{a}	NA	NA
Coriander	**Control**	89±2.0^{p}	89±2.0^{p}	NA	NA
	CP1	90±3.0^{p}	90±2.0^{p}	89±1.0^{p}	87±2.0^{q}
	CP2	90±2.0^{p}	90±3.0^{p}	89±2.0^{p}	87±3.0^{q}
Mint	**Control**	89±2.0^{x}	88±3.0^{x}	NA	NA
	CP1	89±3.0^{x}	88±2.0^{x}	88±2.0^{x}	87±3.0^{x}
	CP2	89±2.0^{x}	88±3.0^{x}	87±2.0^{x}	87±2.0^{x}

Control samples: Non-treated, packed and stored at 4 ºC; CP1 – (Potable water wash, Na-Hypochlorite (200ppm), 2 kGy Gamma), CP2 – (Potable water wash, Sodium hypochlorite treatment (200 ppm, 2kGy EB), and stored at 4 ºC; NA –Not Available for analysis due to spoilage of sample (control); Different superscript ($^{a-b}$ for spinach, $^{p-q}$ for coriander and $^{x\ to\ x}$ for mint) letters across the rows indicates significant difference (p d0.05) between the sample means as analysed by one way ANOVA.

Leaf moisture is a function of the dynamics of air and leaf temperature and relative air humidity. It is very difficult to define which part of leaves is wet or dry at various times in day and night (Huber and Gillespie, 1992). Factor like plant morphological properties, includes leaf topography, composition, trichome density are also responsible for moisture content in leaves (Mogren et al. 2018)

Prithvi Simha and Ashita Gugalia (2013) in the work, showed spinach dried using various different techniques like natural sun microwave and conventional. The experiment carried out by using variable micro wave power and constant temperature and air velocity. They found that in all the treatments moisture diffusivity varied in the range of 10^{-9}- 10^{-10}m2/sec. Santakumari *et al.,* 1989 studied estimation of bound water fraction i.e. apoplast water in the spinach (*Spinacia oleracea* L.) leaves. Study shows using pressure/volume curve approximately 40% bound water. Second technique by using vacuum infiltration shows 50 % bound water.

3.3.3. Biochemical quality attributes of processed leafy greens well retained during storage

3.3.3.1. Combination process retained phenolics and chlorophyll

Biochemical and functional attributes of leafy greens assayed included total phenolic content, and total chlorophyll content.

Total phenolic content

Spinach: In the fresh control spinach samples contents total phenolic was to be ~19 mg GAE/ g on day one. CP1 treated sample shown total phenolic content was to be ~ 23 mg GAE/g (Table 3.18). At the end of 15th day storage period insignificant (p e 0.05) reduction in total phenolic were found. Chlorophyll content in CP2 processed and 15 days stored (4°C) samples was shown ~35 mg GAE/g, which is significantly increased (p d 0.05). This

phenomena attributed could be due to that release of bound phenolics from glycosidic components because of radiation treatment.

Coriander: Amongst the vegetables studied coriander was resulted to have maximum total phenolic content. In fresh control coriander samples total phenolic was to be 446 mg GAE/ g. However total phenolic content in CP1 treated samples on day one of storage was of 438 mg GAE/g. These CP1 treated coriander sample shown minor enhance in the phenolic content to ~ 44 mg GAE/g on seven day of storage, which is further found to be decreased to 38 mg GAE/g on day 15. CP2 treated samples showed total phenolics content of 44 mg GAE/g on day one of storage, which was the further considerably reduced by ~20 % on day fifteen of storage (Table 3.18).

Mint: Total phenolic content in fresh control mint samples was to be ~29 mg GAE/g on day one of storage, whereas in the CP1 treated samples was shown 435 mg GAE/g. During storage period of fifteen days at 4 to 6 °C, there was insignificant (p e 0.05) reduction in the total phenolic content. CP2 a treated sample was shown neither decrease nor increase of total phenolic content at the end of 15 days storage at 4 to 6 °C and RH of 85-90% (Table 3.18).

Fan and Sokorai 2002 also showed similar variation in earlier studies regarding effect of radiation processing on total phenolics in fresh spinach. Previously, González et al., 2003 reported that the percentage of phenolic content of propolis from different area in Argentina ranged between 3.25 and 33.49 GAE (analyzed using Folin-Ciocalteu), and 2.36 and 22.86 GAE (analyzed using Prussian Blue assay). Total phenolic content in red wine was also higher when were determined using Folin-Ciocalteu assay (5.14 to 13.3 mg/L GAE) compared with Prussian-Blue assay (1.8 to 4.8 mg/L GAE).

Chlorophyll content:

Total chlorophyll content in fresh spinach control sample as well as CP1 treated samples was to be 90 mg/g. However CP1 treated samples resulted significant reduction (p d 0.05) of

chlorophyll throughout the fifteen days storage, further it was decreased up to 50 mg/g. This reduction of chlorophyll content may be due to degradation of chlorophyll content during storage period. Study carried out by Gomes et al (2008) also resulted similar decrease in chlorophyll content in electron beam (CP2) treated spinach samples.

In fresh controls coriander samples total chlorophyll content was to be of 67 mg/g and (CP1) treated samples shown 465 mg/g on day one of storage. Chlorophyll content decreased considerably and it reduced up to 30 mg/g at the end of fifteen days storage (Table 3.18). Fresh Control mint samples and CP 1 treated samples resulted total chlorophyll content 4 70 mg/g (Table – 3.19). There were 460% losses in total chlorophyll content CP1 treated mint samples at the end of fifteen days storage. Thus the chlorophyll content decline in leafy vegetables during storage period can be because of the degradation of chlorophyll. This is also supported by the colorimetric data.

Studies carried out by Hsu et al. (2010) was also resulted that, similar decline in total chlorophyll content upon storage of fresh mint samples which was irradiated at 2 kGy. Chlorophyll gives an indirect estimation of the nutrient status because much of leaf nitrogen is incorporated in chlorophyll (Filella *et al.,* 1995). In recent years, chlorophylls, the most abundant pigments in green plants are gaining increasing importance in the human diet, not only as food colorants, but also as healthy food ingredients. (Xue and Yang, 2009). Chlorophyll is a green pigment, which is structurally similar to porphyrin pigments such as heme and it is produced through the same metabolic pathway. Chlorophyll benefits the body in a unique and distinctive ways. It helps to cleanse harmful toxins from the body and it is also used to fight infection. A recommended and regular intake of chlorophyll can keep the circulatory and digestive systems much healthier.

TABLE 3. 18 BIOCHEMICAL AND FUNCTIONAL ATTRIBUTES OF CONTROL AND PROCESED LEAFY VEGETABLES							
Vegetables	**Day of Storage**	**Total phenolics content (mg GAE/g)**			**Total chlorophyll content (mg/10 g)**		
Spinach		Control	CP1	CP2	Control	CP1	CP2
	Day1	19.2±2^{a,u}	22.8±5^{a,v}	19.6±4^{a,v}	90±9^{ax}	90±8^{a,x}	91±8^{a,x}
	Day7	ND	19.8±3^{a,u}	21.3±5^{a,u}	ND	78±8^{b,x}	79±8^{b,x}
	Day13	ND	17.5±4^{b,u}	30.3±4^{b,v}	ND	55±6^{c,x}	56±6^{c,x}
	Day15	ND	19.1±4^{a,u}	34.5±6^{b,v}	ND	50±6^{d,x}	51±6^{d,x}
Coriander	Day1	46.1±7^{b,u}	37.9±5^{c,v}	43.8±6^{c,w}	67±8^{bx}	65±7^{e,x}	64±7^{e,x}
	Day7	ND	43.5±4^{d,u}	36.7±6^{d,v}	ND	50±6^{f,x}	49±6^{f,x}
	Day13	ND	40.8±5^{d,u}	35.4±4^{d,v}	ND	45±5^{g,x}	46±5^{f,x}
	Day15	ND	38.3±4^{d,u}	33.9±5^{d,v}	ND	30±4^{h,x}	32±4^{g,x}
Mint	Day1	29.2±2^{c,u}	34.8±5^{e,v}	29.3±6^{e,w}	70±7^{cx}	70±7^{i,x}	71±7^{h,x}
	Day7	ND	32.7±6^{e,u}	26.1±4^{e,v}	ND	50±6^{j,x}	51±6^{i,x}
	Day13	ND	25.9±4^{f,u}	27.4±5^{e,u}	ND	48±6^{k,x}	47±6^{j,x}
	Day15	ND	28.8±3^{f,u}	27.7±4^{e,u}	ND	29±5^{l,x}	30±5^{k,x}

Control samples: Non-treated; CP1: Potable water wash + Sodium hypochlorite treatment (200 ppm) + Gamma irradiation (2 kGy); CP2: Potable water wash + Sodium hypochlorite treatment (200 ppm) + Electron beam irradiation (2 kGy); $^{a-l}$ Different letters across the columns and $^{\text{(u-w for total phenolic and x to x for chlorophyll)}}$ different letters across the rows indicates significant difference (p d0.05) between the sample means as analysed by one way ANOVA.

3.3.3.2. Nutritional characteristics of processed leafy greens well retained during storage

Spinach

Ash content in control, CP1 and CP2 samples was in the range of 1.4 to 1.8 g/100g. There was no significant difference in ash content between these samples. Total fat content, carbohydrates and total energy content did not show any significant difference between these samples (Table 3. 19). Sugars were absent in all the three vegetable samples. Niacin content was also unaltered in all the spinach samples while vitamin C content was similar in control and combination treated (CP1) spinach samples. Second combination treated samples however showed significant decrease as the vitamin C is sensitive to irradiation and at high dose rate of electron beam it is even more pronounced. Similarly, riboflavin (vitamin B_2) also showed sensitivity towards radiation and both gamma as well as electron beam irradiated samples showed significant loss of this vitamin. Similar effect was observed in vitamin A also (Table 3. 19).

Coriander

Ash content of control, CP1 and CP2 treated coriander samples was in the range of 1.4 to 1.6 g/100g. There was no significant change observed in the ash content in these samples after the treatment. Total fat content and total carbohydrate content also did not show any difference in control and irradiated (gamma or electron beam) coriander samples and the values were in the range of 0.7 – 0.9 g/100 g and 5.0 -5.7 g /100g, respectively. Energy content was determined by calculation and did not vary significantly between control and combination treated samples. Slight variation observed can be attributed to the biological variation among the samples. Vitamin C content was not determined in coriander samples. Similar to spinach samples, niacin (Vitamin B3) content remained unaltered after CP1 and

CP2 treatments and was in the range of 0.7 to 0.8 mg/100g (Table 3.19). Vitamin A was sensitive to some extent and showed slight but insignificant reduction after CP1 and CP2 treatments. Amount of riboflavin (vitamin B_2) in coriander was very small and did not alter by irradiation.

Mint

In case of mint, the ash content was in the range of 1.3 to 1.7 g/100g (Table 3.19). There was no effect of CP1 and CP2 treatments on this parameter. Total fat content and total carbohydrate content in control, both combinations treated (CP1 and CP2) mint samples was in the range of 0.2-0.3 g/100g and 9.1 – 10.0 g/100g, respectively. There was no significant change in these parameters after these combination treatments. Energy content did not alter after irradiation and was in the range of 49 – 57 kcal/ 100g while total protein content was in the range of 2.5 – 3.0 g/100g. Niacin (Vitamin B3) content showed slight but significant reduction unlike other vegetables. Vitamin C content showed insignificant reduction due to irradiation while vitamin A remained unaltered due to combination treatment and was in the range of 1132 – 1269 IU/100 g. Riboflavin (vitamin B_2) was stable and did not vary in samples treated by both these combinations. Thus, except few vitamins, all other nutritional parameters remained unaffected due to the combination treatments.

Nutritive value of commonly consumed leafy vegetables has been studied (Nkafamiya, 2010; Pattan and Usha, 2014). Vegetables play an important role in human nutrition; they offer the most rapid and lowest cost source of fibers, minerals and vitamins to the majority of people in developing countries, where they are frequently consumed in relatively small amounts as side dish or relish with the staple foods (Iyaka, 2007). The wide variation in color, taste and texture of various vegetables is an interesting additional touch to the meals. Hence, the cultivation and consumption of green leafy vegetables cuts across different races because of their nutritional and health benefits (Schmidt, 1994).

Many vegetables in Sri Lanka represent a class of underexploited plants which gives rich sources of natural antioxidants. Antioxidant properties of leaves of *Gymnema lactiferum* not have significant references. Gymnema leaf possesses hypoglycaemic properties (Bandara *et al.,* 2009). The antidiabetic components of this plant also known for its gymnemic acid and phenolic triterpene content (Kanetkar, Singhal, Laddha, & Kamat, 2006; Surveswaran, Cai, Corke, & Sun, 2007). Phenolics are present in the green vegetables and vital role in natural antioxidants as it has major bioactivities (Chandrasekara & Shahidi, 2011). Most studies reported the and focused on phenolic substances and *Sesbania grandiflora, Murraya koenigii Spreng, Gymnema lactiferum, Passiflora edulis, Hemidesmus indicus and Cassia auriculata are content a higher amount of* phenolic content. Leafy vegetables are also used in medicinal formulations in Sri Lanka and also for controlling the chronic diseases. However, many research published on the higher phenolics compared with other leafy vegetables tested. *Passiflora edulis, Murraya koenigii Spreng and Sesbania grandiflora* are famous green leafy vegetables in Asia. Lutein is the type of main Carotenoids present in the in green leafy vegetables and helps to identify the antioxidant activity (Chandrika, Basnayake, Athukorala, Colombagama, & Goonetilleke, 2010), (K.D. Prasanna Priyantha Gunathilake, 2016), (Chandrika UG, 2010)

Table 3.19 Nutritional Analysis

Parameters	Method	Spinach			Coriander			Mint		
		Control	CP1	CP2	Control	CP1	CP2	Control	CP1	CP2
Ash (g/100g)	AOAC 19th Edition 930.05, 2012	1.8±0.3 ^{j}	1.6±0.3 ^{j}	1.4±0.2 ^{k}	1.6±0.3 ^{l}	1.5±0.2 ^{l}	1.4±0.2 ^{l}	1.6±0.3 ^{m}	1.7±0.5^{m}	1.3±0.2 ^{n}
Fat (g/100g)	IS: 12711 – 1989 (Reaffirmed 2010)	0.2±0.1^{j}	0.2±0.1 ^{j}	0.2±0.1 ^{j}	0.9±0.15^{k}	0.8±0.15^{k}	0.7±0.2 ^{k}	0.3±0.1 ^{l}	0.2±0.1 ^{m}	0.2±0.1 ^{m}
Carbohydrate (g/100g)	By Difference	3.0±0.5 ^{j}	2.0±0.3 ^{k}	3.0±0.3 ^{j}	5.7±1 ^{l}	5.4±1.0 ^{l}	5.0±0.8 ^{m}	10.0±1.5 ^{n}	10.0±1.5^{n}	9.1±1.5 ^{n}
Total Sugars (g/100g)	AOAC 19th Edition 923.09, 2012	Nil	Nil	NIL	Nil	NIL	NIL	Nil	Nil	NIL
Energy (kcal/100g)	By Calculation	20.0±3.0^{j}	19.0±3 ^{j}	20.0±3 ^{j}	46.0±7.5^{k}	39.0±6.0^{l}	42.0±6.0^{k}	57.0±9.0^{m}	52.0±7.2^{n}	49.0±7.0^{o}
Protein (g/100g)	IS:7219-1973(Reaffirmed 2010)	1.9±0.3 ^{j}	2.0±0.3^{j}	1.6±0.3^{k}	1.5±0.3 ^{l}	2.0±0.3 ^{m}	2.0±0.2^{m}	2.5±1.0 ^{n}	3.0±0.5 ^{o}	3.0±0.5 ^{o}
Niacin (mg/100g)	IS 5400-1969 (Reaffirmed 2010)	0.2±0.1 ^{j}	0.2±0.1 ^{j}	0.2±0.1^{j}	0.7±0.2 ^{k}	0.8±0.1 ^{k}	0.8±0.2 ^{k}	1.9±0.3 ^{l}	1.2±0.1 ^{m}	1.4±0.2 ^{n}
Vitamin C (mg/100g)	IS 5838-1970 (Reaffirmed 2005)	16.6±3.0^{j}	17.8±3 ^{j}	12.7±2.0^{k}	ND	ND	ND	21.9±3.0^{l}	18.2±2.5^{m}	19.4±3.0^{n}
Vitamin A (IU/100g)	IS 5886-1970 (Reaffirmed 2010)	2282±45^{j}	1893±30^{k}	2102±30^{l}	3331±44^{m}	3050±45^{n}	2979±45^{o}	1185±22 ^{p}	1269±19^{q}	1132±22^{r}
Riboflavin (mg/100g)	IS 5399-1969	1.9± 0.3^{j}	1.0±0.2 ^{k}	1.3±0.1 ^{l}	0.1±.01 ^{m}	0.1±.01 ^{m}	0.1±.01 ^{m}	0.9±0.2 ^{n}	0.9±0.2 ^{n}	0.8±0.2 ^{n}

Control: Unirradiated samples analysed on day 1, CP1: Combination (water + sodium hypochlorite (200 ppm) + gamma irradiation 2 kGy)) treated on day 15; CP2: Combination (water + sodium hypochlorite (200 ppm) + electron beam (2 kGy)) treated day 15; ND: Not determined. All sample analysed in triplicate and mean results shown. Analysis done under NABL accredited lab. Appropriate positives and blanks have been used. Nutritional attributes of control and irradiated samples (CP1and CP2) were evaluated. $^{j\ to\ r}$ Different letters across the rows indicates significant difference (p d0.05) between the sample means as analysed by one-way ANOVA.

3.3.4. Enhancement in functional bio-actives upon processing and storage

3.3.4.1. Kaempferol content in spinach enhanced upon radiation treatment

The major flavonoid in fresh spinach was found to be kaempferol as confirmed by TLC study (Table 3.20, Fig. 3.9). In fresh sample (control) of spinach kaempferol content was found to be 538 µg/100 g fresh weight basis. Samples treated with gamma and electron beam (2 kGy) showed enhancement of kaempferol content up to 881 and 950 µg/100g fresh weight respectively on day one of storage (Fig. 3.10 A-D). Upon storage at 4 °C and RH 85 to 90 % for 15 days kaempferol content was found to be further increased up to 1072 µg/100g fresh weight in gamma and 1204 µg/100g fresh weight in electron beam treated samples (Table 3.20). Results indicated that, after radiation processing (Gamma and EB) and storage at above conditions there was enhancement of kaempferol content of spinach samples. Other source like intense pulsed light (IPL) is also being used for exterior sanitization. This treatment was also resulted enhancement of total polyphenolic content of spinach (range of 5–10 %) treated with IPL 20 kJ m^{-2} and (range of 32–34 %) was treated with 40 kJ $m.^{-2}$ Hussain et al., 2016, have shown that upon radiation treatment ferric reducing power of spinach were enhanced from 44.0 % to 443 % and OH^{*} scavenging activity was also enhanced from 1.5 % to 2.4 %. Thus, significant enhancement of kaempferol content was found in spinach. Aguero et al., 2016 carried out study have shown that, polyphenols content in the fresh spinach leaves was 270 mg/ kg tannic acid equivalent and 390 mg/kg as catechin equivalents and in these kaempferol content was 30 mg/kg (Masood et al., 2010). Many researchers have been studied the role of kaempferol in health protection. They have also shown that kaempferol is present in extensive varieties of plant species and possesses anti-inflammatory properties. Kaempferol plays role as a chemo-preventive and chemotherapeutic agent. Study carried out by Kashyap et al., (2017) also revealed that, kaempferol acts on an extensive range of

extracellular and intracellular targets concerned inside the pathway of cell signalling. It also retards the cancer growth processes like apoptosis, metastasis and cell division.

Leafy vegetables are not the either major source of nutrients or source of high calorific gain. As per reports (Sarker and Oba, 2017; Gautam, S *et al.,* 2016) vegetables are rich in health protecting phytochemicals.

Antioxidant activity mainly driven by Phenolic compounds which are present in plant phytoconstituents. As per the study carried out by Pinela et al 2016, resulted that facilitation of free radical scavenging is done through hydroxyl group present in plant extract which containing phytoconstituents. In earlier study it was found that, in *Tuberaria lignosa* plant antioxidant activity, total flavanoid and tocopherol were retaned well upon radation processing of plant at dose of 5 kGy. Uddin et al., also estimated that, Total phenolic content of 3.6 ± 0.089 mg GAE/g (DW) in the *P. oleracea* (Uddin *et al.,* 2012).. Kumar et al., also reported in the plant *C. tora* contains total flavonoid content of 21.53 mg QE/g (DW). Adebooye et al., estimated total flavanoid contet 0.64 mg catechin eq/gm of (FW), in the aqueous extract of *S. Nigrum.* Burri et al 2017 also states that, phenolic content may vary due to fators like varety of plant, geographical location and presence of other biochemical's present in the plant.

As per the study carried out by Fan et al., 2003 iceberg lettuce was dipped in different two temperatures either 5 or 47 °C water in bath for 2 min. Later it was packed MAP (Modified Atmosphere Packaging) and then further gamma irradiated at doses of 0.5, 1, and 2 kGy. This dipped cut lettuce in 47 °C for 2 minutes resulted in reduced antioxidants. Study also resulted that samples irradiated at doses of 0.5 and 1 kGy shown similar firmness and ascorbic acid content as the control sample at the end of 14 and 21 days storage.

Less tissue browning was observed in lettuce dipped at 47°C degrees C and reated at 0.5 and 1 kGy. As per the study of Ferrante et al., 2004 browning development related to polyphenol

amount. They also found decrease level of carotenoids after storage of one week in all the species used for experiment.

3.3.4.2. Quercetin content in coriander enhanced upon irradiation

Quercetin is a major flavanoid in coriander as per USDA data and as analyzed by TLC (figure 3.7, 3.8 and 3.9). Quercetin content in fresh coriander sample (control) was found to be 413.85 mg/100g (Table 3.20). Vegetable samples irradiated with gamma and electron beam (2 kGy) resulted in improved quercetin content up to 17.42 mg/100g and 18.67mg/100g, respectively on day one of storage (Fig. 3.7, 3.8, 3.9 and 3.11 A-D). Further quercetin content was found to be enhanced to 20.14 mg/100g and 22.45 mg/100g in gamma (2kGy) and EB (2 kGy) treated samples, respectively, which was stored at 4 - 6 °C and RH 85 to 90 % for 15 days (Fig. 3.11 A-D). It was resulted that, EB treated sample showed elevated content of quercetin upon irradiation and storage at 4 °C and RH 85 to 90 % for 15 days compared to gamma treated samples. This may be due to the higher dose rate of EB, resulting in release of free flavonoids from bound ones. Study carried out by Nambiar et al., (2010) have also shown that, coriander leaves have quercetin and kaempferol as main flavonoids. Earlier reports also resulted that, radiation processing of coriander resulted in microbial decontamination and shelf life extension at 8–10 °C (Kamat et al., 2003). Here author also found improvement in extractability of carotenes and chlorophylls due to radiation processing. Quercetin is a major flavonoid in vegetables like *Cardiospermum helicacabum*, *Celosia argentea* and *Alternanther asessilis* and the same study also resulted the total phenolics in the range of 3.89 to 8.55 mg Gallic Acid Equivalent (GAE)/g (dry weight basis)), and flavonoids in the range of 9.0 to 38 mg/g(dry weight basis) quercetin equivalent (Ranganathan et al., 2017). USDA database - 2007 also shown that, quercetin content in the fresh coriander leaves has been reported to be 52.9 mg/100g (fresh weight basis). Some study also indicates that there is considerable variety-

based difference in the phytoconstituents of leafy vegetables (Sivakumar et al., 2018). In one of the study Patel et al., (2018) has been shown that, quercetin displayed pharmacological function in cardiovascular disease and oxidative stress. In systemic circulation, existence of quercetin has importance due to this health defensive property (Leskak et al., 2018).

The effect of irradiation on the content of quercetin in onion (*Allium cepa* L.) has been also reported earlier. Radiation dose of ~10 kGy enhanced the yield of quercetin from 36 to153 µg/ml. Yang et al., 2012 was also shown that optimum gamma irradiation dose of 10 kGy is enough to liberate soluble phenols by breaking the physical and chemical bonds. Here onion commodity had been used as a model system to ascertain the effect of high dose radiation treatment. However, the gamma radiation dose necessary for sprout inhibition of onion is average 60 Gy (FSSAI, USFDA).

3.3.4.3. Rosmarinic acid content in mint increased upon irradiation

Rosmarinic acid is a polyphenol derived from many common herbal plants of the Lamiaceae group: rosemary, oregano, Spanish sage, sage, basil, thyme, marjoram the mint group, lavender, lemon balm and perilla. It is presently being studied for its effects on Alzheimer's disease and other diseases.

As per USDA data rosmarinic acid is the major phenolic acid and present in mint (*Mentha piperitae, spicata*). Rosmarinic acid content in fresh sample (control) of mint was found to be 59 mg/100g (fresh weight basis) (Table 3.20). Fresh mint Samples irradiated with gamma and electron beam (2 kGy) showed the enhanced rosmarinic acid content up to 77 mg/100g and 175 mg/100g (fresh weight basis), respectively on day one of storage. Rosmarinic acid content in 15 day's stored (4 - 6 °C and RH 85 to 90 %) samples was found to be 127 mg/100g (fresh weight basis) in gamma and 184 mg/100g (fresh weight) in EB treated samples. Other study resulted that, upon hydrolysis of crude mint extract of air dried leaves there was enhancement in free rosmarinic acid from 12 mg/g to 17 mg/g in *Mentha piperitae*

(Aleksandra et al., 2011). Lou, et al., 2016 also reported that rosmarinic acid has been plays important role in stimulated activation of the mTOR/S6K pathway which was based on hepatocyte proliferation. As per study of Daniella et al., (2015) reported the rosmarinic acid content in *Rosmarinus officinalis* L. and *Origanum vulgare* L. was 1.33 mg/g and 124 mg/g (dry weight basis), respectively. Trigo et al., (2009) showed that 0.5 kGy of gamma radiation could be reduced microbial count by maintaining organoleptic / sensory attributes and functional properties. Thus, evidences support the concept that radiation can hygienize the leafy vegetables as well as enhance the health protective property by increasing the phytoconstituent's content in numerous cases. Milica et al., (2015) also shown in his study that rosmarinic acid content in *Salvia verbenaca* L. was to be ~ 94 µg/mg of. Phenolics acid plays major role in enzyme inhibitory action which was accountable for control of skin disorders, Diabetes mellitus and Alzheimer's disease (Asghari et al., 2018). Fan, 2005 in his study has been also shown that, gamma irradiation (0.5 to 2 kGy) treatment to the leafy vegetable like iceberg lettuce resulted in increased phenolic content and antioxidant capacity when stored for period of 8 days at 8 °C. Study carried out by Lou et al., 2016 resulted with improved impaired liver function by rosmarinic acid. Thus, consumption of irradiated leafy greens improves the prophylactic properties. Sarker and Oba, 2017 also shown that, leafy vegetables are source of health protective phytoconstituents which mostly includes phenolics acids and flavonoids.

Table 3.20 Content of major phytochemicals in leafy greens as analyzed by HPLC						
Sr. No	**Sample type**	**Control (Day 1)**	**Gamma Radiation Treated Sample (Day 1)**	**Electron Beam Treated Sample (Day 1)**	**Gamma Radiation Treated Sample (Day 15)**	**Electron Beam Treated Sample (Day 15)**
1	**Kaempferol content in spinach (µg/100g)**	538 ± 7.21^{a}	881 ± 9.53^{b}	950 ± 10.06^{c}	1072 ± 13.11^{d}	1204 ± 11.55^{e}
2	**Quercetin content in coriander leaves (mg/100g)**	13.85 ± 0.97^{f}	17.42 ± 0.47^{g}	18.67 ± 0.57^{h}	20.14 ± 0.35^{i}	22.45 ± 0.54^{j}
3	**Rosmarinic acid conc. in mint leaves GAE mg/100g**	59 ± 2.2^{k}	77 ± 4.3^{l}	127 ± 5.4^{m}	175 ± 10.6^{n}	184 ± 12.3^{o}

Different letters across the rows **(a to e for kaempferol), (f to j for quercetin) and (k to o for rosmarinic acid)** indicate significant difference (p d0.05) among the sample means as analysed by ANOVA (one-way).

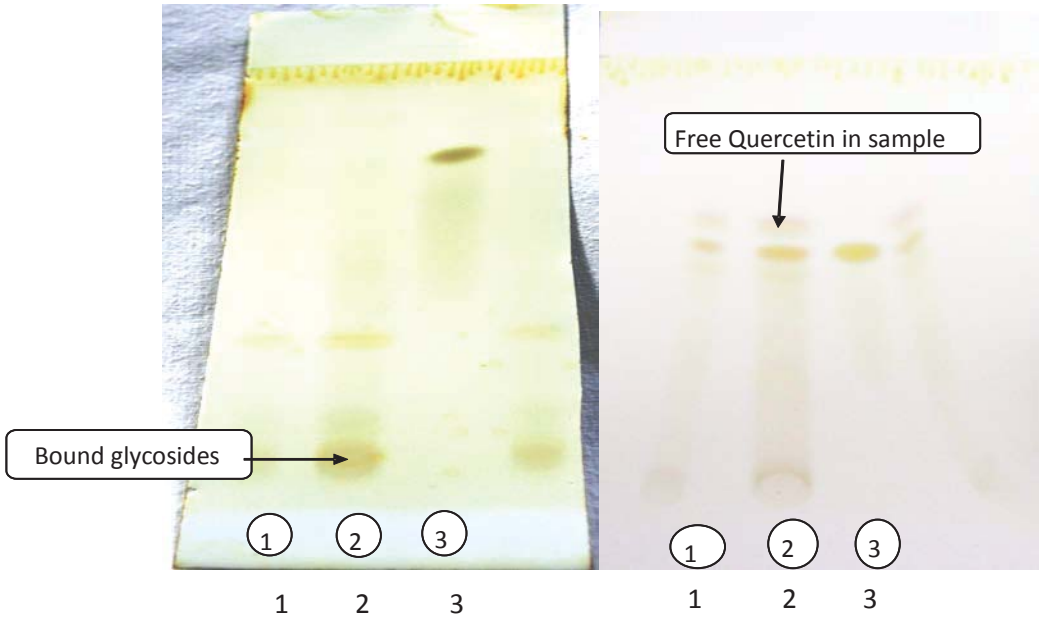


Figure 3.7 TLC analysis of flavonoids extracted from coriander, A (Un-hydrolysed), B (Hydrolysed): spot 1 (10 µl), spot 2 (30 µl), and spot 3 (Standrad quercetin 10 µl). (colour image)

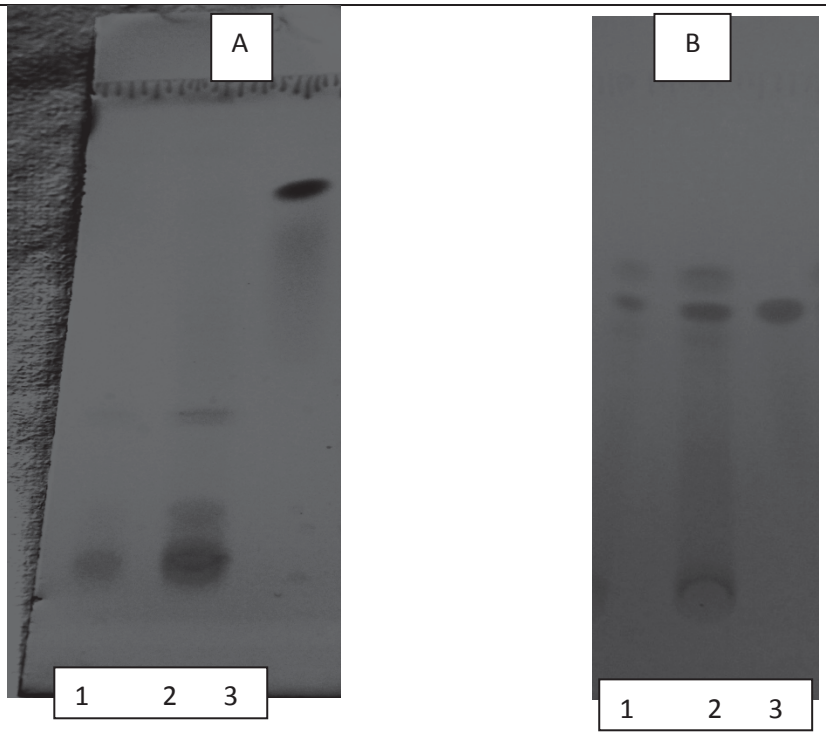


Fig. 3. 8 . TLC analysis of flavonoids extracted from coriander, A (Un-hydrolysed), B (Hydrolysed): spot 1 (10 µl), spot 2 (30 µl), and spot 3 (Standrad quercetin 10 µl). (Black and white image)

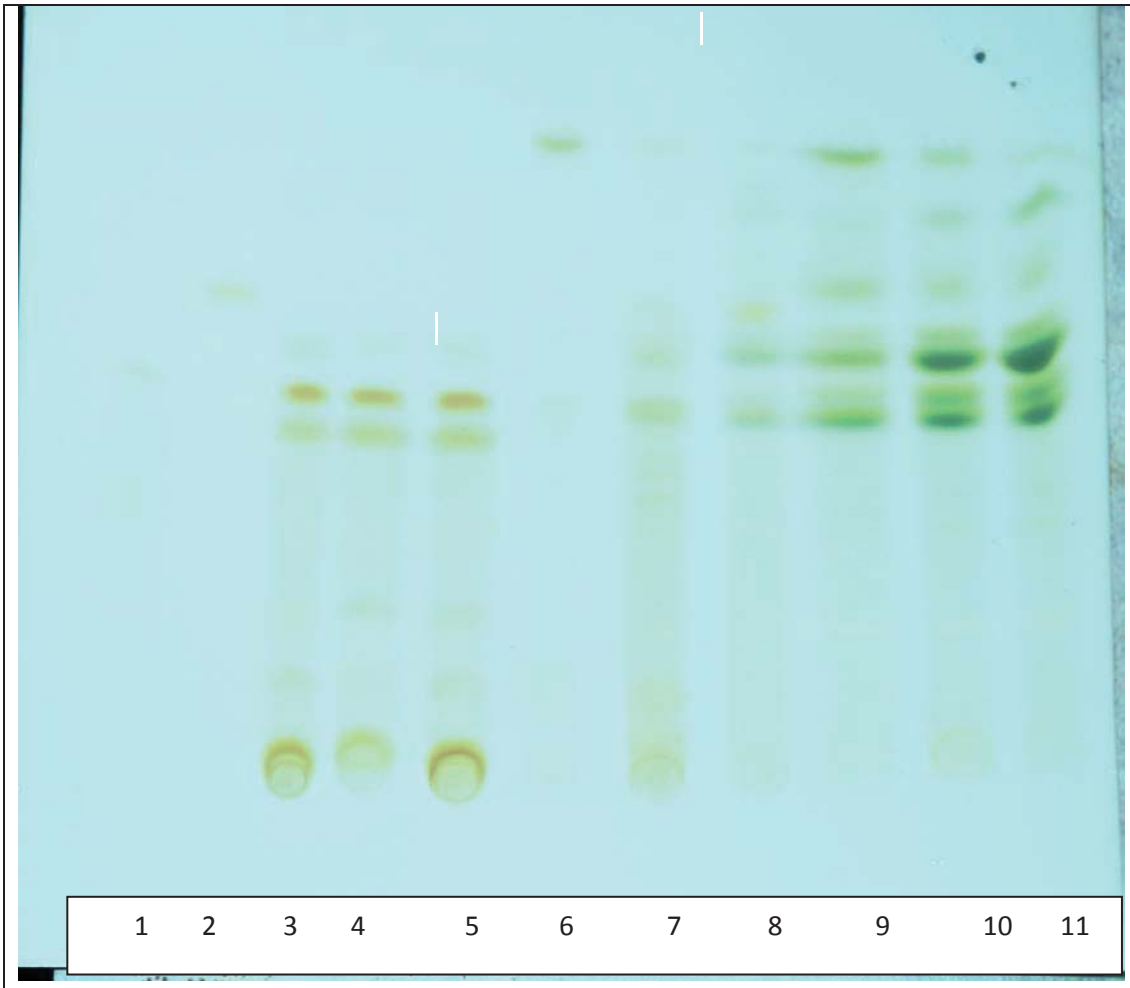


Fig. 3.9. TLC identification of flavonoids extracted from radiation treated coriander and spinach with respective controls and standard: spot 1: Standard quercetin; spot 2. Standard kaempferol; spot 3. coriander control sample; spot 4. coriander 2 kGy gamma radiation treated acid and hydrolysed sample; spot 5. coriander 2 kGy EB treated and acid hydrolysed sample; spot 6. spinach control sample; spot 7. spinach 2 kGy gamma treated and acid hydrolysed sample; spot 8. spinach 2 kGy EB treated and acid hydrolysed sample; spot 9. spinach unhydrolyzed control sample; spot 10. spinach unhydrolyzed 2kGy gamma treated sample; spot 11. spinach unhydrolyzed 2 kGy EB treated sample.

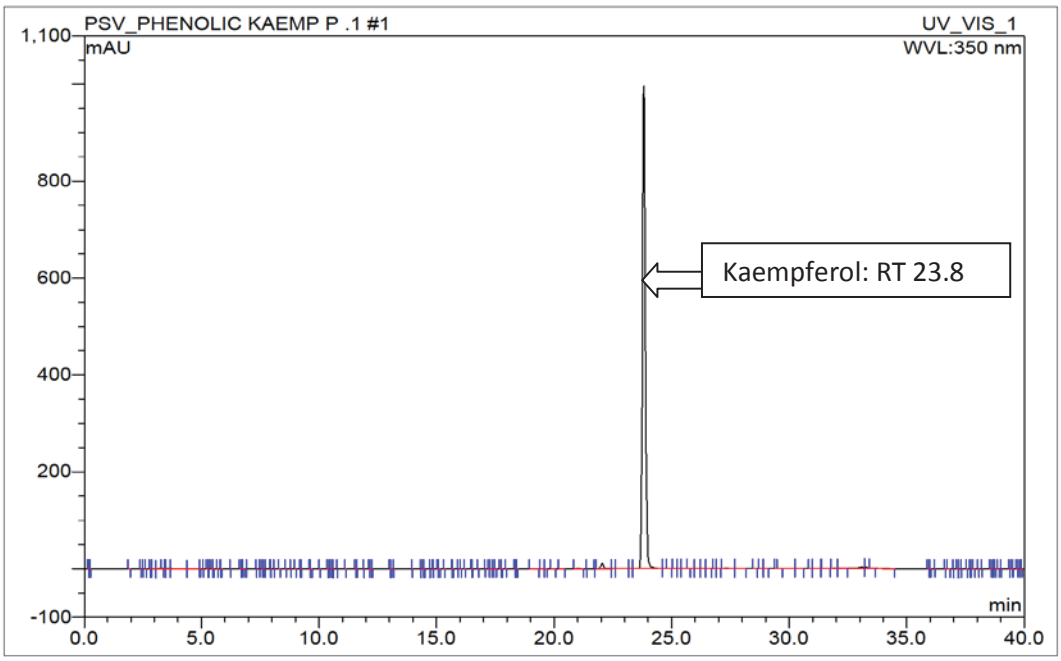
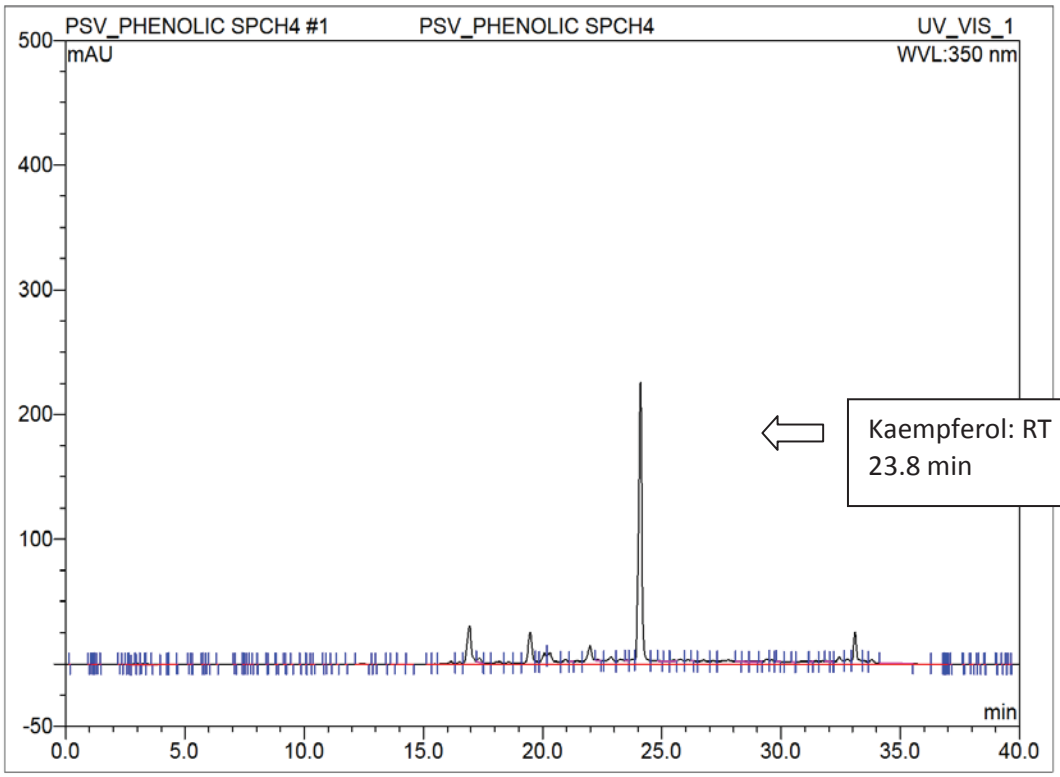


Fig. 3.10A Fig.3.10. HPLC analysis of flavonoids extracted from spinach, A: Kaempferol, B: spinach control acid hydrolysed sample; C: spinach 2 kGy gamma treated acid hydrolysed sample; D: spinach 2 kGy EB treated acid hydrolysed sample.

10B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PSV_PHENOLIC SPCH4
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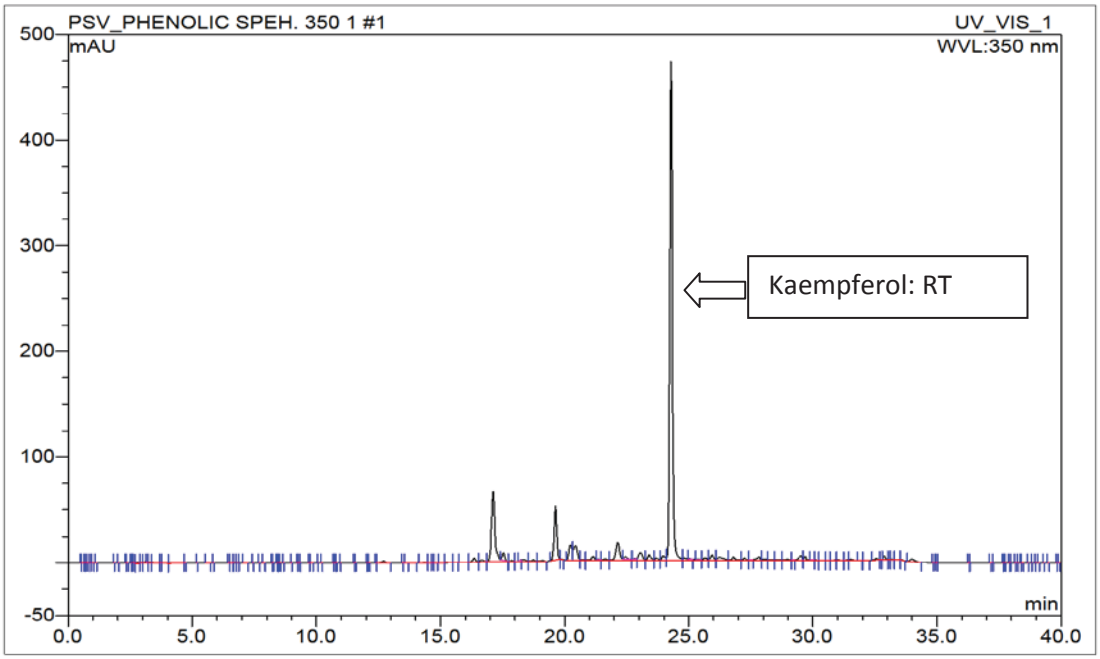
mAU
WVL:350 nm
500
400
300
200
100
-50
min
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
Kaempferol: RT 23.8 min

10C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400
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200
100
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min
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5.0
10.0
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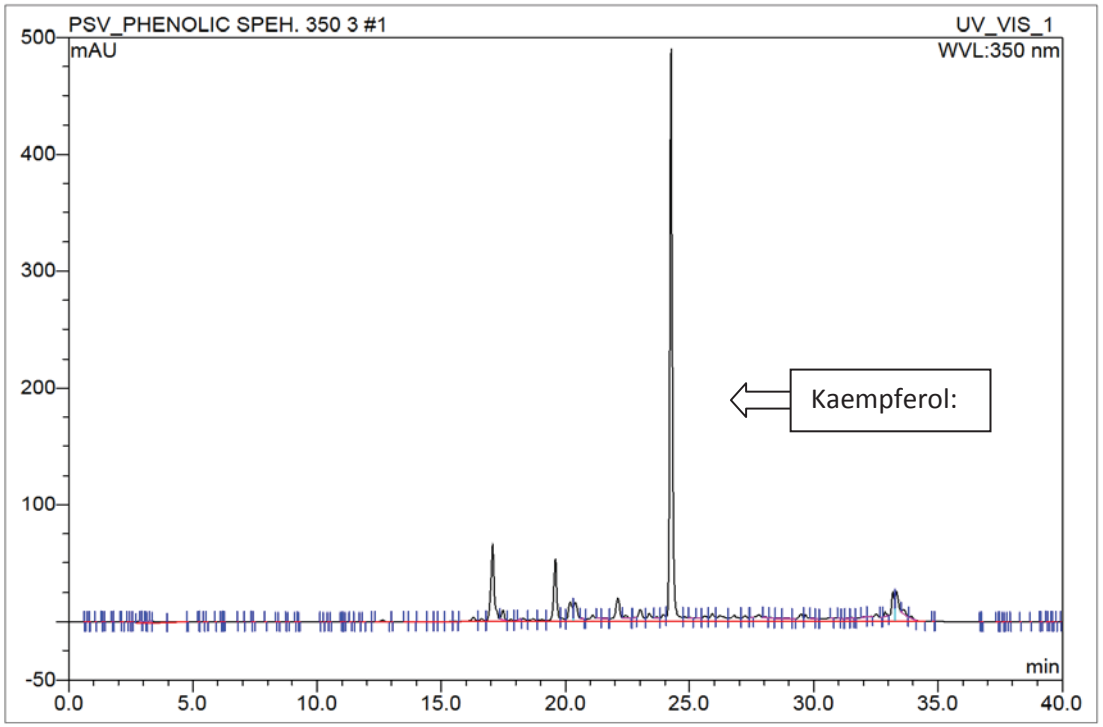
20.0
25.0
30.0
35.0
40.0

10D.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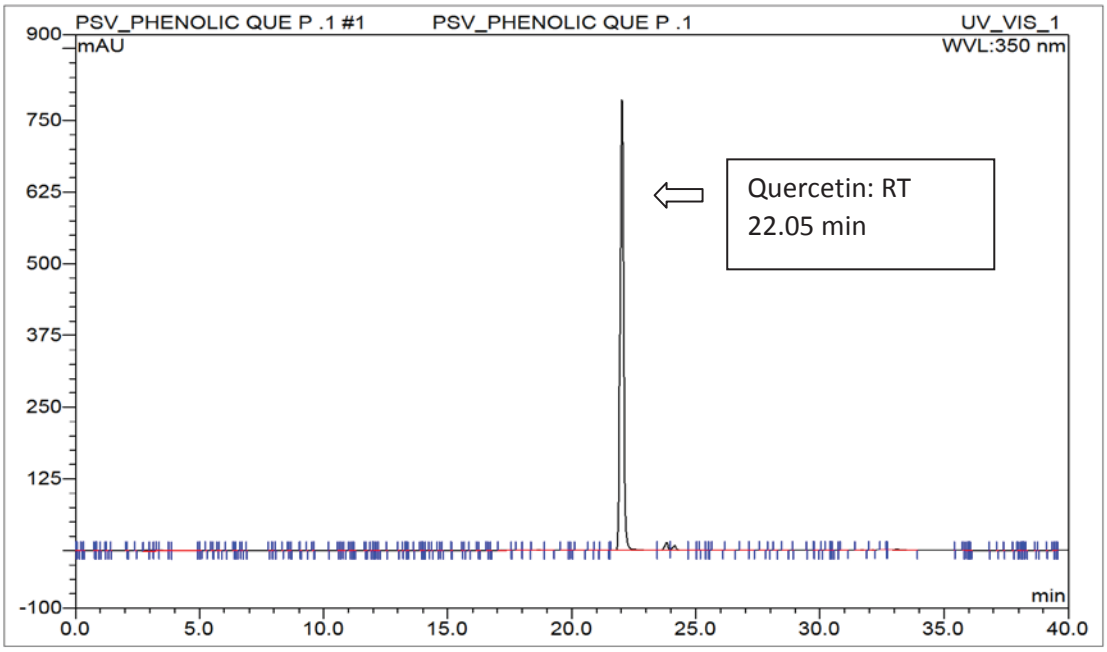
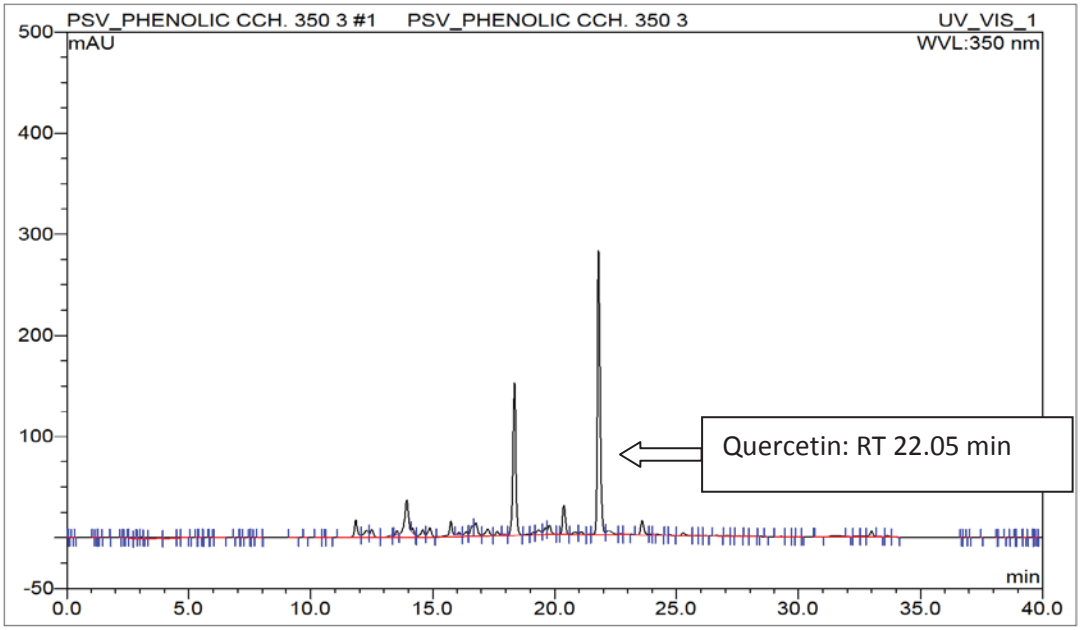
400
300
200
100
-50
Kaempferol:
min
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0

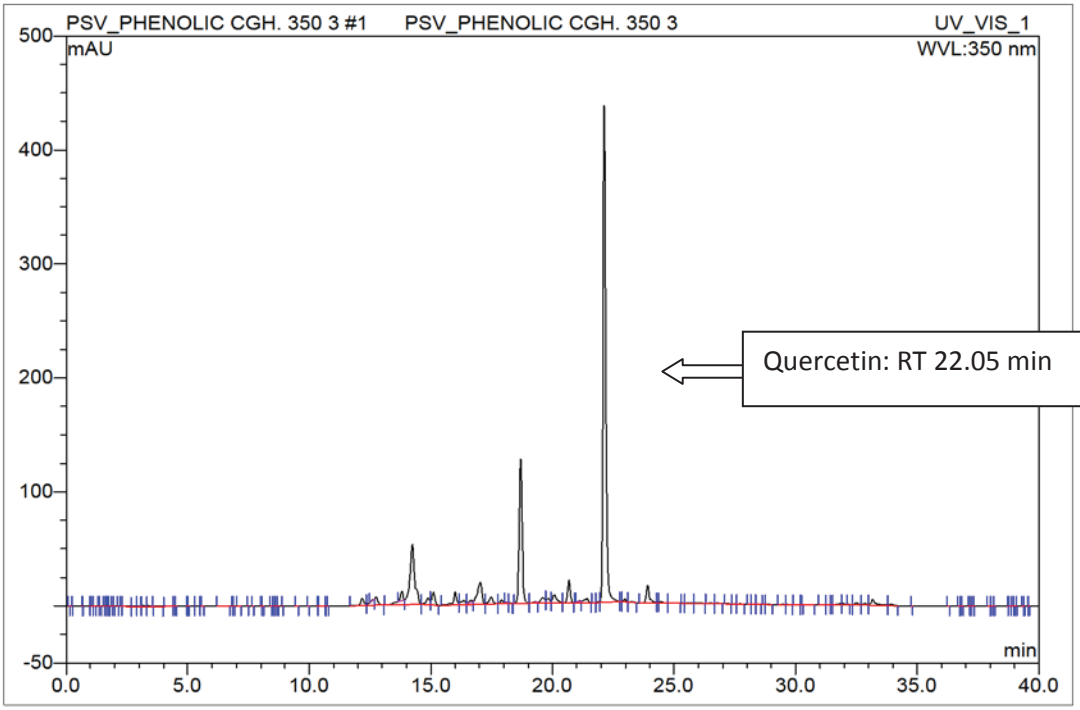
Fig. 3.11A Fig.3.11. HPLC analysis of flavonoids extracted from coriander, A: Quericitin, B: coriander control acid hydroly sed sample; C: coriander 2 kGy gamma treated acid hydrolysed sample; D: coriander 2 kGy EB treated acid hydrolysed sample.



11B



11C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WVL:350 nm
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400
300
200
100
-50
min
0.0
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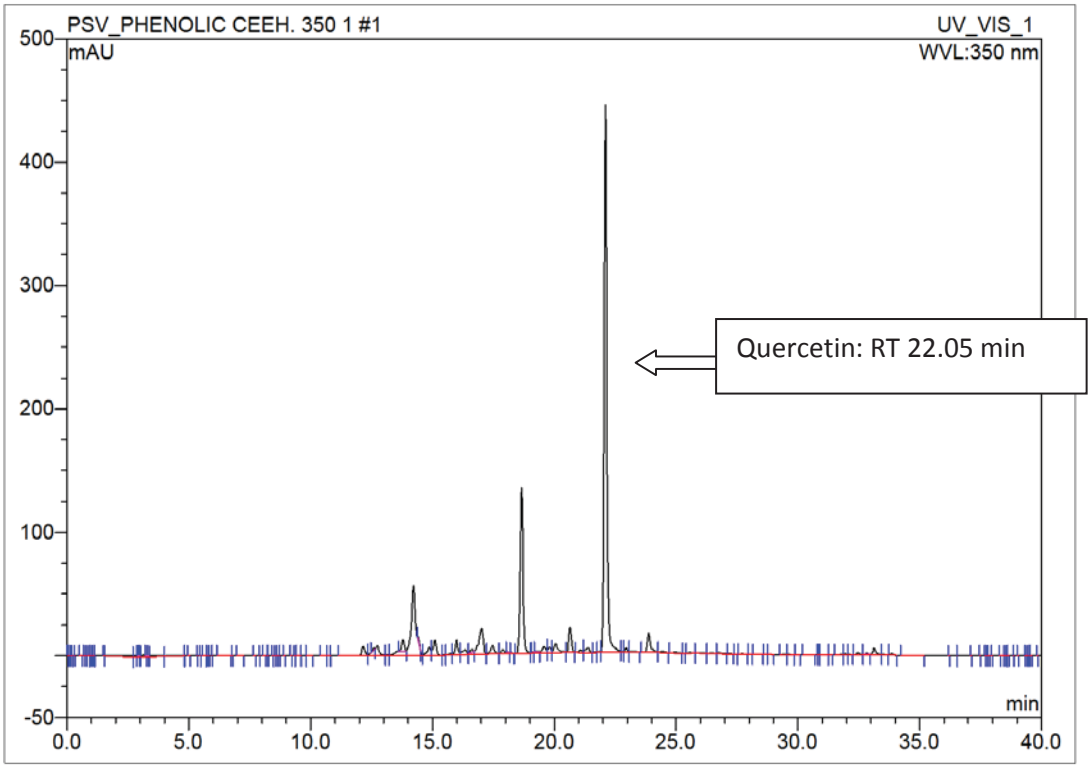
10.0
15.0
20.0
25.0
30.0
35.0
40.0
Quercetin: RT 22.05 min

11D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400
300
200
100
-50
min
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
Quercetin: RT 22.05 min

3.3.5. Enhancement in antimutagenic potential of spinach and coriander upon processing

Spinach and coriander was evaluated by EMS induced Rif^R antimutagenesis assay. The flavonoids extracted from coriander leaves were resulted ~ 46% decrease in mutagenicity. Gamma irradiation (2 kGy) and electron beam (2kGy) irradiated samples was resulted ~57 and ~54% decrease in the mutagenicity. However there was slight raise in the mutagenicity in both gamma as well as electron beam irradiated coriander samples. However, this variation was not significantly different.

Extracted flavonoids from unirradiated control spinach sample showed decrease in about 17% of mutagenicity (Fig. 3.12 A and B). Gamma and EB radiation treatment resulted significant raise in anitmutagenicity by 63% and 93 % as compared to control. (Fig. 3.12 A and B). This may be due to improved extractability of flavonoids because of radiation treatment in spinach as well as the nature of bioactivities.

Free flavonoids seem to possess high antimutagenic potential as seen in case of spinach samples. The antimutagenic potential of control sample, gamma irradiated and electron beam irradiated samples may have various bioactive compounds and act through different molecular mechanism of action. S Kumar et al., 2013 also studied antimutagenicity in polyphenol and anthocyanins extracted from rose (*Rosa centifolia*) petals and tea.

Antimutagenic activity was monitored by phenotype change due to induced mutation (Bandyopadhyay N., et al., 2014). Those cells of *E. coli* cells will not grow on rifampicin selective media which are not exposed to the mutagen. In *E. coli* DNA dependent RNA polymerase is inhibited by rifampicin. *E. coli* (MG1655) culture was selected for study because it response like phenotype cange from rifampicin sensitive to rifampicin resistance.

Cells develop resistance to rifampicin after mutation in the *rpoB* gene which reduces binding affinity of the rifampicin to RNA polymerase present in cell (Campbell EA. et al., (2001). This assay is most robust as compared to others like AMES mutagenicity assay. Iit is very sensitive, specific as well as simple (Lee H, et al., 2012; Garibyan L., 2003; Cupples CG. et al., (1989). The multiple biological activities of medicinal plants indicate their potential as a source of functional foods and nutraceuticals (Han et al., 2007; Singh et al., 2009). A strong correlation (0.976), observed between total phenolics and antimutagenic activity, indicates that the antimutagenic activity of leafy vegetables is directly related with the availability of phenolics. However, antimutagenic property varied among non-leafy vegetable varieties and no relationship could be established with their antioxidant capacity (S Kumar and S Gautam, 2013).

Many studies focused on the health benefits of vegetables act as medicinal properties like anticarcinogenic (Rajesh Kumar *et al.,* 2002). Also have an antibacterial properties (Kubo, Fijita, Kubo, Nehei, & Gura, 2004) and antidiabetic contents (Kesari, Gupta, & Watal, 2005). The main antioxidant containing constituents are Carotenoids and polyphenolic present in green vegetables (Andarwulan et al., 2012; Deng et al., 2013; Khanam, Oba, Yanase, & Murakami, 2012; Subhasree et al., 2009). Many health benefits of vegetables are attributed like the antioxidants content and its properties. Recent studies shows the vegetables are the sources of functional components. In condense, green leafy vegetables plays vital role in the balance diet and provides the significant amounts of vitamins, minerals and antioxidant (Subhasree, Baskar, Keerthana, Susan, & Rajasekaran, 2009). K.D.P.P. Gunathilake et al., 2016

Figure 3.12 Rif^{R} Mutation frequency: A. Coriander and B. Spinach. Different letters across bar indicate that the means for control and irradiated samples were significantly different (P, 0.05) as analysed with one-way ANOVA.

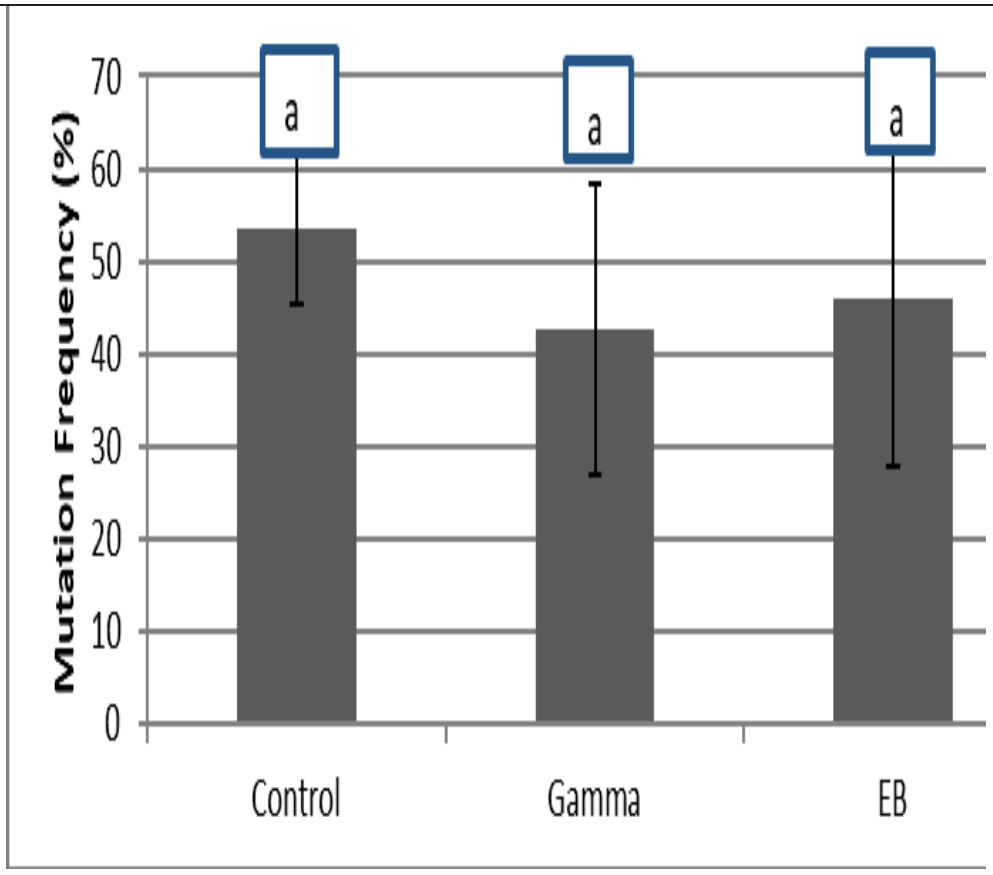
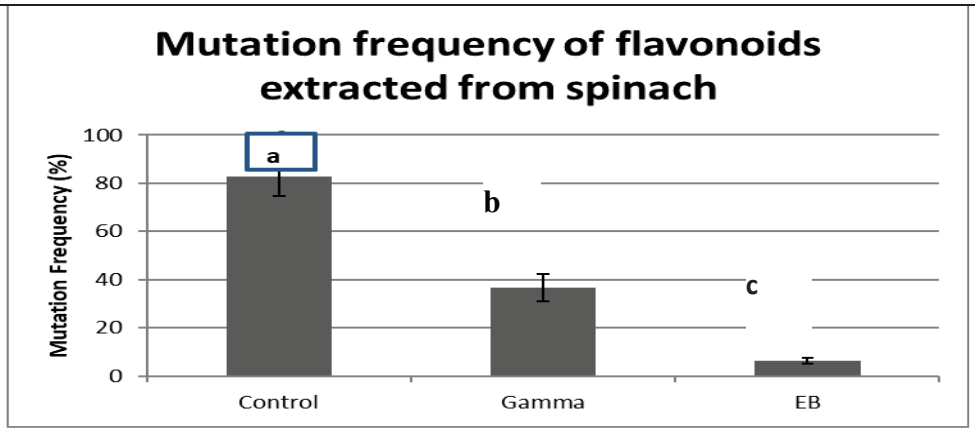


Fig. 12A

Fig 12B



Chapter 4

SUMMARY

AND

CONCLUSION

As per CDC (Centre for Disease Control and Prevention) reports, around 12% of food borne out breaks (reported) are due to consumption of fresh leafy agri-produce. WHO (World Health Organization) has also declared food safety as a most important concern. India's flora covers 6000 species of vegetation which used for consumption purpose, out of this 1/3rd are leafy greens. As leafy agri-produce is easily available source of various minerals and vitamins, dietician's recommends the consumption of leafy vegetable daily. However in India's fresh green leafy agri - produce is most commonly handled in an unorganized manner. Fresh leafy greens like, coriander (*Coriandrum sativum*), mint (*Mentha arvensis*) and spinach (*Spinacia oleracea*) are used for culinary purposes mostly in raw form.

Microbial analysis of above said freshly harvested leafy greens indicated aerobic microbes, yeast and mould, presumptive coliforms and presumptive Salmonella. In the preliminary microbial analysis presumptive coliforms were occurred mostly in all the farm fresh samples collected in various lots. However, presumptive Salmonella were detected in few batches only. Other pathogens like Shigella, *Staphylococcus aureus* and Listeria spps were not detected in these lots of samples.

In the samples collected from various locations of city presumptive coliforms and presumptive salmonella were detected. Amongst presumptive coliform, five numbers of isolates were confirmed to be *Escherichia coli* by PCR (Polymerase Chain Reaction) amplification of the *Escherichia coli* specific uspA gene. Upon further confirmation, these *Escherichia coli* isolates does not shown reactivity / agglutination for antibodies (specific to lipopolysaccharide O157 antigen) of pathogenic type *Escherichia coli* O157:H7. However amongst the presumptive Salmonella isolates none of the isolate shown PCR amplification of the Salmonella specific invA gene.

This study concludes that, none of the screened isolate was confirmed pathogenic *E. coli* O157:H7 but study shows confirmed presence of *Escherichia coli* in the samples and it indicates faecal contamination or *Escherichia coli* contamination could have occurred. To overcome this concern, processing of leafy vegetables is necessary to achieve desired hygiene level.

Combination processes 1 (CP1) and Combination Process 2 (CP2) have been attempted for purpose of hygienization and extension of shelf life (keeping quality). Initially radiation dose optimization was carried out. Packaged fresh green vegetable samples were irradiated at the doses of 1 to 3 kGy. Radiation dose of 1 kGy was not found to be suitable for effectively reduce coliform count. However 2 kGy could reduce the coliform numbers below detection limit.

Higher dose of 3 kGy was able to reduce coliform count but not found suitable to retain the organoleptic qualities. Therefore radiation dose of 2 kGy was fixed for further studies. This dose also has the statutory approvals from US, code of federal regulation, 21 CFR 179.26 as well as food rules recently notified by notified by FSSAI, Government of India.

In the CP1 the vegetables was minimally processed. The processing steps involved washing with potable water for 5 min which was designated as (T1). Further these vegetables were subjected to dip treatment in approved sanitizer like sodium hypochlorite (200 ppm) for 5 min and the treatment was designated as (T2). After sanitizer dip treatment vegetable samples were air dried for 30 min. Packaging of processed vegetables were carried out in a tray with moist cellulose based material which was wrapped in oxygen permeable PVC film. Packed vegetables were gamma irradiated at the dose of 2 kGy and the treatment was designated as T3. The combination comprising of T1+T2+T3 was named as Combination process 1 (CP1).

In the similar way in the second combination process i. e. CP2, the same treatments were used except instead of gamma radiation same dose of electron beam was used and the e – beam treatment was designated as T4. These combination process including T1+T2+T4 was named as combination process 2 (CP2). Individual treatments like T1, T2 and T3 of these combinations was found to be effective up to certain limit but not found to be enough to achieve required hygiene. However, in combination of T1+T2+T3 or T1+T2+T4 displayed synergetic effect and achieved desired hygiene level.

To maintain the freshness throughout the storage period at low temperature (4 to 6 °C), packaging in moist bed trays was optimized where the trays was wrapped using PVC film. As packaging material remains in contact with vegetables during the storage period, effect of radiation dose on packaging material (Tray and film) was also studied. TLC (Thin Layer Chromatography), analysis did not indicate migration of residue from packaging material in both CP1 and CP2 treatment. Thus the packaging materials including tray and film were found to be suitable. Fresh green leafy vegetables packed and processed with CP1 or CP2 was found to retain keeping quality till 15±2 day's storage at 4 to 6 °C and RH (Relative Humidity) of 85 - 90 %. However, control non treated samples stored at ambient (28 to 30°C) temperature were resulted very short shelf life of 1 to 2 days only.

Microbial analysis of CP1 processed fresh spinach samples showed decrease in Total Aerobic Plate Count (TAPC), Yeast and Mold Count (YMC) and Presumptive Coliform (PC) count to 1.2 log CFU/g, 1.4 log CFU/g and to below detection level respectively. Microbial analysis in case of CP2 processed fresh spinach samples showed in reduction of TAPC, YMC and PC Count to 2 log CFU/g, 2 log CFU/g and below detection level, respectively.

CP1 and CP2 treatments effectively hygienize the produce, however during the 15 days storage at 4 - 6 °C, there was an increase in the total aerobic plate count and yeast and mold count by one log cycle. Presumptive coliform count was found to be below detection level in

similar storage condition. Similar trend was observed in case of CP1 and CP2 treated and stored coriander and mint samples. In the present study the extended shelf life of the fresh green leafy vegetables was found to be 15±2 days.

Physico-chemical properties: Moisture loss did not occurred significantly in CP1 & CP2 processed fresh leafy green vegetables samples during the storage up to 15±2 days at 4 to 6 °C. It was found to be in the normal range (~89 - 91 %). The colorimetric values (L* for light, a* for green and b* for yellow) for control, CP1 and CP2 treated vegetables did not show any significant difference (p e 0.05). The sensory or organoleptic score did not change significantly during the 15 days' storage.

Retention of phenolics and chlorophyll contents: Total phenolics content in case of fresh spinach control and CP1 treated samples on day 1 was ~19 and ~ 23 mg GAE/g (fresh weight) respectively. In CP1 treated samples during storage up to 15 days, total phenolics decreased slightly but insignificantly (p e 0.05). In case of CP2 treated samples total phenolics significantly increased (p d 0.05) to ~35 mg GAE/ g (fresh weight), during the storage till 15±2 days at 4 to 6 °C.

Total phenolics content in fresh coriander control samples and CP1 treated samples were ~46 and ~38 mg GAE/g (fresh weight) respectively on 1st day of storage. Further there was minor but insignificant change in the total phenolics content on 7th day of storage as value was found to be ~44 mg GAE/g, however it decreased to ~38 mg GAE/g (fresh weight), on day 15th of storage. In CP2 treated coriander samples total phenolics content was found to be ~44 mg GAE/g (fresh weight), on day 1 which significantly reduced on day 15th of storage.

Total phenolics content in fresh control mint samples and CP1 treated samples was ~29 and 35 mg GAE/g (fresh weight), respectively. During storage of samples for 15 ±2 days at 4 to 6 °C there was an insignificant (p e 0.05) decrease in the phenolics content. However in CP2

treated samples stored at 15 days at 4 to 6 °C no decrease in total phenolics content was observed.

In control spinach samples as well as CP1 treated samples content of chlorophyll was found to be 90 mg/10g (fresh weight). There was significant loss (p d 0.05) of chlorophyll content during 15 days storage in CP1 treated samples, to 50 mg/10g (fresh weight). Chlorophyll content in coriander also decreased significantly during 15 days storage and it reduced to 30 mg/10g (fresh weight). In control mint and CP1 treated samples chlorophyll content was found to be 70 mg/10g (fresh weight), which reduced to about ~60% upon 15 days of storage.

Retention of nutritional parameters: Among the vitamins, niacin, vitamin C (ascorbic acid) and vitamin A were found to be in the range of 0.2 to 1.9 mg/ 100g, ~13 to 22 mg/ 100g and 1132 to 3331 IU/ 100g (fresh weight) respectively. Insignificant changes (p e 0.05) found in vitamins content due to processing or storage was observed. Content of Riboflavin (vitamin B_2) in these leafy vegetables was very less in range of 0.1 to 1.9 mg/ 100g (fresh weight).

Kaempferol, quercetin and rosmarinic acid contents: on day one kaempferol content in fresh control spinach sample, CP1 and CP2 treated samples was found to be 538, 881 and 950 µg/100g respectively, on 15 days of storage at 4 - 6 °C. Kaempferol content was found to be enhanced to 1072 and 1204 µg/100g (fresh weight) in CP1 and CP2 treated samples respectively. Thus the results shown that upon radiation processing and storage there was enhancement in kaempferol content in spinach leaves.

Quercetin content on day one in control coriander sample, CP1 and CP2 treated samples was found to be ~13.85, 17.42 and 18.67 mg/100g respectively. Further quercetin content enhanced to 20.14 and 22.45 mg/100g (fresh weight) in CP1 and CP2 treated samples respectively, on 15 days storage at 4 °C. CP2 treated and stored coriander sample showed increased content of quercetin compared to CP1 treated samples.

Rosmarinic acid content in fresh control irradiated with CP1 and CP2 was found to be 59, 77 and 175 mg/100g (fresh weight) respectively on day one. Further upon storage for 15 days at 4 to 6°C, rosmarinic acid level was found to be elevated in CP1 and CP2 treated samples to 127 and 184 mg/100g (fresh weight) respectively.

Antimutagenic capacity: The flavonoids extracted from coriander showed ~46% reduction in mutagenicity. CP1 and CP2 treated samples resulted in about 57 and 54% decrease in the mutagenicity compared to control.

Thus, study concludes that, Fresh leafy agri-produce such as coriander; mint and spinach were found to be profoundly contaminated with microbes including presumptive coliforms and presumptive *Salmonella* which is mainly responsible for food borne outbreaks. To make sure that the microbiological safety of such fresh agri produce, a combination of processes (CP) including washing with potable water (5 min), sodium hypochlorite (200 ppm) dip treatment (5 min), air drying and radiation processing at 2 kGy dose of radiation (Gamma or Electron Beam) was found to be effective. Study resulted that the microbial load was decreased significantly by about 6 log cycles and the coliform count was remain below detectable level. The combination processes (CP1 and CP2) did not have any adverse effects on physical (colour, moisture and sensory), biochemical (nutritional) and functional attributes of these leafy vegetables. In spinach, coriander and mint kaempferol, quercetin and rosmarinic acid respectively were found to significantly increase upon the CP1 / CP2 treatments. It was also observed that, in case of CP2 increase was more as compared to CP1. Thus these treatments can ensure the safety of leafy greens and also extend their shelf life up to 15 ±2 days at 4 to 6 °C. Use of electron beam in CP2 treatment can make the process very much cost effective at commercial scale due to its high throughput.

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Publications in referred journals:

Application of ionizing radiation (Gamma and Electron Beam) for inactivation of coliforms and shelf life extension of fresh leafy vegetables (spinach, coriander and mint) in combination with water washing and sanitizer treatment

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Short title: Application of Gamma & Electron Beam for shelf life extension and *E. coli* inactivation in leafy vegetables.

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Raw leafy greens often have been associated with global foodborne outbreaks due to pathogenic contaminants. In the current study greens like spinach (*Spinacia oleracea* L.), coriander *(Coriandrum sativum* L*)*, and mint (*Mentha spicata* L.) showed presence of coliforms (including *E. coli*) along with other aerobic microbes, yeast and molds. These vegetables mostly consumed in raw or culinary purpose, so increase the chances of food borne illnesses. Moreover, the leafy greens are perishable. In this context, we optimized the combination process including radiation treatment to achieve hygienization and shelf life extension of leafy green vegetables. A combination treatment comprising of potable water wash, chlorination (NaOCl-200 ppm) followed by irradiation (2 kGy using electron beam or gamma) was developed. The processed samples showed keeping quality up to 15 days at 4 - 6 °C, whereas control samples spoiled within 2 days. The treatment resulted in coliform count below detection level while retaining the nutritional, phenolic content and organoleptic qualities. Thus, the combination treatment could ensure safety, keeping quality enhancement of perishable leafy greens and to control global food outbreaks. Electron beam over gamma processing found to be a commercial viable option due to its high throughput and equal efficacy in microbial decontamination.

Key Words: spinach; mint; coriander; shelf life, chlorination, coliforms

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LETTER OF ACCEPTANCE

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Sub.: "Application of ionizing radiations (Gamma and Electron Beam) for inactivation of coliforms and shelf life extension of fresh leafy vegetables (spinach, coriander and mint) in combination with water washing and sanitizer treatment" by Khade *et al*.-Acceptance-reg.

Dear Dr. Gautam,

I am pleased to inform that your above mentioned manuscript, peer-reviewed and revised on 05th June 2019, has been accepted for publication subject to editorial modifications/corrections.

It shall be published in one of the forthcoming issues of the Indian Journal of Experimental Biology (IJEB) as soon as its finalization. You will be informed once it is taken up for editing.

This Letter of Acceptance is issued to fulfill your professional requirements/needs.

With regards,

Yours Sincerely,

(RS JAYASOMU)

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Radiation Treatment Enhanced the Free Form of Phenolic Acid and Flavonoids in Leafy Greens and therefore Bioactivity in Terms of Antimutagenicity

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Abstract

In the current study, radiation treatment (gamma as well as electron beam of 2 kGy) was used to hygienize leafy greens spinach (cv. Semi-Savoy), mint (cv. menthol mint) and coriander (cv. Co-2), which were rich in microbial load including presumptive *Salmonella* and coliforms. Effect of radiation treatment on major phenolics such as kaempferol (spinach), quercetin (coriander) and rosmarinic acid (mint) contents was studied as phenolics are known to possess functional properties through TLC and HPLC analyses. Kaempferol, quercetin and rosmarinic acid (Gallic Acid Equivalent) content in fresh spinach, coriander and mint leaves was found to be 538 µg/100g, 14 mg/100g, and 59 mg/100g (fresh weight) respectively. There was significant increase ($p \leq 0.05$) in their contents after irradiation which further enhanced during storage at 4-6 ^{o}C for 15 days. Among the radiation sources, electron beam was found to be more effective than gamma rays presumably due to higher dose rate. Thus, radiation treatment besides helping in achieving hygienization, does value addition to leafy greens in terms of its functionality, which was validated in terms of antimutagenicity potential in *E. coli* MG 1655 (wild type) cells using rifampicin resistance forward mutation detection assay.

Practical Application

The radiation treatment was found to enhance phenolic content of the leafy vegetables. Phenolics are known to possess functional attributes such as antimutagenic activity. Thus, the treatment is found to play dual role i.e. hygienization of the leafy vegetables as well as improvement in the functional property.

Short title: Radiation Processing of Leafy Greens led to Nutritional Enrichment

Keywords

Electron beam /Gamma; kaempferol; quercetin; rosmarinic acid; leafy greens.

*****

ABSTRACTS IN CONFERENCEs / SYMPOSIA

Radiation Processing of Fresh Leafy Vegetables by Electron Beam Treatment

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Minimally processed fresh, pre-cut vegetables have limited shelf life and mainly rely on good manufacturing practices (GMP) for preservation and safety. Farm fresh Spinach (semi-Savoy spinach), coriander (Co-2) and mint (Japanese mint, or Menthol mint) procured from Nashik region, washed thoroughly and packed in styrofoam based tray, wrapped with cling film. The packed samples were radiation processed at EB facility, BRIT, Navi Mumbai at the dose of 2 kGy and treated sample were stored at 4°C.

Total aerobic plate counts in Spinach (semi-Savoy spinach), coriander (Co-2) and mint (Japanese mint, or Menthol mint) samples was to be in the order of ~ 10^8 CFU/g. In these samples yeast and mould count and presumptive coliforms was in the order of ~10^5 CFU/g. In the electron beam processed samples the total plate count and yeast mould count was below ~ 10^3 CFU/g and presumptive coliform count was below detectable level. In organoleptic evaluation performed with cooked produce at 9 point hedonic scale, overall acceptability was rated 7.0 ±1 as (like very well).

Colorimeter parameters in terms of a* value was found to be in the range of – 9 to -16±2 in fresh produce as well as processed samples. Further after treatment and storage it remained the same. b*value was found to be in the range of – 13 to -28±5 in fresh produce, treated and stored samples. Moisture content in fresh spinach, coriander and mint was in range of ~ 93, ~ 91 and ~ 89% respectively which reduced to ~ 88, ~ 88 and ~ 87% at the end of storage. Vegetables treated with 2.0 kGy of EB and stored at refrigeration temperature (4°C) in packed condition showed better acceptability in terms of colour, flavour and texture. This also resulted in the extended shelf life up to 2 weeks with acceptable organoleptic attributes over the control which spoiled within 3 days at similar storage conditions.

Title: Screening of Pathogens in Leafy Vegetables sold in retail outlet in Mumbai

H D Khade[1], Dr. Sachin Hajare[2], Dr. S Gautam[3], Dr. S K Ghosh[4].

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Abstract: Leafy green vegetables have been reported to be responsible for numerous outbreaks of foodborne illness worldwide. World Health Organization / FAO of United Nation (WHO/FAO) has ranked leafy vegetables o top priority among fresh fruits and vegetables in terms of microbiological risk. Prevalence of pathogen in leafy vegetables in our country is very common and least reported.

Enteric pathogens, such as presumptive *Salmonella* spps and *Escherichia coli*, spp have been detected upon screening of lots of freshly harvested and retail sold vegetables like spinach coriander and mint. Fresh spinach coriander and mint leaves were collected from Nashik region of Maharashtra state and from retail out lets at Mumbai. Sample were analysed for the presence of *E coli* and *Salomonella, Staphylococcus, Listeria and Shigella* spp. In initial screening presumptive coliforms were observed in all the lots of vegetables, and presumptive *Salmonella* were found in only two lots of vegetables however *Shigella*, *Staphylococcus aureus* and *Listeria* spps was not found in any of the samples / lots procured from farms. Coliform isolates were confirmed by colony morphology, biochemical tests and PCR for 16S rRNA gene amplification. Further confirmed analysis of *E coli* and *salmonella* spp will be done by UAL and invA gene amplification respectively. Thus, results indicate the vegetable samples were found to be contaminated with coliforms in fresh leafy vegetables like coriander and mint which mostly consumed in raw form. Coliforms are reasonably good indicator of poor handling, lack of GAP (Good Agricultural Practice) and GMP (Good Manufacturing Practice).\Key words: Coliform, leafy vegetables, spinach, coriander, mint

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CERTIFICATE OF PARTICIPATION

This is to certify that Dr./Mr./Ms. Hanamant Dattatraya Khade has participated/presented oral/poster in LSS-2015 organised by Bioscience Group, Bhabha Atomic Research Centre at Anushaktinagar, Mumbai, India.

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9th – 10th February 2016

Sponsored By: BCUD Savitribai Phule Pune University

Certificate

This is to certify that Prof./ Dr./ Mr./Ms. H. D. Khade

of Bhabha Atomic Research Center, Mumbai *has participated and presented*

a poster entitled Hygenisation & Shelf life extension of leafy vegetables....

in International Conference on "Innovative Trends In Chemical, Physical And Biosciences" (ITCPB 2016) held at PDEA's Annasaheb Magar Mahavidyalaya, Hadapsar, Pune on 9th and 10th February 2016 as a Teacher / Professional delegate.

Coordinator

Chief Organizer

Organizing Chairman
(Principal)

NATIONAL CONFERENCE

on

Climate Change, Biodiversity and Bio-resource Management

Jointly organized by

Microbiologists Society, India, Jawaharlal Nehru Rajkeeya Mahavidyalaya, Port Blair, A & N Islands and Shree Halari Visa Oswal College, Bhiwandi, Mumbai, Maharashtra.

Certificate

This is to certify that Prof/Dr/Mr/Ms H.D Khade *of* BARC Mumbai *has attended /presented poster/paper/~~chaired Session~~/~~delivered a lead lecture~~/ ~~as a resource person~~ in National Conference entitled* **Climate Change, Biodiversity and Bio-resource Management** *held at* **J.N.R. Mahavidyalaya,** *Port Blair, Andaman from* **1st - 3rd Feb 2017.**

Dr. N Francis Xavier
Principal,
JNR Mahavidyalaya, Port Blair

Dr. A.M Deshmukh
President,
Microbiologist Society, India

Dr. Mrs. Snehal S Donde
Principal & Convenor,
Shree Halari Visa Oswal College

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ICAR – 2018
The Art of Battling Antimicrobial Resistance

International Conference on Antimicrobial Resistance

Sponsored by

Department of BioTechnology, Government of India

CERTIFICATE OF PARTICIPATION

This is certified that Prof./ Dr./ Mr./ Ms. HANAMANT DATTARAYA KHADE

from BHABHA ATOMIC RESEARCH CENTRE has participated and presented (oral/ poster) in this International Conference organized by the Center for Research in Infectious Diseases (CRID), School of Chemical and Biotechnology, SASTRA Deemed University, Thanjavur, Tamil Nadu, India held on 19 and 20 January 2018.

Convenor

Co-convenor

ABBREVATIONS

ANOVA	Analysis of variance
AOAC	The Association of Analytical Chemist
APC	Aerobic Plate Count
APEDA	Agricultural and Processed Food Products Export Development Authority
BAM	Bacteriological Analytical Manual
BFlFC	Bureau of Foods Irradiated Food Committee
BGB	Brilliant Green Bile Broth
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
°C	Degree Celsius
CP1	Combination Process 1
CP2	Combination Process 2
T1	Potable Water Wash
T2	Sodium hypochlorite wash
T3	Gamma Radiation Treatment (2 kGy)
T4	Electron Beam Radiation Treatment (2 kGy)
D min	Dose minimum
D max	Dose maximum
EB	Electron Beam
EU	European Union
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FSSAI	Food Standards & Safety Authority of India

G	gram
“	Gamma radiation
GAE	Gallic Acid equivalent
GAP	Good Agricultural Practices
GCMS	Gas Chromatography Mass Spectrometry
GLV	Green Leafy Vegetables
H	Hour
HPLC	High Performance Liquid Chromatography
HUS	Haemolytic Uremic syndrome
ICGFI	International Consultative Group on Food Irradiation
ICMSF	International Commission on Microbiological Specification for Foods
IR	Ionizing Radiation
IMVIC	Indole Methy Red Vogeus-Proskauer Citrate utilization
Kcal	Kilocalorie
kGy	kilo Gray
mL	milli Liter
LIA	Lysine Iron Agar
MR-VP	Methyl red (MR) Voges - Proskauer (VP) Test
MPN	Most Probable Number
MT	Metric Tons
µg	Microgram
NHB	National Horticulture Board
Nm	Nanometer
PC	Presumptive Coliform

PCR	Polymerase Chain Reaction
Ppm	Part per million
Rif^r	Rifamipicin resistant
TAPC	Total Aerobic Plate Count
TLC	Thin Layer Chromatography
USDA	The United States Department of Agriculture
UV	Ultra Violet
µL	Micro Litre
V/V	volume/volume
VRBA	Violet Red Bile Agar
WHO	World Health Organization
WTO	World Trade Organisation
XLDA	Xylose-Lysine Deoxycholate Agar
YMC	Yeast and Mould count

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