EVALUATION OF *TYLOPHORA INDICA* EXTRACTS AND BACTERIOCIN FOR ANTIMICROBIAL ACTIVITY IN FOOD PACKAGING FORMULATIONS

A

Thesis

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By

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DECLARATION

The research work embodies in this thesis "**Evaluation of *Tylophora indica* extracts and bacteriocin for antimicrobial activity in food packaging formulations"** for the fulfilment of the requirement for the award of the degree of **Doctor of Philosophy in Microbiology** has been accomplished under the supervision of **Dr Shalini Singh, Associate Professor, Department of Microbiology**, **School of Bioengineering and Biosciences**, and co-supervision of **Dr Manish Vyas, Associate Professor, Department of Ayurvedic Pharmacy, School of Pharmaceutical Sciences, Lovely Professional University, Punjab, India.**

The extent of information derived from the existing literature has been indicated in the body of the thesis at appropriate places along with the source of information. The work is original and has not been submitted in part or full any degree or diploma of this or any other University.

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CERTIFICATE

This is to certify that the thesis entitled "**Evaluation of *Tylophora indica* extracts and bacteriocin for antimicrobial activity in food packaging formulations"** submitted for the award of the degree of **Doctor of Philosophy in Microbiology** to **Lovely Professional University,** Punjab, India is a record of bonafide research work carried out by **Ms Charu Khanna** under my guidance/ co-guidance. To the best of my knowledge, the thesis has not been previously submitted elsewhere for the award of any other degree, diploma or dissertation of any kind anywhere before.

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Abstract

Health of an individual is a primary concern as it affects the overall development of a society and a nation. Food is one of the health determinants and consumption of contaminated food might lead to infections and intoxications. The chances of contamination/spoilage by microbes and otherwise, is generally higher for nutrient and moisture rich foods such as, milk and milk products, and meat and meat products, etc. Hence, appropriate preservation methods, involving various preservatives, especially natural & environment friendly agents must be adopted to render the food articles un-contaminated, so that consumption of good quality and healthy food can be ensured. Also, emergence of antibiotic resistant microbial strains, due to improper and injudicious use of these agents in infectious diseases, has further attracted the focus of researchers world-wide towards use of natural and herbal agents, instead of potentially harmful chemical based agents and even resistance prone antibiotics. While focussing upon such eminent and critical issues, this study has been conceived to formulate an active packaging film formulation, involving herbal plant extracts and natural, bacterial borne peptide, bacteriocins.

The plant used in the current study involved was *Tylophora indica*, where the antimicrobial potential of extracts of *T. indica* roots & leaves, and that of bacteriocins, the antimicrobial ribosomally synthesised peptides, produced by bacteria, was checked against different bacterial pathogens. The standardisation of the collected and authenticated plant parts was done and ensured for use of high quality raw material by physico-chemical and phytochemical screening. Further, bacteriocins from indigenously isolated LAB strains were obtained under optimized conditions of fermentation, and its antimicrobial potential compared with that of standard lactic acid bacterial strain of *Lactobacillus plantarum* NCIM2083. The partially purified bacteriocins from *Lactobacillus plantarum* NCIM2083 and *Enterococcus sp.* YT3, the most promising LAB strains amongst the isolates, were obtained, optimised and characterised for further study. Subsequently, the partially purified bacteriocins namely Plantaracin 2083 and Enterocin YT3 along with Nisin, the standard commercially available bacteriocin, were analysed for their antimicrobial potential

with and without plant extracts (methanolic, ethanolic and aqueous) against five selected food pathogens (*E. coli, P. aeruginosa, S. aureus, B. subtilis* and *L. monocytogenes*). The findings indicated a synergism between the plant extracts and bacteriocins under study. Nisin, a standard, known was also used for a comparative analysis and it was found to be most effective against gram positive studied strains as compared to Plantaracin 2083 and Enterocin YT3. Along with plant extracts, nisin was found to be effective against gram negative stains too. This aim of the above part of the study was to screen the minimum concentration of best plant extract and bacteriocin, either alone or in combination, which could inhibit all the selected food pathogens and thus, five such formulations, based on the above criteria, were shortlisted for further investigations. These included, *T. indica* leaf and root extracts (50 mg/ml methanolic and 50 mg/ml ethanolic respectively), nisin (2500 IU/ml) and the combinations of leaf (20 mg/ml of aqueous extract) and roots (20 mg/ml of aqueous extract) with nisin (1000 IU/ml and 500 IU/ml, respectively). The partially purified bacteriocins were unable to inhibit *L. monocytogenes* alone and along with plant extracts, and hence not considered for the further study.

Subsequently, methyl cellulose based coating solutions with selected combinations of antimicrobial agents (as mentioned above), were prepared and coated over sterile low density food grade polyethylene (LDPE) food grade packaging films. The films were dried under standard conditions, and the chicken meat samples were packed for shelf life analysis. Unpacked meat samples, films without any coating, and films coated with coating solution without any antimicrobial agent served as controls. The parameters of shelf life evaluation included sliminess, discoloration, foul odour, water retention, and presence of bubbles and extent of aerobic colony count (performed using spread plate method). Under room temperature, none of the film samples were able to exhibit a significant antimicrobial action for potential use as active meat packaging films, although slightly lesser contamination was observed with combination of root extract and nisin coated films, in comparison to controls. For refrigerated storage conditions, the film with same combination of antimicrobials (T. indica aqueous extracts 20mg/ml and Nisin 500 IU/ml) was able to maintain meat quality up to six days due to lesser contamination as compared to controls which

exhibited contaminated after 2 days. Under deep freezer conditions, same film was able to limit the chicken contamination up to 16 days. Thus, the film coated with *T. indica* root aqueous extract and nisin was found to be the most promising antimicrobial packaging film, based on the shelf life quality of tested chicken raw meat samples.

The best antimicrobial packaging film, as obtained above, was subsequently, characterized for physical properties and it was observed that the given film exhibited increase in thickness, tensile strength and haziness along with decrease of elongation of break, transparency and thermal shrinkage. Toxicity analysis for the best antimicrobial coating formulation (cellulose based solution possessing *T. indica* aqueous extracts 20mg/ml and Nisin 500 IU/ml) confirmed its non-toxicity against HEK 293 cell lines up to the concentration of 10 g/ml, thereby ensuring the safety aspects for usage in food industry.

Hence, the active packaging film, prepared using *T. indica* extracts and nisin has been found to be a potential antimicrobial film option for improvement of shelf life of meat, and can be explored for a pilot scale run. The given films can even be explored for shelf life enhancement of other food products as well, and thus, further studies can be carried with other food items to ensure its large scale commercial exploration and applications in food packaging.

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Chapter 1
Introduction

1.1 Introduction

With fast industrialization, food industry is not far behind in influencing our daily life, across states and countries. Owing to the increasing demand of the fast paced generation, this industry has also progressed immensely to provide ready-to-eat foods, which certainly save time and effort that goes into conventional cooking, yet issues like, microbial security, quality and shelf life assurance for these foods, as with conventional foods, still remain a major concern. Food borne illness/ infections are thus, one of the major public global health concerns (Khare et al., 2018). Food borne diseases are those acute or sub-acute diseases, which are caused by a chemical and/or biological agent entering the body through the ingestion of food (Adams and Moss, 2013). The main causative agents of food poisoning include microbes such as bacteria, fungi, parasites, viruses, and even chemicals which may enter the food chain (Rao et al., 2012). A news release from WHO, estimated that one in every ten people globally, fall sick due to consumption of contaminated food every year, and as a result, nearly 4,20,000 individuals die, annually (Anonymous, 2015). The African and South-east Asian regions of the world (Anonymous, 2015) are found to be the most prone areas to food borne illnesses. As per the recommendations made by WHO (Anonymous, FAO/WHO, 2010), there is a need to increase the awareness towards causes of food spoilage, recognition of food poisoning symptoms, and diseases, along with the development of some effective food spoilage control measures (Anonymous, 2015; Rao et al., 2012), for better tackling of the problems highlighted above.

As indicated above, ensuring the quantity and quality of food items throughout the year, and elongation of their shelf life have been a challenge for industries, worldwide (Fatoki and Onifade, 2013). Raw as well as processed food products has been affected by various pathogenic microbes such as, *Staphylococcus sp., Bacillus sp., E. coli, Salmonella sp., and Aspergillus sp.* etc., causing their spoilage, and even food borne

illnesses, even though numerous ways including, use of preservatives, to preserve food quality, are being adopted (Malhotra et al., 2015). Investigations and records indicate that the major microbiological causes of such outbreaks in India during the period of 1980- 2016, have been contributed *Staphylococcus aureus, Vibrio species, E. coli, Bacillus cereus, Salmonella species, Clostridium botulinum, Listeria monocytogenes* and *Yersinia sp.*, and thus, have been significant contributors to the mortality, morbidity and economical loss (Rao et al., 2012; Khare et al., 2018). Usually, bacterial pathogens enter into foods from environmental exposure during various stages of cultivation, procurement/collection, processing, packaging, transportation, etc. (Sung et al., 2013).

Historically, man has been applying different approaches/techniques for long term storage of food or improving shelf life of foods for availability of foods, especially during off season, and also to limit the spoilage during the transportation to the industry/consumers. Food fermentation is one of those notable techniques which involves micro-organisms, various yeasts and lactic acid bacteria and is used for enhancing digestibility, flavors, and aroma, along with enhanced food shelf life, and preservation (Hintz et al., 2015). The microbes, due to their microbial activity produce organic acids, thereby lowering the pH of the food and making food an unfavorable environment which is for the growth of different pathogenic organisms. Drying, where water activity of foods is reduced, and smoking, for food like, meat and fish, have also been contributing an important role for improving the storage life of the foods (Hintz et al., 2015). Other conventional techniques such as, heating, boiling, deep freezing and addition of excess sugar, salt, oil etc. are also frequently used to minimize the microbial contamination. The introduction of chemical preservatives viz., sorbic acid, benzoic acid and propionic acid (Devlighere et al., 2004) in foods is another major way of food preservation. Despite of the use of a large number of physical and chemical preservatives in foods, newer ways to incorporate these agents, or develop/find new ways to improve shelf life of foods are continuously being tested, as a single method/conventional approaches might not provide satisfactory results in today's times. Also, chemical preservatives might involve health risks of the food consumers, due to left-over chemical residues (Fatoki and Onifade, 2013). The other

reason for this concern is the harmful effects sometimes caused by such chemicals preservatives. Sulphur di oxide, used to preserve wine, may be irritating to the bronchia of asthmatic patients. Nitrites have been shown to be carcinogenic (Davidson and Harrison, 2002; Abdulmumeen et al, 2012). The chemical preservatives in use have been tested for their toxicological effects, and strict legal regulations have challenged their use in foodstuffs so as to render the food item toxic free (Devlighere et al., 2004). Appearance of newer foods in the market almost every day, appearance of newer/resistant varieties of microbial pathogens, to endeavour the food safety demands of the fast growing working generation, and focus on enhancing the shelf life of food are all demands of changing time (Sung et al., 2013).

Currently, with the rising awareness and among masses, education in focus, the mind-set of human population is changing from usage of synthetics to the natural products which has seen a marked rise in the demand for the involvement of organic processing, and application of organic and herbal origin substances for the preservation of food items with natural origin (Malhotra et al., 2015). With the advancement of technology in this modern era, newer approaches in food preservation like, the use of non-thermal strategies like, pulsed electric fields (PEF) and high hydrostatic pressure (HHP), or packaging systems, such as bio-preservation, active packaging, etc. are being evaluated. Techniques like, HHP and PEF have been reported to reduce the vegetative forms of bacteria, but fail to destroy endospores, can be used for liquid food products only, involve high costs and tend to generate resistant bacterial strains which create limitations to usage of their new technologies (Devlighere et al., 2004). Contrarily, the active packaging, based on hurdle technology, has played a crucial role towards rising of shelf life of food items (Malhotra et al., 2015). Using food packaging films, involving antimicrobials, create a barrier to the entry of microbes into the food, and hence reduce chances of food borne infections. Such active packaging formulations are inert in nature, and show no toxicity. The flavor, aromas, aesthetics are better maintained in such active packaging, along with provision of product information, advertisement and enhanced marketing strategies (Sung et al., 2013). Such packaging films involving various

substances, singly, and in combinations, are used to reduce the microbial load in packed food items leading to usage of food for a longer span.

The active packaging involving antimicrobial film formulations have been categorized as non-biodegradable and biodegradable where, the non-biodegradable films are made up of synthetic polymers and have been accepted as superior candidates than degradable for food packaging. The biodegradable films are eco-friendly, as they can be degraded by microbes and have been classified as edible and non-edible films. The edible films are generally prepared from the edible biopolymers such as, polysaccharides, proteins or lipids, along with plasticizers, and some food grade additives. The non-edible films are generally made up of polyvinyl alcohol, polyanhydrides, and polylactic acid (Sung et al., 2013).

Non-biodegradable films are generally made of plastics such as, low density polyethylene films, high density polyethylene films, ethylene vinyl acetate (EVA) and polypropylene (PP), which possess the thickness up to 10 mils (Testin and Vergano, 1997). They are made by different resins such as Low density polyethylene (LDPE), High density polyethylene (HDPE) and Polyvinyl chloride (PVC). The LDPE films are used as packaging materials over foods such as chicken, bakery foods, candies and ice, along with other similar applications such as bubble packaging, garment bags, textile bags, grocery sacks and agricultural films (Testin and Vergano, 1997).

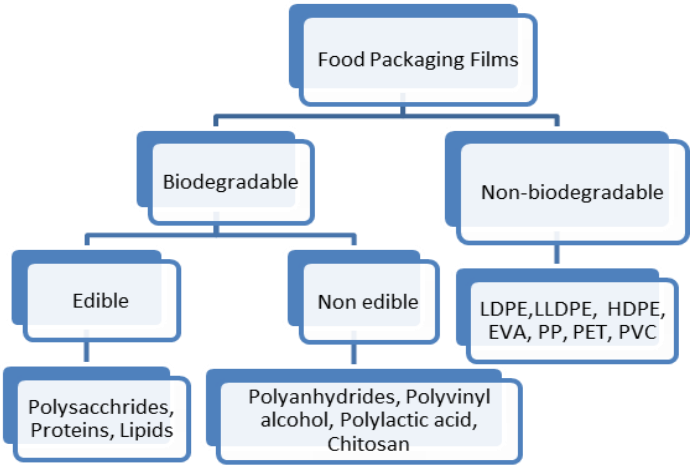

Figure 1.1: Major categories of food packaging films

The various benefits of the LDPE and other plastic packaging films includes source reduction, cost effectiveness, better functionality, containment, convenience, provide information to consumers by printing, versatility and above all protection of the food items from environmental contamination and degradation (Testin and Vergano, 1997). The LDPE films are strong, possess good clarity, provide fair gas barriers and are efficient moisture barrier. They have the ability to heat sealed and are highly flexible (Irkin and Esmer, 2015). The use of these films have helped in lesser waste creation of substances such as, glass and steel packs, along with reducing transportation cost, fuel saving and lesser road stress. Since these films are non-biodegradable within short span, various methods have been adopted for their disposal. The LDPE packages manufactured are thin walled while retaining the similar performance properties. These films are disposed as land filling as it does not pose environmental hazards due to its inertness, non-leaching property and its non-contribution towards production of methane gas. Recycling of LDPE films are also employed to produce variety of different products such as, trash bags, garbage bins and waste baskets. These films even do not pose problems in waste to energy (WTE) combustion facilities because of complete combustion of the hydrocarbons to carbon di oxide and water (Testin and Vergano, 1997). Thus, such packaging formulations with antimicrobials such as, nisin, chitin, EDTA, lysozyme, lacto peroxidases, volatile oils, enzymes, nanoparticles, phytochemicals and plant extracts (such as, Turmeric and Neem extracts) incorporated in the food or, have packaging films, themselves coated with various antimicrobials, to form active packaging are rigorously being tested. (Irkin and Esmer, 2015; Joerger, 2007).

Plants have always been an integral part of human life, as a major component of human diet. Also, nearly 80% of the world population (approximately four billion people) still rely on the herbal products and supplements for the primary healthcare in the developing nations (Bandaranayake, 2006). As mentioned above, different plant oils and extracts have also been in applications as food preservatives, since thousands of years. Spices like ginger, garlic, clove, cumin, and caraway have traditional importance in Indian cuisines, and are known to exhibit antimicrobial actions, apart from being used as flavoring agents. Plants such as, *Murraya koengeii*, *Azadirachta*

indica, Piper longum, Piper nigrum, Zingiber officinale, Allium cepa, Trachyspermum ammi, Mentha spicata, Syzigium aromaticum, Carica papaya, Occimum sanctum, Glycyrrhiza glabra and *Curcuma longa* are the integral part of Indian kitchen (Vaishnavi et al., 2007; Rathore and Shekhawat, 2008; Antolak and Kregiel, 2017). The cereal grains, stored with the Neem leaves, and capsicum peppers for next season availability, in unharmed state, has been practised since ancient ages (Hintz et al., 2015). Plant tubers have been stored under the dried mulch of millets and maize (Hintz et al., 2015). Tremendous surge in consumer demand for the natural preservatives, and the specific limitations of the physical and chemical methods of preserving food stuffs, have thus, brought plants and plant products into limelight for use as natural preservatives (Hintz et al., 2015).

The biological efficacy of these plant based antimicrobials, as food preservatives, is usually determined in reference to bioactive compounds such as, plant antimicrobial peptides (pAMP), phytochemicals phenolics, alkaloids and terpenes (Hintz *et al.*, 2015; Antolak and Kregiel, 2017), present. The pAMP are small molecules which defend the plant from pathogens. Thionins, plant defensins, snakins and lipid transfer proteins are common pAMP, reported inhibit microbial pathogens like, *S. aureus*, *Listeria monocytogenes* and *B. cereus* (Hintz et al., 2015). The phytochemicals such as, tannins, alkaloids, glycosides, gums, essential oils, resins and fixed oils are the inclusions recognized in the plant cells, and are responsible for the different pharmacological, antibacterial, antifungal and even antiviral activities (Verma and Singh, 2008, Zaika, 1988; Cowan, 1999; Dabur et al., 2007; Ignacimuthu et al., 2009; Preethi et al., 2010; Alavijeh et al., 2012; Mohanka and Priyanka, 2014; D' Souza, 2014; Hintz et al., 2015; Mostafa et al., Antolak and Kregiel, 2017; 2018; Brnawi et al., 2019). Interestingly, studies indicate that the efficiency of plant compounds as food preservatives is higher towards Gram positive organisms rather than Gram negative ones (Tiwari et al., 2009). Turmeric, Grape fruit seed extract, Neem extract, Oregano, Rosemary etc. have been evaluated for their shelf life potential, and reduction in the microbial count have been reported (Lee et al., 1998; Theivendran et al., 2006; Pranoto et al., 2005; Joerger, 2007; Mustapha et al., 2017) for all such plants based materials. Undoubtedly, numerous studies appreciate the antimicrobial

potential of plant extracts and their use as preservatives in food packaging formulations, but further research initiatives are needed to evaluate use of wide range of plants/plant products against different types of pathogenic organisms, so as to enable extensive use of these natural substances in food preservation is needed. To check and maintain the quality and efficiency of plants in use, more efforts are required to develop their specific standards and to ensure their toxicological safety. The inadequate knowledge of mode of action of the antimicrobials and adverse reactions in the body still remains a concern with their large scale use and needs extensive scientific investigations (Kunle et al., 2012; Ekor, 2014). The influence of such plant extracts on organoleptic characters of various preserved food items also needs to be evaluated before their large scale and more frequent use in food industry (Mustapha et al., 2017). The incorporation/inclusion of plant extracts in the food packaging films, are thus being investigated extensively.

The use of antioxidants or antimicrobials in food preservation is different from the use of antibiotics and even better than antibiotics, as drug resistant is not witnessed, as in the recent times, a concern for the development of resistance and tolerance of pathogenic bacterial strains towards such chemical antimicrobials has been generated (Davidson and Harrison, 2002; Abdulmumeen et al, 2012). Antibiotics are normally not used as preservatives, but exploitation of these agents to improve the growth of livestock such as birds and animals has been observed globally. The agents are added in low doses in the feed of pigs which in turn helps in achieving the desired growth of pigs with nearly 10-15% (Chattopadhyay, 2014) less feed thereby reducing the rearing expenditure. This helps in improvement of the animal growth rate, better meat quality along with higher amount to protein as compared to fat. Betterment for quality of chicken eggs has been determined with the use of tetracycline and penicillin in chicken feed but studies have illustrated the isolation of antibiotic resistant microbial strain from these animals (Chattopadhyay, 2014). The emergence and rapid spread of antibiotic resistant including, multi drug resistant bacteria in the developing nations, due to greater and easier access to antibiotics (Cleveland et al., 2001; Balciunas et al., 2013, Zaman et al., 2017) along with resistance to the preservatives in use. Hence, the search and demand for organic and natural food preservatives, has also brought

natural and safe antimicrobials like, bacteriocins, into focus along with plant applications. Bacteriocins, the gene encoded, and ribosomally synthesized peptides are the produce of some bacterial species including, Lactic Acid Bacteria, and found to generally act against closely related groups of bacteria. They are being explored for preservation of foods like, pickles, cheese, wine and curd since ages (Gautam and Sharma, 2009).

Bacteriocins such as lacticin, pediocin, plantaracin, subtilin and cerein have been isolated, characterized and evaluated as food preservatives. These applications have led to their evaluation in active antimicrobial food packaging systems. Still, the sole bacteriocin, granted as GRAS (Generally regarded as safe) profile, and is thus, being extensively in application for preservation of many foods commercially is, Nisin (Joerger, 2007; Gautam and Sharma, 2009). Its low toxicity to the humans (toxic dose 6,950 mg/kg), biodegradability in the human gastrointestinal system, hypo allergic nature, thermo stability, and wide spectrum of antimicrobial potential has made it an important preservative for foods such as, cheese, canned foods and sauces (Balciunas et al., 2013). Significant number of investigations reports the use of Nisin in edible and non-edible packaging films against food pathogens. No doubt, Nisin has been highly recognized in the areas of medicine, food preservation and food packaging systems, however, some cases of Nisin resistant bacterial strains are also emerging which needs to be focused upon, and tackled in time (Balciunas et al., 2012). Few integrated approaches, involving bacteriocins along with plant extracts has also been explored for their performance in packaging films. (Joerger, 2007)

The human food is commonly contributed by plant and animal origin matter. The proportion of vegetarian food consumers in western countries is estimated <5% (Shridhar et al., 2014) while in India, it is estimated to be around 35% which actually varies between 10%-62% regionally (Shridhar et al., 2014). These figures indicate that major human population consumes non-vegetarian diet which mainly includes meat, poultry and sea food. The significance of meat as meal has been long established due to its being a source for different amino acids and other essential nutritional components such as Zn, Fe and cyanocobalamin (McAfee et al., 2010). Meats, rich in proteins, are liable to get biologically, physically and chemically

spoiled within short span, if not preserved properly, causing loss to consumer and the producer and consumption of meats contaminated with microbial pathogens might cause serious foodborne illnesses such as Cholera, Salmonellosis, Diarrheal diseases, and Botulinum, Amoebiasis and *E. coli* infections especially in the developing countries. In India, the common food pathogens, causing such illnesses, found in meats includes *S. aureus, E. coli, B. cereus, Vibrio sp., Campylobacter jejuni, Aeromonas, Listeria spp., Clostridia spp.,* and *Salmonella spp*. Hence, the hygiene and proper preservation practices related to meat and meat products must be maintained (Rao et al., 2012). Such changes increased the scope of meat industry, which has responded by manufacturing variety of both processed and unprocessed meat products and made available in the market as per the consumer demand (Grunert, 2006). However, the preservation of such products is still the interesting area for the researchers by use of active packaging films with efficient preservation results may be a solution to preservation of meat products.

High cost involved with packaging film formulations like, those with silver containing nanoparticles and enzymes, toxicity issues with films like, those with Hexamethylenetetramine, and even due to involvement of components which do not comply with the regulatory norms such as, those using triclosan (Joerger, 2007) is another area of concern.

Hence a 'magic bullet' film (Joerger, 2007) is still to be discovered, which is natural, acceptable to user, potent, safe, cost effective and even comply with the regulatory norms.

Considering the aforesaid present day scenario, current study is conceived to examine and analyze the biological behavior with plant extracts of *Tylophora indica*, along with and without its binary combination with Bacteriocins, both standard as well as obtained from standard and isolate, against standard common food borne pathogens. Thereafter, an active packaging film formulation using, the evaluated antimicrobials, will be developed and evaluated for its efficacy to improve shelf life of meat products.

Following are the objectives for the current study:

- To characterize *Tylophora indica* properties for its physico-chemical and phytochemical screening.
- To isolate, optimise, and characterise bacteriocins produced by indigenously isolated as well as standard procured Lactic acid bacterial strains.
- To evaluate bacteriocins, from standard/isolates of Lactic acid bacteria and root/leave extracts from *T. indica* for their antimicrobial potential against test bacterial pathogens.
- To develop anti-microbial active food packaging (LDPE) films using bacteriocins and plant (*T. indica*) extracts for meat products.
- To evaluate formulated product behaviour, and quality at laboratory.

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Chapter 2

Physico-chemical and phyto-chemical characterisation of *Tylophora indica*

2.1 Introduction and review of literature

Use of plants as multifunctional agents such as, food, healers, shelters and preservatives, dates back to pre-historical times, and contributes similarly even today. Food refers to the nutritious substances consumed by the people so as to maintain life and growth. Use of contaminated foods results in the development of various foods borne diseases, which are a major concern world- wide and its safety plays significant role in human health (Hintz et al., 2015). Unnecessary and harmful growth of food pathogens leads to food contamination, and to check their growth, scientific studies are being conducted to render the food items usable and enhance their shelf life. Different plant oils and their extracts have been studied to control the growth of microbes, hence serving as potential natural preservative vehicles in all types of foods including raw or processed meat, milk and milk products, juices, vegetables and fruits (Hintz et al., 2015). Herbs such as, *Murraya koengeii*, *Azadirachta indica, Piper longum, Piper nigrum, Zingiber officinale, Allium cepa, Trachyspermum ammi, Mentha spicata, Syzigium aromaticum, Carica papaya, Ocimum sanctum, Glycyrrhiza glabra* and *Curcuma longa* have already registered their efficacy against food borne pathogens and form an integral part of Indian cuisines (Vaishnavi et al., 2007; Rathore and Shekhawat, 2008; Antolak and Kregiel, 2017). A few studies has been conducted, analysing the efficacy of plant extracts in packaging films to reduce the microbial contaminants, thus enhancing the shelf life for various food items. These studies has been conducted with herbs such as turmeric, grape fruit seed extract, neem extract, oregano, cinnamon, lemon grass, garlic, rosemary and thyme, and the efficacy determined against pathogenic entities such as, *S. enterica, S. aureus, L. plantarum, E. coli, L. monocytogenes*, yeast and moulds (Joerger, 2007). This suggests that the efficacy of plants as preservatives must be explored intensively to provide natural

checks to growth of harmful pathogens in food, without affecting the human health as in cases of artificial preservatives (Chapter 1). To ensure purity, potency, safety, affectivity and stability of the final products involving different herbal raw materials, the attention is focussed on some quality indices which include macroscopic and microscopic evaluation, removal of foreign organic matter, ash values, extractive values, phyto-chemical evaluation, chromatographic examinations and toxicological analysis (Folashade et al., 2012). The various challenges faced by the industry and legal authorities when using the plants as raw material includes, the availability of high quality standard material, over dosages, misidentification, the toxicity effects and adverse reactions (WHO, 2005; Ekor, 2014). Hence, active pharmacovigilance along with proper prior studies conducted at laboratory level can help combating such issues leading to long term effective applications of plants in the food industry (Folashade et al., 2012; Ekor, 2014).

Tylophora indica (Burm.f.) Merr. (*T. indica*), indigenously found in India, has been in use for managing diseases viz, asthma, rheumatoid arthritis and diarrhea (Kumar and Sharma, 2012;). Since the year 1884, this climber, also known as '*Antmool*' has been an integral part of *Bengal Pharmacopoeia (*Kaur and Singh, 2012; Kumar and Sharma, 2012*). Tylophora indica* (Burm.f.) Merr. is also renowned as '*Tylophora asthmatica*' as its leaves have been used in treatment asthma (Shivpuri et al., 1969; Kaur and Singh, 2012). The roots of this plant have been used as a substitute for Ipecac, which induces vomiting, and hence also known as Indian Ipecac. *T. indica* is the member of *Asclepiadiaceae* family, a semi-shrub perennial climber and can be found along the Sub Himalayan tract, primarily in the South and East parts of our homeland. The plant grows well in sandy, well-drained soil, and demonstrates restricted growth in dry and arid areas (Kaur and Singh, 2012). *Tylophora indica* possess knotted stem and has watery latex. The roots are long, fleshy and knotted. The leaves are simple, glabrous, opposite, petiolate (nearly 6-13mm long), has acute apex and has ovate-oblong shape. The yellow- purple colored small flowers are present in lateral cymes along with paired follicular fruit tapering towards the apex to a fine point (Gupta et al., 2010). The seeds are very small with low germinating potential. The plant bears a pleasant odor. Beneath the microscope, the leaves present a layer of

thin cuticle with single layered epidermis below. 2-3 palisade layers along with 6-8 spongy parenchyma layers may be found in the mesophyll layer. Spongy parenchyma is also studded with rosettes of calcium oxalate crystals (Suhas et al., 2011).

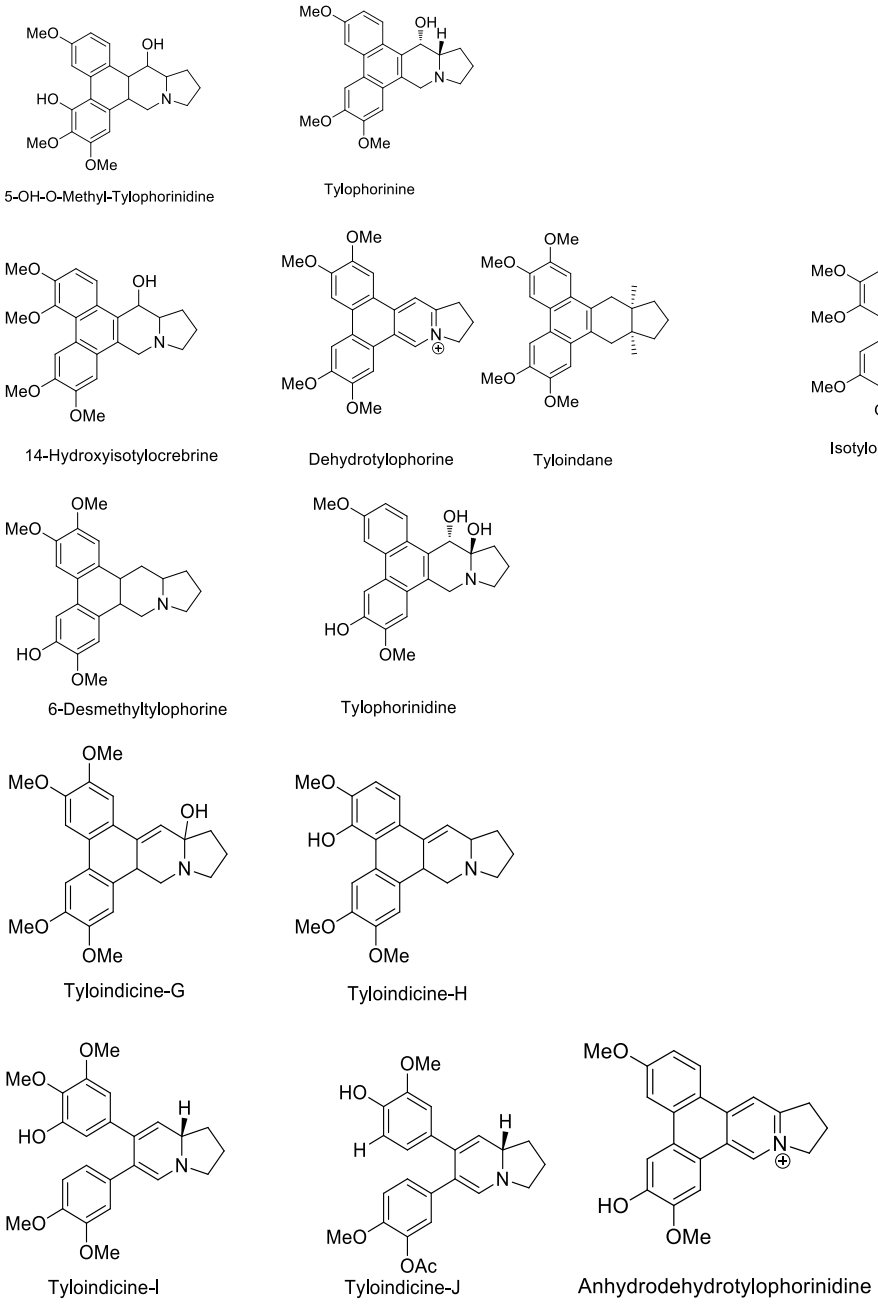

Figure 2.1: Chemical alkaloid structures of found in *Tylophora indica* (Adapted from Kumar and Sharma, 2012)

T. indica possess numerous bioactive components like, phenanthroindolizidine alkaloids and non-alkaloids, formed in the various parts of this plant. The phenanthroindolizidine alkaloids include tylophorine, septidine, tylophorinine, tylophorinidine and tyloindicines A-J and are mainly isolated from roots and leaves.

Other alkaloids include, anhydrous dehydrotylo-phorinine, 4, 6-des-methylisodroxy-o-methyltylophorinindine, desmethyltylophoridine, desmethyltylophorinine, desmethyltylophorine and isotylocrebrine (Kaur and Singh, 2012). Stigma sterol, kaempferol, β-sitosterol, quercetin, tetratriacontanone, α- and β-amyrins, cetyl alcohol, tyloindane, coutchone, octacosanol octacosanoate, resin, tannins, wax, pigments, glucose, potassium chloride and some calcium salts (Kaur and Singh, 2012) are the major non-alkaloids present (Figure 2.1).

Table 2.1: Quantitative Standards of the leaves of *T. indica* (Gupta AK, 2003)

Parameter of Evaluation	Quantitative standards for *T. indica* leaves
Foreign matter	≤ 2.0%
Loss on drying	≤ 6.5 %
Total ash	≤ 27 %
Acid insoluble Ash	≤ 14.4 %
Ethanol soluble extractive	≥ 5 %
Water soluble extractive	≥ 15 %

To ensure the use of quality *T. indica* crude sample, the quantitative standards of *T. indica* leaves (Table 2.1) have been suggested by Indian Council of Medical Research (Gupta AK, 2003).

The plant has been a choice of tribes and traditional practitioners especially in forest and remote places where plants form the major healers (Table 2.2).

Some pharmacological investigations have been performed in different models using varied *T. indica* extracts and support some of the ethno-botanical applications (Table 2.3). Literature suggests that *T. indica* may be used to alleviate lochia in the parturient female, for the purification of the blood and to destroy various worms. *T. indica* may also be used as a preservative of the foodstuffs. The roots possess anti rheumatic and stimulant property. The plant may also be implemented to make fine fabrics as it possesses strong, fine, and silky fibres. Since no toxicity has been reported in some conditions such as pregnancy and breastfeeding, so *T. indica* may be used in these situations (Butani et al., 2007).

Table 2.2: Ethno-botanical uses of *T. indica*

Place	Communities name, if any	Part used	Application	Reference
Bhadra Wildlife Sanctuary, Karnataka	-	Tablets constituted by roots and leaves of *Aadu muttada balli* (*T. indica*), garlic, cardamom and betel leaves	Asthma	Parinitha et al., 2004
Madurai district in Tamil Nadu	Paliyar tribes	Leaves and roots paste of *Nangilai (T. indica*) mixed with *Rauwolfia serpentina*, the juice of T. indica leaves	Bite area of a snake bite	Ignacimuthu et al., 2006
Bidar district of Karnataka, India	Folk practitioners	paste of *Datura stramonium* flowers and *T. indica* leaves (in equal ratio)	Skin ailments	Prashantkumar and Vidyasagar, 2004
Kalahandi district in Orissa, India	The tribes	*T. indica* flowers	Contraceptive	Pandha and Padhya, 2008
Visakhapatnam District in Andhra Pradesh, India	The tribal people-Gangaraju Madugula Mandal	Root powder of *T. indica* with milk	Asthma	Bapuji and Venkatratnam, 2009
Seshachalam Hills of Andhra Pradesh, India	Tribal and non-tribal practitioners	Tender stem of *Meka meyani aku* (*T. indica*) extracts	Urine retention	Reddy et al., 2009
Jhajjar district, Haryana	Snake charmers from Khetawas	Tender stem extracts	Female sexual disorders	Panghal et al., 2010
Villupuram district, Tamil Nadu, India	Folklore practitioners	Root decoction of *Nachapuram (T. indica)*	Asthma, expectorant	Sankaranarayanan et al., 2010
		Leaf decoction	antidote to poisons	
Krishna district, Andhra Pradesh, India	Local medical practitioners and some elderly	Tender tips extracts of *Mekha meyani Aku (T. indica)*	Bronchitis-once a day for fortnight, orally,	Reddy et al., 2010
		Paste of tender tips of *T. indica*	Gastric ailments- once daily, early hours orally for a month,	
Paderu area of Visakhapatna, Andhra Pradesh, India	The tribes	Root	Diarrhoea, dysentery	Padal et al., 2010
Maruthamalai hills in Tamil Nadu, India	An ethnic primitive tribe-*Irulars*	Fresh leaves of *Kaakittam (T. indica*)	Asthma	Sarvalinga et al., 2011
Srikakulam District, Andhra Pradesh, India	41 vaidyas	The leaves	Epilepsy, asthmatic attack	Naidu and Reddy, 2011
Khulna city, Bangladesh	Traditional medicals	The fresh juice from leaves from *Onontomul (T. indica*) and *Ocimum sanctum*	bronchitis, cough and asthma	Akber et al., 2011
		The stem and leaves powder with powder of *Aegle marmelos* (pulp from young fruit)	blood dysentery	

Although studies have supported *T. indica* as a potential drug in different pharmacological conditions, some side effects have also been reported. The patients may experience giddiness, mouth pain, temporary nausea, vomiting, loss of taste for salt with fresh leaf and the tincture (Butani et al., 2007). Other side effects might be dermatitis, eruption, and redness of the skin by tylophorine and tylophorinine (Butani et al., 2007). The preliminary studies demonstrates that the extracts of tylophora are

toxic in very high doses, and quite safe at low dosage, which is actually required to be effective as a therapeutic agent (Butani et al., 2007; Malathi and Gomaz, 2008).

Not only pharmacological potential, but the effects of various parts of *T. indica* have also been studied for its antimicrobial potential (Table 2.4).

Table 2.3: Pharmacological potentials of *T. indica*

Pharmacological action	Model	Dose	Observations	Reference
Anti-asthmatic activity	Human	One leaf, daily	Immense relief to asthmatic symptoms Side effects observed: irritation of mucous membrane in mouth, tongue and the stomach	Shivpuri et al., 1969
		T. indica leaves alcoholic extracts- 40mg for 6 days, daily	Improvement in symptoms of asthma	Shivpuri et al., 1972
		T. indica leaf powder (350 mg) for a week followed by antiasthmatic drug combination	Increase in the lung oxygen capacity, relief from breath shortness at night times	Thiruvengadam et al., 1978
		T. indica leaf powder	Reduced nasal obstruction and sneezing; Ventilator capacity improvement	Gore et al., 1980
	Guinea pigs	Methanolic extracts 100mg/kg	Bronchodilation	Pritesh et al, 2011
Hepatoprotective activity	Swiss albino mice, Wistar albino rats	Alcoholic extracts- 200 and 500 mg/kg Aqueous extracts- 125 and 300 mg/kg	A decrease in wet liver weight and volume, prevention in biochemical changes, minimal fatty alterations of hepatic cells	Gujrati et al., 2007
		methanolic extracts of *T. indica* leaves 200 and 300 mg/kg	lower level of liver enzymes in the serum with higher level of proteins	Mujeeb et al, 2009
	Adult wistar albino rats	Ethanolic extracts of *T. indica* 100, 200, 400, 800, 1600 and 3200 mg/kg	Reverse of elevated levels of alkaline phosphatase, alanine aminotransferase (ALT), gamma-glutamyl transpeptidase, aspartate aminotransferase (AST) and bilirubin, rise of reduced albumin serum level to normal No mortality up to 3200 mg/kg	Shyamajith and Rao, 2013
Anti-inflammatory potential	Mesenteric bits of male albino rats	Tylophora alkaloids 1 µg/mL	Stabilisation of mast cells by raising cyclic AMP level due to inhibition of phosphor-diesterase	Geetha et al, 1981
Cardioprotective	Female wistar	Hydroethanolic extracts of *T. indica*	Decrease in levels for LDH and creatine phosphokinase (CK-MB)	Asdaq and Sowmya,

activity	albino rats	leaves (dose (mg/kg): 100 and 200)	in the serum	2008
Antioxidant	Female wistar albino rats	Hydroethanolic extracts of *T. indica* leaves (dose: 100 mg/kg and 200 mg/kg)	The serum activities of CK-MB and LDH were found to be reduced higher activity of catalase and superoxide dismutase and the removal of cardiac damages	Asdaq and Sowmya, 2010
	DPPH assay	*T. indica* leaves, stems, roots and callus (methanolic extracts)	Free radical scavenging activity found maximum in leaves followed by callus, stem, and root	Mohan et al, 2014
		T. indica leaves (methanolic, ethanolic, aqueous and chloroform)	Most potential- methanolic extracts	Rangaswamy et al, 2014
Anticancer	*Erythroleukaemic* cell lines (K562)	*Tylophora* alkaloids (concentration above 0.1 μg/mL/5 × 10 cells)	inhibit the complete proliferation of the cells along with apoptosis	Ganguly and Khar, 2002
	A549 human cancer cell lines	Tylophorine derivatives	IC_{50} values of 0.27 and 0.16 μM	Wei et al., 2006
	Human colon adenocarcinoma cell lines (HCT-15)	Crude drug samples- 10, 50, 100 μg	Higher cellular damage on cancer cells by 50 μg and 100 μg crude drug samples than 10 μg of the crude extract	Pratheesh et al., 2014
Cytotoxicity	MTT assay- BHK-21 cell lines	alcoholic extracts : 20mg, 2mg, 200μg and 20μg	IC_{50} value approximately to be 20 μg/ mL	Kannan et al, 2013
Immune response	Mice	*T. indica* leaf crude extract	Suppress cellular immune response	Ganguly and Sainis, 2001
	Mice	*T. indica* alkaloid mixture	Suppression of splenocytes proliferation, activation of macrophages and cytostatic effect	Ganguly et al, 2001
Diarrhoea	Albino rats	Aqueous and alcoholic extracts of *T. indica* roots (mg/kg)- 800 and 400	Diminution of intestinal propulsive movement with the aqueous and alcoholic extract dose (mg/kg)- of 400 and 200, respectively	Patel et al, 2006
Intestinal amoebiasis	Adult mice	Tylophorine- 15, 5, 3.5, 2.15 mg/kg	Maximum effective dose- 3.5 mg/kg	Bhutani et al, 1987

Antiviral avtivity		Tylophorine B	$1.0 \times 10^{(-6)}$) g/mL concentration-showed 60% inhibition of tobacco mosaic virus	Xi et al, 2006
Wound healing potency	Rats	Emulsifying ointment (hydro alcoholic extracts of *T. indica* leaves) - 10% and 5% conc	Period of epithelisation reduced significantly	Asdaq et al, 2008b
Diuretic effects	Wister albino rats	*T. indica* leaves (aqueous and alcoholic extracts)	Elevated sodium, potassium, and calcium concentration in urine	Meera et al, 2009
Lipid profile	Female wistar rats	*T. indica* leaves (methanolic extracts)- 500 mg/kg, 100 mg/kg	Reduce the level of low-density lipoprotein, total cholesterol, and triglycerides in the serum, elevated the levels of high-density lipoproteins, also reduced ALT and serum AST levels	Asdaq et al, 2009
Anti-diabetic activity	Diabetic Balb/c mice	*T. indica (*methanolic extracts)- 50 mg/kg	Reduce the blood glucose level	Najafi, 2010
Anti-ulcer (Gastric)	male wistar rat model	*T. indica* leaves (methanolic extracts)- 50, 100, and 200 mg/kg	Antiulcer potential by blocking the hydrogen receptor and reducing the lipid peroxidase level	Ghodekar et al, 2010
Alzheimer's disease	Adult wistar rats	*T. indica* leaves (ethanolic extracts)-	Lower cataleptic activity when compared to the control	Shyamjith et al., 2012
Antianxiety effects	Wistar rat	*T. indica* leaves (ethanolic extracts)- 300 mg/ kg	Rats treated with *Tylophora* extracts possessed anxiolytic effects	Rao et al., 2013
	Rats	*T. indica* leaves (ethanolic extracts) - 100mg/kg	Dopamine levels were raised	Mannikoth et al., 2016
Anti-convulsing effects	Wistar rats	*T. indica* leaves (ethanolic extracts) - 100 mg/kg, orally	Prevention in hind-limb extension and decrease in duration of convulsions	Mannikoth et al, 2017
Toxicity studies (acute and sub chronic)	Male wistar rats	T. asthmatica (methanolic extracts) Acute- oral dose (mg/kg) of 50, 100, 200, 500, and 1000 Sub chronic dose (mg/kg)- 100, 200, and 300	Dose (mg/kg) of 200 is well tolerated Toxin symptoms observed from 500mg/kg dose The LD50 calculated to be 223.6 mg/kg (calculated from acute dose) The plant is mildly toxic with methanolic extracts	Malathi and Gomaz, 2008
Acute oral toxicity	Non-pregnant female mice	Alcoholic extract Aqueous extract of T. indica root	Aqueous root extract non toxicity up to 5000 mg/kg Alcoholic extract LD50 3162 mg/kg	Gujrati et al., 2007

Table 2.4: The antimicrobial potential of *Tylophora indica* extracts

T. indica sample	Microorganism	Extract	Zone of inhibition	Reference
Root extracts	*Bacillus subtilis (B. subtilis)*	Methanolic	+	Reddy et al., 2009
	Staphylococcus aureus (S. aureus)	Methanolic Ethyl acetate	+ +	Reddy et al., 2009 Balasubramanian et al., 2010
	Micrococcus luteus	Methanolic	+	Reddy et al., 2009
	Klebsiella pneumoniae (K. pneumoniae)	Ethyl acetate	+	Balasubramanian et al., 2010
	Escherichia coli (E. coli)	Methanolic Ethyl acetate Methanolic Petroleum ether Aqueous	- + + - +	Reddy et al., 2009) Balasubramanian et al., 2010 Raut et al., 2012 Raut et al., 2012 Raut et al., 2012
	Salmonella typhi	Ethyl acetate	+	Balasubramanian et al.,2010
	Pseudomonas flavescens	Methanolic Petroleum ether Aqueous	+ - -	Raut et al., 2012 Raut et al., 2012 Raut et al., 2012
	Micrococcus roseus (M. roseus)	Methanolic Petroleum ether Aqueous	+ - -	Raut et al., 2012 Raut et al., 2012 Raut et al., 2012
	Pseudomonas aeruginosa (P. aeruginosa)	Methanolic Ethyl acetate	- +	Reddy et al., 2009 Balasubramanian et al., 2010
	Aspergillus niger	Methanolic	+	Reddy et al., 2009
	Trichoderma virdi (T. virdi)	Methanolic	+	Reddy et al., 2009
	Aspergillus fumigatus	Methanolic	-	Reddy et al., 2009
Leaf extracts	*Bacillus subtilis*	Methanolic	+	Reddy et al., 2009
	Staphylococcus aureus	Methanolic Ethanolic Aqueous Ethanolic Ethyl acetate	+ - - - +	Reddy et al., 2009 Reddy BU, 2009 Parekh et al., 2008 Parekh et al., 2008 Balasubramanian et al., 2010
	Staphylococcus epidermis	Aqueous Ethanolic	- -	Parekh et al., 2008) Parekh et al., 2008
	S. subflava	Aqueous Ethanolic	- -	Parekh et al., 2008 Parekh et al., 2008
	Micrococcus luteus	Methanolic	+	Reddy et al., 2009
	K. pneumoniae	Ethanolic Aqueous Ethanolic Ethyl acetate	- - + +	Reddy BU, 2009 Parekh et al., 2007 Parekh et al., 2007 Balasubramanian et al., 2010
	Escherichia coli *E. coli* *E. coli*	Methanolic Ethanolic Aqueous Ethanolic Ethyl acetate	- + - - +	Reddy et al., 2009 Reddy BU, 2009 Parekh et al., 2007 Parekh et al., 2007 Balasubramanian et al., 2010
	Salmonella typhi *S. typhimurium*	Ethanolic Aqueous Ethanolic Ethyl acetate	- - - 	Reddy BU, 2009 Parekh et al., 2007 Parekh et al., 2007 Balasubramanian et al., 2010
	Proteus vulgaris (P.	Ethanolic	+	Reddy BU, 2009

	vulgaris)	Aqueous Ethanolic	- -	Parekh et al., 2007 Parekh et al., 2007
	P. aeruginosa	Methanolic Ethanolic Ethyl acetate	+ + +	Reddy et al., 2009 Reddy BU, 2009 Balasubramanian et al., 2010
	Fusarium species	Ethanolic	+	Reddy BU, 2009
	Enterobacter aerogenes	Aqueous Ethanolic	- -	Parekh et al., 2007 Parekh et al., 2007
	P. mirabilis	Aqueous Ethanolic	- -	Parekh et al., 2007 Parekh et al., 2007
	Plasmodium falciparum MRC-2 (chloroquine sensitive) RKL-9 (chloroquine resistant)	 Aqueous Aqueous	 + +	 Rana et al., 2012 Rana et al., 2012
	Aspergillus niger	Methanolic Ethanolic	+ +	Reddy BU, 2009 Reddy BU, 2009
	T. virdi	Methanolic	+	Reddy BU, 2009
	Aspergillus fumigatus	Methanolic Ethanolic	+ +	Reddy et al., 2009 Reddy BU, 2009
Stem extracts	*Escherichia coli*	Methanolic Pet ether Aqueous	+ + +	Raut et al., 2012
	Pseudomonas flavescens	Methanolic Pet ether Aqueous	- + -	Raut et al.,2012
	Micrococcus roseus	Methanolic Pet ether Aqueous	- - -	Raut et al., 2012
	Aspergillus niger	*In vivo* alcoholic *In vitro* alcoholic	+ +	Deshwal et al., 2013
	Aspergillus fumigatus	*In vivo* alcoholic *In vitro* alcoholic	- +	Deshwal et al., 2013
	Aspergillus flavus	*In vivo* alcoholic *In vitro* alcoholic	- +	Deshwal et al., 2013
	Candida parapsilosis	*In vivo* alcoholic *In vitro* alcoholic	- +	Deshwal et al., 2013
	Candida krusei	*In vivo* alcoholic *In vitro* alcoholic	- +	Deshwal et al., 2013
	Plasmodium falciparum RKL-9	Aqueous Alcoholic	+ +	Rana et al., 2012
	Plasmodium falciparum MRC-2	Aqueous Alcoholic	+ +	Rana et al., 2012

(+): Present; (-): Not present; *In vivo:* Parent plant grown naturally; *In vitro:* Plant grown in artificial conditions; Pet ether: Petroleum ether

To ensure the standardised quality of plant samples, the physico chemical standards of leaves have been prescribed in text such as Quality Standards of Indian Medicinal Plants but for roots, it still needs to be addressed. The antimicrobial potential of *T. indica* against different food borne pathogens has been studied such as *B. subtilis, S. typhi, P. aeruginosa, E. coli and S. aureus,* and *Listeria monocytogenes* is a food

pathogen causing listeriosis especially in refrigerated foods and is of a concern globally (Cleveland et al., 2001).The efficacy of this plant towards this pathogenic organism needs to be exhaustively studied so as to check its potential against this food borne pathogen. The potential of leaves and roots of *T. indica* has not been studied in food packaging formulation individually and when combined other antimicrobial agents such as Bacteriocin, towards bacterial species. Hence, the plant has been selected to understand its potential towards various food pathogens and its behaviour when combined with Nisin, a potent Bacteriocin, along with its potential in food packaging formulations to enhance shelf life of meat product.

2.2 Materials and Methods

2.2.1 Materials

The roots and leaves of *T. indica* were collected during June, 2017 from Ayushya Vatika, Lovely professional University, Punjab, in the morning around 9.30 am. The temperature during the time of collecting plant drug samples was 34 ℃ and the humidity as recorded corresponded nearly 61%. One whole plant was also uprooted, for the preparation of the herbarium for authentication of the climber. The analytical grade chemicals as used in current study, were procured from Loba Chemicals, Mumbai.

2.2.2 Methods

The collected plant matter was thoroughly cleaned using running tap water and subsequently washed with double distilled water twice, so as to remove foreign matter and soil. 500 g of cleaned plant matter (leaves and root each) was dried in shade, was powdered using a mixer grinder and subsequently, stored in sterile plastic containers in a dried place at room temperature for further use. A herbarium (Photograph 2.1) presenting the collected and dried whole plant of *T. indica* including stem, leaves and root was prepared and submitted to the Department of Botanical and Environmental Sciences, Guru Nanak Dev. University, Amritsar for the authentication.

2.2.2.1 Physico-chemical analysis of leaves and roots of *T. indica*

Determination of moisture content

5 g of the weighed powdered leaves/root was added directly in the pre-weighed, dried china dish and exposed at 105°C for 5 h using the hot air oven. China dish along with the content was weighed. Drying, cooling and re-weighing were continued with one hour interval until the difference between the two successive weighs was not more than 0.01g **(**Khandelwal and Sethi, 2013; Anonymous, 2001; World Health Organization, 1998).

The moisture content was thus, determined using the formula (all weights have been taken in grams, g): % Weight loss= [(a+5) – b)] x 100/5= c % (Khandelwal and Sethi, 2013)

Where,

Weight of dried china dish: a

Weight of leaves/root powder taken: 5

Weight of china dish+ Weight of leaves/root powder= (a+ 5)

Final weight leaves/root powder after drying and china dish = b

Loss of weight = [(a+5) – b)]

Determination of total ash content

The silica crucibles were taken, ignited, cooled and then weighed. 2g of the coarsely powdered leaves/root was weighed with the crucible. The incineration was done at 450º C for 8-10h in the muffle furnace (until free from carbon), properly cooled and reweighed **(**Anonymous, 2001; World Health Organization, 1998)

The total ash was thus, determined using the formula (all weights have been taken in grams, g): Total ash% = (y-x) x 100/2= z % (Khandelwal and Sethi, 2013)

Where,

Weight of empty crucible = x

Weight of leaves/root powder taken = 2

Weight of empty crucible+ leaves/root powder= (x+2)

Weight of empty crucible + ash= y

Weight of total ash= (y-x)

Value of ash by 100g of the leaves/root sample= (y-x) x 100/2 (Where 2 is weight of the sample taken)

Determination of acid insoluble ash

Obtained total ash was washed using 25mL dilute HCl in a 100mL glass beaker, followed by 5 min boiling, filtered using an ash-less filter paper and subsequently washed with hot water. This filter paper along with ash was again kept in the dried crucible and ignited at 450ºC for 8-10h in the muffle furnace, cooled and reweighed **(**Khandelwal and Sethi, 2013; Anonymous, 2001; World Health Organization, 1998)

The acid insoluble ash was thus, determined using the formula (all weights have been taken in grams, g): % of acid insoluble ash= (b-a) x 100/2= c % (Khandelwal and Sethi, 2013)

Where,

Weight of empty crucible = a

Weight of leaves/root powder taken = 2g

Weight of empty crucible+ leaves/root powder= (a+2)

Weight of the residue after washing with water and ignition = (b)

Weight of acid insoluble ash= (b-a)

Determination of water soluble ash

Obtained total ash was boiled for 5 min using 25mL water in 100mL dried glass conical flask, followed by filtering using ash-less filter paper. This filter paper along with remaining ash was re-kept in the dried crucible and ignited at 450ºC in the muffle furnace for 8-10h, cooled and reweighed **(**Khandelwal and Sethi, 2013; Anonymous, 2001; World Health Organization, 1998)

The water soluble ash was thus, determined using the formula (all weights have been taken in grams, g): Total water soluble ash % = [(b-a) – (c-a)] x 100/2= d % (Khandelwal and Sethi, 2013)

Where,

Weight of empty crucible = a

Weight of leaves/root powder taken = 2g

Weight of empty crucible+ leaves/root powder= (a+2)

Weight of crucible+ total ash= b

Total ash= (b-a)

Weight of the residue after washing with water and ignition = (c)

Weight water insoluble ash= (c-a)

Weight of water soluble ash= [(b-a) – (c-a)]

Determination of alcohol soluble extractive

2.5 g of *T. indica* leaves/root, coarse dried powder, was taken and dissolved in 50 mL of 90% methanol in 250 ml Erlenmeyer flask. This flask was corked, and was set aside with continuous shaking for 6 h followed by standing for a period of 18 hrs. The solution was then filtered using filter paper and the filtrate was obtained. 25 ml of the collected filtrate was transferred to pre-weighed dried china dish and was allowed to evaporate to dryness using water bath followed by complete drying in the hot air oven

until constant weight was achieved. The dish was cooled in the desiccator and finally weighed. The remaining extract was used for the phytochemical analysis **(**Khandelwal and Sethi, 2013; Anonymous, 2001; World Health Organization, 1998)

The alcohol soluble extractive was thus, determined using the formula (all weights have been taken in grams, g): % of alcoholic soluble extractive= c %

Where,

Weight of china dish= a

Amount of filtrate taken= 25mL

Residual weight (after keeping on water bath and hot air) = b

Wt. of the alcohol soluble extract = (b-a)

Alcoholic soluble extract value by 100 mL of the filtrate= (b-a) x4

Alcoholic soluble extract value by 100gm of the drug= [(b-a) x4] x 100/2.5= c (Where 2.5 is weight of the sample taken) (Khandelwal and Sethi, 2013)

Determination of water soluble extractive

2.5g of the coarse dried powder of *T. indica* leaves/root was taken and dissolved in 50 ml of chloroform water in a 250 mL Erlenmeyer flask. The flask was corked, and was set aside with continuous shaking for 6 h followed by standing for a period of 18 hrs. The solution was then filtered using filter paper, 25 ml of the filtrate, thus, obtained was transferred to pre-weighed dried china dish and was allowed to evaporate to dryness on the water bath followed by complete drying in the hot air oven until constant weight was achieved. The dish was cooled in the desiccator and finally weighed. The remaining extract was used for the phytochemical analysis **(**Khandelwal and Sethi, 2013; Anonymous, 2001; World Health Organization, 1998)

The water soluble extractive was thus, determined using the formula (all weights have been taken in grams, g): % of water soluble extractive= c %

Where,

Weight of china dish= a

Amount of filtrate taken= 25mL

Residual weight (after keeping on water bath and hot air) = b

Wt. of the water soluble extract = (b-a)

Water soluble extract value by 100 mL of the filtrate= (b-a) x4

Water soluble extract value by 100gm of the drug= [(b-a) x4] x 100/2.5= c (Where 2.5 is weight of the sample taken) (Khandelwal and Sethi, 2013)

Thereafter mean and standard deviation was calculated. All the experiments were done in six replicates.

2.2.2.2 Phytochemical screening of leaves and roots of *T. indica*

The methanolic and aqueous extracts left after determination of quantitative standards of the plant under study, was further used for qualitative chemical characterization.

2.2.2.2.1 Qualitative estimation for the presence of alkaloids in the extracts of *T. indica* leaves

Methanolic and aqueous left over extracts of the leaves were dissolved in the dilute hydrochloric acid individually and filtered. This prepared filtrate was used for estimation of alkaloids by subjecting them to Mayer's test, Hager's test and Dragendorff's test **(**Trease and Evans, 1989; Bhandary et al., 2012; Vimal kumar et al., 2014).

2.2.2.2.2 Qualitative estimation for the presence of carbohydrates in the extracts of *T. indica* leaves

Samples used in this estimation were the left over extracts during estimation of alcohol and water soluble extractives. They were subjected to Molisch's test and Benedict's test for determining the presence of carbohydrates **(**Trease and Evans, 1989; Vimalkumar et al., 2014).

2.2.2.2.3 Qualitative estimation for the presence of flavonoids in the extracts of *T. indica* leaves

The samples used in this estimation were the left over extracts during estimation of alcohol and water soluble extractives. They were subjected to ferric chloride test, alkaline reagent test, lead acetate test and Pew's test to assure the presence of flavonoids **(**Trease and Evans, 1989; Vimalkumar et al., 2014).

2.2.2.2.4 Qualitative estimation for the presence of tannins in the extracts of the *T. indica* leaves

The samples used in this estimation were the left over extracts during estimation of alcohol and water soluble extractives. The obtained samples were subjected to ferric chloride test, dilute iodine test and dilute nitric acid test to confirm the presence of tannins **(**Trease and Evans, 1989; Vimal kumar et al., 2014).

2.2.2.2.5 Qualitative estimation for the presence of glycosides in the alcoholic extracts of the *T. indica* leaves

The samples used in this estimation were the left over extracts during estimation of alcohol and water soluble extractives. The obtained samples were subjected to Keller-Kiliani test and Modified Borntrager's test (for C-glycosides) to understand the presence of glycosides (Trease and Evans, 1989; Bhandary et al., 2012).

2.2.2.2.6 Qualitative estimation for the presence of steroids and terpenoids in the extracts of the *T. indica* leaves

The samples used in this estimation were the left over extracts during estimation of alcohol and water soluble extractives. The obtained samples were subjected to Salkowski test and Liebermann Burchard's test to understand the presence of steroids and terpenoids (Trease and Evans, 1989; Vimalkumar et al, 2014).

2.2.2.2.7 Qualitative estimation for the presence of saponin in the extracts of the *T. indica* leaves

The samples used in this estimation were the left over extracts during estimation of alcohol and water soluble extractives. Foam test was conducted to estimate the same **(**Trease and Evans, 1989; Bhandary et al., 2012).

2.2.2.2.8 Qualitative estimation for the presence of amino acids in the extracts of the *T. indica* leaves

The samples used in this estimation were the left over extracts during estimation of alcohol and water soluble extractives. Ninhydrin and Biuret tests were conducted to estimate the same ***(***Trease and Evans, 1989; Bhandary et al., 2012).

2.2.2.3 Analysis for the presence tylophorine in the leaves and roots of *T. indica*

High Performance Thin Layer Chromatography (HPTLC) analysis for the methanolic extracts of leaves and root of *T. indica* was done to ensure the presence of tylophorine, the main alkaloid present in *T. indica*. It was carried out from the Herbal Health Research Consortium, Amritsar, Punjab, India, with CAMAG Linomat 5 instrument with two different solvent systems:

Solvent system 1: Toluene: Ethyl acetate: Diethyl amine (14:2:2)

Solvent system 2: Toluene: Chloroform: Ethyl Acetate (5:90:5)

Rf values has been recorded at 254 nm and 366 nm.

2.2.2.4: XRF analysis for heavy metal detection in roots of *T. indica*

XRF analysis of *T. indica* roots has been done from SAIF, Punjab University, Chandigarh. The fine root powder was provided to the lab for the detection of presence of various inorganic elements by XRF analysis.

2.3 Results and Discussion

2.3.1 Authentication of the *T. indica*

The Department of Botanical and Environmental Sciences, GNDU, Amritsar has authenticated the plant as *Tylophora indica* belonging to family Asclepiadiaceae with voucher reference no. 2350 dated 23/6/2017 and issued a certificate for the same (Photograph 2.3). Photograph 2.2 presents the various parts of *T. indica* growing in Ayushya Vatika, Lovely Professional University, Jalandhar, Punjab, India

2.3.2 Physico-Chemical analysis of leaves and roots of *T. indica*

The leaves and roots of *T. indica* were subjected to physicochemical analysis (Photograph 2.4) and the respective evaluation was done (Table 2.5). The values for loss on drying (LOD), total ash, acid insoluble ash, water soluble ash, methanol soluble extractive and water soluble extractive for dried leaves samples was found to be 4.5 ± 0.5 %, 11.08 ± 0.9 %, 12.8 ± 1.16 %, 6.6 ± 0.4 %, 29.0 ± 2.35 % and 38.9 ± 2.4 % respectively. The fresh leaves were also evaluated for LOD, methanol soluble extractive and water soluble extractive which was found to be 81.6 ± 0.27 %, 7.7 ± 2.1 % and 1..4 ± 2.18 % respectively. The observations for LOD, total ash, acid insoluble ash, water soluble ash, methanol soluble extractive and water soluble extractive of dried root samples was found to be 11.3 ± 0.6%, 6.8 ± 1.32 %, 1 ± 1.0 %, 4.16 ± 0.98 %, 30.4 ± 1.75% and 20 ± 1.6 % respectively. The availability of standard quality crude drug is a big challenge to the food and herbal drug industry for the formation of effective and high quality food and medicinal products (Ekor M, 2014). In order to deal with this issue, studies have been investigated and the standardization parameters have been elaborated by the controlling authorities. This has enabled the users such as researchers and industry to recognize the quality of raw material. The physicochemical analysis plays a significant role in understanding the purity and standard of the crude drug (Mulla and Swamy, 2010). In this study, the physicochemical values of the *T. indica* dried leaves were found within the suggested limits (Gupta, 2003). The values of loss on drying for the available dried leaves and roots samples were observed to be 4.5 ± 0.5 % and 11.3 ± 0.6%, respectively. The significance of proper drying of the herbal drugs is to reduce the moisture content

which helps reducing the biological contamination by inhibiting the growth of microbes and fungi (Bignoniya et al., 2011). This allows the extended retention of the bioactive constituents which may have been degraded by the microbes and thus enabling the drug to be used for a longer span. The ash values are an important tool to determine the authenticity and purity of any crude drug. They may also contribute in knowing the adulteration and extent of impurities present in the raw drug (Nayak and Patel, 2010). This can also help the industries in analyzing the quality of the procured raw material involved in large scale production of medicines. The total ash observed in the leaves and root samples has been 11.08 ± 0.9% and 6.8 ± 1.32%, respectively. Acid-insoluble along with water soluble ash were higher in dried leaves 12.8 ± 1.16% and 6.6 ± 0.4%, respectively. In the roots it was found to be 1± 1.0% and 4.16±0.98 % respectively. The acid insoluble and water soluble ash values indicate the presence of various inorganic matters and metallic salts such as carbonates, phosphates, silicates, sand, and so on and are soluble in different solvents like water and acids (Thomas et al., 2008). The different extractive values of a crude drug elucidate solubility of the various chemical constituents present in that drug in the solvent. These values can be used as a reliable diagnostic tool for detecting the adulteration of different samples (Kunle et al., 2012). The methanolic soluble extractive (Photograph 2.5) values of dried leaves and roots were comparable (29.0 ± 2.35% and 30.4 ± 1.75%) whereas water soluble extractive of leaves and roots were 6.6 ± 0.4% and 4.16 ± 0.98%. No reference has been found in the literature for physicochemical analysis of *T. indica* dried root. It has been reported first time in the present study.

2.3.3.Phytochemical screening of leaves and roots of *T. indica*

The aqueous and methanolic crude extracts (leaves and roots) were analyzed qualitatively to determine the presence of various phytochemicals (Table 2.6). During the analysis of *T. indica* leaves extracts, white and yellow precipitates were formed during Hager's and Mayer's test respectively which confirmed the presence of alkaloids. No orange precipitates were found in Dragendorff's test for both extracts. The violet ring appeared in Mohlisch's test and the solution turned green in Benedict's test for both the extracts which confirmed the presence of reducing sugars. During alkaline reagent test, the formation of intense yellow was developed which

indicated the presence of flavonoids in both the samples. During lead acetate testing, yellow precipitates were formed in the aqueous extract sample only. No other test was positive for flavonoids. In ferric chloride test, the colour of both the solutions turned blue black, transient colour developed in dilute iodine test, and reddish yellow colour developed in dilute nitric acid test which confirmed the presence of tannins in both the samples. The upper layer appeared greenish blue and there was a formation of reddish brown colour at the junction suggesting the presence of glycosides in Keller kiliani test. The development of pinkish red colour of ammoniacal layer indicated the presence of C-glycosides in modified Borntragger's test. The presence of glycosides was indicated in both the tests. There was no appearance of red or yellow colour in the Salkowski test and no development of red colour in Liebermann Buchard's test indicating the absence of steroids in the water and methanolic extracts. The presence of saponin was confirmed in the water extract as the layer of foam was well developed in foam test. There was no development of purple colour in Ninhydrin test and no formation of violet or pink colour in Biuret test indicating the absence of proteins and amino acids in the extracts.

During the analysis of *T. indica* root extracts (Table 2.6), white and yellow precipitates were formed during Hager's and Mayer's test respectively which confirmed the presence of alkaloids. No orange precipitates were found in Dragendorff's test for both extracts. The violet ring appeared in Mohlisch's test which confirmed the presence of reducing sugars but the solution did not turned green in Benedict's test for both the extracts. During alkaline reagent test, the formation of intense yellow was developed which indicated the presence of flavonoids in both the samples. During lead acetate testing, yellow precipitates were formed in the aqueous extract sample only. No other test was positive for flavonoids. In ferric chloride test, the colour of none of the the solutions turned blue black. The transient colour developed in dilute iodine test, and reddish yellow colour developed in dilute nitric acid test for aqueous extract which confirmed the presence of tannins in this extract of the root samples. Tannins were altogether absent ion the methanolic extracts. The upper layer appeared greenish blue and there was a formation of reddish brown colour at the junction in the root aqueous extract only, suggesting the presence of glycosides

in Keller kiliani test. The development of pinkish red colour of ammoniacal layer indicated the presence of C-glycosides in modified Borntragger's test in both extracts. There was the appearance of red colour in the Salkowski test indicating the presence of steroids in the water and methanolic extracts but no development of red colour in Liebermann Buchard's test. The presence of saponin was confirmed in the water extract as the layer of foam was well developed in foam test. There was no development of purple colour in Ninhydrin test and no formation of violet or pink colour in Biuret test indicating the absence of proteins and amino acids in the root extracts.

Various chemical constituents play a significant role in establishing pharmacological and antimicrobial properties of any drug. Literature has suggested that *T. indica* possess various alkaloid and non-alkaloid compounds (Kaur and Singh, 2012). The solubility of active constituent is depended on its chemical nature and the solvent. Hence, methanolic and aqueous extracts of leaf and roots were evaluated for the presence of different phytochemicals. The qualitative analysis of leaf revealed the presence of alkaloids, carbohydrates, glycosides, and tannins. However, amino acids, steroids, and terpenoids were not detected in any extracts of the leaf. The root extracts revealed the presence of alkaloids, carbohydrates, glycosides, tannins, steroids, and amino acids. However, saponin was present in the aqueous extracts of both, i.e. leaves and roots. This study indicates the presence of various secondary metabolites which are responsible for multiple pharmacological and antimicrobial effects of the plant (Sangeetha et al., 2012).

2.3.4 Analysis for the presence tylophorine in the leaves and roots of *T. indica*

The TLC (Photograph 2.6) and HPTLC (Figure 2.2-2.5) of the methanolic extracts were conducted with two different solvent systems and correspondingly the peaks were recorded at two wavelengths (254 nm and 366 nm).

In solvent system 1, the number of peaks for the leaf and root extract at wavelength 254 nm was found to be 9 and 6 respectively (Table 2.8). The R_f values analyzed for the leaf extract are 0.02, 0.08, 0.12, 0.22, 0.46, 0.51, 0.54, 0.88 and 0.96. The R_f values observed for the root extract are 0.02, 0.07, 0.13, 0.23, 0.47 and 0.59.

In the same solvent system, six peaks for both the leaf and root extract at wavelength 366 nm were found. The R_f values analyzed for the leaf extract were 0.01, 0.37, 0.50, 0.57, 0.75 and 0.89. The R_f values observed for the root extract were 0.02, 0.07, 0.24, 0.37, 0.47 and 0.59.

In solvent system 2, the number of peaks for the leaf and root extract at wavelength 254 nm was found to be 11 and 7 respectively. The R_f values analyzed for the leaf extract were 0.03, 0.04, 0.20, 0.26, 0.33, 0.37, 0.47, 0.71, 0.74, 0.88 and 0.94. The R_f values observed for the root extract at this wavelength are 0.04, 0.21, 0.30, 0.45, 0.46 and 0.79. In the same solvent system, the numbers of peaks for the leaf and root extract observed at wavelength 366 nm were 6 and 5. The R_f values analyzed for the leaf extract are 0.03, 0.04, 0.11, 0.15, 0.21 and 0.27. The R_f values observed for the root extract are 0.04, 0.21, 0.22, 0.30 and 0.95.

The different number of peaks has been observed in the chromatograms and presented as various Rf values and they determine the presence of different compounds in the extracts. The Rf of the tylophorine has been reported 0.59 in solvent system 1 at 254 nm. One of the RF values of root extract, 0.59, corresponds to that of standard, expressing the presence of tylophorine (Chaturvedi and Chowdhary, 2014). Chromatographic techniques are the advancements which help to separate and analyze the various molecules of a drug due to their differences in composition and/or structure (Senguttuvan et al., 2014) and thus, help in identifying the genuine sample.

2.3.5: XRF values of various components detected in root sample of *T. indica*

In this analysis (Table 2.8), *T. indica* root sample was found to be composed of organic matter which mainly includes cellulose (95.69 %) followed by calcium (1.03%), potassium (0.89 %), silica (0.74 %), magnesium (0.44 %), aluminium (0.32%), sodium (0.27 %), iron (0.17%), phosphorus (0.14%), chlorine (0.12%), sulphur (0.12%), titanium (0.02%), strontium (0.01%), barium (0.01%), zinc (75 ppm), copper (56 ppm), nickel (15 ppm), ruthenium (13 ppm), chromium (10 ppm), molybdenum (7 ppm), rubidium (7 ppm), and zirconium (5 ppm). This assay indicates the presence of 22 elements of metallic (calcium, potassium, silicon, magnesium, etc.) and non-metallic (chlorine, sulphur, phosphorus, etc.) origin.

The fine root powder was tested for the presence of various organic and inorganic elements by XRF analysis. The heavy metals such as mercury, arsenic, lead, and cadmium were not present in the root sample. XRF analysis is a helpful technique in confirming and estimating the presence of various matters of mineral origin in the fine powder sample (Chauhan and Chauhan, 2014). This technique plays a significant role in assessing the presence of heavy metals such as mercury and lead which are of high concern in health issues.

2.4 Statistical Analysis

All the experiments have been performed in triplicates. The results have been expressed as mean± standard deviation (SD). The software, Excel 2010 in Microsoft Office programme has been used to calculate the same.

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Tables

Table 2.5: Physico-chemical Analysis of leaves and roots of *T. indica*

S. No	Parameter	Observation		
		Leaves		Root
		Dried leaves	Fresh leaves	
1	Loss on drying	4.5 ± 0.5 %	81.6 ± 0.27 %	11.3 ± 0.6%
2	Total Ash	11.08 ± 0.9 %	-	6.8 ± 1.32 %
3	Acid insoluble ash	12.8 ± 1.16 %	-	1 ± 1.0 %
4	Water soluble ash	6.6 ± 0.4 %	-	4.16 ± 0.98 %
5	Methanol soluble extractive	29.0 ± 2.35 %	7.7 ± 2.1 %	30.4 ± 1.75%
6	Water soluble extractive	38.9 ± 2.4 %	1..4 ± 2.18 %	20 ± 1.6 %

Table 2.6: Phyto-chemical screening of leaves and roots of *T. indica*

S. No	Component analyzed	Test Performed	Leaves		Root	
		Extract	Aqueous	Methanolic	Aqueous	Methanolic
1	**Alkaloid**	Mayer's test	+	+	+	+
2		Hager's test	+	+	+	+
3		Dragendorff's test	_	_	_	_
4	**Carbohydrates**	Mohlisch's test	+	+	+	+
5		Benedict's test	+	+	_	_
6	**Flavonoids**	Ferric chloride test	_	_	_	_
7		Alkaline reagent test	+	+	+	+
8		Lead acetate test	+	_	+	+
9		Pew's test	_	_	_	_
10	**Tannins**	Ferric chloride test	+	+	_	_
11		Dilute iodine test	+	+	+	_
12		Dilute nitric acid test	+	+	+	_
13	**Glycosides**	Keller-Kiliani test	+	+	+	_
14		Modified Borntragger's test	+	+	+	+
15	**Steroids and Terpenoids**	Salkowski test	_	_	+	+
16		Liebermann Buchard's test	–	–	–	–
17	**Saponins**	Foam test	+	–	+	–
18	**Amino acids**	Ninhydrin test	_	_	+	+
19		Biuret test	_	_	_	_
+: Present - : Absent						

Table 2.7: Analysis for the presence tylophorine in the leaves and roots of *T. indica*

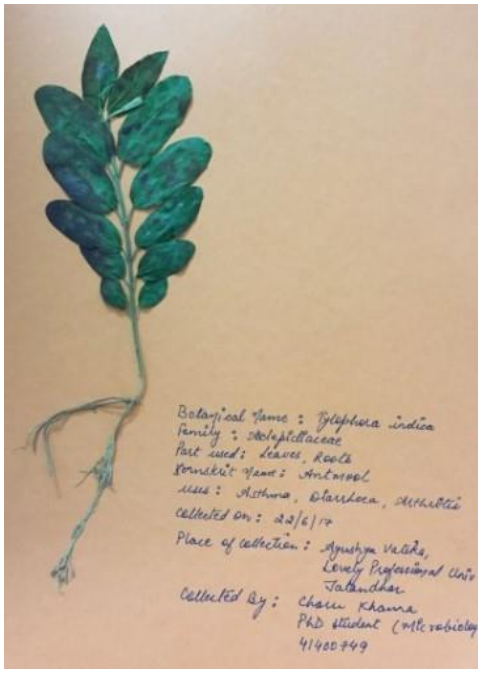
Root extract				Leaf extract			
Solvent system 1 (Rf values)		Solvent system 2 (Rf values)		Solvent system 1 (Rf values)		Solvent system 2 (Rf values)	
254 nm	366 nm	254 nm	366 nm	254 nm	366 nm	254 nm	366 nm
0.02	0.02	0.04	0.04	0.02	0.01	0.03	0.03
0.07	0.07	0.21	0.21	0.08	0.37	0.04	0.04
0.13	0.24	0.30	0.22	0.12	0.50	0.20	0.11
0.23	0.37	0.45	0.30	0.22	0.57	0.26	0.15
0.47	0.47	0.46	0.95	0.46	0.75	0.33	0.21
0.59	0.59	0.79	-	0.51	0.89	0.37	0.27
-	-	0.84	-	0.54	-	0.47	-
-	-	-	-	0.88	-	0.71	-
-	-	-	-	0.96	-	0.74	-
-	-	-	-	-	-	0.88	-
-	-	-	-	-	-	0.94	-

Solvent system 1: Toluene: Ethyl acetate: Diethyl amine (14:2:2)
Solvent system 2: Toluene: Chloroform: Ethyl Acetate (5:90:5)
Conducted at Herbal Health Research Consortium, Amritsar, Punjab, India

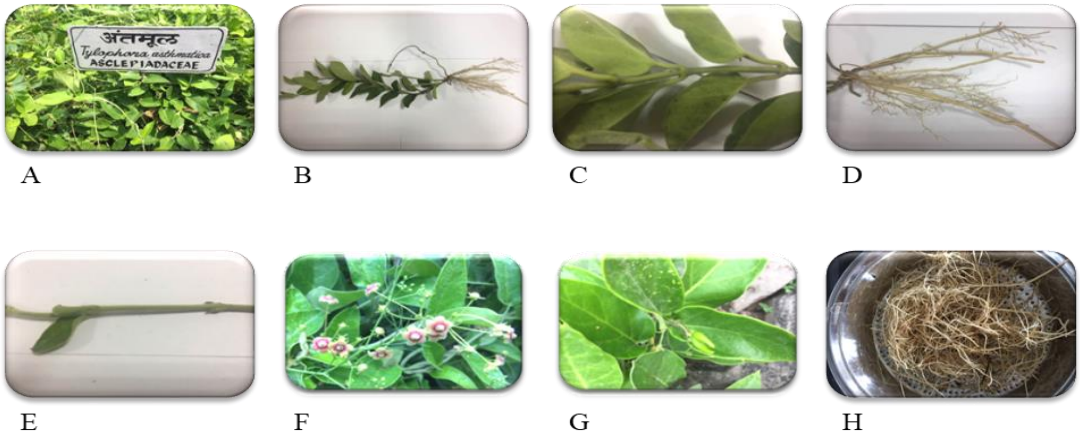
Table 2.8: XRF values of various components detected in root sample of *T. indica*

Component detected	Value	Component detected	Value
Organic components (containg C, H, O and N)	95.69%		
Mineral components:	4.31%	Sr	0.01%
Ca	1.03%	Ba	0.01%
K	0.89%	Zn	75ppm
Si	0.74%	Mn	56 ppm
Mg	0.44%	Cu	26 ppm
Al	0.32%	Ni	15 ppm
Na	0.27%	Ru	13 ppm
Fe	0.17%	Cr	10 ppm
P	0.14%	Mo	7 ppm
Cl	0.12%	Rb	7 ppm
S	0.12%	Zr	5 ppm
Ti	0.02%	Sum	100%
Conducted at SAIF, Punjab University, Chandigarh			

Photographs

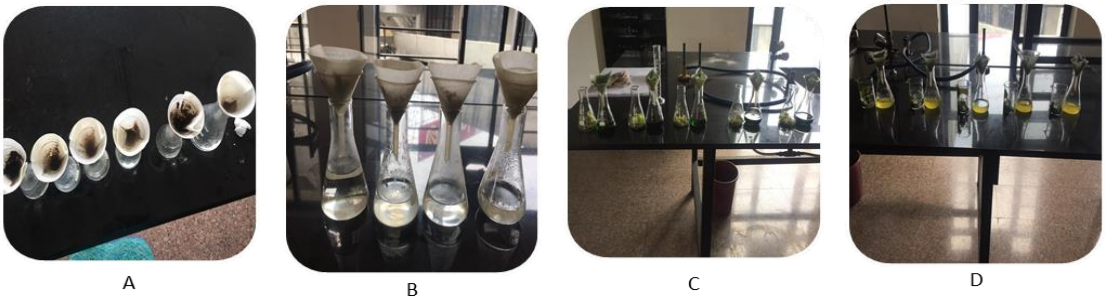


Photograph 2.1: Herbarium of *T. indica* whole plant deposited at Department of Botanical and Environmental Sciences GNDU, Amritsar



Photograph 2.2: A: *Tylophora indica* growing in the Ayushya Vatika of Lovely Professional University; B: Complete plant of *Tylophora indica*; C: The simple leaves of *Tylophora indica* arranged with opposite phyllotaxy; D: The tap root along with rootlets of *Tylophora indica*, E: The hairy green colored stem of *Tylophora indica*; F: The yellow-purple colored flowers of *Tylophora indica*; G: The fruit of *Tylophora indica*; H: Washed and dried roots of *T. indica*

Photograph 2.3: Authentication certificate issued for *T. indica* by the Department of Botanical and Environmental Sciences GNDU, Amritsar



A B C D

Photograph 2.4: Physicochemical analysis of *T indica* leaves, and roots A: Estimation of total ash; B: Estimation of water insoluble ash; C: Estimation of alcohol soluble extractives; D: Estimation of water soluble extractives

A B

Photograph 2.5: The methanolic extracts of *T. indica* stored in the capped bottles, A: *T. indica* leaves methanolic extract, B: *T. indica* root methanolic extract

A B C

Photograph 2.6: TLC plate illustrating spots of constituents, A: Spots in daylight; B: Spots in UV spectrophotometer at 344nm; C: Spots in UV spectrophotometer 369 nm

Figures

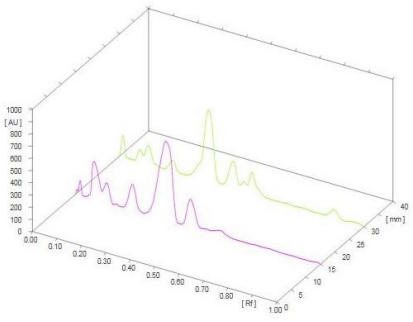


Figure 2.2: 3D view of Methanolic extract of *T. indica* with solvent system 1 (254 nm)

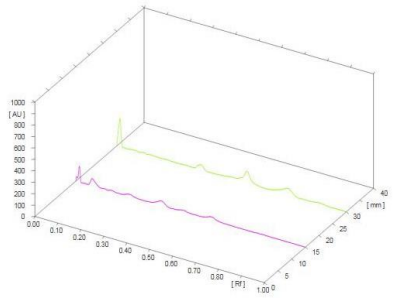


Figure 2.3: 3D view of Methanolic extract of *T. indica* with solvent system 1 (366 nm)

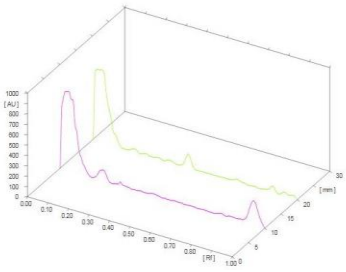


Figure 2.4: 3D view of Methanolic extract of *T. indica* with solvent system 2 (254 nm)

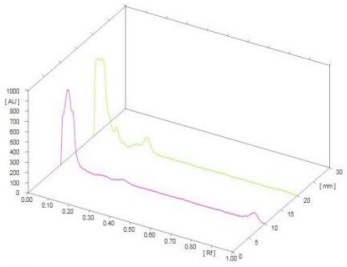


Figure 2.5: 3D view of Methanolic extract of *T. indica* with solvent system 2 (366 nm)

Chapter 3

Isolation, optimisation and characterisation of bacteriocins produced by indigenously isolated and standard Lactic acid bacterial strains

3.1 Introduction and review of literature

Chemical preservatives, although extensively being used, may show harmful responses such as, nitrites have shown to be carcinogenic; and sulphur di oxide may precipitate bronchitis or asthma in consumers (Davidson and Harrison, 2002; Abdulmumeen et al, 2012). The inappropriate and excessive use of antibiotics during treating patients along with their exploitation in livestock industry, to achieve desired level of meat and other products, has generated ringing alarms due to developments of antibiotic resistant microbial strains. Increasing consumer concerns about use of chemicals in food preservation, and correspondingly inclination towards the use of natural, minimally processed food items, have drawn scientific attention to natural techniques of preservation in past few decades. Thus, the biological preservation approaches have a higher attraction for food safety due to use of reduced and potential antimicrobial additives as compared to the regular chemical preservatives (Gharsallaoui et al., 2016).

Bacteriocins, the gene encoded and ribosomally synthesized peptides, are naturally produced by some species of the bacteria including, Lactic Acid Bacteria. The first bacteriocin discovered was colicin, obtained and characterized in *E. coli* (Lazdunski, 1988). The most comprehensively studied and evaluated bacteriocin producing microbes belong to the genera of *Lactobacillus*. These antibacterial peptides have been categorized into Class 1, II and III (Table 3.1) (Balciunas et al., 2013).

Lactic Acid Bacteria (LAB), have been in applications for the production of different dairy food items since thousands of years, and have received Generally Regarded as Safe (GRAS) status (Cleveland et al., 2001). They usually act against and affect the

viability of generally closely related groups of bacteria and are being used for preservation of foods such as, pickles, cheese, wine and curd (Gautam and Sharma, 2009). Isolation and characterization of bacteriocins from diverse LAB strains includes genera from groups such as *Lactobacillus, Lactococcus, Enterococcus* and *Streptococcus* (Khan, 2013). Nisin, as highlighted in chapter 1, is the only bacteriocin that has been approved for food applications along with its safety ensured by the Food and Agriculture Organization/ World Health Organization (FAO/WHO) since 1969 (Gharsallaoui et al., 2015). It is the antimicrobial product of the Lactic Acid Bacteria, *Streptococcus lactis*, and widely used in the food industry for its preservative role. This bacteriocin is quite a biological potential against the Gram positive bacteria along with their spores. The extensive studies on nisin includes three dimensional structure, genetics involved, mode of action, mechanisms of extracellular transport and the mechanisms of self- immunity (Cleveland et al., 2001).

Table 3.1: Classification of Bacteriocin (Adapted from Balciunas et al., 2013)

Classification (Molecular weight)	Number of amino acids	Features	Sub categories	Examples
Class I or Lantibiotics (<5kDa)	19-38	Lantionine or peptides containing b-lantionine	Type A (linear molecules)	Nisin, subtilin, epidermine
			Type B (globular molecule)	Mersacidin
Class II (<10 kDa)	37-48	Heterogeneous class of small thermostable peptides	Subclass II a (antilisterial-pediocine bacteriocins type)	Pediocin, enterocin, sakacin
			Subclass II b (composed of two peptides)	Plantaricin, lactacin F
			Subclass II c (other bacteriocins)	Lactococcin
Class III (>30kDa)	-	Large thermolabile peptides		Helveticin J, millericin B

Some issues with nisin have also come into limelight as it exhibits very low anti-microbial activity towards gram negative bacterial strains. Emergence of nisin resistant bacterial strains is another focus which insisted the researchers for need of biologically potential another bacteriocin (Balciunas et al., 2013).

Amongst reported Lactic Acid Bacteria, *Lactobacillus* genera generally include bacteriocin producing species such as *Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus sakei, Lactobacillus rhamnosus, Lactobacillus casei* and

Lactobacillus brevis. Among different LAB strains, *Lactobacillus plantarum (L. plantarum)* is one of the most versatile species, because of its competency to ferment wide types of carbohydrates and rendering it to inhabit different ecological niches including human gastrointestinal tract (Behera et al., 2018). Additionally, the amount of valuable substances obtained from this organism (Figure 3.1) such as organic acids, bioactive peptides, vitamins, and enzyme systems enables it to be a potential antioxidant, antimicrobial and probiotic (Sabo et al., 2014; Behera et al., 2018) and thus, justifies it's use for industrial applications. *L. plantarum* is a facultative and hetero-fermentative bacterium acid tolerant microbe and considered as a safe microbe. *L. plantarum* has been found to be multi-potential for different enzymes production, thereby enhancing functional properties and quality of fermented foods. The variety of enzymes such as α- amylase, esterase, lipase, α- glucosidase, β- glucosidase, phosphoketolase- 2, enolase, and lactate- dehydrogenase (LDH) are responsible for its multiple actions such as energy metabolism, starch degradation, production of phenolic compounds etc. In fermented foods, *L. plantarum* helps increasing the levels of vitamins such as vitamin B-12 and riboflavin (Sabo et al., 2014). This organism possesses an outstanding effect in developing aroma, flavours and textures in the fermented food stuffs along with health promoting benefits. *L. plantarum* has also been studied for its bio surfactant property, thus enabling it to be used as an anti-adhesive agent opposite to different pathogenic microbes (Zacharof and Lovitt, 2012). The exopolysacchrides produced by *L. plantarum* possess anti-tumour, cholesterol lowering, Immunomodulatory and anti-oxidant effects which enable it to be a potential for health industry. Various other compounds such as ascorbic acid and phenols help improving the bioavailability of substances such as iron (Behera et al., 2018). Another most interesting feature and of wide interest is the bacteriocin producing nature of this species. Different strains of *Lactobacillus plantarum* has been isolated from sources (Figure 3.2) such as milk and other dairy products, meat, fish, fruits and vegetables, and studied for the antimicrobial efficacy by its bacteriocin (Table 3.2).

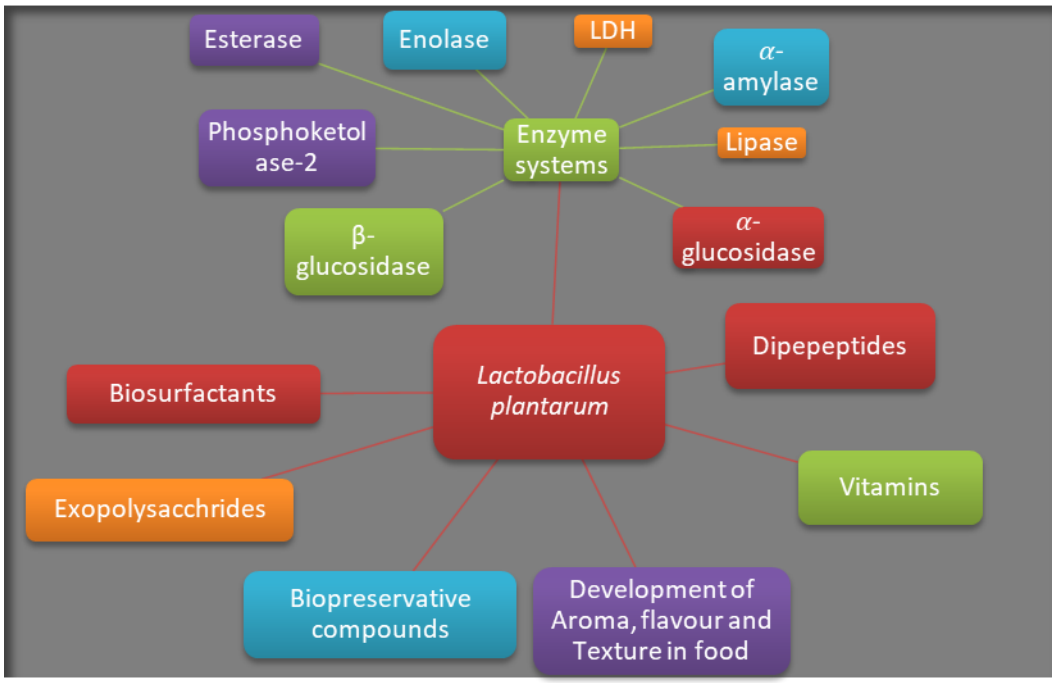


Figure 3.1: Functional applications of *L. plantarum*

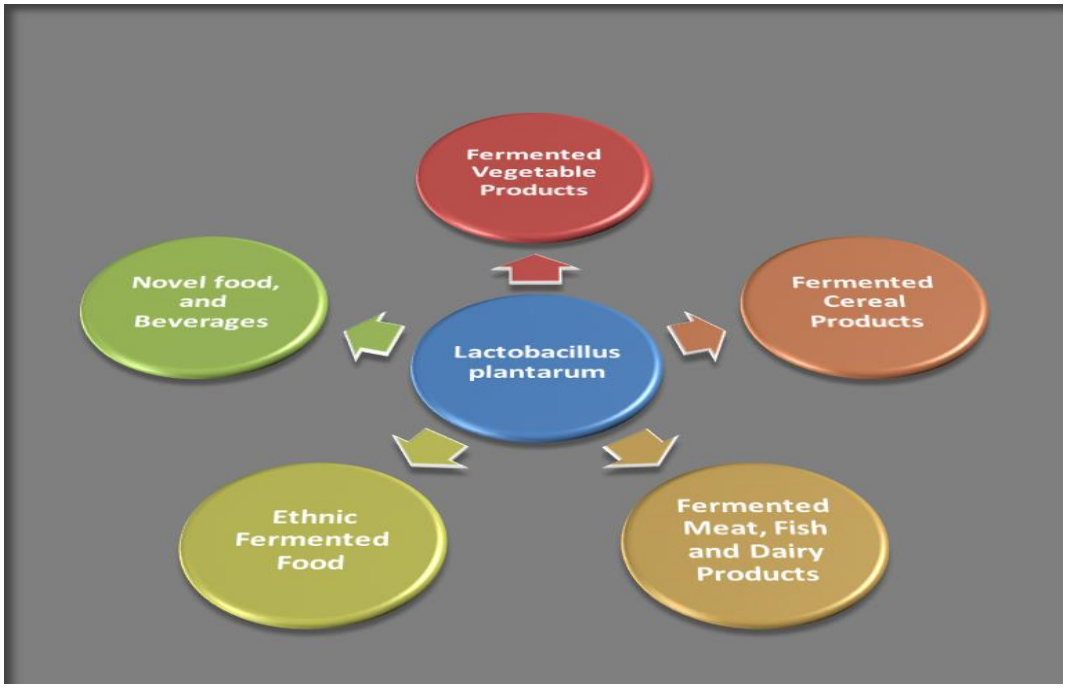


Figure 3.2: Different sources of isolation of *L. plantarum*

Table 3.2: Bacteriocins from different strains of *L. plantarum* (Adapted from Sabo et al., 2014)

Organism	Source	Test strain	Bacteriocin and molecular weight (MW)	Reference
L. plantarum C-11	Fermented cucumbers	*Staphylococcus aureus*	Plantaricin EF and Plantaricin JK	Daeschel et al., 1990
L. plantarum LPC010	Fermented green olives	Bacteriocinogenic	Plantaracin T (2.5 KDa)	Diaz et al., 1993
L. plantarum BF001	Flesh of refrigerated and processed catfish	*Lactococcus, Lactobacillus, Micrococcus, Listeria, Leuconostoc, Staphylococcus, Pediococcus, Streptococcus, Pseudomonas and Salmonella*	Plantaracin F (between 0.4-6.7 kDa)	Fricourt et al., 1994
L. plantarum LL441	Cabrales cheese	Bactericidal and cause cell lysis	Plantaracin C	Gonalez et al., 1994
L. plantarum LT154	Dry sausages	Bacteriocinogenic	Plataracin 154 (3.0 kDa)	Kanatani and Oshimura, 1994
L. plantarum NRIC 149	Pineapple	Bacteriocinogenic	Plantaricin-149	Kato et al., 1994
L. plantarum UG1	Dry sausages	*Lactobacillus, Lactocossus, B. cereus, L. monocytogenes, C. sporogenes , C. perfringens*	UG1 (between 3.0-10.0 kDa	Enan et al., 1996
L. plantarum BFE 905	Waldorf" salad	Baceriocinogenic	Plantaricin D	Franz et al., 1998
L. plantarum sp.	Vegetable origin	Bacteriocinogenic	Plantaricin NA	Olasupo, 1998
L. plantarum C19	Fermented cucumbers	Baceriocinogenic	Plantaricin C19	Atrih, et al., 2001
L. plantarum (LAB isolate)	Italian sausages	S. aureus, *L. monocytogenes, A. hydrophila*	Plantaricin 35d (4.5kDa)	Messi et al., 2001
L. plantarum	molasses	Gram-negative bacteria: *P. aeruginosa, A. baumanii and E. coli*	ST28MS (5.5kDa) ST26MS (2.8 kDa)	Todorov & Dicks, 2004a
L. plantarum ST194BZ	Boza, a fermented beverage from eastern Balkan countries	*P. aeruginosa , E. faecalis, E. cloacae, E. coli*	ST194BZ (3.3 KDa) ST194BZ (14 kDa)	Torodov & Dicks, 2005
L. plantarum PMU 33	Somfak (Thai product made of fish with low salt contents)	*B.cereus, L. monocytogenes, S. aureus, E. faecalis,*	Plwα (MW: 3.2KDa) Plwβ (3.0 kDa)	Noonpakdee et al., 2009
L. plantarum ST202Ch *L. plantarum* ST216Ch	Beloura or chorizo (a traditional Portuguese product made of pork meat)	Gram positive Gram negative	bacST202Ch (3.5 kDa) bacST216Ch (10 kDa)	Todorov et al., 2010
L. plantarum LB-B1	Koumiss, a Chinese traditional dairy product	*E. coli, Enterococcus, Listeria, Pediococcus , Lactobacillus, Streptococcus,*	Pediocin LB-B1 (between 2.5-6.5 kDa)	Xie et al., 2010
L. plantarum ST16Pa	*Carica papaya*	*Pseudomonas, Staphylococcus, Enterobacter, Enterococcus, Lactobacillus, Streptococcus, Listeria species*	ST16Pa (6.5 kDa)	Todorov et al., 2011
L. plantarum 163	Chinese fermented vegetables	*S. aureus. B.pumilus, L. monocytogenes, L. rhamnosus, B. cereus, M. luteus, L. thermophiles, P. fluorescens, E. coli, P. aeruginosa*	Plantaricin 163 (3.5 kDa)	Hu, et al., 2013

Enterococcus species are also of wide interest to scientists due to their potential in producing enterocins (Figure 3.3), a bacteriocin, which may possess wide spectrum of anti-microbial activity (M'hir et al., 2012). Certain strains of Enterococcus have been linked to pathogenicity and also indicate the fecal contamination. On the contrary, various species exhibit important role as a protector and starters in dairy and fermentation products (Fisher and Philips, 2009; M'hir et al. 2011). These organisms are Gram positive, spore non-forming, oxidase negative, catalase negative and facultative anaerobic cocci which may occur singly, in pairs or in chains (M'hir et al., 2012). Nearly 38 species have been known on the basis of phylogenetic studies which includes *E. faecalis, E. faecium, E. gallinarium, E. hirae* and *E. mundtii* (Vu and Carvarlho, 2011). Enterococci are present in good numbers in milk and milk products, meats and even in different fermented foods, such as olives and sausages (Table 3.3). However, their role in these food items has not been fully elucidated (M'hir et al., 2012). The biological potential of bacteriocins by *Enterococci*, is documented (Table 3.3), but still exhaustive studies needs to be focused so that they can be in application at industrial level.

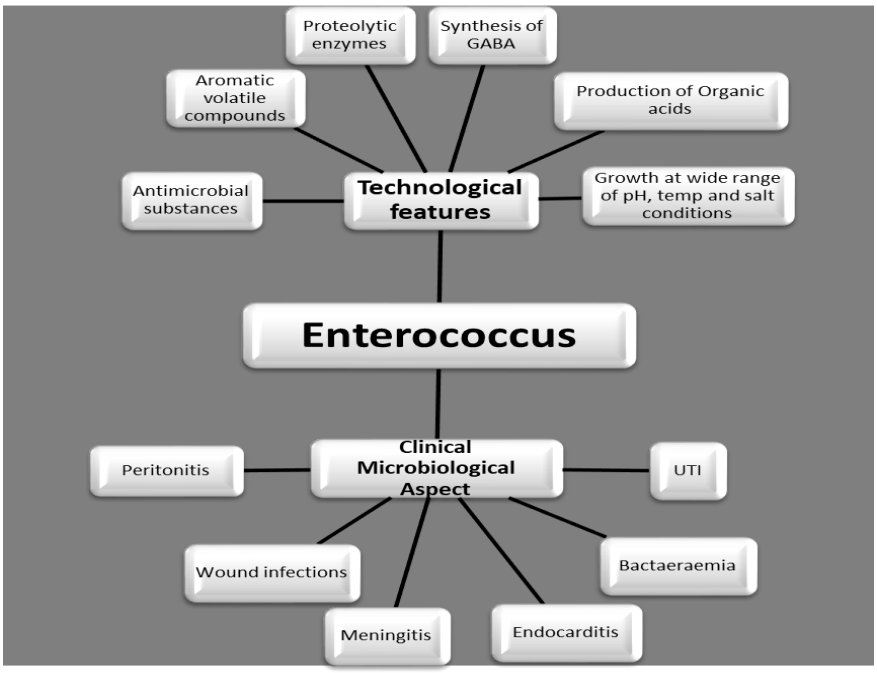


Figure 3.3: Economic importance of *Enterococcus* sp.

Table 3.3: Enterocin producing studies conducted on *Enterococcus strains* (Adapted from Fisher and Philips, 2009; M'hir et al., 2012)

Organism	Source	Test strain	Bacteriocin and molecular weight (MW)	Reference
E. faecium P21	Chorizo	*Listeria monocytogenes Clostridium butyricum*	Enterocin A and B	Fisher and Philips, 2009
E. faecalis S-47	Human wound exudate	*E. coli, Enterobacter cloacae, Kleibsella pneumoniase, Salmonella typhimurium, P. aeruginosa, P. fluorescens, Pasteurella sp., B. subtilis, B. cereus, Nocardia corrallina, Micrococcus luteus, S. aureus, S. faecium*	Enterocin Ej97	Fisher and Philips, 2009
E. faecium A2000	Cheese	Gram positive and gram negative bacteria	Enterococcin A 2000	Fisher and Philips, 2009
E. faecium CRL35	Cheese	*Listeria monocytogenes*	Enterocin CRL35 (3500 Da)	Fisher and Philips, 2009
E. faecium N15	Nuka	*Listeria monocytogenes Bacillus circulans*	Bacteriocin N15 (3000-4000 Da)	Fisher and Philips, 2009
E. faecium WHE81	Cheese	*Listeria monocytogenes*	Enterocins A and B (4833 Da and 5462 Da)	Fisher and Philips, 2009
E. faecium RZS C5*; E. faecium* DPC1146	Milk, cheese or farm (silage, faeces)	*Listeria spp. Clostridium perfringens Bacillus spp.*	Undefined bacteriocin (±3.0 kDA)	Fisher and Philips, 2009
E. faecalis Subsp. liquefaciens S-48	Porcine intestinal tract	*Bacillus coagulans, B. cereus, Bacillus weihenstephanensis, Bacillus licheniformis*	AS-48	Fisher and Philips, 2009
E. gallinarium strain 012	Ostrich duodenum	*Ent. faecalis, Lact. sakei, Lactobacillus acidophilus, Propionibacterium acidipropionici, Listeria innocua, Propionibacterium sp., Pseudomonas aeruginosa, Clostridium perfringens and Salmonella typhimurium*	Enterocin 012 (3400 Da)	Fisher and Philips, 2009
E. faecium CRL 1385	Free-range chicken	*Listeria monocytogenes*	Undefined bacteriocin	Fisher and Philips, 2009
E. faecium P13	Dry fermented sausage	*Staphylococcus aureus, Listeria monocytogenes, Clostridium botulinum and Clostridium perfringens*	Enterocin P	Fisher and Philips, 2009
E. faecalis BFE 1071	Faeces of mini pigs	*Listeria spp., Enterococcus spp., Propionibacterium spp. and Clostridium spp.*	Enterocins 1071A and 1071B (4285 and 3899 Da)	Fisher and Philips, 2009
E. mundtii AT06	Vegetables	-	Mundticin AT06 (4287 Da)	Fisher and Philips, 2009
E. mundtii NFRI 7393	Grass silage	*Listeria monocytogenes*	Mundticin KS (4290 Da)	Fisher and Philips, 2009
E. faecium BFE900	Black olives	*Listeria monocytogenes, Lactobacillus sakei Clostridium perfringens, Clostridium butyricum*	Enterocin 900	Franz et al. 1996
E. faecalis	Porcine intestinal tract	*Salmonella enterica, Escherichia coli O157:H7, Shigella spp., Enterobacter aerogenes, Yersinia enterolitica, Aeromonas hydrophila, Pseudomonas fluorescens*	Enterocin AS-48+ additional treatment	Molinos et al. 2008b
E. mundtii ST4V	Soya beans	*L. sakei*	Enterocin	Todorov et al. 2009
*E. faecium (*C1 and M2d strains)	Honey	*L. monocytogenes*	Enterocin	Ibarguren et al. 2010
E. faecalis EJ97	Municipal waste water	*Bacillus macrolides, Bacillus maroccanus*	Enterocin EJ97	Garcia et al. 2004
E. faecium, E. mundtii	Wheat	*Listeria innocua 4202*	BLIS	Corsetti et al. 2008
E. faecium L50	Dry fermented sausages	*L. brevis, Pediococcus pentosaceus*	Enterocin L50 A and B	Basanta et al. 2008

The current investigation was thus, involved isolation of a bacteriocin producing strain from the randomly collected food samples in search of a novel bacteriocin possessing wide spectrum antimicrobial potential. The standard strains, *L. plantarum* NCIM 2083 and *Enterococcus hirae* MTCC 3612 were used to compare the action of bacteriocin, potentially obtained from indigenous isolates.

3.2 Materials and Methods

3.2.1 Materials

The different growth media used in this study were obtained from Himedia, Mumbai, India. The chemicals used were also of analytical grade and were obtained from Loba Chemicals, Mumbai, India. The glassware used was of borosilicate, obtained from Borosil, India.

3.2.2 Procurement and maintenance of bacterial cultures

The lyophilized culture strains of *Enterococcus hirae* (MTCC 3612), *E. coli* (MTCC1687) and *Listeria monocytogenes* (MTCC1143) were purchased from MTCC, Chandigarh, Punjab, India. The agar slant of *Lactobacillus plantarum* (NCIM 2083) was procured from NCIM, Pune, India. *Enterococcus hirae* (MTCC 3612) and *E. coli* were revived in sterilized Nutrient broth incubated at 37^{o}C for 24 hrs. *Listeria monocytogenes (L. monocytogenes)* and *Lactobacillus plantarum (L. plantarum)* were revived in sterilized Brain Heart Infusion (BHI) broth and MRS broth respectively by incubating at 37^{o}C for 24 hrs.

Staphylococcus aureus (S. aureus; MTCC 96), *Bacillus subtilis (B. subtilis*; MTCC 121) and *Pseudomonas aeruginosa* (*P. aeruginosa;* MTCC 4673) were obtained from the glycerol stocks maintained at -20^{o}C in the laboratory of Department of Microbiology, Lovely Professional University, Punjab, India. They were revived in Nutrient Broth at 37^{o}C for 24 hrs.

The working cultures of all the bacterial strains were sub cultured from the broth by streaking on the appropriate media (Table 3.4) and incubated at 37^{o}C for 24 hours.

The plates were stored at 4±2^{o}C for short term preservation and were periodically refreshed during the complete research period at 37^{o}C monthly. For the long term preservation, 1 ml of the fresh culture broth of all strains were added separately to 1 ml 30% sterilized glycerol aseptically in the 2ml sterilized vial and stored at -20^{o}C.

Different food samples were procured from local shops from Jalandhar, Punjab, India which included pasteurized milk, raw chicken and chicken salami. The curd samples were procured from local residents of Jalandhar, Punjab, India (Table 3.5). The samples were collected in the sterilized plastic containers and transported over ice in the icebox to the laboratory and were processed immediately.

3.2.3 Methods

3.2.3.1 Isolation of Lactic acid bacteria (LAB) and preliminary characterization of reference and isolated strains of LAB

1 ml of milk was added in 9 ml double distilled sterilized water followed by preparation of serial dilutions. Spread plating was done on Bile Aesculine Azide Agar aseptically. The plates were incubated at 37°C for 24 hrs. Similarly, the curd was homogenized, raw chicken and salami were macerated and homogenized, and the three homogenized samples were serially diluted, spread plated and incubated. Small biomass of the obtained colonies was subjected to catalase test. Similarly, the colonies of standard LAB strains, *E. hirae* and *L. plantarum*, as procured from collection centers, were also subjected to catalase test (Sharpe et al., 1979; Kandler and Weiss, 1986; Manero and Blanch, 1999; Reiner K, 2010; Ahirwar et al., 2017).

The LAB isolates along with *E. hirae* and *L. plantarum* were characterized for colony characters (color, shape and texture) and microscopic features (Gram's reaction, bacterial shape and arrangement). Standard strains and isolates possessing catalase negative character were shortlisted for the further study (Claus, 1992).

3.2.3.2 Biochemical characterization of reference and isolated strains of LAB

Different biochemical test illustrating the reactions of test organisms was analyzed.

3.2.3.2.1 Qualitative estimation of presence of urease by the test organisms

Urease test: Some microbes possess the ability to produce an enzyme urease, which act over urea to produce ammonia and carbon di oxide. Urea agar base was dissolved in distilled water followed by sterilization by autoclaving at 10 lbs pressure (115°C) for 20 minutes and cooled to 45-50°C. Thereafter, 40% Urea solution was filter sterilized and added aseptically. The formed media was dispensed in sterilized test tubes and was allowed to set in slanting positions. Inoculations of the isolates were done aseptically by streaking and tubes were labeled accordingly. The tubes were incubated at 37°C up to 7 days. Examination of slants was done to observe for the development of pink color indicating the presence of urease (Sharpe et al., 1979; Kandler and Weiss, 1986; Manero and Blanch, 1999).

3.2.3.2.2 IMViC reaction by the test organisms

IMViC test: This is a series of four individual tests performed to identify the bacterial species. These are indole production, methyl red, Vogue's Prausker and Citrate utilization test. For first three tests, MRVP media was dissolved in distilled water, dispensed in test tubes, sterilized, inoculated aseptically and incubated for 24-48 h at 37°C. Sterilized petri plates of Simmons citrate agar were prepared aseptically and streaked different test strains followed by incubation at 37°C for 48 hrs. The presence of growth with change of media to blue was observed (Sharpe et al., 1979; Kandler and Weiss, 1986; Manero and Blanch, 1999; Ahirwar et al., 2017).

3.2.3.2.3 Characterization of the test strains by carbohydrate fermentation

Bacterial species possess ability to utilize different sugars as the carbon and energy source. This fermentation produces acids and hence reducing the pH of the solution indicated by the indicator. Phenol red broth base was prepared and 5ml of the solution was dispensed in different test tubes. To these tubes, 0.1% of different sugars was added (Table 3.8) and sterilized at 121°C, 15 lbs. pressure for 15 minutes except for glucose which was separately sterilized at 10 lbs. for 15min. The tubes were cooled and inoculated aseptically followed by incubation at 37°C for 24 hrs. The change of color from red to yellow indicated the production of acid and thereby indicating fermentation of the sugar by the organism (Sharpe et al., 1979; Kandler and Weiss, 1986; Manero and Blanch, 1999; Ahirwar et al., 2017).

3.2.3.2.4 Characterization of the isolates by growth on specific and differential media

Petri plates with bile aesculine azide agar, EMB agar, Tryptic soy agar, Triple sugar iron agar, Skim milk agar and Gelatin agar were prepared aseptically and streaked with different test strains followed by incubation at 37°C for 48 hrs. The presence of growth with change of media was observed (Reuter G, 1992). Similarly, test tubes with MRS broth supplemented with 6.5% sodium chloride were prepared aseptically, inoculated and incubated for 48h at 37°C. The presence of turbidity was indicative of positive growth (Facklam, 1972; Devriese et al., 1987; Facklam and Collins, 1989; Manero et al., 1999; Domig et al., 2003).

3.2.3.2.5 Growth at different temperatures: Test tubes with MRS broth were prepared aseptically, inoculated with different test strains and incubated for 48h at 25°C, 37°C and 45°C. The presence of turbidity was indicative of positive growth (Facklam, 1972; Devriese et al., 1987; Facklam and Collins, 1989; Manero et al., 1999; Domig et al., 2003).

3.2.3.3 Preliminary screening of LAB strains for production of Bacteriocin

2-3 colonies of *L. plantarum* and selected isolates were transferred aseptically into 10 ml of seed culture media (MRS broth) in glass test tubes and incubated at 37°C for 24 hrs. The cultured broth was centrifuged at 10,000 rpm for 10 minutes and the pellets were separated. The cell free supernatant (CFS) was collected and the pellets discarded. This obtained CFS was used to detect the presence of bacteriocin preliminarily by well diffusion technique against the indicator strains (*E. coli, P. aeruginosa, S. aureus, B. subtilis* and *L. monocytogenes*). Subsequently, sterilized Muller Hinton Agar was poured aseptically in sterilized 90 mm petri plates and cooled to solidify. A thick lawn of indicator pathogenic strains (15-16 hrs. old) was prepared with the sterilized swabs separately. The wells of 8 mm diameter were cut with the sterilized borer. 100 µl of the obtained CFS was poured into the wells and these plates were kept at 4°C in refrigerator to allow the diffusion of the samples in the media. The plates were kept for the incubation at 37°C the zones recorded after 24 h. Inhibition was recorded vertically and horizontally and the average of the two reported. The half-moon shaped or full moon shaped haloes and/or clear areas around the wells were considered as the zone of inhibition. The experiments were performed

in triplicates and the values are the mean of the readings found (Torodov, 2008; Torodov et al., 2009; Kaur and Arora 2009; Kalpana Rana, 2012; Yang et al., 2012).

Of the six isolates, the isolate exhibiting anti-bacterial activity against maximum number of strains was selected for the further study. Of the five test organisms, most sensitive strain was selected as the indicator strain for further analysis.

Thereafter, the CFS obtained from the most influential isolate and *L. plantarum* were checked to confirm the source of anti-microbial activity. Portions of obtained CFS were subjected to pH 6.5 and treatment with the catalase (300 IU/ml) followed by determination of the biological activity by well diffusion assay as discussed earlier (Torodov, 2008; Torodov et al, 2009; Yang et al., 2012).

3.2.3.4 **Taxonomic analysis of the selected LAB isolate**

In order identify the isolate, 16S ribosomal DNA sequencing was done. The plate with proper streaking and isolated colonies was sent at Chromous Biotech, Bangalore. The genomic DNA (~1.5kb) was isolated and the fragment was amplified using PCR polymerase. This product was sequenced bi-directionally and the obtained data was aligned and analyzed for the identification and its closest neighbor.

3.2.3.5 Optimization of production of bacteriocin for standard and isolated strain

The optimization ensures the best environmental and nutritional conditions for maximum bacteriocin production by the organism.

3.2.3.5.1 Influence of time on bacteriocin production

100 µl of the overnight broth culture of *L. plantarum* and identified test isolate was inoculated in each sterilized 10 ml of MRS broth in different test tubes and was incubated at 37°C for variable time periods (12 h, 24 h, 36 h, 48 h, 66 h and 72 h). The CFS of both the strains was obtained from the samples as discussed earlier. Subsequently, the wells (8mm) were cut on the sterilized Muller Hinton agar media plates, swabbed with selected indicator strain to prepare a thick lawn. 100 µl of the obtained CFS samples were added in each well and incubated for 24 h at 37°C. Appropriate control included the un-inoculated sterilized MRS broth tube were exposed to similar conditions and analyzed for biological potential. The inhibitory zones were recorded horizontally and vertically and the average of the two reported.

The time length illustrating maximum zone was selected for further study (Yang et al., 2012; Torodov, 2008; Torodov et al., 2009).

3.2.3.5.2 Influence of temperature on bacteriocin production

The influence of variable temperatures was studied for the bacteriocin production by both the test strains. 100 µl of the broth culture was inoculated in each sterilized 10 ml of MRS broth in different test tubes, were incubated at variable temperatures of 25°C, 30°C, 37°C, 45°C and 50°C for optimized time duration. The biological potential of the CFS as obtained from both the strains were observed against the selected indicator strain as discussed above and ZOI (mm) reported. Appropriate control included the un-inoculated sterilized MRS broth tube were exposed to similar conditions and analyzed for biological potential. The temperature illustrating maximum bacteriocin production was selected for further study (Yang et al., 2012; Torodov, 2008; Torodov et al., 2009).

3.2.3.5.3 Influence of pH on bacteriocin production

Similarly, the effect of different pH on the bacteriocin production was determined. 100 µl of the broth culture was inoculated in each sterilized 10 ml of MRS broth adjusted at 4.5, 5, 5.5, 6, 6.5 and 7 pH with 1N HCl or 1N NaOH in different test tubes followed by incubation for optimized time and temperature. An un-inoculated sterilized MRS broth tube exposed to similar conditions was taken as the control. The anti-microbial activity was subsequently determined as explained earlier. (Yang et al., 2012; Torodov, 2008; Torodov et al., 2009).

3.2.3.5.4 Influence of different sugars on bacteriocin production

Succeeding, the 10 ml MRS broth was supplemented with 1%, 2%, 3%, 4% and 5% of glucose, fructose, lactose and sucrose, and adjusted to optimized pH in the test tubes. Thereafter, the media was sterilized, cooled and inoculated with 100 µl of the broth culture aseptically. The inoculated test tubes were incubated for optimized time and temperature. Inoculated MRS broth without any supplemented sugar, adjusted to optimized pH and exposed to similar conditions was taken as the control. The anti-microbial activity was subsequently determined as explained earlier (Yang et al., 2012; Torodov, 2008; Torodov et al., 2009).

3.2.3.5.5 Influence of different nitrogen sources on bacteriocin production

Variable concentrations of two organic nitrogen sources, tryptone and beef extract (1%, 2%, 3%, 4% and 5%) and two inorganic nitrogen source, triammonium citrate and ammonium sulphate (0.1%, 0.2%, 0.3%, 0.4% and 0.5%) were supplemented in the MRS broth, adjusted to optimized pH, sterilized, cooled, inoculated aseptically, incubated for optimized time and temperature. Thereafter, the evaluation of antibacterial potential, by obtained CFS was studied by well diffusion method as discussed earlier. Inoculated MRS broth without any supplemented nitrogen source, adjusted to optimized pH and exposed to similar conditions was taken as the control. The anti-microbial activity was subsequently determined as explained earlier (Yang et al., 2012; Torodov, 2008; Torodov et al., 2009).

3.2.3.5.6 Production of bacteriocin with optimized conditions

Finally, each 20 ml MRS broth in 100 ml conical flask was supplemented with optimized sugar and nitrogen source, adjusted to optimized pH, sterilized and inoculated with 200 µl of two test strains under study, followed by their incubation at optimized time and temperature. Thereafter, the anti-bacterial effect was studied by well diffusion method as explained earlier (Yang et al., 2012; Torodov, 2008; Torodov et al., 2009).

3.2.3.6 Influence of ammonium sulphate on precipitation of bacteriocin

Each 100 ml of MRS broth in 250 ml conical flask was supplemented with optimized sugar and nitrogen source, adjusted to optimized pH, was sterilized and inoculated with 1 ml of the two test strains, followed by incubation at optimized time and temperature. Thereafter, the media was centrifuged at 10,000 rpm for 10 minutes and the pellets were separated. The obtained CFS was then subjected to variable ammonium sulphate concentrations (30%, 40%, 50 %, 60%, 70% and 80%) for the precipitation of the proteins at 20°C. The required amount of ammonium sulphate (1.76g, 2.43g, 3.14g, 3.9g, 4.71g and 5.59g respectively) was added slowly in each 10ml of CFS on the magnetic stirrer with continuous stirring for 2 h. This solution was then centrifuged at 10,000 rpm for 20 minutes, supernatant discarded, the pellets were dissolved in 0.02M potassium phosphate buffer (pH 6.5) where amount of buffer

used was 0.5% the total volume of the CFS taken. The anti-microbial activity was subsequently determined by well diffusion method, as explained earlier.

Finally, 25 ml of partially purified bacteriocin was prepared with optimized ammonium sulphate concentration and stored in a 50 ml sterilized capped glass containers at -20°C for further use. This partially purified bacteriocin obtained from *L. plantarum* NCIM2083 was renamed as plantaracin 2083 and that obtained from *Enterococcus sp. YT3* was renamed as Enterocin YT3 (Yang et al., 2012; Torodov, 2008; Torodov et al., 2009).

3.2.3.7 Characterization of partially purified Bacteriocin

3.2.3.7.1 Influence of temperature on activity of bacteriocin

Each 5 ml of cell free supernatant, as obtained with optimized conditions, was taken in test tubes and was exposed to different temperatures (37°C, 60°C, 80°C, and 100°C) for 20 minutes and cooled to room temperature thereafter. One set was exposed in the autoclave at 121°C for 20 min, cooled and filter sterilized. These treated CFS samples were collected and the residual activity was then checked against the selected indicator strain by well diffusion method as explained earlier. Untreated CFS was taken as the control (Yang et al., 2012; Torodov, 2008; Torodov et al., 2009).

3.2.3.7.2 Influence of pH on activity of bacteriocin

Each 5ml CFS was taken in test tubes and adjusted to different pH (2, 4, 5, 9 and 10) with 1N HCl or 1N NaOH, followed by incubation at 37°C for 2 hr. Thereafter, it was filter sterilized and residual bacteriocin potential was studied against the selected indicator strain as discussed earlier and compared to untreated CFS, which was taken as the control (Yang et al., 2012; Torodov, 2008; Torodov et al., 2009).

3.2.3.7.3 Influence of enzymes on activity of partially purified bacteriocin

Each 300 µl of plantaracin 2083 and Enterocin YT3 were subjected to trypsin, pepsin, proteinase K, a-amylase and lysozyme enzyme solutions at the final concentration of 1mg/ml and incubated at 37°C for 2 hr. Thereafter, it was filter sterilized and residual bacteriocin potential was studied by well diffusion method and compared to the initial sample without any enzymatic treatment, which was considered as the control (Yang et al., 2012; Torodov, 2008; Torodov et al., 2009).

3.2.3.8 Statistical Evaluation

All the experiments have been performed in triplicates.The data obtained after one factor analysis of optimization (time duration, temperature, pH, sugar and nitrogen source supplementation and ammonium sulphate precipitation) have been expressed as mean± standard deviation (SD). Thereafter, the obtained data was further statistically determined and subsequently analysed using one way ANOVA at 5% level of significance.

3.3 Results and Discussion

3.3.1 Procurement and maintenance of bacterial cultures

The standard strains of different procured bacteria were cultured on different nutrient media at 37 C for 24 h, as already explained and stored appropriately (Table 3.4).

3.3.2 Bacterial isolation and preliminary characterization

Nearly 680 colony growths were observed on the BAAA plates from the procured food samples. Of these only six isolates (I1, I2, I3, I4, I5 and I6) were found to be catalase negative. Isolate 1 and 2 were isolated from pasteurised milk, isolate 3 from raw chicken, isolate 4 and 5 from salami and isolate 6 obtained from the curd (Table 3.6). The colonies of all isolates were gram positive, typically pin pointed, small, convex and greyish coloured on MRS agar. Where isolate1, 4, 5 and 6 was were cocci, occurring in pairs, tetrads or short chains, the isolate 2 and 3 were determined rod shaped bacteria, in short chains (Table 3.7; Figure 3.7).

The standard strain *E. hirae* (MTCC3612) was procured for analysing its bacteriocin property. The colonies of *E. hirae* were very small, pin pointed, greyish on Brain Heart Infusion agar (Table 3.7, Photograph 3.1) and catalase positive. The cells of *E. hirae* (MTCC 3612) were found to be gram positive cocci, present in chains. Similar characters were studied for other strains of *Enterococcus* as suggested in the literature (Facklam, 1972; Devriese et al., 1987; Facklam and Collins, 1989; Manero et al., 1999; Domig et al., 2003).

The colonies of other standard strain, *L. plantarum*, were wide, white, raised, smooth and mucoid. On subjecting to catalase test, the biomass was determined to be catalase negative (Table 3.7; Photograph 3.2). The cells were found to be gram positive, rod

shaped bacilli. Similar characters were studied for other strains of *L. plantarum* (Qian et al., 2018; Wheater, 1955; Lim et al. 2014).

3.3.3 Biochemical characterization of reference and isolated strains of LAB

On biochemical analysis, *E. hirae* was found to be negative for urease, vogues prausker test, tryptophan test, growth on tryptone soy agar, citrate utilisation and triple sugar iron test. It showed methyl red positive and exhibited growth in glucose, lactose, sucrose, galactose, mannose, xylose, MRS broth with 6.5% NaCl, EMB agar, Bile aesculine azide agar (Photograph 3.3) and determined growth at all tested temperatures (Table 3.8). Similar results determined the organism to be an *Enterococcus* as suggested in the literature (Facklam, 1972; Devriese et al., 1987; Facklam and Collins, 1989; Manero et al., 1999; Domig et al., 2003).

Similarly, *L. plantarum* was found to negative for urease, vogues prausker test, tryptophan test, growth on tryptone soy agar and triple sugar iron test. The strain was positive for methyl red test, was able to grow in MRS with 6.5% NaCl, grew at all tested temperatures, had the ability to tolerate bile, and aesculine and was able to ferment tested sugars such as glucose, lactose, sucrose, arabinose, mannitol, raffinose, pyruvate, sorbitol, galactose, ribose, salicin, mannose and xylose (Table 3.8; Photograph 3.5). These results also determined the organism to be *L. plantarum* (Qian et al., 2018; Wheater, 1955; Lim et al. 2014).

On subjecting the isolates to biochemical analysis, none of them could produce ammonia in urease test and resulted negative for Tryptophan and Vogues Prausker test. All the strains were able to utilize different sugars for fermentation. Only isolate 1 was determined to utilize citrate as the source of carbon as indicated by citrate utilization test. No isolate determined the pigmentation over Tryptic soy agar along with no growth and hydrogen sulphide observed in triple sugar iron test. Isolate 2 and 3 produced caseinase as determined by the presence of zones around the colonies on skim milk agar. Only isolate 1 and 2 were able to hydrolyze gelatin. All the isolates presented their colonies on bile aesculine azide agar (Photograph 3.3) depicting their ability to use aesculine and grow in alkaline pH. No colonies of isolate 2 and 3 were found on EMB agar. All isolates showed growth at 25° C and 37° C but only isolate 6

was able to grow at 45° C. Isolate 4, 5 and 6 determined their growth in MRS with 6.5% NaCl. All the strains were able to ferment tested sugars except that isolate 1 and 2 were not able to ferment lactose (Table 3.8; Photograph 3.6).

3.3.4 Preliminary screening of strains for Bacteriocin production

Following the identification, the preliminary analysis to detect the production of bacteriocin production was performed to determine the anti-microbial potential of different test strains against all indicator microbes (*E. coli* and *P. aeruginosa, S. aureus, B. subtilis* and *L. monocytogenes)*

Since *E. hirae* was found to be catalase positive, it was not pursued for the bacteriocin production. Some microbes produce catalase for their defense mechanism, to repair the damage caused by oxidation from hydrogen peroxide produced during metabolism in the cell. Its concentration has been related with the pathogenicity of the organism (Reiner, 2010).

The investigations on CFS obtained from *L. plantarum* exhibited zone of inhibition against *E. coli, P. aeruginosa, S. aureus and B. subtilis* except for *L. monocytogenes* (Table 3.9). *B. subtilis* was found to be the most sensitive strain.

Similarly, no zones of inhibition were exhibited by CFS from isolate 1, 2, 3, and 4 against all the test strains. While, isolate 5 was selectively effective against *B. subtilis* only, the CFS of isolate 6 exhibited zones against all test strains (Table 3.9). Hence, only isolate 6, sourced from home-made curd, was selected for further studies.

Since *B. subtilis* was revealed to be the most sensitive, hence it was selected as the indicator strain for further analysis.

Investigations on the CFS adjusted to pH 6.5, sourced from *L. plantarum* and isolate 6 against *B. subtilis,* revealed no loss of biological activity (Table 3.10). The lactic acid bacteria such as *L. plantarum* and *Enterococcus* strains are highly applied in different fermentation processes and are potent for the production of organic acids such as lactic acid, phenyl lactic acid, acetic acid and propionic acid along with diacetyl. These acids are the causative factors of reducing the pH of the environment thereby

causing inhibition of growth of the microbes which may be causative factor for the biological potential of CFS (Fisher and Philips, 2009; Behera et al., 2018).

Further, on treatment of CFS with catalase, sourced from both the test strains, revealed no loss of anti-bacterial activity. This was suggestive of non- production of potential hydrogen peroxide by the organism. Some microbes are a potential producers of hydrogen peroxide during metabolism and subsequently produce catalase for their defense mechanism to repair the damage caused by oxidation from hydrogen peroxide. This may be the cause of biological potential of CFS, besides production of acids. The concentration of hydrogen peroxide has also been related with the pathogenicity of the organism (Reiner, 2010; Yang et al., 2012).

Therefore, it was preliminarily verified that the biological activity of *L. plantarum* and test isolate 6 was due to some bacteriocin in the cell free supernatant and not acids or hydrogen peroxide produced by the two test organisms.

3.3.5 Taxonomic analysis of the isolated strain

On subjecting the isolated strain 6 to 16S ribosomal DNA sequencing, it was revealed as an *Enterococcus* species and was designated as *Enterococcus sp. YT3* for the future reference. The closest homologue to this identified strain were *Enterococcus gallinarum strain 46W* and *Enterococcus casseliflavus strain SBMAX24* and has been presented in the phylogenetic tree (Figure 3.4).

3.3.6 Optimisation for bacteriocin production by standard and isolated strain

The purpose of optimisation is to ensure the optimum conditions for maximum bacteriocin production (Abbasiliasi et al., 2017). Hence, the influence of different parameters was selected to ensure the higher yield of bacteriocin.

3.3.6.1 Influence of time on bacteriocin production

The ZOI (mm) from CFS, as obtained from *L. plantarum* after 12 h of incubation, was determined to be 17±0.8, after 24 h it was 17.3±0.5, after 36 h it was 21.7±0.5, after 48 h it was 26.7±0.9, after 66 h it was 24.3±0.5 and after 72h it was found to be 23±0.8. Maximum bacteriocin production was determined at 48 h, where the zone was 9.7 mm larger than the smallest zone observed (Table 3.11; Photograph 3.7).

Similarly, the ZOI (mm) with CFS, as obtained from *Enterococcus sp. YT3,* after 12 h of incubation was found to be 20.7±0.9, after 24 h it was 22.7±0.9, after 36 h it was 25.3±0.4, after 48 h it was 27±0, after 66 h it was 25±0, and after 72h it was found to be 23±0.5. The maximum antimicrobial activity was exhibited after 48 h of incubation, beyond which there was a reduction of 9.8% of the activity (Table 3.11; Photograph 3.8; Figure 3.5).

This gradual loss of bacteriocin activity may be due to adsorption of the antibacterial compounds on the surface of the producer cells or proteolytic degradation by the extracellular proteases (Zamfir et al., 2000; Ogunbanwo et al., 2003). Studies with *Lactobacillus lactis* and *Lactobacillus fermentum* KNO2 depict that maximum bacteriocin production was determined after 48 h of incubation (Rajaram et al., 2010; Nithya, 2012).

3.3.6.2 Influence of temperature on bacteriocin production

After optimising the time duration of 48 h, the ZOI (in mm) against *B. subtilis* determined by the CFS obtained from *L. plantarum* by incubation at 25°C was found to be 12±2.4, at 30°C it was 21±0.5, at 37°C it was 25.7±1.7, at 45°C it was 27.6±0.5, at and at 50°C it was found to be 24±0.9. The optimum temperature for the bacteriocin production was found to be 45°C (Table 3.12; Photograph 3.7; Figure 3.6).

Thereafter, the ZOI (mm) against *B. subtilis* CFS obtained from *Enterococcus sp. YT3* incubated for 48 h at 25°C was found to be 20.7±0.9, at 30°C it was 22.7±0.9, at 37°C it was 27±0.8, at 45°C it was found to be 25.3±0.4 and at 50°C it was found to be 23±0.5. Highest zones were found at 37°C which was 6.3 mm larger than the smallest zone observed at 25°C and this suggests the most optimum temperature for bacteriocin production for *Enterococcus sp. YT3* is 37°C (Table 3.12; Photograph 3.8; Figure 3.6).

In various studies held, different LAB strains produced bacteriocin at variable temperatures. Some microbial strains have been found to be producing bacteriocin at 25°C to 37°C such as *Lactobacillus plantarum* UG1 and *Lactobacillus plantarum* ST8KF, *Lactobacillus Plantarum* Jw3bz And Jw6bz, And *Lactobacillus Fermentum* Jw11bz and Jw15bz (Enan et al., 1996; Powell et al. 2007; Todorov and Mollendorff,

2009; Aguilar et al., 2011; Yusuf and Hamid, 2012) Studies with *L. plantarum* NCIM2084 and *Enterococcus faecalis* K-4 exhibited maximum bacteriocin at 40°C and 43°C respectively (Suma et al. 1998; Eguchi et al., 2001).

3.3.6.3 Influence of pH on bacteriocin production

Succeeding, *L. plantarum* exhibited growth at all tested pH when the inoculated media was incubated for 48h at 45°C (Table 3.13). The ZOI (in mm) from the CFS obtained when initial media was adjusted to pH 4.5 was found to be 21±0.8, at pH 5 ZOI (in mm) was 23.3±0.5, at pH 5.5 it was 26±1.6, at pH 6 it was 28.7±1.2, at 6.5 it was 26.7±0.5 and at pH 7 was 23.3±1.2. Maximum bacteriocin production has been recorded with the media adjusted to initial pH 6 which was 5.1 mm larger than the smallest zone observed (Table 3.13, Photograph 3.7; Figure 3.7).

Similarly, *Enterococcus sp. YT3* also exhibited growth at all tested pH, when incubated for 48 h at 37°C. At pH 4.5 the ZOI (in mm) was found to be 24.6±0.9, at pH 5 ZOI was 25.6±0.9, at pH 5.5 it was 26.3±0.5, at pH 6 it was 28 ±1.6, at 6.5 it was 26.7±0.9 and at pH 7 ZOI was analysed to be 23.3±0.5. The most influential pH as analysed by the results was 6, where the difference between largest and smallest zone was 4.7 mm (Figure 3.13, Photograph 3.8; Figure 3.7).

The growth and metabolism of LAB strains along with the ability to produce bacteriocin is highly pH dependant. It affects the cellular aggregation, bacteriocin absorption and its proteolytic degradation. Bacteriocins are produced at specific range of pH which else controls the enzymatic reactions of the cells. Sometimes, at low pH, which may be due to production of organic acids, the growth of cells stops, thereby affecting the bacteriocin production. During the process of fermentation, this acidified media, may also affect the solubility of different proteins and polypeptide solubility. pH may even regulate the expression of different biosynthetic genes, where slower growth of bacterial cells enhance the bacteriocin production (Abbasiliasi et al, 2017).

Similar results can be witnessed in the literarture where *Lactobacillus acidophilus* ATCC 4356, *Enterococcus faecium* CWBI-B1430, *Enterococcus mundtii* CWBI-B1431, *Enterococcus faecium* B3L3 and *Enterococcus faecium* SD2 has produced maximum bacteriocin at pH 6 (Aguilar et al., 2011; Yusuf and Hamid., 2012; Schirru et al., 2014; Mohamed et al., 2016).

3.3.6.4 Influence of different sugars on bacteriocin production

Proceeding, different sugars were supplemented in the initial media, adjusted with pH 6, sterilized, inoculated by *L. plantarum* and were incubated for 48 h at 45°C (Table 3.14; Photograph 3.7). The ZOI (in mm) with CFS from MRS broth with 1%- 5% of glucose supplementation was 16.3±0.5, 25±0.8, 22±0.8, 21.3±0.5 and 13±2.2 (Figure 3.8). The ZOI (mm) with 1%-5% of lactose supplementation was 27.3±0.5, 24±1.4, 22.7±0.9, 21.6±2.1 and 21.7±1.2. The ZOI (in mm) with CFS from MRS broth with 1%-5% of fructose supplementation was 20.7±0.9, 22.3±1.9, 18.7±3.7, 16.7±0.9 and 16±0. The ZOI (in mm) with CFS from MRS broth with 1%-5% of sucrose supplementation was 20.7±0.9, 22.3±1.8, 21.3±0.5, 18.7±3.7 and 16.7±0.9 (Photograph 3.7; Figure 3.8). Amongst the various carbon source supplemented, the bacteriocin production has been found to be maximum with 1% lactose as compared to glucose, fructose or sucrose. The difference between the highest and the lowest zones was 11 mm.

Similarly, the influence of different sugars supplemented in the initial media adjusted to pH 6 and inoculated with *Enterococcus sp. YT3* was incubated for 48 h at 37°C was analysed on bacteriocin production (Table 3.14, Photograph 3.8; Graph 3.5). The ZOI (in mm) with CFS from MRS broth with 1%-5% of glucose supplementation was 21±0.8, 24.7±0.9, 23.3±1.9, 22.7±0.9 and 22.3±1.9. The ZOI (in mm) with CFS from MRS broth with 1%-5% of lactose supplementation was 25.3±0.5, 25±0.8, 24.7±0.9, 24.3±0.5 and 22.6±1.7. The ZOI (in mm) with CFS from MRS broth with 1%-5% of fructose supplementation was 24.3±1.7, 29±0.8, 25.7±0.5, 25±1.6 and 24±1.4. The ZOI (in mm) with CFS from MRS broth with 1%-5% of sucrose supplementation was 23.3±0.5, 24.6±0.5, 24.3±1.2, 23.3±0.5 and 22.7±0.9. The most influential carbon source was found to be 2% fructose when compared to other sugar (glucose, lactose and sucrose). The difference of 8 mm could be observed between the highest and lowest zones.

During the process of fermentation, carbon plays a significant role for growth and product formation. Mostly, the microbes possess the ability to ferment mono and di-saccharides as compared to complex sugars. The nature of carbon in the media influences the microbe metabolism. Higher yield of bacteriocins by some LAB strains

with different sugars in the media may be attributed because of complex enzymatic system which is able to use various carbohydrates. Additionally, whey, an important by product from the dairy industries, is a rich source of lactose, soluble proteins and some mineral salts and has been considered as waste. The bacteriocin producing microbes may be studied with such by products for the required product (bacteriocin) yield (Abbasiliasi et al, 2017).

Study with *Lactobacillus sp. MSU3IR* exhibited maximum bacteriocin with lactose in the media. Some LAB strains such as *Leuconostoc mesenteroides* E131 and *Lactococcus lactis subsp. lactis* HV219 yielded higher bacteriocin when media was supplemented with fructose (Drosinos et al., 2005; Torodov et al., 2006; Iyyaparaj et al., 2013).

3.3.6.5 Influence of different nitrogen source on bacteriocin production

The influence of different organic and inorganic nitrogen sources were determined by their supplementation in the initial media (MRS broth) adjusted to pH 6, inoculated by *L. plantarum*, incubated for 48 h at 45°C (Table 3.15, Photograph 3.7; Figure 3.10; Figure 3.12). The ZOI (in mm) by the obtained CFS from MRS broth with 1%-5% of tryptone supplementation was 19.6±0.5, 20±1.4, 20.7±0.9, 22.3±0.5 and 22.7±0.5 (Figure 3.10). With 1%- 5% beef extract supplementation, the ZOI (in mm) was 17.6±1.2, 20±1.4, 20±1.6, 22±1.4 and 21.7±0.9, with 0.1%- 0.5% triammonium citrate supplementation, the ZOI (in mm) was 20.3±2.1, 22.3±0.5, 25.3±1.2, 27.3±1.9 and 24.3±0.5 respectively. With 1%- 5% ammonium sulphate supplementation, the ZOI (in mm) was 13.6±1.7, 14±1.6, 14.3±0.5, 16.3±1.2 and 15±0.8. The initial media supplemented with 0.4% triammonium citrate as the nitrogen source exhibited maximum bacteriocin production as compared to tryptone, beef extract and other ammonium salts.

Similarly, the influence of different nitrogen sources on the bacteriocin production by *Enterococcus sp.* YT3 was studied (Table 3.15; Photograph 3.8; Figure 3.11, 3.13). The ZOI (in mm) with CFS from MRS broth with 1%-5% of tryptone supplementation was 23.3±0.5, 25.7±0.9, 26±0.8, 28.6±1.2 and 27.3±0.4. With 1%-5% beef extract supplementation, the ZOI (in mm) were 24.3±1.7, 26.3±0.5, 25.7±0.5, 23.6±1.2 and 23.6±1.7. With 1%-5% triammonium citrate supplementation

was 17.3±1.2, 20.3±1.2, 22.0±0.8, 24.6±0.9 and 25.7±1.2. With 1%-5% ammonium sulphate supplementation was 14.6±1.2, 18.7±0.4, 21.3±1.2, 24.7±0.9 and 25.3±0.4. The initial media supplemented with 4% tryptone as the nitrogen source exhibited maximum bacteriocin production.

Along with carbon, nitrogen contributes significantly towards biological growth such as cellular protein and nucleic acid formation, and product formation from the microbes. In the process of fermentation, appropriate nitrogen source in the media is required for proper metabolism of the micro-flora, thereby enabling better yield of the targeted product. In some microbes, the cellular growth is enhanced with higher production of bacteriocin when supplemented with organic nitrogen sources such as tryptone, beef or yeast extract which may be due to the presence of essential sources such as amino acids, vitamins and minerals. Interestingly, the inorganic sources have determined conflicting results where both increase as well as decrease of bacterial growth and/ or bacteriocin yield has been witnessed. The enhanced production of bacteriocin with increase in nitrogen amount may be due to elevation in the peptide and.or growth factors in the nitrogen sources. Addition of nitrogen source to slight excess as compared to consumption has resulted in lesser biomass and bacteriocin production, also constituting additional cost. Instead use of low cost proteins may help towards bringing down the large scale cost of production with some better product yield (Iyyaparaj et al., 2013, Abbasiliasi et al, 2017; Lim et al., 2019).

The result in this study for higher bacteriocin production by *L. plantarum* is similar to that studied for *Lactobacillus fermentum* in which the ammonium citrate stimulated the higher production of the bacteriocin bacJW15BZ (Torodov et al., 2009).

The higher yield of bacteriocin from *Enterococcus sp.* YT3 with tryptone are in coherence with other LAB strains such as *Lactococcus lactis* and *Lactobacillus plantarum AMA-K* (Torodov and Dicks, 2004; Torodov and Dicks, 2005; Torodov, 2008).

3.3.6.6 Production of bacteriocin with optimized conditions

Optimisation for fermentation to achieve maximum yield is definitely a time consuming and laborious process involving high expenditure and experiment trials. But it ultimately helps in developing conditions essential for economical, viable and cost-effective large scale production of bacteriocins. It is a complex approach but essential critically for large scale production of high performing bacteriocins. In estimation, in commercial fermentations, culture media could account for nearly 30% of production cost (Abbasiliasi et al, 2017).

In this study, the production of bacteriocin by sourcing CFS from *L. plantarum* with optimized conditions revealed the ZOI (mm) to be 29±1.6 (Table 3.16). Similarly, the production of bacteriocin from *Enterococcus sp. YT3* with optimized conditions by analysing obtained CFS revealed the ZOI (mm) to be 29.7 ±0.5 (Table 3.16).

3.3.7 Influence of ammonium sulphate on bacteriocin precipitation

On precipitating the CFS as obtained from *L. plantarum* with ammonium sulphate determined variable inhibition zones against *B. subtilis* (Table 3.17, Photograph 3.7; Figure 3.14). At 30% -80% precipitations, the ZOI (in mm) were revealed to be 17.7±0.9, 16.7±0.9, 16±0, 16.7±0.9, 18.3±0.5 and 19.33±0.5.

Similarly, for *Enterococcus sp. YT3*, 30% -80% precipitation revealed the ZOI (in mm) to be 19±0, 18.7±0.9, 17±1.4, 17.3±0.5, 19.7±0.9 and 20.3±0.5 (Table 3.17; Photograph 3.8; Figure 3.14). Both the test organisms revealed maximum bacteriocin precipitation with 80% ammonium sulphate.

Ammonium sulphate precipitation as the first step for the purification is suggested of certain advantages such salt stability of proteins to avert denaturing, cost effectiveness, to limit the proteolysis and to reduce the sample application time for successive chromatographic steps. So as to concentrate the activity from cell free supernatant, ammonium sulphate precipitation is done and maximum activity observed (Simha et al., 2012).

Studies confirmed the maximum potential of partially purified bacteriocin by 80% ammonium sulphate precipitation from organisms such as *Lactobacillus lactis, Lactococcus lactis, Lactobacillus plantarum* ZJ5, *Lactobacillus sp.* MSU31R, *Enterococcus faecium* 3D, *Enterococcus faecium* LM-2, *Enterococcus faecalis* N1-33

and *Pediococcus pentosaceous* NCDC273 (Ivanova et al., 2000; Hata et al., 2009; Rajaram et al., 2010; Bayoub et al, 2011; Liu et al., 2011; Iyapparaj et al., 2013; Song et al., 2014).

This semi purified bacteriocin from *L. plantarum* NCIM2083 and *Enterococcus sp.* YT3 strain were subsequently named as Plantaracin 2083 (P-2083) and Enterocin YT3 (E-YT3) respectively.

3.3.8 Characterization of partially purified Bacteriocin

3.3.8.1 Effect of temperature

Subjecting the bacteriocin from *L. plantarum* and *Enterococcus sp. YT3* to different temperatures along with autoclaving, revealed the retention of biological potential (Table 3.18, Photograph 3.9-3.10). This was suggestive of thermal stability of the anti-bacterial compound by both the test strains at all tested temperatures.

Although some thermo-labile bacteriocin producers (*Enterococcus faecium* CK21 and *Lactobacillus fermentum* PP17) has been reported in the literature (Reda et al, 2018), but still studies have confirmed the thermo stable bacteriocins from different LAB strains such as *Lactobacillus plantarum* ATCC8014, *Lactobacillus acidophilus* GP1B, *Lactobacillus acidophilus* DSM 20079, *Lactobacillus plantarum*, *Lactobacillus plantarum* LP6SH, *Lactobacillus plantarum* C8, *Enterococcus lactis* 4CP3, *Enterococcus faecium* S6 and S9, *Enterococcus faecium* LR/6 and *Enterococcus hirae* IM1, *Enterococcus faecium* AQ71. (Han et al., 2007; Kumar et al., 2010; Deraz et al., 2011; Sankar et al., 2012; Marie et al., 2012; Ahmadova et al., 2013; Fang et al., 2014; Mohamed et al., 2016; Braiek et al., 2018; Rahmeh et al., 2018). This potential of the bacteriocin like substance may have an advantage potential as a bio preservative in addition to other processing such as thermal treatment for the preservation of food items (Yang et al., 2012).

3.3.8.2 Effect of pH

When subjected to variable pH (2-10), no complete loss of activity was found with bacteriocin from *L. plantarum* and *Enterococcus sp.* YT3 (Table 3.19, Photograph 3.9-3.10), suggestive of stability of antimicrobial component in acidic and alkaline conditions although lesser activity was observed in pH 2 and 10 treated samples.

Stability of bacteriocins towards broader range of environmental acidic or alkaline conditions suggests that these antimicrobial compounds may be a better option when compared to the most commonly used bacteriocin, Nisin, as a bio preservative which is inactive at the alkaline pH (Ivanova et al., 2000). Hence, such natural substances may have applications in large scale of food products including meat and meat products as preservatives.

These findings are in coherence with other studies where bacteriocins have presented similar character (Ahmad et al., 2004; Kumar et al., 2010; Braiek et al., 2018; Rahmeh et al., 2018; Reda et al., 2018)

3.3.8.3 Effect of enzymes

No zones were observed with Plantaracin 2083 when treated with trypsin and pepsin. Some haloes were observed with Plantaracin 2083 already treated with proteinase K. Clear zones were observed with Plantaracin 2083 treated with lysozyme, a-amylase and untreated samples (Table 3.20, Photograph 3.9-3.10). No ZOI were observed with Enterocin YT3 when treated with trypsin, pepsin and proteinase K. Clear zones were observed with this semi purified bacteriocin on treatment with lysozyme, α-amylase and in untreated samples.

Bacteriocins are susceptible to be broken down by the various proteolytic enzymes causing their loss of antimicrobial potential. Upon treatment with various enzymes, complete loss of activity was observed with trypsin and pepsin along with reduction of activity (with treated Lactacin 2083 from *L. plantarum*) or loss of activity (with treated Enterocin YT3) with proteinase K. Zones were found with untreated sample, and the samples treated with α-amylase and lysozyme. These observations also support the protein nature of the antimicrobial compound and not the carbohydrate moiety. Many studies have reported that bacteriocin activity is reduced or lost upon the treatment with trypsin, pepsin and proteinase-K (*Klanhammer,* 1988; Jack et al., 1995; Ahmad et al., 2004; Kumar et al., 2010; Yusuf and Hamid, 2012; Reda et al., 2018).

The results determined in this study depict the antimicrobial potential of Plantaracin 2083 and Enterocin YT3. Hence, they must be evaluated for their efficiency towards other microbes as well for their large scale application in food and health industry.

On analyzing the obtained data by one way ANOVA for different factors of time duration, temperature, pH, sugar source supplementation, nitrogen source supplementation and ammonium sulphate precipitation for *L. plantarum*, the P- value was found to be 3.77E-08, 1.57E-06, 0.0001, 9.15E-12, 1.17E-15 and 0.005, respectively. Similarly, for *Enterococcus sp.* YT3, the P- value was found to be 3.8E-06, 5.2E-05, 0.011, 2.69E-05, 9.77E-16 and 0.029, respectively. Hence, the aforesaid factors showed significant difference on the zone of inhibition as the P-value has been determined less than 0.05, and thus, it illustrates their significant effect on bacteriocin production/ precipitation (Table3.11- 3.17).

3.4 Statistical Analysis

The software, Excel 2010 in Microsoft Office programme has been used to calculate the mean along with standard deviation. Thereafter, same software was applied to determine statistical significance using one way ANOVA at 5% level of significance.

3.5 References

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Tables:

Table 3.4: Bacterial strains used in the current study

Bacterial strain	Source	Incubation Temperature	Growth Media	Application
Enterococcus hirae (MTCC 3612)	MTCC, Chandigarh	37^{o}C	BHI Broth/ Agar	Bacteriocin production
Lactobacillus plantarum (NCIM 2083)	NCIM, Pune	37^{o}C	MRS Broth/ Agar	Bacteriocin production
E. coli (MTCC1687)	MTCC, Chandigarh	37^{o}C	Nutrient Broth/ MacConkey agar	Indicator strain
Listeria monocytogenes (MTCC1143)	MTCC, Chandigarh	37^{o}C	BHI Broth/ Agar	Indicator strain
S. aureus (MTCC96)	ML, LPU*	37^{o}C	Nutrient Broth/ Mannitol Salt Agar	Indicator strain
B. subtilis (MTCC 121)	ML, LPU*	37^{o}C	Nutrient Broth/ Nutrient Agar	Indicator strain
P. aeruginosa (MTCC4673)	ML, LPU*	37^{o}C	Nutrient Broth/ Cetrimide Agar	Indicator strain
BHI: Brain heart Infusion media MTCC: Microbial Type Culture Collection, Chandigarh, Punjab, India ML, LPU*: Microbiology Laboratory, Department of Microbiology, Lovely Professional University, India				

Table 3.5: Collection of samples for bacteriocin producers

S. No	Types of Sample	Place of purchase	Number of samples
1	Pasteurized milk	Local shop, Jalandhar, India	5
2	Homemade Curd	Residence, Jalandhar, India	2
3	Raw Chicken	Easy day store, Jalandhar, India	5
4	Salami	Local shop, Jalandhar, India	5

Table 3.6: Source of isolation of isolates

Bacterial strain	Source	Incubation Temperature	Growth Media	Application
Isolate 1 (I1)	Milk	37^{o}C	MRS Broth/ Agar	Bacteriocin production
Isolate 2 (I2)	Milk	37^{o}C	MRS Broth/ Agar	Bacteriocin production
Isolate 3 (I3)	Raw chicken	37^{o}C	MRS Broth/ Agar	Bacteriocin production
Isolate 4 (I4)	Salami	37^{o}C	MRS Broth/ Agar	Bacteriocin production
Isolate 5 (I5)	Salami	37^{o}C	MRS Broth/ Agar	Bacteriocin production
Isolate 6 (I6)	Curd	37^{o}C	MRS Broth/ Agar	Bacteriocin production
I1-I6: Designation of selected LAB isolates from different food samples				

Table 3.7: Preliminary characterization of reference and isolated strains of LAB

Character		***E. hirae***	***L. plantarum***	**I 1**	**I 2**	**I 3**	**I 4**	**I 5**	**I 6**
Catalase test		+	—	—	—	—	—	—	—
Colony	Color	Greyish	White	Cream	Greyish	Greyish	Greyish	Greyish	Greyish
	Shape	Pin pointed	Wide, raised, smooth	Pin pointed, convex	Pin pointed, convex	Pin pointed, convex	Pin pointed, convex	Pin pointed, convex	Pin pointed, convex
	Texture	Smooth	Mucoid	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Microscopy	Gram staining	+	+	+	+	+	+	+	+
	Shape of microbe	Coccus	Bacillus	Coccus	Bacillus	Bacillus	Coccus	Coccus	Coccus
	Arrangement	Short chains	Pairs or in chains	Diplococcus or tetrads	Short chains	Short chains	Singly or in pairs	Diplococcus	Short chains or in pairs

+ve: Positive; -ve: Negative
I1-I6: Designation of selected LAB isolates from different food samples

Table 3.8: Biochemical characterization of reference and isolated strains of LAB

Test performed		***E. hirae***	***L. plantarum***	**I 1**	**I 2**	**I 3**	**I 4**	**I 5**	**I 6**
Urease test		—	—	—	—	—	—	—	—
IMViC	Methyl red test	+	+	+	+	+	+	+	+
	Vogues Prausker test	—	—	—	—	—	—	—	—
	Citrate utilisation test	—	—	+	—	—	—	—	—
	Tryptophan test	—	—	—	—	—	—	—	—
Carbohydrate fermentation test	Glucose	+	+	+	+	+	+	+	+
	Lactose	+	+	—	—	+	+	+	+
	Sucrose	+	+	+	+	+	+	+	+
	Arabinose	+	+	+	+	+	+	+	+
	Mannitol	+	+	+	+	+	+	+	+
	Raffinose	+	+	+	+	+	+	+	+
	Pyruvate	+	+	+	+	+	+	+	+
	Sorbitol	+	+	+	+	+	+	+	+
	Galactose	+	+	—	—	—	+	+	+
	Ribose	+	+	—	—	—	+	+	+
	Salicin	+	+	—	—	—	+	+	+
	Mannose	+	+	—	—	—	+	+	+
	Xylose	+	+	—	—	—	+	+	+
Growth on specific and Differential media	Growth on BAA Agar	—	—	—	+	+	+	+	+
	Growth on EMB Agar	—	—	+	+	—	—	—	—
	Growth on TSA	+	+	+	+	+	+	+	+
	Growth in MRS broth+ 6.5% NaCl	+	+	+	—	—	—	—	+
	Triple sugar iron test	—	—	—	—	—	—	—	—
	Skim milk test	+	+	—	—	—	+	+	+
	Gelatinase test	—	—	—	—	—	—	—	—
Growth at different temperatures	25 °C	+	+	+	+	+	+	+	+
	37 °C	+	+	+	+	+	+	+	+
	45 °C	+	+	—	—	—	—	—	+

- +ve: Positive test result -ve: Negative test result
- I1-I6: Designation of selected LAB isolates from different food samples
- BAA: Bile Aesculin Agar; EMB: Eosin Methylene Blue; TSA: Tryptic soy agar; NaCl: Sodium Chloride; MRS: Lactobacillus MRS broth

Table 3.9: Preliminary screening of LAB strains for production of Bacteriocin

Isolate	Test Organisms				
	E. coli	***P. aeruginosa***	***S. aureus***	***B. subtilis***	***L. monocytogenes***
I 1	—	—	—	—	—
I 2	—	—	—	—	—
I 3	—	—	—	—	—
I 4	—	—	—	—	—
I 5	—	—	+	+	—
I 6	+	+	+	++	+
LP	+	+	+	++	—

- Antibacterial analysis: Well- Diffusion Method (Well size, mm: 8); Amount of sample used: 100 µl; Incubation period: 24h; Incubation temp: 37°C; Media used: Muller Hinton Agar; pH: 7.3±0.1
- I 1-6: Isolates 1 to 6; LP: *L. plantarum*
- +: Zone of inhibition present —: Zone of inhibition absent

Table 3.10: Preliminary characterization of strains to ensure the presence of bacteriocin

S. No		Antibacterial potential	
		L. plantarum	**Isolate 6**
1	Untreated CFS	++	++
2	CFS adjusted to pH 6.5	++	++
3	CFS treated with catalase	++	++

- Antibacterial analysis: Well- Diffusion Method (Well size, mm: 8); Amount of sample used: 100 µl; Incubation period: 24h; Incubation temp: 37°C; Media used: Muller Hinton Agar; pH: 7.3±0.1
- Test strain: *B. subtilis*; +: Zone of inhibition present

Table 3.11: Influence of time on bacteriocin production

S. No	Time (h)	Zone of inhibition of *Bacillus subtilis*	
		L. plantarum	***Enterococcus sp. YT3* (I6)**
1	12	17±0.8**	20.7±0.9**
2	24	17.3±0.5**	22.7±0.9**
3	36	21.7±0.5**	25.3±0.4**
4	48	26.7±0.9**	27±0**
5	66	24.3±0.5**	25±0**
6	72	23±0.8**	23±0.5**

- For bacteriocin production: Incubation temp: 37°C; Production Media: MRS broth (Himedia); pH: 6.5±0.2
- Antibacterial analysis: Well- Diffusion Method (Well size, mm: 8); Amount of sample used: 100 µl; Incubation period: 24h; Incubation temp: 37°C; Media used: Muller Hinton Agar; pH: 7.3±0.1
- **: P- value <0.001

Table 3.12: Influence of temperature on the bacteriocin production

S. No	Temperature (°C)	Zone of inhibition of *Bacillus subtilis*	
		L. plantarum	***Enterococcus sp. YT3***
1	25	12±0.8**	20.7±0.9**
2	30	21± 0.5**	22.7±0.9**
3	37	25.7±1.7**	27±0.8**
4	45	27.6±0.5**	25.3±0.4**
5	50	24±0.9**	23±0.5**

- For bacteriocin production: Incubation period: 48h; Production Media: MRS broth (Himedia); pH: 6.5±0
- Antibacterial analysis: Well- Diffusion Method (Well size, mm: 8); Amount of sample used: 100 µl; Incubation period: 24h; Incubation temp: 37°C; Media used: Muller Hinton Agar; pH: 7.3±0.1
- **: P- value <0.001

Table 3.13: Influence of pH in initial medium on the bacteriocin production

S. No	pH	Zone of inhibition of *Bacillus subtilis*	
		L. plantarum	***Enterococcus sp. YT3***
1	4.5	21±0.8**	24.6±0.9*
2	5.0	23.3±0.5**	25.6±0.9*
3	5.5	26±1.6**	26.3±0.5*
4	6.0	28.7±1.2**	28 ±1.6*
5	6.5	26.7±0.5**	26.7±0.9*
6	7.0	23.6±1.2**	23.3±0.5*

- For bacteriocin production: Incubation period: 48h, Incubation temp: *L. plantarum* : 45°C, *Enterococcus sp. YT3*: 37° C; Production Media: MRS broth (Himedia)
- Antibacterial analysis: Well- Diffusion Method (Well size, mm: 8); Amount of sample used: 100 µl; Incubation period: 24h; Incubation temp: 37°C; Media used: Muller Hinton Agar; pH: 7.3±0.1
- *: P-value <0.05; **: P- value <0.001

Table 3.14: Influence of different sugar on the bacteriocin production

Sugar		Zone of inhibition of *Bacillus subtilis*	
	% supplementation	***L. plantarum***	***Enterococcus sp. YT3***
Glucose	1	16.3±0.5**	21±0.8**
	2	25±0.8**	24.7±0.9**
	3	22±0.8**	23.3±1.9**
	4	21.3±0.5**	22.7±0.9**
	5	13±2.2**	22.3±1.9**
Lactose	1	27.3±0.5**	25.3±0.5**
	2	24±1.4**	25±0.8**
	3	22.7±0.9**	24.7±0.9**
	4	21.6±2.1**	24.3±0.5**
	5	21.7±1.2**	22.6±1.7**
Fructose	1	20.7±0.9**	24.3±1.7**
	2	22.3±1.9**	29±0.8**
	3	18.7±3.7**	25.7±0.5**
	4	16.7±0.9**	25±1.6**
	5	16±0**	24±1.4**
Sucrose	1	20.7±0.9**	23.3±0.5**
	2	22.3±1.8**	24.6±0.5**
	3	21.3±0.5**	24.3±1.2**
	4	18.7±3.7**	23.3±0.5**
	5	16.7±0.9**	22.7±0.9**
Control	-	26±0.8**	26.7±1.2**

- For bacteriocin production: Incubation period: 48h,; pH: 6; Incubation temp: *L. plantarum* : 45°C, *Enterococcus sp. YT3*: 37° C
- Different sugar has been supplemented in initial media (MRS broth, already possessing 2% Dextrose)
- Control: CFS of organism grown in initial media without any additional sugar
- Antibacterial analysis: Well- Diffusion Method (Well size, mm: 8); Amount of sample used: 100 µl; Incubation period: 24h; Incubation temp: 37°C; Media used: Muller Hinton Agar; pH: 7.3±0.1; **: P- value <0.001

Table 3.15: Influence of different nitrogen source on the bacteriocin production

Nitrogen source			Zone of inhibition (mm)	
			L. plantarum	***Enterococcus sp. YT3***
Organic	Tryptone	1%	19.6±0.5**	23.3±0.5**
		2%	20±1.4**	25.7±0.9**
		3%	20.7±0.9**	26±0.8**
		4%	22.3±0.5**	28.6±1.2**
		5%	22.7±0.5**	27.3±0.4**
	Beef extract	1%	17.6±1.2**	24.3±1.7**
		2%	20±1.4**	26.3±0.5**
		3%	20±1.6**	25.7±0.5**
		4%	22±1.4**	23.6±1.2**
		5%	21.7±0.9**	23.6±1.7**
Inorganic	Triammonium citrate	0.1%	20.3±2.1**	17.3±1.2**
		0.2%	22.3±0.5**	20.3±1.2**
		0.3%	25.3±1.2**	22.0±0.8**
		0.4%	27.3±1.9**	24.6±0.9**
		0.5%	24.3±0.5**	25.7±1.2**
	Ammonium sulphate	0.1%	13.6±1.7**	14.6±1.2**
		0.2%	14±1.6**	18.7±0.4**
		0.3%	14.3±0.5**	21.3±1.2**
		0.4%	16.3±1.2**	24.7±0.9**
		0.5%	15±0.8**	25.3±0.4**
Control		-	26.6±1.7**	27±1.6**

- Bacteriocin production: Incubation period: 48h,; pH: 6; Incubation temp: *L. plantarum* : 45°C, *Enterococcus sp. YT3*: 37° C
- Nitrogen sources supplemented in MRS broth (initial N_2 sources- 1% Proteose peptone, 1% Beef extract, 0.5% yeast extract, 0.2% ammonium citrate)
- Control: CFS of organism grown in initial media (MRS broth) without any nitrogen source supplement.
- Well- Diffusion Assay: Well size, mm: 8; Amount of sample used: 100 µl; Incubation period: 24 h; Incubation temp: 37°C; Media used: Muller Hinton Agar; pH: 7.3±0.1.
- Indicator strain: *B. subtilis;* **: P- value <0.001

Table 3.16: Production of bacteriocin with optimized conditions

S. No	Organism	Optimized Conditions	ZOI (mm)
1	*L. plantarum*	Media: MRS broth; Time: 48 h; Temperature: 45°C; pH: 6; Sugar supplemented: Lactose (1%); Nitrogen source supplemented: Triammonium citrate (0.4%)	29±1.6
2	*Enterococcus sp. YT3*	Media: MRS broth; Time: 48 h; Temperature: 37°C; pH: 6; Sugar supplemented: Fructose (2%); Nitrogen source supplemented: Tryptone (4%)	29.7±0.5

- Antibacterial analysis: Well- Diffusion Method (Well size, mm: 8); Amount of sample used: 100 µl; Incubation period: 24h; Incubation temp: 37°C; Media used: Muller Hinton Agar; pH: 7.3±0.1
- Sample: Cell free supernatant (CFS); indicator strain: *B. subtilis*

Table 3.17: Influence of ammonium sulphate on precipitation of bacteriocin

S. No	% of Ammonium sulphate	Zone of inhibition (mm) of *Bacillus subtilis*	
		L. plantarum	***Enterococcus sp. YT3***
1	30	17.7±0.9*	19±0*
2	40	16.7±0.9*	18.7±0.9*
3	50	16±0*	17±1.4*
4	60	16.7±0.9*	17.3±0.5*
5	70	18.3±0.5*	19.7±0.9*
6	80	19.33±0.5*	20.3±0.5*

- For antibacterial analysis: Well- Diffusion Method (Well size:8mm); Amount of sample used: 100 µl; Incubation period: 24h, Incubation temp: 37°C; Media used: Muller Hinton Agar (Himedia); pH: 7.3±0.1
- *: P- value <0.05

Table 3.18: Influence of temperature on activity of bacteriocin

S. No	Temperature (°C)	Zone of inhibition of *Bacillus subtilis*	
		L. plantarum	***Enterococcus sp. YT3***
1	37	+	+
2	60	+	+
3	80	+	+
4	100	+	+
5	121	+	+
6	Control	+	+

- For antibacterial analysis: Well- Diffusion Method (Well size:8mm); Amount of sample used: 100 µl; Incubation period: 24h, Incubation temp: 37°C; Media used: Muller Hinton Agar (Himedia); pH: 7.3±0.1
- +: Activity Present —: Activity Absent

Table 3.19: Influence of pH on activity of bacteriocin

S. No	pH	Zone of inhibition of *Bacillus subtilis*	
		L. plantarum	***Enterococcus sp. YT3***
1	2	+	+
2	4	++	++
3	5	++	++
4	9	+	+
5	10	+	+
6	Control	+	+

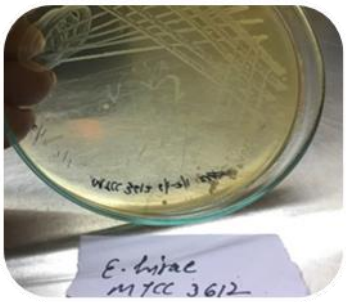
- For antibacterial analysis: Well- Diffusion Method (Well size:8mm); Amount of sample used: 100 µl; Incubation period: 24h, Incubation temp: 37°C; Media used: Muller Hinton Agar (Himedia); pH: 7.3±0.1
- +: Activity Present —: Activity Absent

Table 3.20: Influence of enzymes on activity on bacteriocin

S. No	Enzyme	Zone of inhibition of *Bacillus subtilis*	
		L. plantarum	***Enterococcus sp. YT3***
1	Trypsin	—	—
2	Pepsin	—	—
3	Proteinase K	Partial loss	—
4	Lysozyme	+	+
5	a-amylase	+	+
6	Catalase	+	+

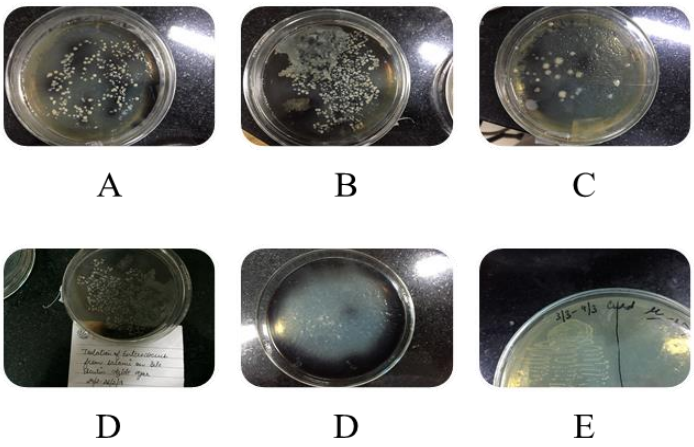
- For antibacterial analysis: Well- Diffusion Method (Well size:8mm); Amount of sample used: 100 µl; Incubation period: 24h, Incubation temp: 37°C; Media used: Muller Hinton Agar (Himedia); pH: 7.3±0.1
- +: Activity Present — : Activity Absent

Photographs

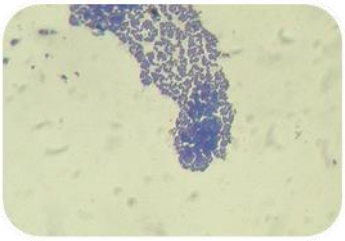


Photograph 3.1: Colonies of *E. hirae* on Brain heart infusion agar

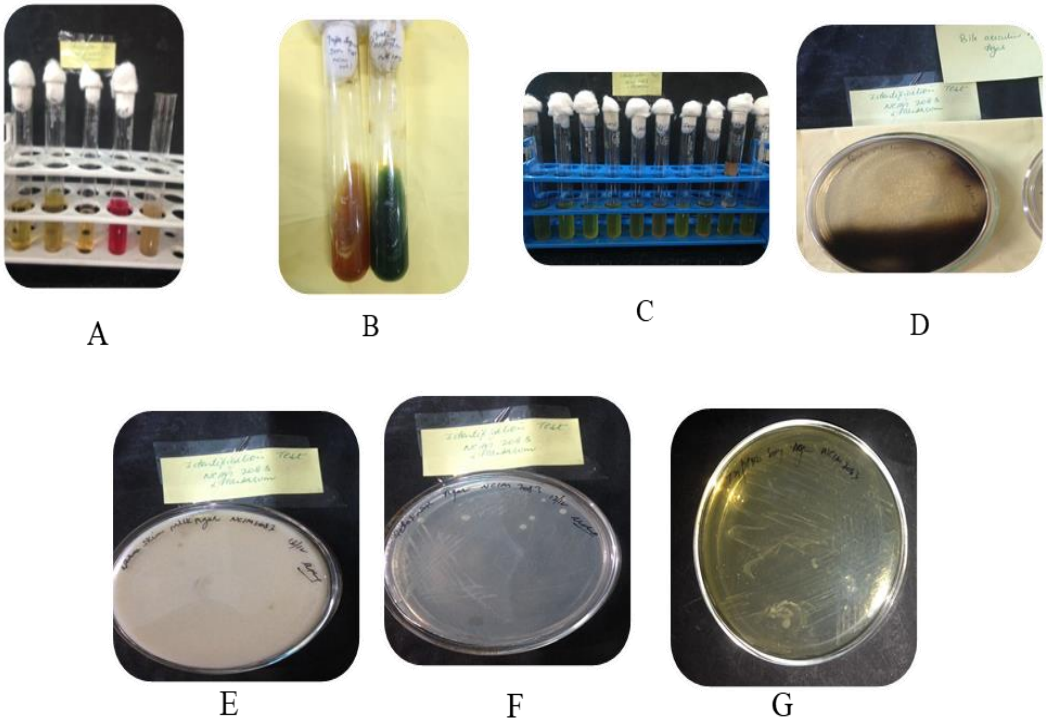
Photograph 3.2: Identification and maintenance of *L. plantarum* (NCIM2083); A: *Lactobacillus plantarum* purchased from NCIM, Pune; B-C: *L. plantarum* colonies on MRS agar; D: Gram positive rod shaped



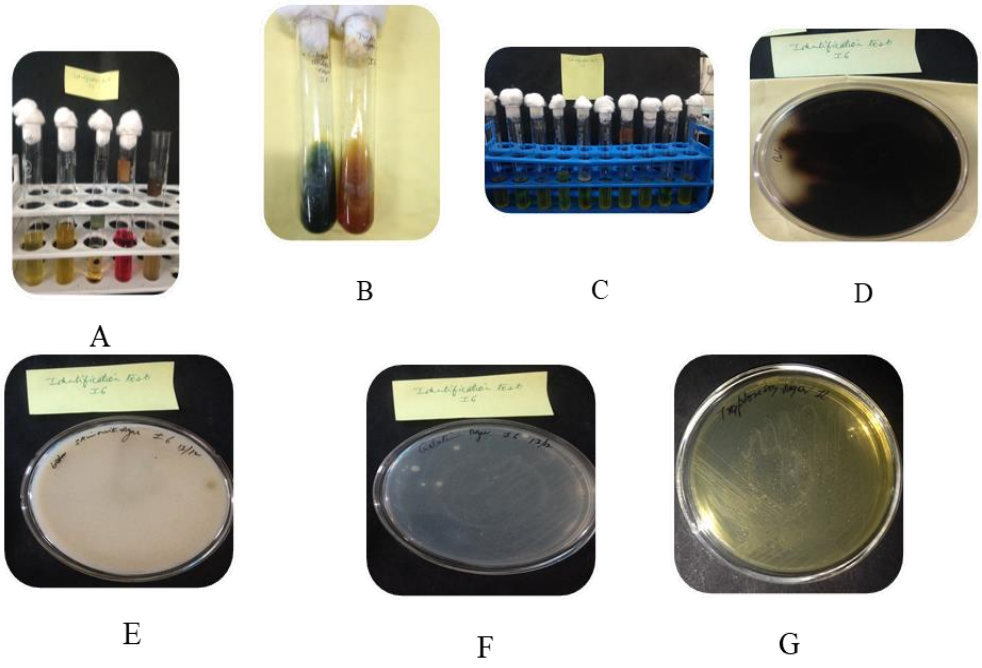
Photograph 3.3: Bacterial isolation on Bile Aesculine Azide Agar; A- D: Isolates on bile aesculine azide agar; E: Isolate on MRS agar



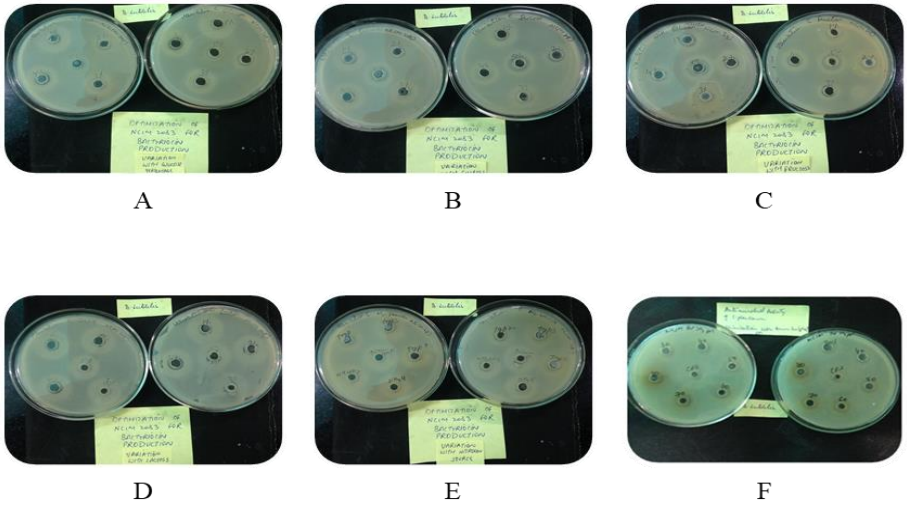
Photograph 3.4: Gram positive cocci of *Enterococcus sp. YT3*



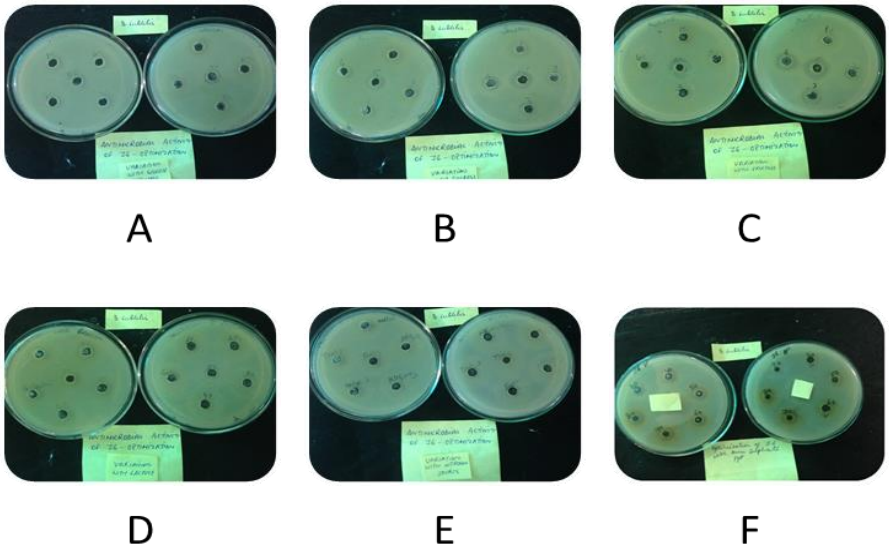
Photograph 3.5: Biochemical characterization of *L. plantarum* (NCIM2083); A: MRVP test; B: Triple sugar iron test, citrate utilization test; C: Fermentation of different sugars in phenol red broth; D: Growth on bile aesculine azide Agar; E: Growth on skim milk agar; F: Growth on gelatin agar; G: Growth on Tryptic soy agar



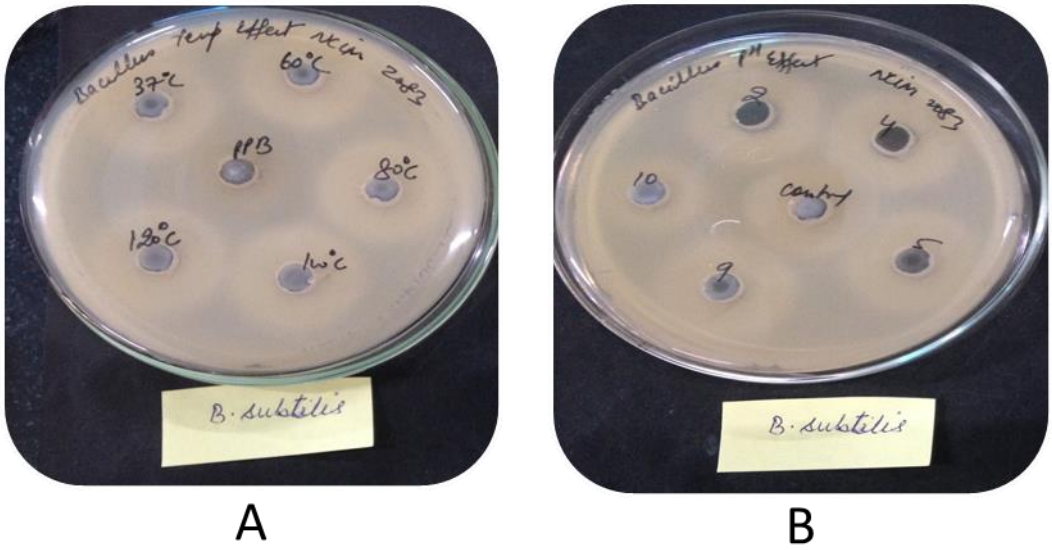
Photograph 3.6: Biochemical characterization of *Enterococcus sp. YT3;* A: MRVP test; B: Triple sugar iron test, citrate utilization test; C: Fermentation of different sugars in phenol red broth; D: Growth on bile aesculine azide Agar; E: Growth on skim milk agar; F: Growth on gelatin agar; G: Growth on Tryptic soy agar



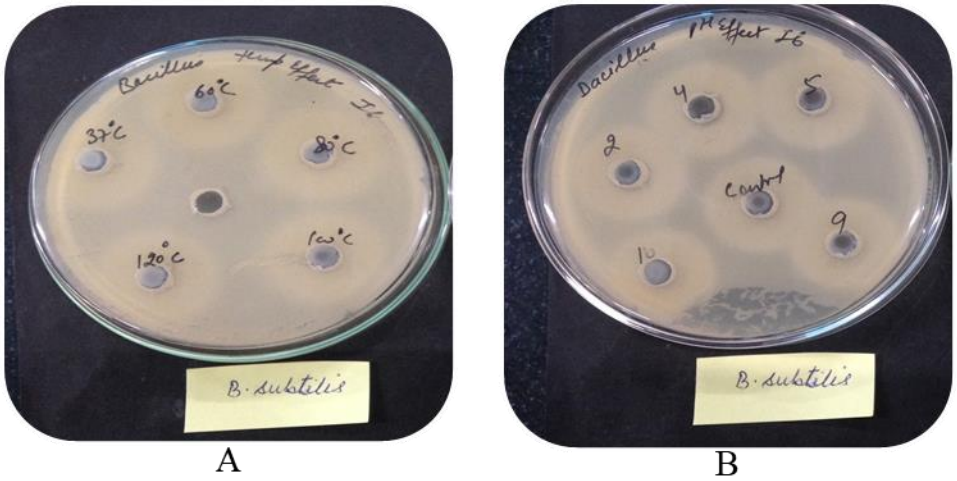
Photograph 3.7: Optimization for bacteriocin from *L. plantarum* (NCIM2083); A: Variation with glucose; B: Variation with sucrose; C: Variation with fructose; D: Variation with lactose; E: Variation with nitrogen source; F: Variation with ammonium sulphate precipitation



Photograph 3.8: Optimization for bacteriocin from *Enterococcus sp. YT3*; A: Variation with glucose; B: Variation with sucrose; C: Variation with fructose; D: Variation with lactose; E: Variation with nitrogen source; F: Variation with ammonium sulphate precipitation



Photograph 3.9: Characterization of partially purified Bacteriocin from *L. plantarum* (NCIM2083); A: Effect of Temperature; B: Effect of pH



A B

Photograph 3.10: Characterization of partially purified Bacteriocin from *Enterococcus sp. YT3;* A: Effect of Temperature; B: Effect of pH

Figures:

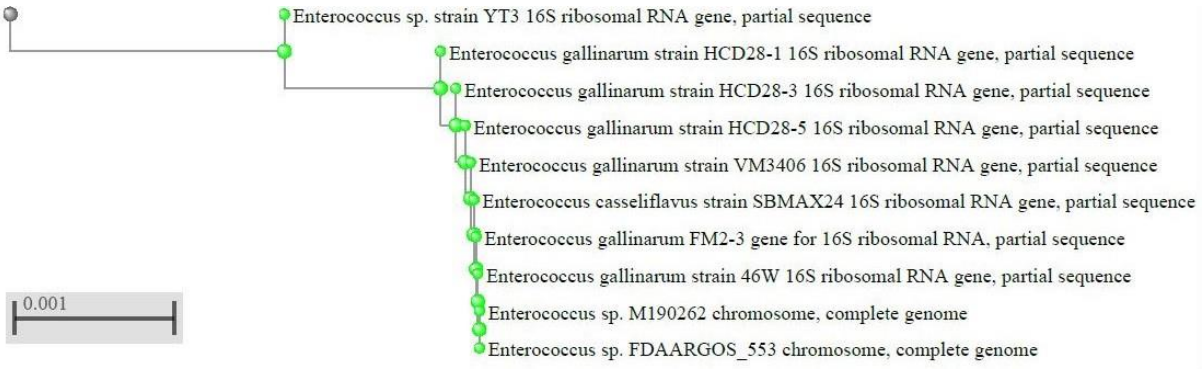


Figure 3.4: Phylogenetic tree for *Enterococcus* sp. strain YT3

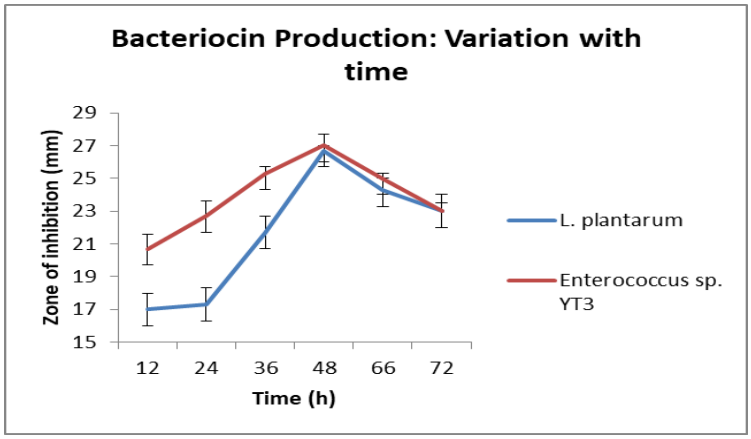


Figure 3.5: Influence of time on bacteriocin production

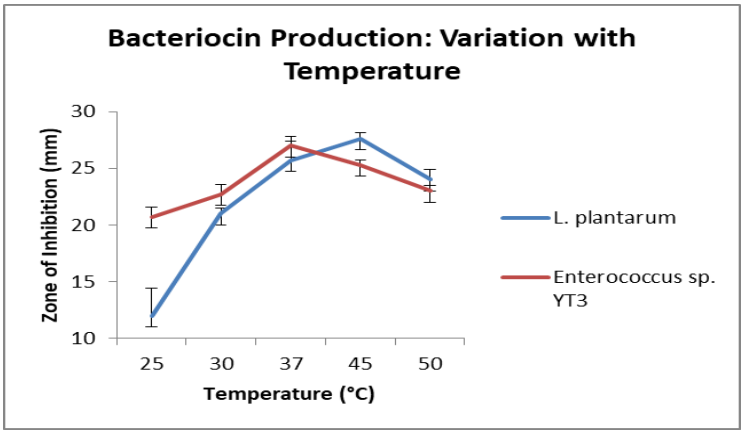


Figure 3.6: Influence of temperature on the bacteriocin production

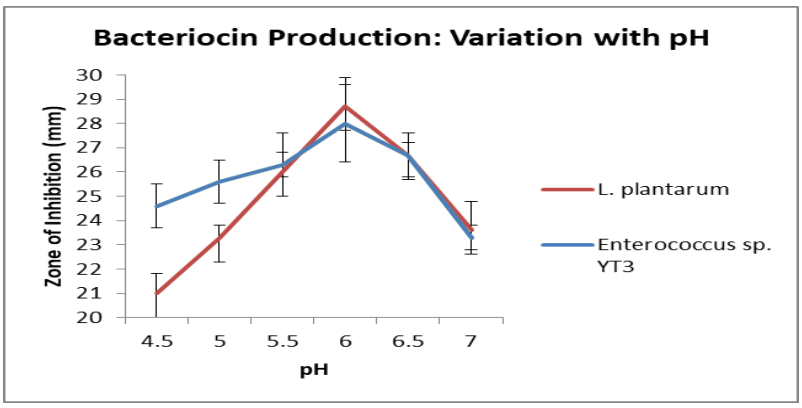


Figure 3.7: Influence of pH in initial medium on the bacteriocin production

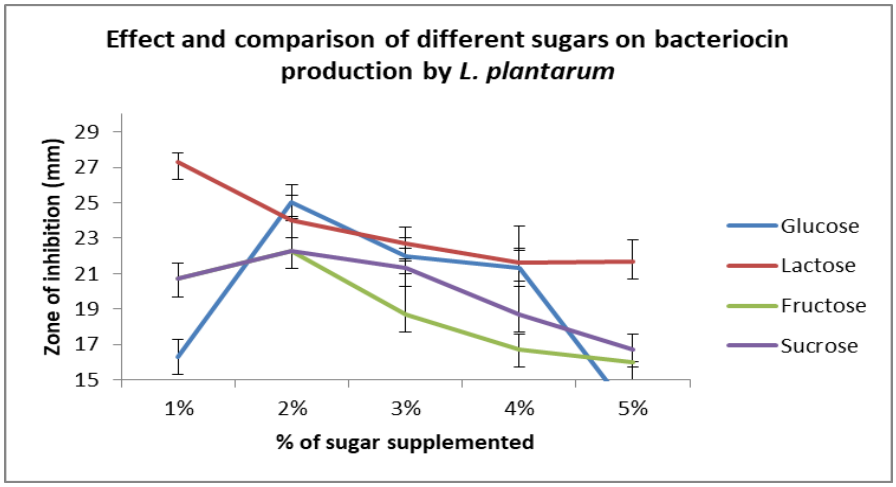


Figure 3.8: Influence of different sugar on the bacteriocin production by *L. plantarum*

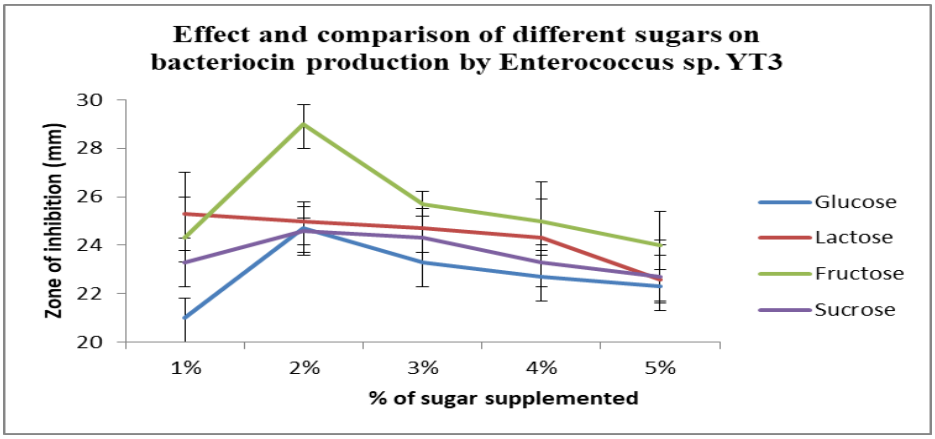


Figure 3.9: Influence of different sugar on the bacteriocin production by *Enterococcus sp. YT3*

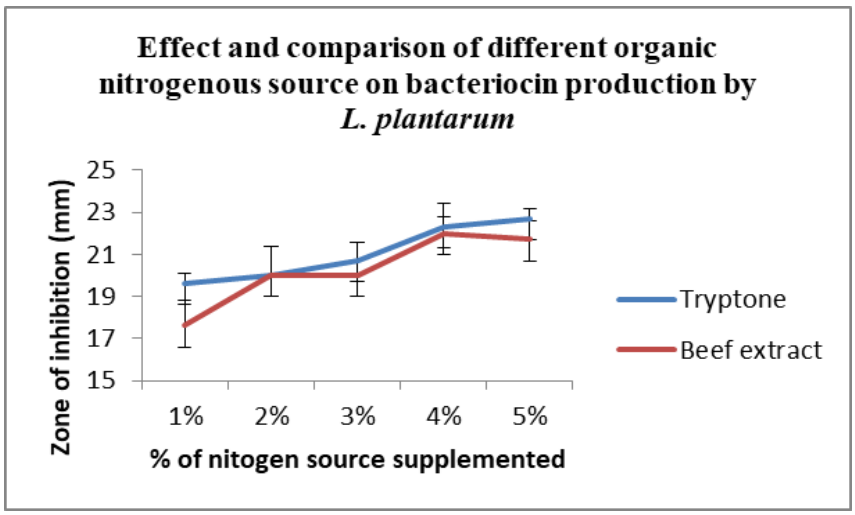


Figure 3.10: Influence of different organic nitrogen source supplemented in initial media (MRS broth) on the bacteriocin production by *L. plantarum*

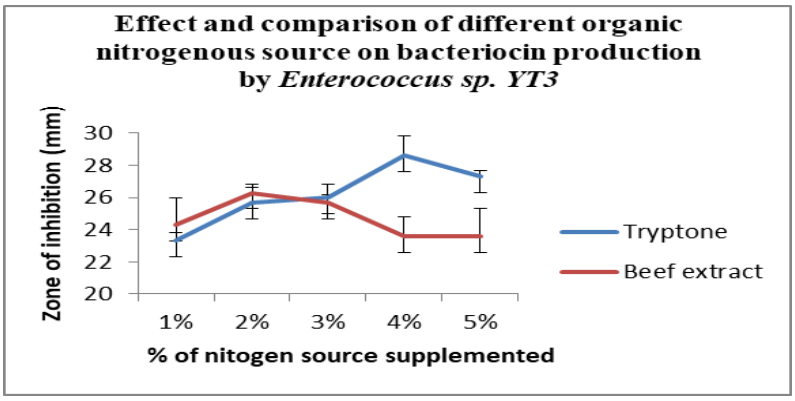


Figure 3.11: Influence of different organic nitrogen source supplemented in initial media (MRS broth) on the bacteriocin production by *Enterococcus sp. YT3*

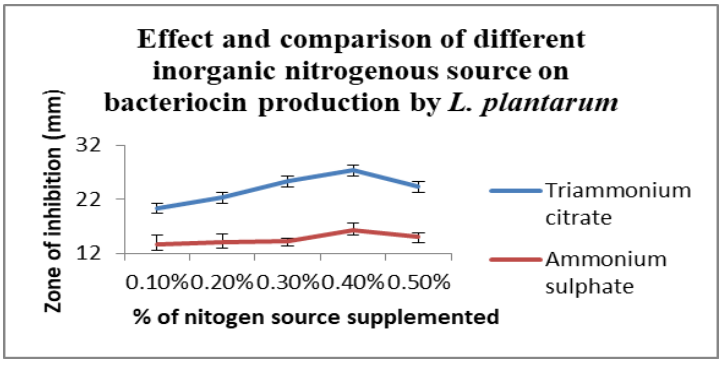


Figure 3.12: Influence of different inorganic nitrogen source supplemented in initial media (MRS broth) on the bacteriocin production production by *L. plantarum*

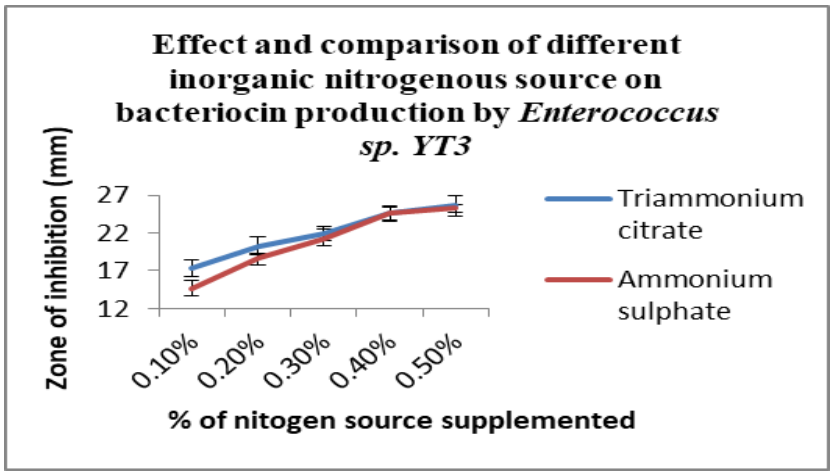


Figure 3.13: Influence of different inorganic nitrogen source supplemented in initial media (MRS broth) on the bacteriocin production by *Enterococcus sp. YT3*

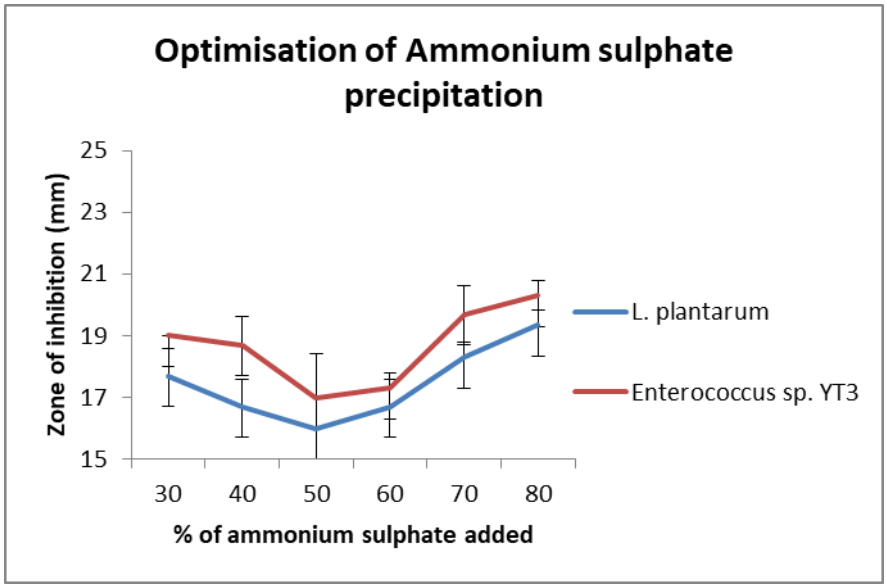


Figure 3.14: Influence of ammonium sulphate concentration on precipitation of bacteriocin

Chapter 4

Antimicrobial potential of *T. indica* extracts and bacteriocins

4.1 Introduction and review of literature

Food borne infections/ intoxications has always been for consideration to the humans as poor quality of food including microbiologically or other-wise contaminated food plays a symbolic role in maintaining the mental health of a person. Due to the favorable environmental conditions, incidences of food borne illnesses increase, especially in tropical regions of the world, including South East Asian origin countries such as, India. A study (Tambekar et al., 2008) was conducted to analyze the extent of pathogenic microbes in ready to eat food in Amravati city, India, which investigated the presence of *P. aeruginosa, Salmonella, E. coli, S. aureus* and *Proteus* as the major pathogens. The other food contaminating microbes of major concern include species of *Campylobacter*, *Clostridium*, *Listeria*, *Bacillus*, *Vibrio,* and *Shigella* (Felsenfeld, 1965; Biswas et al., 2011) In order to treat the diseases caused by consumption of such contaminated food, use of antibiotics further complicate the situation owing to the emergence of multidrug resistant microbial strain. This, in the recent global perspective, shifted the focus on natural and herbal origin medicines (Kunle et al., 2012). Many spices and different herbs are used as additives in food where these natural substances not only elevate/ improve the organoleptic characters, but also retard the flourishing of food borne pathogens thereby, extending the usability of the food products. The spices and herbs have registered their applications as flavoring agents and agents for remedial therapy in different ailments in countries like, India and China since centuries. The active ingredients present in such as hydrocarbons, phenols, ketones, aldehydes, alcohols, alkaloids and esters have been found to be responsible for such potentials (Bor et al., 2016). Many scientific studies and reviews have been witnessed which depict the herbal origin substances as

antimicrobials for use in infectious diseases and as preservatives (Verma and Singh, 2008). The first ever scientific study was done in 1880's which depicted the antimicrobial action of cinnamon oil against *Bacillus anthracis* spores thereby, illustrating the preservative role of the spices (Bor et al., 2016). Beneficial properties (antioxidant, antimicrobial and their health maintaining role) of herbs and spices such as thyme, basil, rosemary, turmeric, clove, onion, black pepper and fenugreek have been extensively reviewed in the last three decades (Valdes et al., 2015). Mint extracted from *Mentha piperita* has been found to be a potential against *S. aureus* and *S. enteritidis* (Bor et al., 2016). Thyme, Bay leaves and mint have been found to show antimicrobial potential against *Vibrio parahaemolyticus* and *S. aureus* (Bor et al., 2016). Galangal extract and finger root extract are the potential against *Bacillus cereus, E. coli* O157:H7 and *S. aureus* (Bor et al., 2016). Carvacrol and p-cymene exhibited antagonistic effects against *Campylobater spp., Nandina domestica, L. monocytogenes, B. subtilis, E. coli* O157:H7, *Enterobacter aerogenes, P. aeruginosa, S. typhimurium, S. entridis, S. aureus,* and *E. coli O157* (Bor et al., 2016). Studies indicate that anti-quorum sensitivity of *Citrus limonoids* shows an antagonistic influence on the biofilm formation of *E. coli O157:H7,* thereby decreasing pathogenicity of the given microbe. The grapefruit juice also inhibits the biofilm formation in food pathogens viz. S. *typhimurium, P. aeruginosa* and *E. coli* O157:H7. Cinnamaldehyde has the potential to affect the bacterial quorum sensing processes. Similarly, the garlic extracts blocks the production of molecules generating quorum sensing, and inhibit the extracellular virulence factors thereby inhibiting the pathogenicity of *P. aeruginosa* (Sultanbawa, 2011). *Tylophora indica* is a well-known climber from Asclepiadiaceae family (Reddy et al., 2009) with antimicrobial potentials against various disease causing microbes such as *B. subtilis, P. aeruginosa, S. aureus and E. coli*.

The crude, methanolic root extracts were observed to be effective against *Bacillus subtilis, Micrococcus luteus, Staphylococcus aureus, Aspergillus niger* along with *Trichoderma viridae* with a zone of inhibition (ZOI) <5mm diameter while leaf crude methanolic extracts were also effective against *P. aeruginosa* and *A. fumigatus* (Reddy et al., 2009). The ethyl acetate root and leaf extracts, at variable

concentrations (1mg/mL, 10mg/mL and 50mg/mL) were evaluated against *Klebsiella pneumoniae, E. coli, S. aureus, Salmonella typhi* and *P. aeruginosa* isolated from HIV patients. While, no antibacterial activity was observed with 1mg/mL of the root extract, other concentrations were effective against all microbes with ZOI above 10mm. The best inhibitory activity was found against *K. pneumonia*, with ZOI of 18mm. The ZOI with leaf extracts were found were between 11-19 mm at all concentrations against all test pathogens, while antimicrobial resistance was observed with 10µl of 1mg/mL concentration for *Klebsiella pneumoniae* and *E. coli* only *(*Balasubramanian et al., 2010*)*. The aqueous, methanolic, and petroleum ether root extracts of *T. indica* were evaluated against *E. coli, Pseudomonas flavescens* and *Micrococcus roseus* by disc diffusion method. The methanolic extract was able to inhibit the growth of all three species tested, while the aqueous extract was effective only against *E. coli*. The petroleum ether extract of the roots were ineffective for all three microbial species evaluated (Raut et al., 2012*).* In last few decades, the plant tissue culture and related bio techniques has provided a significant platform for enabling the propagation of herbs which are endangered or of high medicinal value, so that their large biomass can be made available throughout the year along with the maintenance of the species. However, for large scale applications, the evaluation of the plant matter produced by such *in vitro* techniques must be carried out for different aspects such as the presence of required phytochemicals and antimicrobial efficiency as compared to the parent naturally growing plant (Bharat and Parmar, 2010). With similar objective, a comparative antimicrobial study with the petroleum ether, aqueous, methanol and chloroform extracts for leaves and roots of *T. indica* was conducted against *Bacillus cereus, Micrococcus luteus, Escherichia coli* and *Salmonella typhi*. The inhibitory effects were found more in the extracts of naturally grown plants as compared to that of *in vitro* developed plant samples (Bharat and Parmar, 2010*)*. The antimicrobial potency of the crude ethanolic extract of dried *T. indica* leaves, at different concentrations (20mg/mL, 30mg/mL and 40mg/mL) was tested by agar well diffusion technique for different microbes (*K. pneumoniae, S. aureus, S. typhi, Proteus vulgaris, P. aeruginosa, E coli, Aspergillus niger, Aspergillus fumigatus* and *Fusarium* species). No activity was found against *K.*

pneumoniae and *S. typhi* with any of the tested concentrations. The ZOI was >5mm for all other test species with a maximum ZOI of 10.5 mm against *Fusarium* species at 40mg/mL concentration (Reddy, 2009). The activity of the aqueous and ethanolic extracts of *T. indica* leaves was evaluated by disc diffusion and well diffusion techniques respectively. The only inhibition was found for *K. pneumonia* with ethanolic extract (ZOI= 3mm) while no activity was demonstrated against P. *aerogenes, K. pneumoniae, E. coli, P. vulgaris, P. mirabilis,* and *S. typhimurium* (Parekh et al., 2007*).* With another study conducted similarly, the aqueous and ethanolic leave extracts of *T. indica* were estimated not to be effective against *S. aureus, S. epidermidis and S. subflava*, as no ZOI was observed (Parekh et al., 2008). The antimicrobial assays were conducted for *T. indica* leaf ethanolic extracts against gram positive microbes (*Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, Enterococcus faecalis)* and gram negative microbes *(Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus vulgaris, Salmonella typhi, Salmonella typhimurium, Shigella dysenteriae type 1, Vibrio cholera, S. aureus, and P. aeruginosa)* with 100mg/mL concentration by well diffusion method. A comparison was observed amongst the activity of the leaf extracts of the cultivated parent plant, *in-vitro* raised plant and the leaf callus. The leaves extract of the parent plant were effective only against some gram negative strains while *in-vitro* raised plant and the leaf callus presented zones against both the categories of microbes (Jahan et al., 2013). A study conducted with aqueous (cold, warm, hot and boiling water) and organic solvent (methanol, ethanol, petroleum ether and chloroform) extracts of *T. indica* stem and leaves expressed no efficiency against *E. coli* and *P. aeruginosa* and multi drug resistant bacterial strains of *E. coli, P. aeruginosa* and *S. aureus*. Methanolic extracts were able to inhibit *E. coli* and petroleum ether and chloroform extracts were effective against *P. aeruginosa*. Higher ZOI against the normal bacterial strains were observed when the extracts were tested with the antibiotic (tetracycline) exhibiting synergism (Kannan et al., 2013*).* The extracts (aqueous, methanol, ethanol, petroleum ether and chloroform) of *T. indica* crude leaf and callus were evaluated against both Gram positive, Gram negative bacteria (*Staphylococcus aureus, Streptococcus faecalis, Micrococcus luteus, Bacillus*

subtilis, Escherichia coli, Salmonella typhi, Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter aerogenes), and yeast (*Candida albicans*). The gram positive bacterial strains were more sensitive as compared to gram negative which may be due to the presence of external lipopolysaccride layer. Overall, the leaf extracts showed more promising antimicrobial and antifungal effects, as compared to the callus extracts (Sellathurai et al., 2013). The antimicrobial activity of ethanolic and aqueous extracts using fresh leaves of *T. indica* against *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli* (*bla* amp C), *Klebsiella* spp. (*bla* CTX-M) and *Klebsiella* spp. (*bla* SHV) was analysed. The ethanolic leaf extracts were observed to be more potent when compared to the aqueous extracts. The aqueous extracts were effective only against *E. coli* and *K. pneumoniae* with inhibitory zones measuring 12.33 and 11.00 mm respectively while the ethanolic extracts were the potential against *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli* (*bla* amp C) with inhibitory zones 12.67, 11.67, 11.33 and 11.67 mm respectively (Jahan et al., 2013*).* The antimicrobial efficiency of aqueous and ethanolic extracts of *T. indica* leaves and stem of naturally grown parent plant and *in-vitro* grown plant was studied against different microbes (*Candida albicans, Candida parapsilosis, Candida krusei, Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus, Rhizopus* species, *Alternaria* species, *Penicillium* species, *Fusarium* species and *Mucor* species). The comparative analysis depict that the alcoholic leaf extract of naturally grown plant was selectively better against *Candida krusei, Aspergillus flavus, Aspergillus niger* and *Aspergillus fumigatus* than *in vitro* grown leaf extracts. The alcoholic stem extracts of *in vitro* grown samples were better potential against *Candida parapsilosis, Candida krusei, Aspergillus flavus* and *Aspergillus fumigates* as compared to naturally grown sample extract which showed better activity against *Aspergillus niger* (Deshwal et al., 2013)

The activity of *T. indica* stem extracts (methanolic, petroleum ether and aqueous) were analysed against three bacterial strains (*E. coli, Micrococcus roseus* and *Pseudomonas flavescens).* All the tested extracts exhibited ZOI against *E. coli* while

the petroleum ether extract also produced ZOI (2 mm) against *Pseudomonas flavescens.* No activity was observed against any of the other bacterial species by other solvents (Raut et al., 2012). The efficacy of an aqueous and hydro-alcoholic crude extract of the shoot of *T. indica* was studied against 3D7 and RKL-9 strains of *Plasmodium falciparum (P. falciparum).* For *P. falciparum* 3D7, the EC_{50} value was demonstrated to be 2.542 μg/ mL and 2.364 μg/ mL for the aqueous and hydro-alcoholic extracts, respectively. For *P. falciparum,* RKL-9, the EC_{50} value was determined to be 15.240 μg/ mL and 9.717 μg/ mL respectively for both the extracts. These values represent that the extracts are effective in killing malaria causing *P. falciparum* (George et al., 2015). The ZOI with 20 μg/mL of crude alkaloid extract from *T. indica* leaves was found to be 0.341, 0.326 and 0.371 mm against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* respectively. The alkaloid compound was then isolated and was found to be tylophorinidine alkaloids cation which has been found to inhibit the bacterial protein synthesis along with the leakage of protein and sugars from the bacterial membranes (Sathyabama et al., 2013). In an analysis, the secondary metabolites (tylophorine, tylophorinine and kaemferol) present in methanolic extracts of *T. indica* leaves were tested for their cytotoxicity assay against Madin Darbin Canine Kidney cell lines by MTT assay. The CC50 was analysed to be 30mg/ml and was not cytotoxic below this concentration. Based on this observation, the anti-influenza activity was observed at 20 mg/mL concentration (Chaturvedi et al., 2014) by haem-agglutination assay. As highlighted above, *T. indica* seems to be a safe and a potent antimicrobial agent.

As elaborately emphasized in chapter 1, Bacteriocins are also potent natural antibiological substances which can be of potential value to the food industry. A rich literature is available where, bacteriocins produced by diverse Lactic acid bacterial strains viz., *Lactobacillus lactis, L. plantarum* and *E. faecium* are found to be effective against various pathogenic food related to organisms such as *E. coli, Micrococcus, L. monocytogenes, S. aureus, Streptococcus*, and *Bacillus* and so on (Gautam and Sharma, 2009). A bacteriocin produced from the *Lactococcus lactis*, an isolate from dry fermented sausage, exhibited antilisterial potential (Rodriguez et al., 1995). *L. brevis SB27* produced bacteriocin effective against *P. pentosus, L.*

plantarum, L. sake and L. acidophilus (Gautam and Sharma, 2009). *L. curvatus LTH1174* produced bacteriocin effective against *L. innocua* (Gautam and Sharma, 2009). *L. lactis* subsp *cremoris R* produced bacteriocin effective against *Clostridium, Listeria, Enterococcus, Leuconostoc, Staphylococcus, Lactobacillus, Bacillus, Streptococcus* and *Micrococcus* (Gautam and Sharma, 2009).

A bacteriocin from *P. acidilactici* was effective against *E. faecalis*. Another bacteriocin from the strain *S. warneri* has been effective against *L. luteus*. *L. plantarum* and *L. brevis OG1* produced bacteriocin against *E. coli* and *E. faecalis* (Gautam and Sharma, 2009). The enterocins A and B produced by *E. faecium WHE81* has shown antibacterial activity against *L. monocytogenes*. Another enterocins AS-48 produced by *E. faecalis A-48-32* has been effective against *B. cereus* and *S. aureus*. Enterocins A and B produced by *E. faecium CTC492* has been a potential against *L innocua* and *L. sakei CTC746* (Cisneros *et al.,* 2011). Not only against food pathogens, but different LAB species have been found to produce bacteriocins against uropathogens such as *S. aureus, Proteus spp., Enterococcus species, P. aeruginosa,* uropathogenic *E. coli, C. albicans, Neisseria gonorrhoea, S. typhimurium* and *B. cereus* (*Mokoena,* 2017). Such studies have further widened the field of applications of bacteriocins in health and food industry. Nisin, as explained (Chapter 2), is produced by *Lactococcus lactis* and, is available commercially for its application as a preservative. It belongs to lantibiotics, the class 1 bacteriocin and is a small, hydrophobic, cationic, 34 amino acid peptide with 3.5 kDa weight (Hagiwara et al., 2010). It has the ability to exhibit broad spectrum antimicrobial potential especially against Gram positive bacteria and their spores (Delves Broughton, 1990). Still, it is not generally found to be effective against Gram negative bacteria, fungi and yeasts because of the presence of their outer membrane permeability barrier. It has been suggested that this barrier may be altered by the use of some chelating agent or by some physical treatment which may lead to an induced sensitivity to nisin (Sanlibaba et al., 2009; Chung et al., 1989). Although nisin is immensely being used, however, some cases of Nisin resistant strains are also emerging which needs to be focused and tackled in time (Balciunas et al., 2013). Nisin and other bacteriocins are normally synthesized as pre-peptides which have an N-terminal sequence guide (Macwana &

Muriana, 2012). During exponential phase, these preformed bacteriocin molecules are then transported to the bacterial cell surface which are converted to their active form with energy expenditure (Aucher et al., 2005). Nisin has the ability to interact with the anionic lipids of the host membrane and forms pores by interacting with peptidoglycan precursor Lipid II causing depolarization of the trans-membrane electrical potential, and disruption of the lipid bilayer, leading to killing the bacteria (Rodrieguez et al., 2000; Rodriguez et al., 2003). The importance of Nisin, thus, has already been recognized in the areas of medicine, food preservation and food packaging systems. Its low toxicity to the humans (toxic dose 6,950 mg/kg), biodegradability in the human gastrointestinal system, hypo-allergic nature, thermo-stability and wide spectrum of antimicrobial potential has made it an important leading bio-preservative in industry to preserve foods such as, pasteurized cheese, canned food and sauces (Balciunas et al., 2013). Nisin has been designated as GRAS (generally regarded as safe) and is available commercially for its role as bio preservative (Balciunas et al., 2013). Nisin is being used in skim and whole fat milk, cottage cheese, milk pudding, cow milk and yogurt to increase its shelf life (Cleveland et al., 2001). The milk products especially, cheeses and other ready to eat products are normally stored in the refrigerators, where the contamination of psychotropic organisms such as, *Listeria*, is a challenge. Not only as the preservative, but bacteriocin producing strains are also helpful in, fermentations and enhance the flavours of the food items, as well (Behera et al., 2018). Nisin is the only Bacteriocin approved for food applications being considered to be safe by the Food and Agriculture Organization/ World Health Organization (FAO/WHO) in 1969 (Gharsallaoui et al., 2015). It is permitted to be used as food additive in at least 69 different countries (Sanlibaba et al., 2009). Pediocin PA1 is another example of bacteriocin which has been commercialised as a food preservative and is available as the name: Alta 2341^{TM} or Microgard^{TM} (Balucinas et al., 2013). This bacteriocin shows wide spectrum of anti-microbial activity against both Gram positive and Gram negative microbes, higher stability at wider pH range and resistance to heat and freeze (Silva et al., 2018). The antimicrobial efficacy of Pediocin PA-1 has been well studied in meat and it has been found to be effective against food pathogens such as *L.*

innocua, *L. monocytogenes*, *Clostridium laramaie*, *Clostridium perfringens and Leuconostoc spp.* (Balucinas et al., 2013) but its exhaustive study needs to be done.

Although not extensively, but some attempts has been made to observe the behavior of bacteriocins along with plant extracts. The antimicrobial potential of a bacteriocin, enterocins AS-48 produced by *E. faecalis*, was enhanced against *S. aureus* in vegetable sauces when it was combined with phenolic such as carvacrol against *S. aureus.* Alone with 25 ug/ml, Enterocin AS-48 was able to inhibit the growth of Staphylococci at 10C, while 80ug/ml along with 126mM carvacrol decreased the counts of test organism below detectable limits for up to 30 days (Grande et al., 2007). The biological activity of nisin Z was enhanced when combined with thymol against *L. monocytogenes* and *Bacillus subtilis.* A very slight retardation of growth for *L. monocytogenes* and *Bacillus subtilis* was observed when nisin Z was added in the concentration of 10-25 IU/ml and 25-50 IU/ml, respectively, where optical density value was determined above 0.25. No thymol test concentration (0.05%, 0.04%, 0.03% and 0.02%) could inhibit both test strains to OD value <0.25. in combination, 40 IU/ml with 0.02% of nisin Z and thymol, respectively, determined great reduction of *L. monocytogenes* (OD value: <0.02). Similarly, 75 IU/ml with 0.03% of nisin Z and thymol, respectively, determined great reduction (OD value <0.02) of *B. subtilis* (Ettayebi et al., 2000). Nisin along with grape seed extracts, or green tea extracts, which are rich in phenolic, were effective causing cell damage in *L. monocytogenes* as observed, under electron microscope (Wolska et al., 2012). Another study reported no synergy between *Mentha pulegium* essential oil with Bacteriocin like inhibition substance, E204, against *E. coli* and *L. monocytogenes* (E.O Khay et al., 2016). Instead, antagonistic effects were observed between E204 and honey (E.O Khay et al., 2016).

After having a glance at the above scientific work available, an attempt has been made to determine the behavior and as combinations of Bacteriocin and *T. indica* crude extracts towards the food contaminating pathogens. This study includes Nisin (purchased from market), partially purified bacteriocin from two organisms: *L. plantarum* (Plantaracin 2083) and *Enterococcus sp. YT3* (Enterocin YT3) as the source of bacteriocin.

4.2 Materials and Methods

4.2.1 Materials

The obtained powdered leaves and roots of *Tylophora indica* (as already discussed in Ch. 2) were used for the preparation of the extracts. Nisin E234 was obtained from Hangzhou Bioactive Yeast Company Ltd, China, with nearly 10% purity and near 1070 IU/mg potency. Plantaracin 2083 and Enterocin YT3 were obtained as discussed in chapter 3. The indicator strains included in this study were *E. coli* MTCC1687, *Pseudomonas aeruginosa* MTCC 4673, *Staphylococcus aureus* MTCC 96, *Bacillus subtilis* MTCC 121 and *Listeria monocytogenes* MTCC1143 and were obtained as discussed in Ch. 3 (Table 4.1). The complex nutrient media used in the study was procured from Himedia, Mumbai, India as discussed in Ch. 3. The analytical grade chemicals were procured from Loba Chemicals, Mumbai, India. The borosilicate glassware used, was purchased from Borosil, India.

4.2.2 Methods

4.2.2.1 Preparation of crude extracts of *T. indica* leaves and roots

Extraction of 15 g of dried and powdered *T. indica* crude drug (leaves and roots separately) was done with 200ml of the solvent (Methanol: Water: 9:1). The powders were completely submerged and covered with aluminum foil in the washed, dried and sterilized 500 ml conical flask for 48 h (including continuous stirring for 6 hrs.). Obtained extract was filtered, and dried at the temperature of 45^{o}C in the pre-weighed dried china dish on water bath till the evaporation of the solvent (methanol). The residue was a thick syrupy mass (extract) which was cooled and weighed. Subsequently, the reconstitution of the crude extracts to formulate a stock solution with concentration (mg/ml) of 200 in 100% sterilized DMSO was done followed by their storage in 100 ml sterilized capped glass bottles.

Similarly, the ethanolic, aqueous and ethyl extracts were prepared using ethanol, double distilled water and ethyl acetate respectively as the solvents (Chandrasekara and Venkatesalu, 2003; Babayi et al., 2004; Anibijuwon and Udeze, 2009). 5%

sterilized DMSO served as the control. The extractive value has been calculated as below (Khandelwal and Sethi, 2013):

$$\textbf{Extractive value (\%)} = \frac{\text{Weight of crude extract obtained (g)} \times 100}{\text{Weight of powder drug taken (g)}}$$

To each 5 ml *T. indica* leaf methanolic stock extract, sterilized 5% DMSO was added in 15 ml sterilized capped test tube to obtain required concentration (100 mg/ ml, 50 mg/ml, 25 mg/ml, 20 mg/ml, 15 mg/ml, 10 mg/ml, 5 mg/ml) of working test sample for leaf extracts. Similarly, the dilutions for ethanolic and aqueous were prepared from their respective stock solutions.

The test working dilutions for *T. indica* root extracts were also prepared in the same manner using its corresponding stock solution.

4.2.2.2 Preparation of Bacteriocin samples

Undiluted partially purified bacteriocin, as obtained and subsequently discussed earlier in chapter 3 for Plantaracin 2083 and Enterocin YT3, were used as the stock solution. The different dilutions (50%, 75%, 82.5%, 90%, 95% and 99%) were prepared in 0.02M acetic acid (v/v) as the test samples.

To 2.74 g of nisin, 20 ml of sterilized 0.02M acetic acid was added in the capped test tubes, to obtain the nisin stock solution of 20,000 IU/ml. To each 5 ml of nisin stock, sterilized 0.02M acetic acid was added in the capped test tubes, to obtain different dilutions (50%, 75%, 80%, 87.5%, 90%, 95% and 97.5%).

Stocks of obtained bacteriocin namely, Plantaracin 2083, Enterocin YT3 and Nisin were stored in refrigerated conditions of 4± 2^{o}C (Sanchez et al., 2007).

4.2.2.3 Preparation of combinations of *T. indica* extract and bacteriocin

To 2 ml of methanolic extract of *T. indica* leaf extracts, 2 ml of Plantaracin 2083 was added, in a sterilized test tube to obtain required combination and incubated at room temperature for 2 h under static conditions. Similarly, binary combination of ethanolic and aqueous extracts with Plantaracin 2083 was prepared and incubated (Table 4.4) (Ettayebi et al., 2000).

Similarly, the combination of plant extracts with Enterocin YT3 and nisin were prepared and incubated to obtain the required combination (Table 4.7) (Ettayebi et al., 2000).

Thereafter, the binary combinations of *T. indica* root extracts and bacteriocins were prepared similarly. The obtained test solutions were then analyzed for their potential against the indicator pathogens (*E. coli*, *P. aeruginosa*, *S. aureus, B. subtilis* and *L. monocytogenes)* by well diffusion method, as discussed ahead (Table 4.7).

4.2.2.4 Evaluation of antimicrobial activity (Agar well diffusion technique)

Thick lawn of each indicator pathogenic strains (15-16 hrs. old) was prepared with sterilized swabs on sterile MHA plates. Subsequently, wells of 8 mm diameter were cut with sterilized borer and 100 µl of each test sample (plant extract, bacteriocin, combinations of extract and bacteriocin) was poured into the different wells and kept at 4^{o}C in refrigerator to allow the diffusion of the samples in the media. Further, the inoculated plates were incubated at 37^{o}C for 24 h and subsequently, the zones of inhibition were measured, vertically and horizontally, and average of the two was recorded. Appropriate controls comprising of 5 % DMSO and 0.02 M acetic acid were used (Rana, 2012).

As and when needed, to compare the potential of nisin and partially purified bacteriocin, the activity unit per ml has been calculated by using the formula (Iyapparaj et al., 2013):

$$\textbf{AU/ml} = \frac{\text{Diameter of zone of inhibition (in mm)} \times 1000}{\text{Volume of sample used in each well (µl)}}$$

4.2.2.5 Statistical Evaluation

The antimicrobial agents were analysed for their antimicrobial activity, individually and in combinations All the experiments have been performed in triplicates..The data obtained have been expressed as mean± standard deviation (SD). The agents illustrating best results were shortlisted for their application in further analysis. The antimicrobial data obtained by these selected agents was statistically analysed using one way ANOVA at 5% level of significance.

4.3 Results and Discussion

4.3.1 Extractive values for *T. indica* crude extracts

The yield of leaves of *T. indica* methanolic, ethanolic, aqueous and ethyl acetate crude extracts were (%) 28, 25, 21 and 5, respectively (Table 4.2). Similarly, the yield for roots of *T. indica* methanolic, ethanolic, aqueous and ethyl acetate crude extracts were (%) 25, 24, 15 and 4, respectively (Table 4.8). A yield for 6% -9% (Reddy et al., 2009) has been reported for *T. indica* methanolic extract indicating better yields in our study. As indicated above, the yield for ethyl acetate for both leaves and roots were too low, hence, it was not considered for further study. Similar low yields with ethyl acetate (3.5%) for *Moringa oleifera* pods have been reported as compared to other solvents viz. ethanol (22%) and water (14%) (Sharma and Paliwal, 2013).

DMSO was used as the solvent for the crude plant extracts as it has been suggested to be an aprotic solvent that enables the dissolution of polar and non-polar substances. It is miscible with wide range of organic solvents and with water as well. DMSO itself is a non-biological potential against organisms such as *B. subtilis, S. aureus* and *E. coli* (Ghosh et al., 2008; Antonioswamy et al., 2012) and hence, one of the considerably used solvent for dissolving extractives (Chah et al., 2006; Ghosh et al., 2008; Antonioswamy et al., 2012).

4.3.2 Preparation of bacteriocin samples

The test dilutions (50%, 75%, 80%, 87.5%, 90%, 95% and 97.5%) of nisin, as prepared from the stock was expected to possess 10,000 IU/ml, 5,000 IU/ml, 4,000 IU/ml, 2500 IU/ml, 2000 IU/ml, 1000 IU/ml and 500 IU/ml of nisin (Table 4.3).

4.3.3 Antimicrobial activity of leaves of *T. indica*

When methanolic extracts with 5 mg/ml concentration were evaluated, no activity was found against *E. coli* (TO1E*), P. aeruginosa (*TO2P*), B. subtilis (*TO4B*)* and *L. monocytogenes (*TO5L*)* but zone of inhibition (in mm) against *S. aureus* (TO3S) was observed to be 9.3±0.5. Up to 25 mg/ml, no activity was found against TO4B (*L. monocytogenes.* At 10mg/ml, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 10.7±0.9, 9.6±0.4, 10.3±0.5 and 9.3±0.5 respectively. At 15

mg/ml, the zones (in mm) against TO1E, TO2P TO3S and TO4B were found to be 11±1.6, 10±0.8, 11.7±0.9 and 10±0.8 respectively. At 20mg/ml, the zones (in mm) against TO1E, TO2P TO3S and TO4B were found to be 11.7±0.9, 10.7±0.5, 12.3±0.5 and 10.3±0.5, respectively. At 25 mg/ml, the zones (in mm) against TO1E, TO2P TO3S and TO4B were found to be 11.3±0.5, 11.7±0.4, 12.7±0.5 and 10.7±0.9 respectively. At 50 mg/ml, the zones (in mm) against TO1E, TO2P TO3S, TO4B and TO5L were found to be 14±1.4, 14.7±0.4, 15±1.4, 12.7±0.9, 9.3±0.4 respectively. At 100 mg/ml of methanolic extracts, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 15.7±1.7, 16.7±0.9, 16±1.4, 14.3±0.5 and 9.7±0.4 respectively. No activity of DMSO was exhibited against any of the indicator organisms (Table 4.5; Photograph 4.1; Figure 4.1).

With 5 mg/ml, 10mg/ml and 15 mg/ml of ethanolic extracts, no activity was found against any of the indicator organisms. At 20 mg/ml, no zones were found against TO1E, TO2P, TO3S and TO5L. The zone against TO4B was found to be 9±0 mm. At 25 mg/ml, no activity was found against TO1E, TO2P and TO3S by ethanolic extracts. The zones (in mm) against TO4B and TO5L were found to be 10.3±0.5, and 11.3±0.5 respectively. Again at 50 mg/ml, no activity was found against TO1E, TO2P and TO3S. The zones (in mm) against TO4B and TO5L were found to be 13.3±0.4, and 13±0.8 respectively. At 100 mg/ml of ethanolic extracts, no activity was found against TO3S. The zones (in mm) against TO1E, TO2P, TO4B and TO5L were found to be 14.7±0.5, 11±0.8, 14±0.8 and 18.3±0.5 respectively. No activity of DMSO was exhibited against any of the indicator organisms (Table 4.5; Photograph 4.2; Figure 4.1).

With 5 mg/ml of aqueous extracts of leaves, no activity was found against TO1E, TO2P, TO4B and TO5L. The zones (in mm) against TO3S were found to be 9.6±0.4. At 10mg/ml, no activity was found against TO2P, TO4B and TO5L. The zones (in mm) against TO1E and TO3S were found to be 9.7±0.9 and 10.3±0.5 respectively. At 15 mg/ml, no activity was found against TO2P and TO5L by the aqueous extracts of leaves. The zones (in mm) against TO1E, TO3S and TO4B by this extract were found to be 11.3±0.5, 10.7±0.5 and 9.3±0.5 respectively. At 20mg/ml, no activity was found against TO2P and TO5L. The zones (in mm) against TO1E, TO3S and TO4B were

found to be 12±0, 11.3±0.5 and 9.7±0.4 respectively. At 25 mg/ml, no activity was found against TO2P and TO5L. The zones (in mm) against TO1E, TO3S and TO4B were found to be 13.7±0.4, 11.3±0.5 and 9.7±0.4 respectively. With 50mg/ml of aqueous extracts of leaves, no activity was found against TO5L. The zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 16±0.8, 12.3±0.5, 11.7±0.5 and 10.7±0.4 respectively. At 100mg/ml the zones (in mm) against TO1E, TO2P, TO4B, TO3S and TO5L were found to be 16.7±1.2, 13.3±0.4, 12.3±0.5, 12.7±1.2 and 13.3±0.5 respectively. No activity of DMSO was exhibited against any of the indicator organisms (Table 4.5; Photograph 4.3; Figure 4.1).

Three different extracts of *T. indica* leaves were studied for their biological potential. The methanolic extracts were determined to be most effective followed by aqueous and ethanolic extracts. Only *S. aureus* was sensitive to 5 mg/ml of methanolic and aqueous extract. While 100 mg/ml and 50 mg/ml of methanolic extract was potential against all test strains, 10 mg/ml, 15 mg/ml, 20 mg/ml and 25 mg/ml of same extract was effective except for *L. monocytogenes*. Of Gram negative organisms, *E. coli* was more sensitive to extracts than *P. aeruginosa*. Amongst Gram positive test strains, higher to lower sensitivity was determined in the order *S. aureus* > *B. subtilis*> *L. monocytogenes*.

E. coli was observed to resistant to 1 mg/ml concentration of crude methanolic *T. indica* leaf extracts while zones were found for *E. coli, P. aeruginosa* and *S. aureus* with 10 mg/ ml and 50 mg/ml (Balasubramanian et al., 2010). *P. aeruginosa, B. subtilis* and *S. aureus* were determined to be sensitive to *T. indica* leaves methanolic crude extracts with 50 to 1000 µg /ml concentration while *E. coli* exhibited in-sensitivity at same concentration (Reddy et al., 2009). The aqueous and ethanolic extracts exhibited no activity against *E. coli, P. aeruginosa* and *S. aureus* whereas *E. coli* was sensitive to methanolic extracts (Kannan *et al., 2013).* These studies support our research findings. Some studies reported the insensitivity of alcoholic extracts of *T. indica* leaves to *E. coli* and *S. aureus* (Parekh and Chanda, 2007; Parekh and Chanda, 2008) which does not comply with the results of our study as the methanolic extracts showed zones against these two organisms at 10mg/ml concentration. Interestingly, the aqueous extracts in this study also revealed selective efficacy against

test pathogens. No study has been conducted by *T. indica* leaf extracts on *L. monocytogenes*. This is the first report of such findings. The plant extracts exhibited low potential against *L. monocytogenes.* 100 mg/ml of ethanolic extracts determined maximum zone (18.3±0.5) against this organism.

The mode of action of crude alkaloid has been reported in the literature. The crude alkaloid extract of *T. indica* leaves (which possess phenanthroindolizidine alkaloids such as tylophorine and tylophorinine) inhibit protein synthesis, cause leakage of proteins and sugars from the bacterial cell membranes and cause cell death (Sathyabama et al., 2013). The performance of different extracts at same concentration, exhibited quite variation against tested strains in this work. This may be because of the difference in solubility of active compounds with solvents of different polarity (Gami and Parmar, 2010).

4.3.4 Antimicrobial activity of roots of *T. indica*

With 5 mg/ml of the methanolic extracts, no activity was found against TO1E, TO2P, TO4B and TO5L. The zone (in mm) against TO3S was found to be 9.7± 0.5. At 10 mg/ml, no activity was found against TO1E, TO2P and TO5L. The zones (in mm) against TO3S and TO4B were found to be 11.7±0.5 and 9.7± 0.5 respectively. At 15 mg/ml, no activity was found against TO1E, TO2P and TO5L. The zones (in mm) against TO3S and TO4B were found to be 12.7±0.5 and 10.3± 0.5 respectively. At 20mg/ml, no activity was found against TO1E, TO2P and TO5L by the methanolic roots extract. The zones (in mm) against TO3S and TO4B were found to be 13.6±0.9 and 10.7±0.5 respectively. At 25 mg/ml, no activity was found against TO1E, TO2P and TO5L. The zones (in mm) against TO3S and TO4B were found to be 14.7±0.9 and 11.3±0.47 respectively. At 50 mg/ml, no activity was found against TO1E and TO2P. The zones (in mm) against TO3S, TO4B and TO5L were found to be 15.6± 0.9, 14.3±0.9 and 10.7±0.5 respectively. With 100 mg/ml of the methanolic extracts, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 11.3±0.9, 13.3± 0.5, 16.3±0.5, 15.6±0.5 and 15.3±0.5 respectively. No activity of DMSO was exhibited against any of the indicator organisms (Table 4.10; Photograph 4.4; Figure 4.2).

With 5 mg/ml of the ethanolic extracts, no activity was found against TO1E, TO2P and TO5L. The zones (in mm) against TO3S and TO4B were found to be 9.3±0.5 and 10.3±0.5 respectively. At 10mg/ml, no activity was found against TO1E, TO2P and TO5L. The zones (in mm) against TO3S and TO4B were found to be 9.6±0.4, and 11.3±1.24 respectively. At 15 mg/ml, no activity was found against TO2P and TO5L. The zones (in mm) against TO1E, TO3S and TO4B were found to be 9.3±0.5, 10.3±0.5 and 12±0 respectively. At 20mg/ml of the ethanolic roots extract, no activity was found against TO2P and TO5L. The zones (in mm) against TO1E, TO3S and TO4B were found to be 9.7±0.4, 10.7±1.2 and 12.3±0.5 respectively. At 25mg/ml, no activity was found against TO2P and TO5L. The zones (in mm) against TO1E, TO3S and TO4B were found to be 10.7±0.4, 11.3±0.5 and 12.7±0.9 respectively. At 50mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 13.6±0.9, 10.3±1.2, 12.7±1.7, 14.3±1.2 and 10.6±0.5 respectively. With 100 mg/ml of the ethanolic root extract, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 18.3±0.5, 11.7±1.2, 13±0.8, 17.3±1.2 and 16.3±0.5 respectively. No activity of DMSO was exhibited against any of the indicator organisms (Table 4.6; Photograph 4.5; Figure 4.2).

With 5 mg/ml and 10mg/ml, no activity was found against any of the indicator organisms by the aqueous roots extract. At 15 mg/ml the zone against B. subtilis exhibited was 10.7±0.5. At 20mg/ml, no activity was found against TO1E, TO2P, TO3S and TO5L. The zone (in mm) against TO4B was found to be 13.3±0.5. At 25mg/ml, no activity was found against TO1E, TO2P, TO3S and TO5L. The zone (in mm) against TO4B was found to be 13.7±1.2 by the aqueous roots extract. At 50 mg/ml, no activity was found against TO3S and TO5L. The zones (in mm) against TO1E, TO2P and TO4B were found to be 10.3±0.5, 10±1.4 and 14.3±0.5 respectively. With same extracts at 100 mg/ml concentration, no activity was found against TO5L. The zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 12.7±0.5, 12.3±0.5, 11.3±0.9 and 17.3±0.9 respectively. No activity of DMSO was exhibited against any of the indicator organisms (Table 4.6; Photograph 4.6; Figure 4.2).

Not much literature is found for the antimicrobial potentials of *T. indica* roots with different pathogenic strains. The methanolic extracts with concentration of 120 μg/ ml was found to inhibit gram positive organisms such as *B. subtilis* and *S. aureus* while no activity was found against gram negative organisms such as *E. coli* and *P aeruginosa* (Reddy *et al.*, 2009). 50 mg/ml methanolic and aqueous concentration of *T. indica* root was found to inhibit *E. coli* and *B. cereus* (Gami and Parmar, 2010). 1 mg/ml concentration of methanolic root extracts of *T. indica* was ineffective towards *E. coli, S. aureus* and *P. aeruginosa* whereas 10 mg/ml and 50 mg/ml concentrations were determined effective (Balasubramanian et al., 2010). In another similar study, the methanolic and aqueous extracts were found to inhibit *E. coli* (Sangeetha *et al.*, 2012; Raut *et al.*, 2012). This is the first report of activity of *T. indica* root extracts against *L. monocytogenes*.

4.3.5 Antimicrobial potential of Partially Purified Bacteriocins of standard (Plantaracin 2083) and isolated LAB (Enterocin YT3) strains

4.3.5.1 Antimicrobial potential of Plantaracin 2083

The partially purified bacteriocin from *L. plantarum* NCIM2083 exhibited wide spectrum antimicrobial activity. Various dilutions of the partially purified bacteriocin were prepared (50%, 75%, 87.5%, 90%, 95% and 99%), analyzed for its antimicrobial potential against different food pathogens and has already been reported in the last chapter (Table 4.7; Photograph 4.7; Figure 4.3). In undiluted form, the zones against TO1E, TO2P, TO3S and TO4B were exhibited to be 14±0.81, 12.33±0.47, 12.3±0.9 and 18.33±0.47, respectively. With 50% dilution, the zones against TO1E, TO2P, TO3S and TO4B were exhibited to be 11.0±0.81, 10.33±0.47, 11±0.8 and 9.33±0.47, respectively. With 75% dilution, the zones (mm) against TO2P and TO3S were exhibited to be 9.33±0.47, and 10±0.8, respectively. With 87.5%, 90% and 95% dilution, the zones against TO3S was exhibited to be 9.7±0.5, 9.3±0.4 and 9±0.

The activity unit/ml (in AU/ml) against TO1E, TO2P, TO3S and TO4B was found to be 140, 123.3, 183.3 and 103.3 respectively. At 50% dilutions, the AU/ml against TO1E, TO2P, TO3S and TO4B and was determined to be 110, 103.3, 103.3 and 93.3 respectively. With 75% dilution, the AU/ml against TO2P and TO3S was calculated

to be 93.3. Only TO3S was sensitive at 87.5%, 90% and with 95% dilution and the AU/ml was calculated as 93.3, 93.3 and 90 respectively. 99% dilution did not determine any activity (Table 4.7).

Although the bacteriocins are generally effective against gram positive organisms, few LAB strains, has been reported to possess wide spectrum of antimicrobial activity (De Kwaadsteniet et al., 2006; Drieder et al., 2006; Line et al., 2008). Different strains of *L. plantarum* can be cited where broad spectrum of the antimicrobial behaviour of the obtained bacteriocin has been determined. Plantaracin F, sourced from *L. plantarum* BF001, exhibited strong inhibition of strains such as *S. aureus, L. monocytogenes* and *P. aeruginosa* (Fricourt et al., 1994). Plantaracin ZJ5, sourced from *L. plantarum* ZJ5, was found to inhibit *E. coli, B. subtilis, P. aeruginosa, L. monocytogenes, Salmonella spp., Shigella dysenteriae* and *S. aureus (*Song et al., 2014).

4.3.5.2 Antimicrobial potential of Enterocin YT3

Similarly, partially purified bacteriocin from *Enterococcus sp. YT3* exhibited wide spectrum antimicrobial activity where different dilutions of the semi purified bacteriocin were prepared (50%, 75%, 87.5%, 90%, 95% and 99%), and analyzed for its antimicrobial potential against different food pathogens which has already been reported in the last chapter (Table 4.11; Photograph 4.8; Figure 4.3). In undiluted form, the zones against TO1E, TO2P, TO3S and TO4B and TO5L were exhibited to be 17.7±0.9, 20.3±0.5, 17±0, 18.3±0.5 and 14.7±0.9, respectively. With 50% dilution, the zones against TO1E, TO2P, TO3S and TO4B were exhibited to be 16.7±2.3, 14.3±0.5, 31±0 and 14.3±0.5, respectively. With 75% dilution, the zones (mm) against TO3S were exhibited to be 10.3±0.5. 87.5%, 90%, 95% and 99% dilutions were ineffective against any test organism (Table 4.7).

The activity unit/ml (in AU/ml) against TO1E, TO2P, TO3S and TO4B was found to be 177, 203, 170 and 183 respectively in undiluted state. At 50% dilutions, the AU/ml against TO1E, TO2P, TO3S and TO4B and was determined to be 167, 143, 310.3 and 143 respectively. With 75% dilution, the AU/ml against TO3S was calculated to be

103. Further dilutions did not determine any activity against any test strains (Table 4.7).

Variations in antimicrobial attitude of bacteriocins towards different bacteria, including pathogens, from Enterococcus species could be determined in the literature. The bacteriocin from *Enterococcus mundtii* QU2 was ineffective against Gram negative bacteria such as *E. coli* but inhibited Gram positive strains such as *Listeria innocua* and *S. aureus* (Zendo et al., 2005). The bacteriocins from *E. gallinarium* strain 012, *E. faecalis* S-47, *E. faecalis Subsp. liquefaciens S-48*, *E. faecalis* BFE 1071, *E. faecium* A2000, *E. faecium* CRL 1385 and *E. faecium* BFE900 exhibited wide spectrum biological potential (Franz et al. 1996; Fisher and Philips, 2009).

It has already been reported that the electrostatic interactions of the bacteriocin from LAB with the bacterial membranes cause the primary binding leading to pore formation and the change of potential of cytoplasmic membrane. There is a trigger in this mechanism when the bacteriocin of class II group binds to the protein receptor of the cell membrane of the host bacteria. (Sabo et al., 2014) The PPB has the ability to inhibit the spore forming organism, *Bacillus*, which is more often found in the contamination of food products especially meats. (Yamamoto et al., 2013) These characteristics enable the promising nature of the biological component from *L. plantarum* and *Enterococcus sp. YT3* as a natural preservative.

The results observed in this study depict the wide spectrum antimicrobial potential of both semi purified bacteriocins. Hence, the results are in coherence with the studies as unfolded in the literature.

4.3.6 Antimicrobial Activity of Nisin

No zones were observed against TO1E and TO2P by nisin at 500 IU/ml concentration. The zones (in mm) against TO3S, TO4B and TO5L were found to be 14.3±0.5, 10.3±1.7 and 16.3±0.5 respectively. At 1000 IU/ml concentration, no zones were observed against TO1E and TO2P. The zones (in mm) against TO3S, TO4B and TO5L were found to be 16.3±0.5, 11±1.4 and 19.3±0.9 respectively. At 2000 IU/ml concentration, no zones were observed against TO1E. The zones (in mm) against TO2P, TO3S, TO4B and TO5L were found to be 11±1.4, 16.7±0.5, 12±0.8 and

20.6±0.5 respectively. At 2500 IU/ml concentration, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 10±0.8, 11±0, 17.3±0.5, 12.7±0.4 and 21±0.8 respectively. The zones (in mm) at 5000 IU/ml concentration against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 10.7±0.9, 14±1.4, 17.7±0.4, 16.7±1.9 and 22.3±1.2 respectively. The zones (in mm) at 10,000 IU/ml concentration against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 11.7±1.7, 18.3±0.5, 19.3±0.5, 17.3±2.5 and 24.3±2.6 respectively. No activity of 0.02M acetic acid, taken as the control, was exhibited against any of the indicator organisms (Table 4.8; Photograph 4.9; Figure 4.4).

The activity unit/ml (AU/ml) for nisin at 500 IU/ml against TO3S, TO4B and TO5L has been calculated to be 143, 103 and 163, respectively. At 1000 IU/ml concentration, the activity unit/ml (AU/ml) against TO3S, TO4B and TO5L has been 163, 103 and 193, respectively. At 2000 IU/ml concentration, the activity unit/ml (AU/ml) against TO2P, TO3S, TO4B and TO5L has been 110, 166, 120 and 206 respectively. At 2500 IU/ml concentration, the activity unit/ml (AU/ml) against TO1E, TO2P, TO3S, TO4B and TO5L has been 100, 110, 173.3, 126.6 and 210 respectively. At 5000 IU/ml concentration, the activity unit/ml (AU/ml) against TO1E, TO2P, TO3S, TO4B and TO5L has been 106.6, 140, 176, 166.6 and 223.3 respectively. At 10,000 IU/ml concentration, the activity unit/ml (AU/ml) against TO1E, TO2P, TO3S, TO4B and TO5L has been 103.3, 183.3, 193.3, 166.6 and 243.3, respectively (Table 4.8; Photograph 4.9; Figure 4.4).

The maximum level of nisin that has been approved by FDA for pasteurized processed cheese products is 10,000 IU/ml, while 153.6 IU/ml has been found to be MIC for *L. monocytogenes* which was too low for inhibition for this microbe in the food product (Franklin et al., 2004). 100 IU/ml, 200 IU/ml, 300 IU/ml, 400 IU/ml and 500 IU/ml concentrations of nisin was ineffective against different organisms such as *B. subtilis, Salmonella, L. bulgaricus* and *S. thermophiles* (Li et al., 2005). 1000 IU/ml and 100 IU/ml concentration of nisin has been determined to be MIC for *E. coli* and *L. monocytogenes,* respectively (Murdock et al., 2007). 2000 IU/ml, >2000 IU/ml 500 IU/ ml and 500 IU/ml concentration of nisin has been determined to be MIC for

Bacillus cereus, S. aureus , *P. fluorescens* and *E. coli,* respectively (Nissa et al., 2014). Hence, six different ranges have been tested in this study ranging from 500 IU/ml to 10,000 IU/ml. The efficacy of different concentrations of nisin against test pathogens has been presented in table 1.1. No zone of inhibition for *E. coli* and *P. aeruginosa* was found at 500 IU/ml and 1000 IU/ml. With 2500 IU/ml, 5,000 IU/ml and 10,000 IU/ml concentration, nisin exhibited 10±0.8 mm, 10.7±0.9 mm and 11.7±1.7 mm zones respectively for *E. coli*. Maximum potential was found against *L. monocytogenes* followed by *S. aureus* and *Bacillus subtilis.* These results comply with the literature where nisin has been found to be effective against gram positive microbes such as *S. aureus, L. monocytogenes* and *B. subtilis* but not against gram negative organisms as *E. coli and P. aeruginosa* at lower concentrations (Jamuna *et al* 2005, Chung *et al* 1989, Gharsallaoui *et al* 2015). Nisin has the ability to cause cell death in gram positive organisms due to its dual action to cause interference of cell wall synthesis along with pore formation in cytoplasmic membrane leading to changes in membrane permeability (Balucinas *et al* 2013, Bower *et al* 1995). Gram negative bacteria have an outer membrane which acts as the hurdle and arrests the molecules to reach the cell membrane thereby protecting the organism from antibacterial agents such as nisin (Stevens *et al.,* 1991).

4.3.7 Antimicrobial activity of binary combination of extracts of *T. indica* and Plantaracin 2083

4.3.7.1 Antimicrobial activity of leaf extracts of *T. indica* and Plantaracin 2083

With 50 mg/ml of *T. indica* methanolic extracts of leaves, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 20.6±0.9, 16±0.8, 25.3±0.5 and 23.3±0.5 respectively. At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 21.3±0.5, 15.7±0.4, 21.3±0.5 and 21±0. At 10 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 19.3±0.5, 15.3±0.4, 18±0 and 18±0. No zones were found against *TO5L* with any of the combinations (Table 4.9; Photograph 4.10; Figure 4.5).

With 50 mg/ml of *T. indica* ethanolic extracts of leaves, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 22.3±0.4, 19±0, 24.6±0.5 and 22.3±0.5 respectively. At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S

and TO4B were found to be 20.7±0.9, 18.7±1.7, 22.3±0.5 and 20±0. At 10 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 20.3±0.4, 16.7 ±2.5, 20.7±0.9 and 16.7±0.9. No zones were found against TO5L with any of the combinations (Table 4.9; Photograph 4.11 ; Figure 4.5).

With 50 mg/ml of *T. indica* aqueous extracts of leaves, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 20.3±2, 16.7±0.9, 19.3±0.4 and 19.7±0.9 respectively. At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 20.3±3.4, 16.3±1.9, 18±0 and 18±0. At 10 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 19.7±0.9, 15.3±0.5, 15±0 and 16.3±0.4. No zones were found against TO5L with any of the combinations (Table 4.9; Photograph 4.12; Figure 4.5).

4.3.7.2 Antimicrobial activity of root extracts of *T. indica* and Plantaracin 2083

With 50 mg/ml of methanolic root extracts, the zones (in mm) against TO1P, TO2P, TO3S and TO4B were found to be 21.3±1.2, 16.3±0.4, 22.7±0.9 and 22.3±0.5 respectively. At 20 mg/ml, the zones (in mm) against TO1P, TO2P, TO3S and TO4B were found to be 20.3±0.4, 14.3±0.4, 21.3±0.5 and 18.3±0.5. At 10 mg/ml of same extracts, the zones (in mm) against TO1P, TO2P, TO3S and TO4B were found to be 20±1.4, 14±2.1, 18.3±0.5 and 16±0. No zones were found against TO5L with any of the combinations (Table 4.10; Photograph 4.10).

With 50 mg/ml of ethanolic root extracts, the zones (in mm) against TO1P, TO2P, TO3S and TO4B were found to be 23±0, 20±0, 23.7±1.7 and 21.3±0.5 respectively. At 20 mg/ml of same extracts, the zones (in mm) against TO1P, TO2P, TO3S and TO4B were found to be 20.7±0.5, 19±0.8, 20.7±0.9 and 18±0. At 10 mg/ml, the zones (in mm) against TO1P, TO2P, TO3S and TO4B were found to be 20.3±0.9, 18.3±1.2, 16.3±0.5 and 16±0. No zones were found against TO5L with any of the combinations (Table 4.10; Photograph 4.11).

With 50 mg/ml of aqueous root extracts, the zones (in mm) against TO1P, TO2P, TO3S and TO4B were found to be 18.3±1.2, 18±2.1, 19.3±0.5 and 18.7±0.9 respectively. At 20 mg/ml, the zones (in mm) against TO1P, TO2P, TO3S and TO4B were found to be 16.7±1.7, 16.3±2.6, 17.7±0.9 and 16.7±0.9. At 10 mg/ml of same extracts, the zones (in mm) against TO1P, TO2P, TO3S and TO4B were found to be

15.7±3, 13.3±2, 16±0 and 14.3±0.5. No zones were found against TO5L with any of the combinations (Table 4.10; Photograph 4.12).

4.3.7 Antimicrobial activity of binary combination of extracts of *T. indica* and Enterocin YT3

4.3.7.1 Antimicrobial activity of leaf extracts of *T. indica* and Enterocin YT3

With 50 mg/ml of *T. indica* methanolic leaf extracts, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 14±0.8, 11±0, 11.7±0.5 and 20.3±0.5, respectively. At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 11.7±0.5, 10±0.47, 10.6±0.5 and 13.3±0.5. At 10 mg/ml of methanolic leaf extracts, the zones (in mm) against TO1E, TO3S and TO4B were found to be 9.7±0.5, 9.6±0.5 and 10.3±0.47 while it was ineffective against TO2P. No zones were found against TO5L with any of the combinations (Table 4.11; Photograph 4. 13; Figure 4.6).

With 50 mg/ml of ethanolic extracts, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 16.7±0.9, 10.3±0.5, 11.6±0.47 and 21.6±1.24 respectively. At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 14.7±0.4, 10±0.8, 10.3±0.5 and 20±8. At 10 mg/ml of same extracts, the zones (in mm) against TO1E, TO3S and TO4B were found to be 11.3±0.47, 9.3±0.5 and 17.3±2 while it was ineffective against TO2P. No zones were found against TO5L with any of the combinations (Table 4.11; Photograph 4.14; Figure 4.6).

With 50 mg/ml of aqueous extracts, the zones (in mm) against TO1E, TO2P, TO3S, TO4B were found to be 16±0.8, 11.3±0.47, 15.3±0.47 and 18±0 respectively. At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 12±0, 10±0.8, 11.3±0.4 and 14.3±0.5 respectively. With 10 mg/ml of aqueous extracts, the zones (in mm) against TO1E, TO3S and TO4B were found to be 11.3±0.5, 9.3±0.47, 12.7±0.5, respectively. No zones were found against TO2P at this concentration. . No zones were found against TO5L with any of the combinations (Table 4.11; Photograph 4.15; Figure 4.6).

4.3.7.2 Antimicrobial activity of root extracts of *T. indica* and Enterocin YT3

With 50 mg/ml of methanolic root extracts and Enterocin YT3, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 12.3±1.2, 12.3±0.9, 11±0 and 16.7±0.9 respectively. At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 11±1.4, 9.3±0.47, 10.3±0.47 and 14.7±0.9, respectively. At 10 mg/ml of methanolic root extracts with same bacteriocin concentration, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 10.3±0.5, 9±0, 9.7±0.47 and 11.7±0.47 respectively. No zones were found against L. monocytogenes with any of the combinations (Table 4.12; Photograph 4.13; Figure 4.7).

With 50 mg/ml of ethanolic root extracts and Enterocin YT3, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 15.7±1.7, 16±0.8, 10.3±0.5 and 22.3±0.47 respectively. At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 13.7±0.5, 12.3±0.5, 9.3±0.5 and 20.3±0.5 respectively. At 10 mg/ml of ethanolic root extracts and Enterocin YT3, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 11.3±0.5, 10.3±0.5, 9±0 and 16.7±0.9 respectively. No zones were found against L. monocytogenes with any of the combinations (Table 4.12; Photograph 4.14; Figure 4.7).

With 50 mg/ml of aqueous root extracts and Enterocin YT3, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 14.3±0.5, 14±0.8, 12.3±0.5 and 15±0 respectively. At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 12±0, 12.3±0.5, 10.3±0.47 and 12.7±0.9 respectively. At 10 mg/ml of ethanolic root extracts and Enterocin YT3, the zones (in mm) against TO1E, TO2P, TO3S and TO4B were found to be 10.3±0.5, 9.7±0.5, 9±0 and 11.3±0.47 respectively. No zones were found against L. monocytogenes with any of the combinations (Table 4.12; Photograph 4.15; Figure 4.7).

Enterocin A and thyme essential oils were evaluated for their antimicrobial potential against *E. coli* and *L. monocytogenes*. Individually, Enterocin A and thyme essential oils, solely, exhibited MIC for *L. monocytogenes* with 457µg/ml and 3.6µg/ml, respectively and for *E. coli*, Enterocin A was not effective but thyme essential oil inhibited the organism with 2.2µg/ml concentration. In combination, concentration

(μg/ml) of Enterocin A with thyme essential oils exhibited MIC for *E. coli* and *L. monocytogenes* was 0.9/1.2 and 34/0.71, respectively (Ghrairi and Hani, 2015).

4.3.9 Antimicrobial activity of binary combination of *T. indica* and Nisin

4.3.9.1 Antimicrobial activity of leaf extracts of *T. indica* and Nisin

Combination of nisin at 250 IU/mg and at 10 mg/ml of *T. indica* methanolic leaf extracts, no activity was found against TO1E, TO2P and TO4B. Same zones (mm) against TO3S and TO5L were determined as 9.7±0.4. With 20 mg/ml, zones (mm) against TO3S and TO5L were determined to be 11.7±0.9 and 10.3±1.2. With 50 mg/ml, zones (mm) against TO1E, TO3S, TO4B and TO5L were determined to be 11.7±0.5, 14.3±0.5, 9.7±0.9 and 11.7±0.5. With nisin at 500 IU/mg and at 10 mg/ml of *T. indica* methanolic leaf extracts, no activity was found against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 13.3±2.6, 11±0.8, 9.3±0.4 and 10.3±0.4 respectively. At 20 mg/ml, no activity was found against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 14.3±2.6, 12.7±0.9, 10.3±1.2 and 11.6±0.9 respectively. At 50 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 15±0.8, 11.7±0.5, 17.3±0.4, 11±0.8 and 12.3±0.4 respectively (Table 4.13; Photograph 4.16; Figure 4.8). With nisin at 1000 IU/ml and 10 mg/ml of *T. indica* methanolic leaf extracts, no activity was found against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 15±2.9, 13±0.8, 10.3±0.5 and 11.3±0.5 respectively. At 20 mg/ml, no activity was found against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 15.3±2.6, 13.7±1.7, 10.7±0.5 and 12.7±0.9 respectively. At 50 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 15.7±0.5, 11.7±0.5, 16±2.1, 11±0.8 and 13±0.8 respectively (Table 4.13; Photograph 4.17; Figure 4.8). With nisin at 2000 IU/mg and 10 mg/ml of *T. indica* methanolic leaf extracts, no activity was found against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 16±2.8, 13.7±0.9, 10.7±0.5 and 13.3±0.4 respectively. At 20 mg/ml, no activity was found against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 16.7±1.7, 15±2.1, 11.7±0.5 and 14±3.7 respectively. At 50 mg/ml,

the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 16.7±0.5, 12±0.8, 18.7±0.9, 12.3±0.4 and 15.3±0.5 respectively (Table 4.13; Figure 4.8). With nisin at 2500 IU/mg and 10 mg/ml of *T. indica* methanolic leaf extracts, no activity was found against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 17±2.1, 14.3±0.9, 11.7±0.4 and 13.7±2.6 respectively. At 20 mg/ml, no activity was found against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 18±0.8, 15.7±3, 12±0.8 and 14.3±0.4 respectively. At 50 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 18.3±0.4, 12.3±0.5, 19±0.8, 12.3±0.4 and 15.3±0.5 respectively (Table 4.13; Photograph 4.18; Figure 4.8).

Combination of nisin at 250 IU/mg and at 10 mg/ml of *T. indica* ethanolic leaf extracts, no activity was found against TO1E, TO2P, TO3S and TO4B. The zone (mm) against TO5L was determined as 15.3±1.9. With 20 mg/ml, zones (mm) against TO3S and TO5L were determined to be 10.3±0.4 and 16±0.8. With 50 mg/ml, zones (mm) against TO1E, TO3S, TO4B and TO5L were determined to be 10.7±0.9, 11.6±0.9, 12.3±0.5 and 17±0.With nisin at 500 IU/mg and 10 mg/ml of ethanolic leaf extracts, no activity was found against TO1E and TO2P. The zones (in mm) against TO3S, TO4B and TO5L were found to be 11±0.8, 11.3±0.5 and 18±1.4 respectively. At 20 mg/ml, no activity was found against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 11.3±0.4, 14±0, 12±0 and 18.3±1.2 respectively. At 50 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 12.3±1.8, 10.3±4.0, 14.7±0.9, 14.7±0.5 and 19.3±0.5 respectively (Table 4.13; Photograph 4.19; Figure 4.8). Combination with nisin at 1000 IU/mg and 10 mg/ml of ethanolic leaf extracts, no activity was found against TO1E and TO2P. The zones (in mm) against TO3S, TO4B and TO5L were found to be 12.3±0.5, 11.7±0.4 and 18.7±1.2 respectively. At 20 mg/ml, no activity was found against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 12.7±1.6, 14.7±0.4, 12.7±0.5 and 19±1.6 respectively. At 50 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 15±0.8, 10.7±0.5, 15.3±0.5, 15±0 and 20±1.6 respectively. Binary combination of nisin at 2000 IU/mg with 10 mg/ml of ethanolic leaf extracts, no activity was found against

TO1E and TO2P. The zones (in mm) against TO3S, TO4B and TO5L were found to be 14±0.8, 12.3±1.2 and 19.7±2.6 respectively. At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 15±0.8, 9.7±0.9, 15.7±0.9, 13.3±0.4 and 20.3±2.5 respectively. At 50 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 16.7±0.5, 11.3±0.5, 16.7±1.2, 15.3±0.5 and 20.7±0.5 respectively (Table 4.13; Photograph 4.20, Figure 4.8). On combining nisin at 2500 IU/mg with 10 mg/ml of ethanolic leaf extracts, no activity was found against TO1E and TO2P. The zones (in mm) against TO3S, TO4B and TO5L were found to be 15.7±0.5, 12±0 and 21±2.4 respectively. At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 16±0.8, 10±0.8, 16±1.6, 14±0.8 and 21.7±1.2 respectively. At 50 mg/ml with same nisin concentration, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 17.3±0.5, 12±0, 17.7±1.2, 15.7±0.9 and 22±0.8 respectively (Table 4.13; Photograph 4.21; Figure 4.8).

Combination of nisin at 250 IU/mg and at 10 mg/ml of *T. indica* aqueous leaf extracts, no activity was found against TO1E, TO2P, and TO4B (Photograph 4.22). The zone (mm) against TO3S and TO5L was determined as 10.7±0.5 and 10±0, respectively. With 20 mg/ml, zones (mm) against TO3S, TO4B and TO5L were determined to be 12±0, 9.7±0.5 and 13.3±0.4, respectively. With 50 mg/ml, zones (mm) against TO2P, TO3S, TO4B and TO5L were determined to be 11.6±0.9, 13±0.8, 10.3±0.4 and 14.3±1.2, respectively (Photograph 4.23). Binary combination of nisin at 500 IU/mg with 10 mg/ml of aqueous leaf extracts, no activity was found against TO1E and TO2P. The zones (in mm) against, TO3S, TO4B and TO5L were found to be 13±1.4, 10.7±0.9 and 15.7±4.4 respectively. At 20 mg/ml, no activity was found against TO1E and TO2P. The zones (in mm) against TO3S, TO4B and TO5L were found to be 14.3±0.9, 12.3±1.2 and 16.3±5.4 respectively. At 50 mg/ml of aqueous leaf extracts with same nisin concentration, the zones (in mm) against TO2P, TO3S, TO4B and TO5L were found to be 13±1.4, 16.3±1.2, 13.7±1.7 and 18±4.9 respectively (Table 4.13; Figure 4.8; Figure 4.8). Combining nisin at 1000 IU/mg with 10 mg/ml of aqueous leaf extracts, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 13±0.5, 10.3±1.4, 14.3±0.9, 11.3±0.4 and 16.3±4

respectively. The zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 13.3±0.5, 10.3±1.8, 15±0.8, 13±0.8 and 16.7±3.8 respectively. At 50 mg/ml with same nisin concentration, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 12.7±0.4, 14.3±0.4, 17.3±0.5, 14±0.8 and 18.7±5.0 respectively (Table 4.13; Figure 4.8). With nisin at 2000 IU/mg and aqueous leaf extracts at 10 mg/ml, the zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 14±0.4, 16.3±2.8, 12.7±0.9 and 18±4.9 respectively. The zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 14.3±1.2, 11±2.1, 16.7±1.2, 14±1.6 and 19±6.3 respectively. At 50 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 14±0, 14.7±0.9, 17.7±0.4, 14.3±1.2 and 21.3±5.7 respectively (Table 4.13; Photograph 4.24; Figure 4.8). Combination of nisin at 2500 IU/mg with 10 mg/ml of aqueous leaf extracts, the zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 14.7±0.5, 17±1.4, 13.7±1.2 and 19±3.6 respectively. The zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 15.3±1.2, 11.7±1.7, 17±2.1, 14.7±1.2 and 20±4.5 respectively. At 50 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 14.3±0.5, 15.3±1.9, 18±1.4, 15±1.6 and 21.7±7.4 respectively (Table 4.13; Figure 4.8).

Three concentrations of *T. indica* different extracts have been tested with six different concentrations of nisin. The nisin alone at the concentration of 2500 IU/ml and above is able to inhibit all the five food pathogens under study. When used in the combination, the 500 IU/ml concentration along with 20mg/ml of *T. indica* root water extract is able to inhibit all the pathogens. From the results is concluded that the spectrum of the activity is enhanced by the binary combination. This is the first reporting of nisin with *T. indica* crude extracts.

Nisin is one of the potential natural preservative being widely used worldwide. Synergism of nisin with various volatile oils containing plants has been reported such as carvacrol and Cinnamaldehyde. These studies indicate the potential of the combination against gram positive organisms such as *B. cereus, L. monocytogenes* and *B. subtilis* and gram negative organisms such as *E. coli* (Field *et al.,* 2017). The use of *Arctostaphylos uva-ursi* (bearberry) ethanolic extract enhanced the activity of

nisin against gram positive organisms only although it did not exhibited any biological potential alone (Carpenter *et al.,* 2007). When thymol and nisin were tested together, the results presented much better antilisterial and anti-bacillus effects as compare to when used alone (Ettayebi *et al.,* 2000). When nisin was used with garlic extracts, enhanced antilisterial potential was exhibited (Singh *et al.,* 2001). Such synergistic results also help reducing the dose of nisin with better efficacy and reduction in emergence of nisin resistant cases. The synergism, as shown by the results of this study, suggest that nisin with *T. indica* extracts may be a potential such as bio preservation of food products by their incorporation in packaging films.

4.3.9.2 Antimicrobial activity of binary combination of root extracts of *T. indica* and Nisin

Binary combination of nisin at 250 IU/mg with 10 mg/ml of methanolic root extracts, no activity was found against TO1E, TO2P, TO4B and TO5L. The zone (in mm) against TO3S was found to be 10±0. At 20 mg/ml, the zones (in mm) against TO3S and TO4B were found to be 11.7±0.9 and 9.7±0.9 respectively. At 50 mg/ml, the zones (in mm) against TO3S, TO4B and TO5L were found to be 12.3±0.4, 11.3±0.5 and 10.3±1.2, respectively. Binary combination of nisin at 500 IU/mg with 10 mg/ml of methanolic root extracts, no activity was found against TO1E, TO2P and TO5L (Photograph 4.25). The zones (in mm) against TO3S and TO4B were found to be 12.6±0.5 and 10.7±0.5, respectively. At 20 mg/ml, no activity was found against TO1E, TO2P and TO5L. The zones (in mm) against TO3S and TO4B were found to be 13.3±0.5 and 11.7±0.9 respectively (Photograph 4.26). At 50 mg/ml, no activity was found against TO2P. The zones (in mm) against TO1E, TO3S and TO4B and TO5L were found to be 11.7±0.5, 15.3±0.5, 13.3±1.2 and 11±0.8, respectively (Photograph 4.27). Nisin at concentration of 1000 IU/mg with 10 mg/ml of methanolic root extracts, no activity was found against TO1E and TO2P and TO5L. The zones (in mm) against TO3S and TO4B were found to be 13.7±0.9 and 11.3±0.5 respectively. At 20 mg/ml, no activity was found against TO1E, TO2P and TO5L. The zones (in mm) against TO3S and TO4B were found to be 14.6±0.9 and 12.7±0.5 respectively. At 50 mg/ml of methanolic root extracts with same nisin concentration,

no activity was found against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 13.7±0.5, 16.3±0.5, 13±0.8 and 11.3±0.5 respectively. Combination of nisin at 2000 IU/mg with 10 mg/ml of methanolic root extracts, no activity was found against TO1E and TO2P. The zones (in mm) against TO3S, TO4B and TO5L were found to be 14.3±0.5, 13.3±1.2 and 10.3±0.5 respectively. At 20 mg/ml, no activity was found against TO1E and TO2P. The zones (in mm) against TO3S, TO4B and TO5L were found to be 16.7±0.5, 14.3±3.3 and 10.7±0.5, respectively. At 50 mg/ml, no activity was found against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 14.3±0.5, 17.3±0.5, 14.7±1.2 and 13±1.6, respectively. Combining nisin at 2500 IU/mg with 10 mg/ml of methanolic root extracts, no activity was found against TO1E and TO2P. The zones (in mm) against TO3S, TO4B and TO5L were found to be 16.3±0.9, 17.3±1.9 and 12.3±1.2, respectively. At 20 mg/ml, no activity was found against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 12.7±0.9, 15±1.6, 17.3±2.1 and 13±0.8 respectively. At 50 mg/ml, no activity was found against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 15.3±0.5, 18±0.8, 17.7±1.7 and 14.7±0.5 respectively (Table 4.14; Figure 4.9).

Binary combination of nisin at 250 IU/mg with 10 mg/ml of ethanolic root extracts, no activity was found against TO1E, TO2P and TO5L. The zone (in mm) against TO3S and TO4B was found to be 10.3±0.4 and 9.7±0.9. At 20 mg/ml, the zones (in mm) against TO3S, TO4B and TO5L were found to be 11.6±0.9, 10.7±3.1 and 9±0 respectively. At 50 mg/ml, the zones (in mm) against TO3S, TO4B and TO5L were found to be 12.3±0.5, 11±0 and 11.7±0.4, respectively. On combining nisin at 500IU/mg with 10 mg/ml of ethanolic root extracts, the zones (in mm) against TO3S and TO4B were found to be 11±1.4 and 13±0, respectively (Photograph 4.28). At 20 mg/ml, no activity was found against TO1E. The zones (in mm) against TO2P, TO3S, TO4B and TO5L were found to be 9.3±0.4, 14±0.8, 14±0 and 13.7±0.9, respectively (Photograph 4.29). At 50 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 12.3±1.2, 12±0, 15±0.8, 14.3±0.5 and 14±1.6, respectively (Photograph 4.30). Binary combination of nisin at 1000 IU/mg with 10 mg/ml of ethanolic root extracts, the zones (in mm) against TO1E, TO3S, TO4B and

TO5L were found to be 10.7±3.1, 11.3±0.4, 13.7±0.5 and 14.7±0.9, respectively. At 20 mg/ml, zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 11.7±3.5, 9.7±0.9, 14.3±0.4, 14.3±0.5 and 15±1.4 respectively. 50 mg/ml of ethanolic root extracts with same nisin concentration zones (in mm) against TO1E, TO2P TO3S, TO4B and TO5L were found to be 14.3±0.5, 12.3±0.4, 15.3±0.5, 15±0.8 and 15.3±1.2 respectively. With nisin at 2000 IU/mg along with 10 mg/ml of ethanolic root extracts, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 13.7±2.3, 9.7±0.9, 12.3±0.5, 14.7±0.5 and 15.7±2.6, respectively. At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 15.7±0.5, 10±0.8, 14.7±0.9, 15±0 and 16±0.9, respectively. At 50 mg/ml, zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 16.3±0.5, 12.7±0.9, 16±0.8, 15.3±1.2 and 16.7±0.9, respectively. With nisin at 2500 IU/mg along with 10 mg/ml of ethanolic root extracts, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 15.3±0.4, 9.3±0.4, 13±0.8, 15.3±0.5 and 16.3±1.2 respectively. At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 16.3±1.2, 11±0.8, 15±0.8, 15.7±0.5 and 17±0.8 respectively. At 50 mg/ml, zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 17.3±0.5, 13±0.8, 15.3±0.5 and 16±2.2 and 17.7±1.2, respectively (Table 4.14; Figure 4.9).

Binary combination of nisin at 250 IU/mg with 10 mg/ml of aqueous root extracts, no activity was found against TO1E, TO2P and TO4B. The zone (in mm) against TO3S and TO5L was found to be 12±0 and 9.3±0.5. At 20 mg/ml, the zones (in mm) against TO3S, TO4B and TO5L were found to be 13.3±0.4, 9.7±0.5 and 10.3±0.5 respectively. At 50 mg/ml, the zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 14.6±0.91, 14±0, 10.7±0.5 and 11±0.8, respectively. Binary combination of nisin at 500 IU/mg along with 10 mg/ml of aqueous root extracts, exhibited no activity against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 12.6±0.9, 14±1.4, 10±0 and 10.3±0.4, respectively. At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 15±1.4, 10.3±0.5, 15.3±0.4, 13.3±0.4 and 11.3±0.4 respectively. At 50 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be

15.7±0.9, 13±2.1, 18±1.4, 14.3±1.2 and 12.7±1.7, respectively. The combination of nisin at 1000 IU/mg with 10 mg/ml of aqueous root extracts, exhibited no activity against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 13.7±2.3, 16.3±1.2, 9.7±0.9 and 13.7±1.7 respectively (Photograph 4.31). At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 16.6±0.9, 11.7±0.9, 17.3±0.4, 11.3±0.5 and 13.3±0.5 respectively (Photograph 4.32). At 50 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 16.3±0.5, 13.3±0.4, 19.3±3.6, 12±1.6 and 12.3±0.5 respectively (Photograph 4.33). Nisin at 2000 IU/mg with aqueous root extracts at 10 mg/ml, showed no activity against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 16±1.4, 17.3±1.2, 10.3±0.5 and 16±0.8 respectively. At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 17.7±0.9, 12.7±0.5, 18.7±1.7, 12±0 and 15±0.8 respectively. At 50 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 18.7±0.5, 14±0.8, 19.7±2.6, 12.37±1.2 and 15.3±0.9 respectively. Combining nisin at 2500 IU/mg with 10 mg/ml of aqueous root extract exhibited no potential against TO2P. The zones (in mm) against TO1E, TO3S, TO4B and TO5L were found to be 18.7±0.5, 18±0.8, 11.3±0.4 and 15±2.1 respectively. At 20 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 19.6±0.5, 13.3±1.2, 20.3±1.2, 12.7±0.5 and 16.7±1.7, respectively. At 50 mg/ml, the zones (in mm) against TO1E, TO2P, TO3S, TO4B and TO5L were found to be 20.7±0.5, 15±0.8, 20.3±2.9, 12.7±1.7 and 16±0.8 respectively (Table 4.14; Figure 4.9).

4.3.10 Screened and selected combinations of antimicrobials for preparation of active packaging films

In order to pursue for preparation of active packaging film and shelf life analysis of food, the minimum concentration of nisin with/without plant extract and plant extract with/ without nisin exhibiting activity against all test food pathogens has been considered. For only *T. indica* leaf extract, 50mg/ml methanolic extract, for *T. indica* root extract, 50mg/ml ethanolic extract, for only nisin, 2500 IU/ml nisin; for Nisin and *T. indica* leaf extract in combination, 1000 IU/ml of Nisin + 20mg/ml water extract of

T. indica leaf; and for Nisin and *T. indica* root combination, 500 IU/ml Nisin + 20mg/ml water extract of *T. indica* root has been selected (Table 4.15).

On analyzing the obtained antimicrobial data, the P- value for selected agents viz. *T. indica* leaf extract, *T. indica* root extract, nisin, nisin + *T. indica* leaf extract combination, and nisin + *T. indica* root combination, against *E. coli* , *P. aeruginosa, S. aureus, B. subtilis* and *L. monocytogenes* was found to be 0.014, 0.008, 0.023, 0.0108 and 0.0002, respectively. Therefore, the selected antimicrobial agents showed significant difference on the zone of inhibition against test indicator food pathogens (Table 4.15).

4.4 Statistical Analysis

The software, Excel 2010 in Microsoft Office programme has been used to calculate the mean along with standard deviation. Thereafter, same software was applied to determine statistical significance using one way ANOVA at 5% level of significance.

4.5 References

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Tables:

Table 4.1: Bacterial cultures used as indicator strains

Bacterial strain	Designated as	Source	Growth Media
S. aureus (MTCC96)	TO3S	ML, LPU*	Nutrient Broth/ Mannitol Salt Agar
B. subtilis (MTCC 121)	TO4B	ML, LPU*	Nutrient Broth/ Nutrient Agar
L. monocytogenes (MTCC1143)	TO5L	MTCC, Chandigarh	BHI Broth/ Agar
E. coli (MTCC1687)	TO1E	MTCC, Chandigarh	Nutrient Broth/ MacConkey agar
P. aeruginosa (MTCC4673)	TO2P	ML, LPU*	Nutrient Broth/ Cetrimide Agar
Incubation temp: 37^{o}C; Incubation time: 24 h MTCC: Microbial Type Culture Collection, Chandigarh, Punjab, India ML, LPU*: Microbiology Laboratory, Department of Microbiology, Lovely Professional University, Punjab, India.			

Table 4.2: Preparation of crude extractives of *T. indica* leaves and roots

S. No.	*T. indica* crude extract (Leaves and Roots)	Extractive yield		Working Concentration (mg/ml)
		Leaves	Roots	
1	Methanolic extract	28%	25%	100, 50 , 25, 20, 15, 10 5
2	Ethanolic extract	25%	24%	
3	Aqueous extract	21%	15%	
4	Ethyl acetate extract	5%	4%	-
Crude powder drug (g): Solvent (ml) =15: 200 Concentration of stock solution used: 200mg/ml Solvent used: Stock preparation: 100% DMSO; Working samples: 5% sterilized DMSO				

Table 4.3: Preparation of working samples of bacteriocin

Bacteriocin stock solution	Working test samples	Obtained concentration (IU/ml)
Undiluted Plantaracin 2083	100%, 50%, 75%, 82.5%, 90%, 95%, 99%	-
Undiluted Enterocin YT3	100%, 50%, 75%, 82.5%, 90%, 95%, 99%	-
20,000 IU/ml Nisin	50%, 75%, 80%, 87.5%, 90%, 95% and 97.5%	10,000, 5,000 4,000, 2500, 2000, 1000 and 500
Solvent used: Sterilized 0.02M acetic acid		

Table 4.4: Preparation of combinations of *T. indica* extract and bacteriocin

S. No	Bacteriocin		*T. indica* extract (leaves and roots)	
	Concentration used	**Concentration obtained (IU/ml) in the combination**	**Concentration used (mg/ml)**	**Concentration obtained (mg/ml) in the combination**
1	Undiluted Plantaracin 2083	50%	100	50
			40	20
			20	10
2	Undiluted Enterocin YT3	50%	100	50
			40	20
			20	10
1	20,000 (IU/ml)	10,000	100	50
			40	20
			20	10
2	10,000 (IU/ml)	5,000	100	50
			40	20
			20	10
3	5,000 (IU/ml)	2,500	100	50
			40	20
			20	10
4	2,500 (IU/ml)	2,000	100	50
			40	20
			20	10
5	2,000 (IU/ml)	1,000	100	50
			40	20
			20	10
6	1,000 (IU/ml)	500	100	50
			40	20
			20	10

- Double strength of samples were added in the test tube
- Bacteriocin: Plant extract added is 1:1
- Binary combination incubated at room temperature for 2 h

Table 4.5: Antimicrobial activity of leaves of *T. indica*

Sample	Conc. (mg/ml)	Organism	Methanolic Extract	Ethanolic Extract	Aqueous Extract
			ZOI (mm)	ZOI (mm)	ZOI (mm)
1	5	TO1E	Nil	Nil	Nil
		TO2P	Nil	Nil	Nil
		TO3S	9.3±0.5	Nil	9.6±0.4
		TO4B	Nil	Nil	Nil
		TO5L	Nil	Nil	Nil
2	10	TO1E	10.7±0.9	Nil	9.7±0.9
		TO2P	9.6±0.4	Nil	Nil
		TO3S	10.3±0.5	Nil	10.3±0.5
		TO4B	9.3±0.5	Nil	Nil
		TO5L	Nil	Nil	Nil
3	15	TO1E	11±1.6	Nil	11.3±0.5
		TO2P	10±0.8	Nil	Nil
		TO3S	11.7±0.9	Nil	10.7±0.4
		TO4B	10±0.8	Nil	9.3±0.5
		TO5L	Nil	Nil	Nil
4	20	TO1E	11.7±0.9	Nil	12±0
		TO2P	10.7±0.5	Nil	Nil
		TO3S	12.3±0.5	Nil	11.3±0.5
		TO4B	10.3±0.5	9±0	9.7±0.4
		TO5L	Nil	Nil	Nil
5	25	TO1E	11.3±0.5	Nil	13.7±0.4
		TO2P	11.7±0.4	Nil	Nil
		TO3S	12.7±0.5	Nil	11.3±0.5
		TO4B	10.7±0.9	10.3±0.5	9.7±0.4
		TO5L	Nil	11.3±0.5	Nil
6	50	TO1E	14±1.4	Nil	16±0.8
		TO2P	14.7±0.4	Nil	12.3±0.5
		TO3S	15±1.4	Nil	11.7±0.5
		TO4B	12.7±0.9	13.3±0.4	10.7±0.4
		TO5L	9.3±0.4	13±0.8	Nil
7	100	TO1E	15.7±1.7	14.7±0.5	16.7±1.2
		TO2P	16.7±0.9	11±0.8	13.3±0.4
		TO3S	16±1.4	Nil	12.3±0.5
		TO4B	14.3±0.5	14±0.8	12.7±1.2
		TO5L	9.7±0.4	18.3±0.5	13.3±0.5
8	DMSO (Control)	TO1E	Nil	Nil	Nil
		TO2P	Nil	Nil	Nil
		TO3S	Nil	Nil	Nil
		TO4B	Nil	Nil	Nil
		TO5L	Nil	Nil	Nil

- Dilutions of plant extracts have been prepared in 5% Dimethyl sulphoxide
- TO1E: Test organism 1, *E. coli;* TO2P: Test organism 2, *P. aeruginosa;* TO3S: Test organism 3, *S. aureus;* TO4B: Test organism 4, *B. subtilis;* TO5L: Test organism 5, *L. monocytogenes*
- **For antibacterial analysis:** Well- Diffusion Method (Well size:8mm); Amount of sample used: 100 µl; Incubation period: 24h, Incubation temp: 37°C; Media used: Muller Hinton Agar (Himedia); pH: 7.3±0.1, For *L. monocytogenes*: Listeria selective agar; pH: 7.3±0.2

Table 4.6: Antimicrobial activity of roots of *T. indica*

Sample	Conc. (mg/ml)	Organism	Methanolic extract	Ethanolic Extract	Aqueous Extract
			ZOI (mm)	ZOI (mm)	ZOI (mm)
1	5	TO1E	Nil	Nil	Nil
		TO2P	Nil	Nil	Nil
		TO3S	9.7± 0.5	9.3±0.5	Nil
		TO4B	Nil	10.3±0.5	Nil
		TO5L	Nil	Nil	Nil
2	10	TO1E	Nil	Nil	Nil
		TO2P	Nil	Nil	Nil
		TO3S	11.7±0.5	9.6±0.4	Nil
		TO4B	9.7± 0.5	11.3±1.24	Nil
		TO5L	Nil	Nil	Nil
3	15	TO1E	Nil	9.3±0.5	Nil
		TO2P	Nil	Nil	Nil
		TO3S	12.7±0.5	10.3±0.5	Nil
		TO4B	10.3±0.5	12±0	10.7±0.5
		TO5L	Nil	Nil	Nil
4	20	TO1E	Nil	9.7±0.4	Nil
		TO2P	Nil	Nil	Nil
		TO3S	13.6±0.9	10.7±1.2	Nil
		TO4B	10.7±0.5	12.3±0.5	13.3±0.5
		TO5L	Nil	Nil	Nil
5	25	TO1E	Nil	10.7±0.4	Nil
		TO2P	Nil	Nil	Nil
		TO3S	14.7±0.9	11.3±0.5	Nil
		TO4B	11.3±0.47	12.7±0.9	13.7±1.2
		TO5L	Nil	Nil	Nil
6	50	TO1E	Nil	13.6±0.9	10.3±0.5
		TO2P	Nil	10.3±1.2	10±1.4
		TO3S	15.6± 0.9	12.7±1.7	Nil
		TO4B	14.3±0.9	14.3±1.2	14.3±0.5
		TO5L	10.7±0.5	10.6±0.5	Nil
7	100	TO1E	11.3±0.9	18.3±0.5	12.7±0.5
		TO2P	13.3± 0.5	11.7±1.2	12.3±0.5
		TO3S	16.3±0.5	13±0.8	11.3±0.9
		TO4B	15.6±0.5	17.3±1.2	17.3±0.9
		TO5L	15.3±0.5	16.3±0.5	Nil
8	DMSO (Control)	TO1E	Nil	Nil	Nil
		TO2P	Nil	Nil	Nil
		TO3S	Nil	Nil	Nil
		TO4B	Nil	Nil	Nil
		TO5L	Nil	Nil	Nil

- Dilutions of the plant extracts have been prepared in 5% Dimethyl sulphoxide
- TO1E: Test organism 1, *E. coli;* TO2P: Test organism 2, *P. aeruginosa;* TO3S: Test organism 3, *S. aureus;* TO4B: Test organism 4, *B. subtilis;* TO5L: Test organism 5, *L. monocytogenes*
- For antibacterial analysis: Well- Diffusion Method (Well size:8mm); Amount of sample used: 100 µl; Incubation period: 24h, Incubation temp: 37°C; Media used: Muller Hinton Agar (Himedia); pH: 7.3±0.1, For *L. monocytogenes*: Listeria selective agar; pH: 7.3±0.2

Table 4.7: Antimicrobial potential of Partially Purified Bacteriocin

Sample	Dilutions of PPB	Test Organism	Activity Observed			
			L. plantarum		***Enterococcus sp. YT3***	
			ZOI(mm)	AU/ml	**ZOI(mm)**	AU/ml
1	Nil (No dilution)	TO1E	14±0.81	140	17.7±0.9	177
		TO2P	12.33±0.47	123.3	20.3±0.5	203
		TO3S	12.3±0.9	123	17±0	170
		TO4B	18.33±0.47	183	18.3±0.5	183
		TO5L	Nil	Nil	14.7±0.9	147
2	50%	TO1E	11.0±0.81	110	16.7±2.3	167
		TO2P	10.33±0.47	103.3	14.3±0.5	143
		TO3S	11±0.8	110	13±0	130
		TO4B	9.33±0.47	93.3	14.3±0.5	143
		TO5L	Nil	Nil	Nil	Nil
3	75%	TO1E	Nil	Nil	Nil	Nil
		TO2P	9.33±0.47	93.3	Nil	Nil
		TO3S	10±0.8	100	10.3±0.5	103
		TO4B	Nil	Nil	Nil	Nil
		TO5L	Nil	Nil	Nil	Nil
4	87.5%	TO1E	Nil	Nil	Nil	Nil
		TO2P	Nil	Nil	Nil	Nil
		TO3S	9.7±0.5	97	Nil	Nil
		TO4B	Nil	Nil	Nil	Nil
		TO5L	Nil	Nil	Nil	Nil
5	90%	TO1E	Nil	Nil	Nil	Nil
		TO2P	Nil	Nil	Nil	Nil
		TO3S	9.3±0.4	93	Nil	Nil
		TO4B	Nil	Nil	Nil	Nil
		TO5L	Nil	Nil	Nil	Nil
6	95%	TO1E	Nil	Nil	Nil	Nil
		TO2P	Nil	Nil	Nil	Nil
		TO3S	9±0	Nil	Nil	Nil
		TO4B	Nil	Nil	Nil	Nil
		TO5L	Nil	Nil	Nil	Nil
7	99%	TO1E	Nil	Nil	Nil	Nil
		TO2P	Nil	Nil	Nil	Nil
		TO3S	Nil	Nil	Nil	Nil
		TO4B	Nil	Nil	Nil	Nil
		TO5L	Nil	Nil	Nil	Nil

- Dilutions of PPB has been prepared in 0.02M Acetic acid
- TO1E: Test organism 1, *E. coli;* TO2P: Test organism 2, *P. aeruginosa;* TO3S: Test organism 3, *S. aureus;* TO4B: Test organism 4, *B. subtilis;* TO5L: Test organism 5, *L. monocytogenes*
- For antibacterial analysis: Well- Diffusion Method (Well size:8mm); Amount of sample used: 100 µl; Incubation period: 24h, Incubation temp: 37°C; Media used: Muller Hinton Agar (Himedia); pH: 7.3±0.1, For *L. monocytogenes*: Listeria selective agar; pH: 7.3±0.2

Table 4.8: Antimicrobial activity of Nisin

Sample	Nisin Conc. (IU/ml)	Organism	ZOI (mm)	AU/ml
1	500 IU	TO1E	Nil	Nil
		TO2P	Nil	Nil
		TO3S	14.3±0.5	143.3
		TO4B	10.3±1.7	103.3
		TO5L	16.3±0.5	163.3
2	1000 IU	TO1E	Nil	Nil
		TO2P	Nil	Nil
		TO3S	16.3±0.5	163.3
		TO4B	11±1.4	103.3
		TO5L	19.3±0.9	193.3
3	2000 IU	TO1E	Nil	Nil
		TO2P	11±1.4	110
		TO3S	16.7±0.5	166.6
		TO4B	12±0.8	120
		TO5L	20.6±0.5	206.6
4	2500 IU	TO1E	10±0.8	100
		TO2P	11±0	110
		TO3S	17.3±0.5	173.3
		TO4B	12.7±0.4	126.6
		TO5L	21±0.8	210
5	5000 IU	TO1E	10.7±0.9	106.6
		TO2P	14±1.4	140
		TO3S	17.7±0.4	176.6
		TO4B	16.7±1.9	166.6
		TO5L	22.3±1.2	223.3
6	10,000IU	TO1E	11.7±1.7	117
		TO2P	18.3±0.5	183
		TO3S	19.3±0.5	193
		TO4B	17.3±2.5	173
		TO5L	24.3±2.6	243

- Dilutions of Nisin has been prepared in 0.02M Acetic acid
- TO1E: Test organism 1, *E. coli;* TO2P: Test organism 2, *P. aeruginosa;* TO3S: Test organism 3, *S. aureus;* TO4B: Test organism 4, *B. subtilis;* TO5L: Test organism 5, *L. monocytogenes*
- For antibacterial analysis: Well- Diffusion Method (Well size:8mm); Amount of sample used: 100 µl; Incubation period: 24h, Incubation temp: 37°C; Media used: Muller Hinton Agar (Himedia); pH: 7.3±0.1, For *L. monocytogenes*: Listeria selective agar; pH: 7.3±0.2
- AU/ml: Activity unit/ml
- Test Organism: TO1E: *E. coli;* TO2P*: P. aeruginosa;* TO3S: *S. aureus;* TO4B: *B. subtilis;* TO5L: *L. monocytogenes*

Table 4.9: Antimicrobial activity of leaf extracts of *T. indica* and Plantaracin 2083

Bacteriocin	Conc. of Plant Extract (mg/ml)		Test Organism				
			TO1E	**TO2P**	**TO3S**	**TO4B**	**TO5L**
50% diluted Plantaracin 2083			**ZOI (mm)**	**ZOI (mm)**	**ZOI (mm)**	**ZOI (mm)**	**ZOI (mm)**
	Methanolic	**50**	20.6±0.9	16±0.8	25.3±0.5	23.3±0.5	Nil
		20	21.3±0.5	15.7±0.4	21.3±0.5	21±0	Nil
		10	19.3±0.5	15.3±0.4	18±0	18±0	Nil
	Ethanolic	**50**	22.3±0.4	19±0	24.6±0.5	22.3±0.5	Nil
		20	20.7±0.9	18.7 ±1.7	22.3±0.5	20±0	Nil
		10	20.3±0.4	16.7±2.5	20.7±0.9	16.7±0.9	Nil
	Aqueous	**50**	20.3±2	16.7±0.9	19.3±0.4	19.7±0.9	Nil
		20	20.3±3.4	16.3±1.9	18±0	18±0	Nil
		10	19.7±0.9	15.3±0.5	15±0	16.3±0.4	Nil

- Plantaracin 2083: Partially purified bacteriocin from *L. plantarum* (80% ammonium sulphate ppt.)
- TO1E: Test organism 1, *E. coli;* TO2P: Test organism 2, *P. aeruginosa;* TO3S: Test organism 3, *S. aureus;* TO4B: Test organism 4, *B. subtilis;* TO5L: Test organism 5, *L. monocytogenes*
- ZOI (mm): Zones of inhibition (mm)
- Dilutions preparation: plant extracts in 5% Dimethyl sulphoxide
- Plant extract: Plantaracin:: 1:1
- For antibacterial analysis: Well- Diffusion Method (Well size:8mm); Amount of sample used: 100 µl; Incubation period: 24h, Incubation temp: 37°C; Media used: Muller Hinton Agar (Himedia); pH: 7.3±0.1, For *L. monocytogenes*: Listeria selective agar; pH: 7.3±0.2

Table 4.10: Antimicrobial activity of root extracts of *T. indica* and Plantaracin 2083

Bacteriocin	Conc. of Plant Extract (mg/ml)		Test Organism				
			TO1E	**TO2P**	**TO3S**	**TO4B**	**TO5L**
			ZOI (mm)	**ZOI (mm)**	**ZOI (mm)**	**ZOI (mm)**	**ZOI (mm)**
50% diluted Plantaracin 2083	**Methanolic**	**50**	21.3±1.2	16.3±0.4	22.7±0.9	22.3±0.5	Nil
		20	20.3±0.4	14.3±0.4	21.3±0.5	18.3±0.5	Nil
		10	20±1.4	14±2.1	18.3±0.5	16±0	Nil
	Ethanolic	**50**	23±0	20±0	23.7±1.7	21.3±0.5	Nil
		20	20.7±0.5	19±0.8	20.7±0.9	18±0	Nil
		10	20.3±0.9	18.3±1.2	16.3±0.5	16±0	Nil
	Aqueous	**50**	18.3±1.2	18±2.1	19.3±0.5	18.7±0.9	Nil
		20	16.7±1.7	16.3±2.6	17.7±0.9	16.7±0.9	Nil
		10	15.7±3	13.3±2	16±0	14.3±0.5	Nil

- Plantaracin 2083: Partially purified bacteriocin from *L. plantarum* (80% ammonium sulphate ppt.)
- TO1E: Test organism 1, *E. coli;* TO2P: Test organism 2, *P. aeruginosa;* TO3S: Test organism 3, *S. aureus;* TO4B: Test organism 4, *B. subtilis;* TO5L: Test organism 5, *L. monocytogenes*
- ZOI (mm): Zones of inhibition (mm)
- Dilutions preparation: plant extracts in 5% Dimethyl sulphoxide
- Plant extract: Plantaracin:: 1:1
- **For antibacterial analysis**: Well- Diffusion Method (Well size:8mm); Amount of sample used: 100 µl; Incubation period: 24h, Incubation temp: 37°C; Media used: Muller Hinton Agar (Himedia); pH: 7.3±0.1, For *L. monocytogenes*: Listeria selective agar; pH: 7.3±0.2

Table 4.11: Antimicrobial activity of leaf extracts of *T. indica* and Enterocin YT3

Bacteriocin	Conc. of Plant Extract (mg/ml)		Test Organism				
			TO1E	**TO2P**	**TO3S**	**TO4B**	**TO5L**
			ZOI (mm)	**ZOI (mm)**	**ZOI (mm)**	**ZOI (mm)**	**ZOI (mm)**
50% diluted Enterocin YT3	**Methanolic**	**50**	14±0.8	11±0.8	11.7±0.5	20.3±0.5	Nil
		20	11.7±0.5	10±0.47	10.6±0.5	13.3±0.5	Nil
		10	9.7±0.5	Nil	9.6±0.5	10.3±0.4	Nil
	Ethanolic	**50**	16.7±0.9	10.3±0.5	11.6±0.47	21.6±1.24	Nil
		20	14.7±0.4	10±0.8	10.3±0.5	20±8	Nil
		10	11.3±0.47	Nil	9.3±0.5	17.3±2	Nil
	Aqueous	**50**	16±0.8	11.3±0.47	15.3±0.5	18±0	Nil
		20	12±0	10±0.8	11.3±0.4	14.3±0.5	Nil
		10	11.3±0.5	Nil	9.3±0.47	12.7±0.5	Nil

- Enterocin YT3: Partially purified bacteriocin of *Enterococcus sp. YT3* (80% ammonium sulphate ppt.)
- TO1E: Test organism 1, *E. coli;* TO2P: Test organism 2, *P. aeruginosa;* TO3S: Test organism 3, *S. aureus;* TO4B: Test organism 4, *B. subtilis;* TO5L: Test organism 5, *L. monocytogenes*
- ZOI (mm): Zones of inhibition (mm)
- Dilutions preparation: plant extracts in 5% Dimethyl sulphoxide
- Plant extract: Plantaracin:: 1:1
- **For antibacterial analysis**: Well- Diffusion Method (Well size:8mm); Amount of sample used: 100 µl; Incubation period: 24h, Incubation temp: 37°C; Media used: Muller Hinton Agar (Himedia); pH: 7.3±0.1, For *L. monocytogenes*: Listeria selective agar; pH: 7.3±0.2

Table 4.12: Antimicrobial activity of root extracts of *T. indica* and Enterocin YT3

Bacteriocin	Conc. of Plant Extract (mg/ml)		Test Organism				
			TO1E	**TO2P**	**TO3S**	**TO4B**	**TO5L**
			ZOI (mm)	**ZOI (mm)**	**ZOI (mm)**	**ZOI (mm)**	**ZOI (mm)**
50% diluted Enterocin YT3	**Methanolic**	**50**	12.3±1.2	12.3±0.9	11±0	16.7±0.9	Nil
		20	11±1.4	9.3±0.47	10.3±0.47	14.7±0.9	Nil
		10	10.3±0.5	9±0	9.7±0.47	11.7±0.47	Nil
	Ethanolic	**50**	15.7±1.7	16±0.8	10.3±0.5	22.3±0.47	Nil
		20	13.7±0.5	12.3±0.5	9.3±0.5	20.3±0.5	Nil
		10	11.3±0.5	10.3±0.5	9±0	16.7±0.9	Nil
	Aqueous	**50**	14.3±0.5	14±0.8	12.3±0.5	15±0	Nil
		20	12±0	12.3±0.5	10.3±0.47	12.7±0.9	Nil
		10	10.3±0.5	9.7±0.5	9±0	11.3±0.47	Nil

- Enterocin YT3: Partially purified bacteriocin of Enterococcus sp. YT3 (80% ammonium sulphate ppt.)
- TO1E: Test organism 1, *E. coli;* TO2P: Test organism 2, *P. aeruginosa;* TO3S: Test organism 3, *S. aureus;* TO4B: Test organism 4, *B. subtilis;* TO5L: Test organism 5, *L. monocytogenes*
- ZOI (mm): Zones of inhibition (mm)
- Dilutions preparation: plant extracts in 5% Dimethyl sulphoxide
- Plant extract: Plantaracin:: 1:1
- **For antibacterial analysis**: Well- Diffusion Method (Well size:8mm); Amount of sample used: 100 µl; Incubation period: 24h, Incubation temp: 37°C; Media used: Muller Hinton Agar (Himedia); pH: 7.3±0.1, For *L. monocytogenes*: Listeria selective agar; pH: 7.3±0.2

Table 4.13: Antimicrobial activity of leaf extracts of *T. indica* and Nisin

Sample	Nisin (AU/ml)		Methanolic Extract			Ethanolic Extract			Aqueous Extract		
		Conc. of Plant Extract (mg/ml)	50	20	10	50	20	10	50	20	10
		Organism	ZOI (mm)	ZOI (mm)	ZOI (mm)	ZOI (mm)	ZOI (mm)	ZOI (mm)	ZOI (mm)	ZOI (mm)	ZOI (mm)
1	250	TO1E	11.7±0.5	Nil	Nil	10.7±0.9	Nil	Nil	Nil	Nil	Nil
		TO2P	Nil	Nil	Nil	Nil	Nil	Nil	11.6±0.9	Nil	Nil
		TO3S	14.3±0.5	11.7±0.9	9.7±0.4	11.6±0.9	10.3±0.4	Nil	13±0.8	12±0	10.7±0.5
		TO4B	9.7±0.9	Nil	Nil	12.3±0.5	Nil	Nil	10.3±0.4	9.7±0.5	Nil
		TO5L	11.7±0.5	10.3±1.2	9.7±0.4	17±0	16±0.8	15.3±1.9	14.3±1.2	13.3±0.4	10±0
2	500	TO1E	15±0.8	14.3±2.6	13.3±2.6	12.3±1.8	Nil	Nil	Nil	Nil	Nil
		TO2P	Nil	Nil	Nil	Nil	Nil	Nil	13±1.4	Nil	Nil
		TO3S	17.3±0.4	12.7±0.9	11±0.8	14.7±0.9	14±0	11±0.8	16.3±1.2	14.3±0.9	13±1.4
		TO4B	11±0.8	10.3±1.2	9.3±0.4	14.7±0.5	12±0	11.3±0.5	13.7±1.7	12.3±1.2	10.7±0.9
		TO5L	12.3±0.4	11.6±0.9	10.3±0.4	19.3±0.5	18.3±1.2	18±1.4	18±4.9	16.3±5.4	15.7±4.4
3	1,000	TO1E	15.7±0.5	15.3±2.6	15±2.9	15±0.8	12.7±1.6	Nil	12.7±0.4	13.3±0.5	13±1.4
		TO2P	11.7±0.5	Nil	Nil	10.7±0.5	Nil	Nil	14.3±0.4	10.3±1.8	Nil
		TO3S	16±2.1	13.7±1.7	13±0.8	15.3±0.5	14.7±0.4	12.3±0.5	17.3±0.5	15±0.8	14.3±0.9
		TO4B	11±0.8	10.7±0.5	10.3±0.5	15±0	12.7±0.5	11.7±0.4	14±0.8	13±0.8	11.3±0.4
		TO5L	13±0.8	12.7±0.9	11.3±0.5	20±1.6	19±1.6	18.7±1.2	18.7±5.0	16.7±3.8	16.3±4
4	2,000	TO1E	16.7±0.5	16.7±1.7	16±2.8	16.7±0.5	15±0.8	Nil	14±0	14.3±1.2	14±0.4
		TO2P	12±0.8	Nil	Nil	11.3±0.5	9.7±0.9	Nil	14.7±0.9	11±2.1	Nil
		TO3S	18.7±0.9	15±2.1	13.7±0.9	16.7±1.2	15.7±0.9	14±0.8	17.7±0.4	16.7±1.2	16.3±2.8
		TO4B	12.3±0.4	11.7±0.5	10.7±0.5	15.3±0.5	13.3±0.4	12.3±1.2	14.3±1.2	14±1.6	12.7±0.9
		TO5L	15.3±0.5	14±3.7	13.3±0.4	20.7±0.5	20.3±2.5	19.7±2.6	21.3±5.7	19±6.3	18±4.9
5	2,500	TO1E	18.3±0.4	18±0.8	17±2.1	17.3±0.5	16±0.8	Nil	14.3±0.5	15.3±1.2	14.7±0.5
		TO2P	12.3±0.5	Nil	Nil	12±0	10±0.8	Nil	15.3±1.9	11.7±1.7	Nil
		TO3S	19±0.8	15.7±3	14.3±0.9	17.7±1.2	16±1.6	15.7±0.5	18±1.4	17±2.1	17±1.4
		TO4B	12.3±0.4	12±0.8	11.7±0.4	15.7±0.9	14±0.8	12±0	15±1.6	14.7±1.2	13.7±1.2
		TO5L	15.3±0.5	14.3±0.4	13.7±2.6	22±0.8	21.7±1.2	21±2.4	21.7±7.4	20±4.5	19±3.6

- Dilutions preparation: plant extracts in 5% Dimethyl sulphoxide; Nisin in 0.02M Acetic acid; Plant extract: Nisin:: 1:1
- TO1E: Test organism 1, *E. coli;* TO2P: Test organism 2, *P. aeruginosa;* TO3S: Test organism 3, *S. aureus;* TO4B: Test organism 4, *B. subtilis;* TO5L: Test organism 5, *L. monocytogenes*
- For antibacterial analysis: Well- Diffusion Method (Well size:8mm); Amount of sample used: 100 µl; Incubation period: 24h, Incubation temp: 37°C; Media used: Muller Hinton Agar (Himedia); pH: 7.3±0.1, For *L. monocytogenes*: Listeria selective agar; pH: 7.3±0.2

Table 4.14: Antimicrobial activity of root extracts of *T. indica* and Nisin

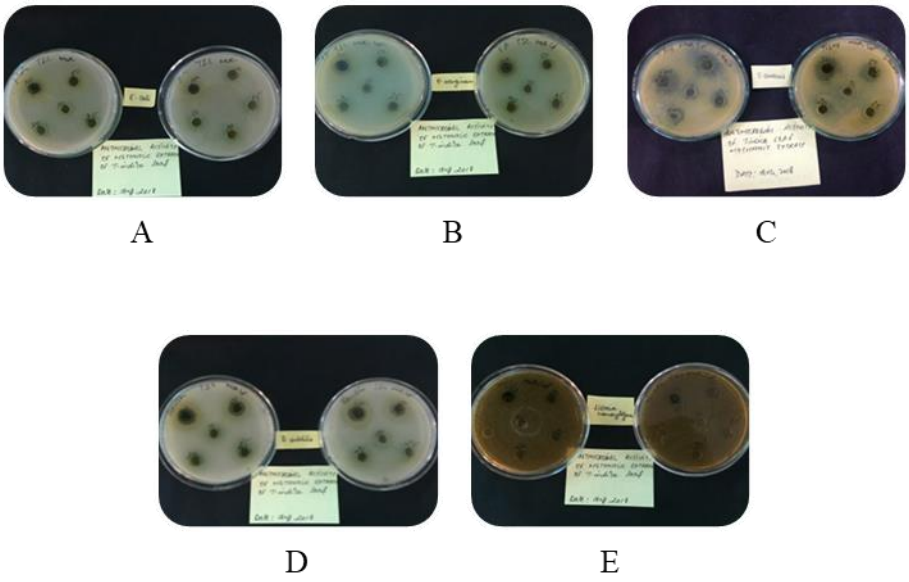
Sample	Nisin (AU/ml)		Methanolic Extract			Ethanolic Extract			Aqueous Extract		
		Conc. of Plant Extract (mg/ml)	50	20	10	50	20	10	50	20	10
		Organism	ZOI (mm)	ZOI (mm)	ZOI (mm)	ZOI (mm)	ZOI (mm)	ZOI (mm)	ZOI (mm)	ZOI (mm)	ZOI (mm)
1	250	TO1E	Nil	Nil	Nil	Nil	Nil	Nil	14.6±0.9l	Nil	Nil
		TO2P	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
		TO3S	12.3±0.4	11.7±0.9	10±0	12.3±0.5	11.6±0.9	10.3±0.4	14±0	13.3±0.4	12±0
		TO4B	11.3±0.5	9.7±0.9	Nil	11±0	10.7±3.1	9.7±0.9	10.7±0.5	9.7±0.5	Nil
		TO5L	10.3±1.2	Nil	Nil	11.7±0.4	9±0	Nil	11±0.8	10.3±0.5	9.3±0.5
2	500	TO1E	11.7±0.5	Nil	Nil	12.3±1.2	Nil	Nil	15.7±0.9	15±1.4	12.6±0.9
		TO2P	Nil	Nil	Nil	12±0	9.3±0.4	Nil	13±2.1	10.3±0.5	Nil
		TO3S	15.3±0.5	13.3±0.5	12.6±0.5	15±0.8	14±0.8	11±1.4	18±1.4	15.3±0.4	14±1.4
		TO4B	13.3±1.2	11.7±0.9	10.7±0.5	14.3±0.5	14±0	13±0	14.3±1.2	13.3±0.4	10±0
		TO5L	11±0.8	Nil	Nil	14±01.6	13.7±0.9	Nil	12.7±1.7	11.3±0.4	10.3±0.4
3	1,000	TO1E	13.7±0.5	Nil	Nil	14.3±0.5	11.7±3.5	10.7±3.1	16.3±0.5	16.6±0.9	13.7±2.3
		TO2P	Nil	Nil	Nil	12.3±0.4	9.7±0.9	Nil	13.3±0.4	11.7±0.9	Nil
		TO3S	16.3±0.5	14.6±0.9	13.7±0.9	15.3±0.5	14.3±0.4	11.3±0.4	19.3±3.6	17.3±0.4	16.3±1.2
		TO4B	13±0.8	12.7±0.5	11.3±0.5	15±0.8	14.3±0.5	13.7±0.5	12±1.6	11.3±0.5	9.7±0.9
		TO5L	11.3±0.5	Nil	Nil	15.3±1.2	15±1.4	14.7±0.9	12.3±0.5	13.3±0.5	13.7±1.7
4	2,000	TO1E	14.3±0.5	Nil	Nil	16.3±0.5	15.7±0.5	13.7±2.3	18.7±0.5	17.7±0.9	16±1.4
		TO2P	Nil	Nil	Nil	12.7±0.9	10±0.8	9.7±0.9	14±0.8	12.7±0.5	Nil
		TO3S	17.3±0.5	16.7±0.5	14.3±0.5	16±0.8	14.7±0.9	12.3±0.5	19.7±2.6	18.7±1.7	17.3±1.2
		TO4B	14.7±1.2	14.3±3.3	13.3±1.2	15.3±1.2	15±0	14.7±0.5	12.37±1.2	12±0	10.3±0.5
		TO5L	13±1.6	10.7±0.5	10.3±0.5	16.7±0.9	16±0.9	15.7±2.6	15.3±0.9	15±0.8	16±0.8
5	2,500	TO1E	15.3±0.5	12.7±0.9	Nil	17.3±0.5	16.3±1.2	15.3±0.4	20.7±0.5	19.6±0.5	18.7±0.5
		TO2P	Nil	Nil	Nil	13±0.8	11±0.8	9.3±0.4	15±0.8	13.3±1.2	Nil
		TO3S	18±0.8	15±1.6	16.3±0.9	15.3±0.5	15±0.8	13±0.8	20.3±2.9	20.3±1.2	18±0.8
		TO4B	17.7±1.7	17.3±2.1	17.3±1.9	16±2.2	15.7±0.5	15.3±0.5	12.7±1.7	12.7±0.5	11.3±0.4
		TO5L	14.7±0.5	13±0.8	12.3±1.2	17.7±1.2	17±0.8	16.3±1.2	16±0.8	16.7±1.7	15±2.1

- Dilutions preparation: plant extracts in 5% Dimethyl sulphoxide; Nisin in 0.02M Acetic acid; Plant extract: Nisin:: 1:1
- TO1E: Test organism 1, *E. coli;* TO2P: Test organism 2, *P. aeruginosa;* TO3S: Test organism 3, *S. aureus;* TO4B: Test organism 4, *B. subtilis;* TO5L: Test organism 5, *L. monocytogenes*
- For antibacterial analysis: Well- Diffusion Method (Well size:8mm); Amount of sample used: 100 µl; Incubation period: 24h, Incubation temp: 37°C; Media used: Muller Hinton Agar (Himedia); pH: 7.3±0.1, For *L. monocytogenes*: Listeria selective agar; pH: 7.3±0.2

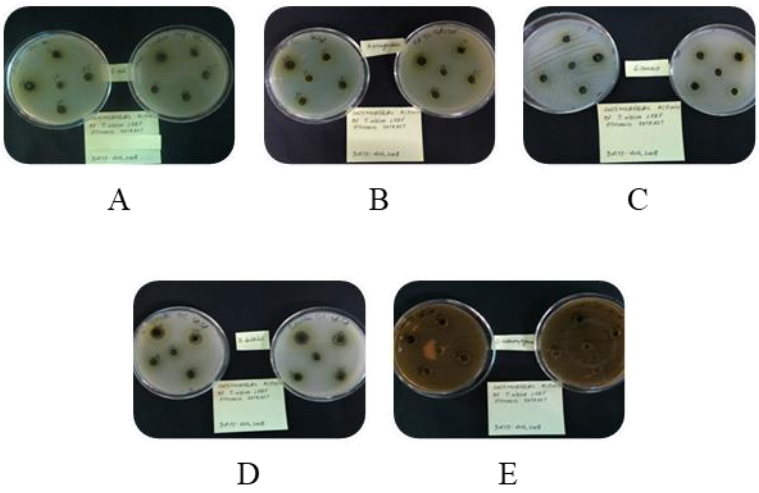
Table 4.15: Screened and selected combinations of antimicrobials for preparation of active packaging films along with statistical analysis

Antimicrobial agent	**Antimicrobial concentration selected to be tested in active packaging film**	**Organism**				
		E. coli	***P. aeruginosa***	***S. aureus***	***B. subtilis***	***L. monocytogenes***
T. indica leaf methanolic extract	50mg/ml	14±1.4*	14.7±0.4*	15±1.4***	12.7±0.9*	9.3±0.4**
T. indica root ethanolic extract	50mg/ml	13.6±0.9*	10.3±1.2*	12.7±1.7***	14.3±1.2*	10.6±0.5**
Nisin	2500 IU/ml	10±0.8*	11±0*	17.3±0.5***	12.7±0.4*	21±0.8**
T. indica leaf (Aqueous extract) +Nisin	20 mg/ml+ 1000 IU/ml	13.3±0.5*	10.3±1.8*	15±0.8***	13±0.8*	16.7±3.8**
T. indica root (Aqueous extract) + Nisin	20 mg/ml+ 500 IU/ml	15±1.4*	10.3±0.5*	15.3±0.4***	13.3±0.4*	11.3±0.4**
• Statistical tool: One way ANOVA; *: P- value <0.01; **: P- value < 0.001; ***: P- value <0.05						

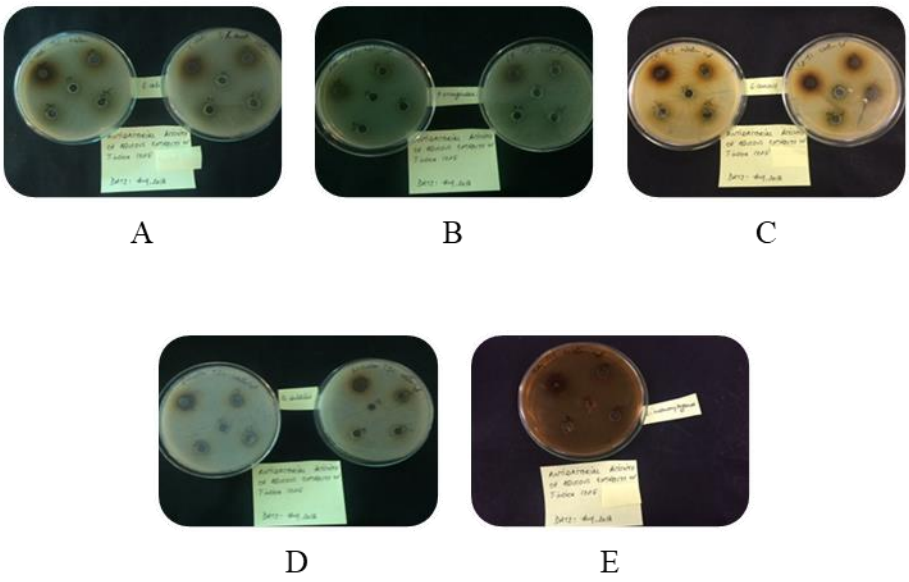
Photographs:



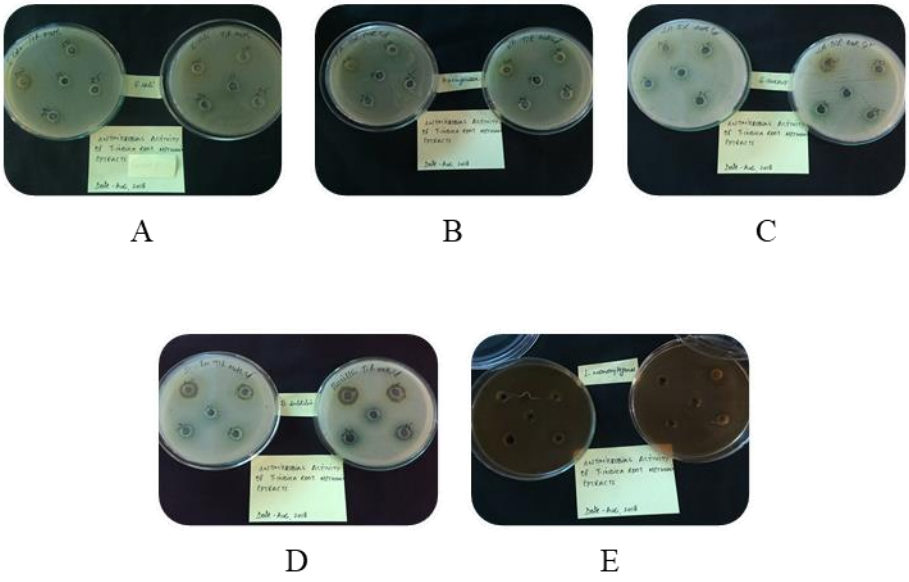
Photograph 4.1: Antimicrobial potential of *T. indica* leaf methanolic extracts against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



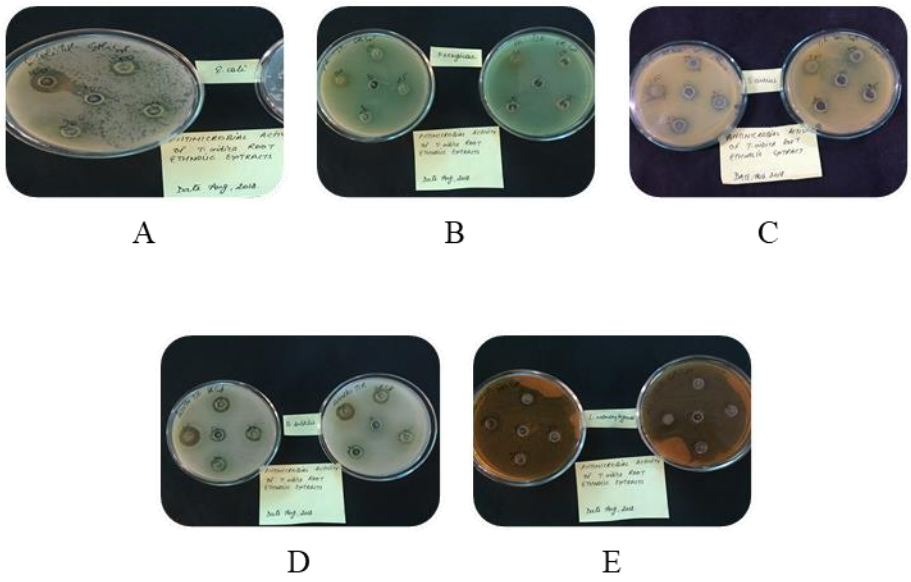
Photograph 4.2: Antimicrobial potential of *T. indica* leaf ethanolic extracts against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



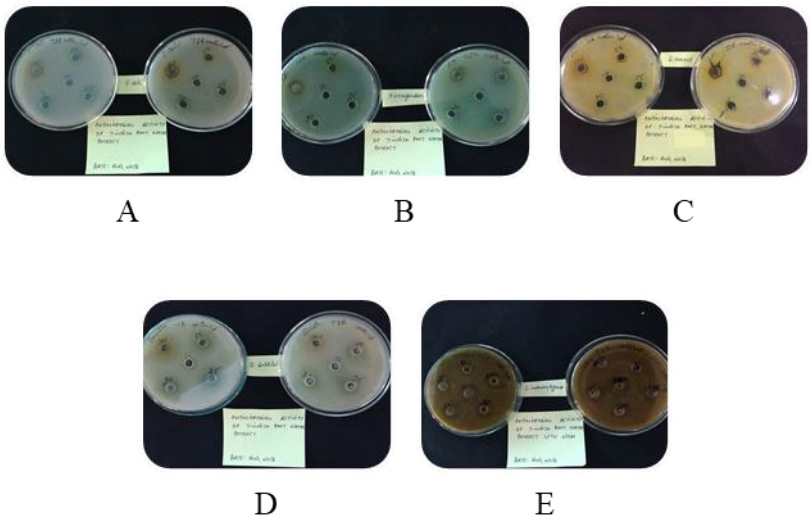
Photograph 4.3: Antimicrobial potential of *T. indica* leaf aqueous extracts against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



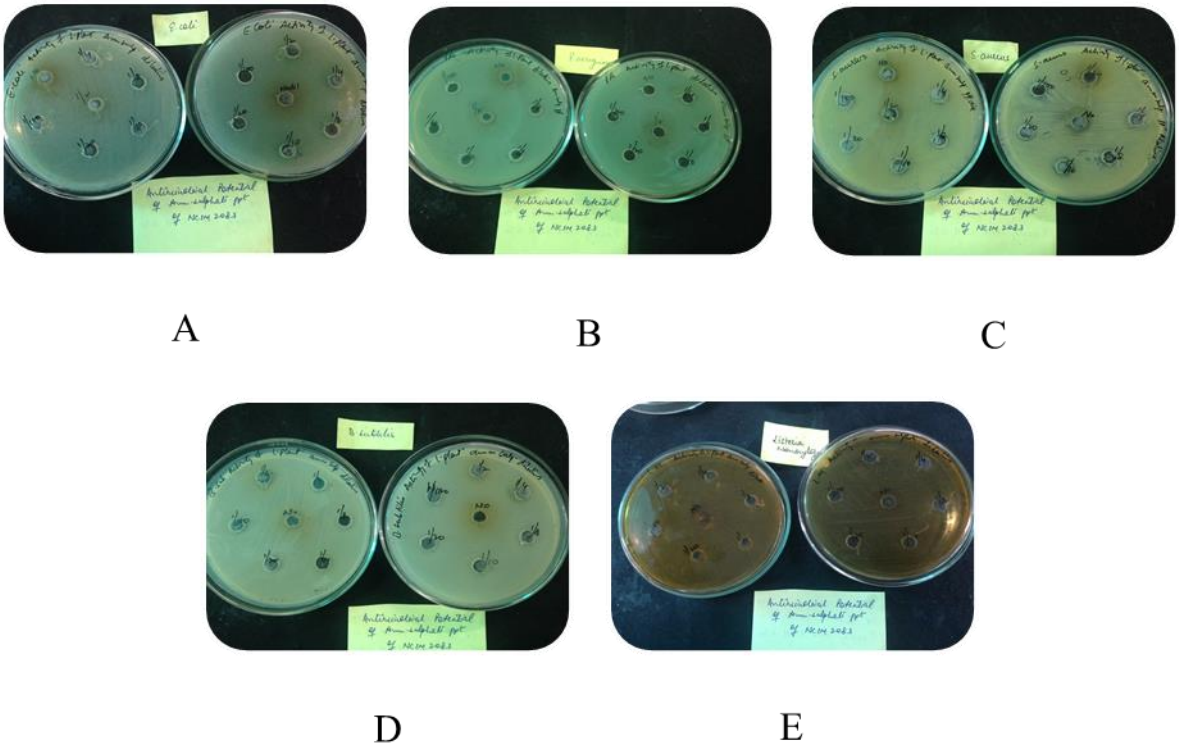
Photograph 4.4: Antimicrobial potential of *T. indica* root methanolic extracts against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



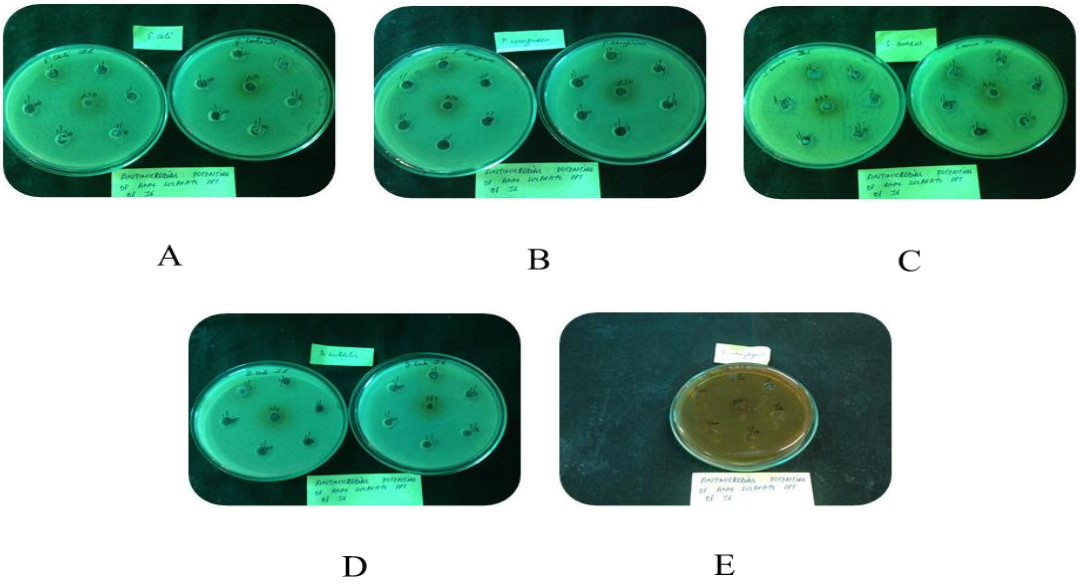
Photograph 4.5: Antimicrobial potential of *T. indica* root ethanolic extracts against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



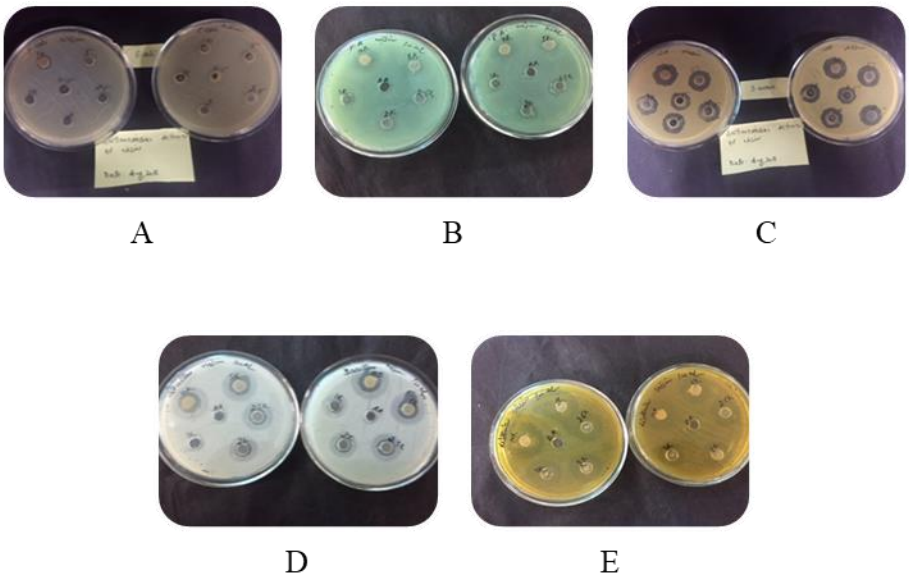
Photograph 4.6: Antimicrobial potential of *T. indica* root aqueous extracts against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



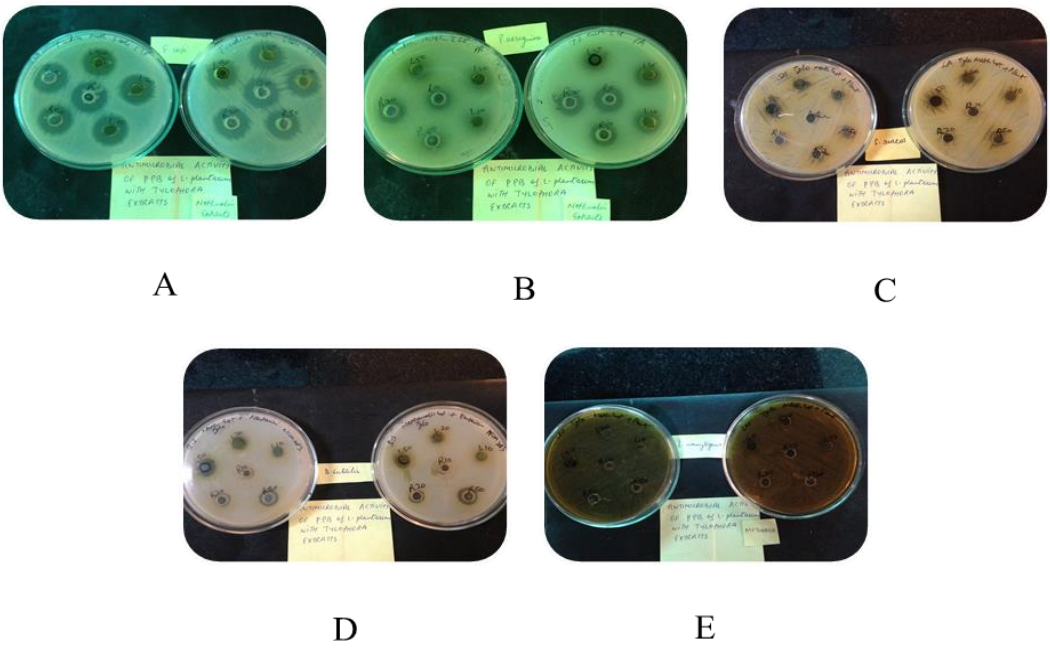
Photograph 4.7: Antimicrobial potential of partially purified bacteriocin from *L. plantarum* (NCIM2083) against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



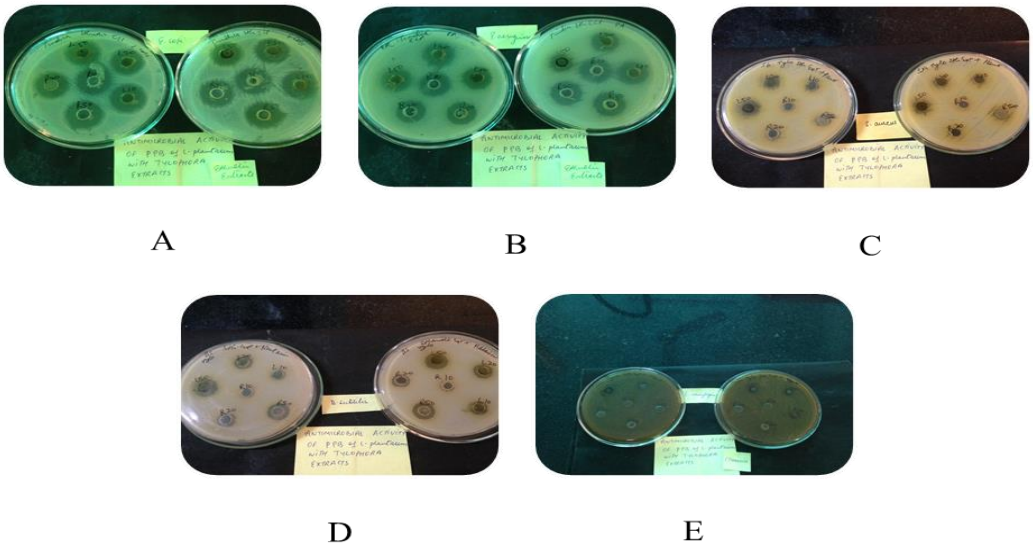
Photograph 4.8: Antimicrobial potential of partially purified bacteriocin from *Enterococcus sp. YT3* against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



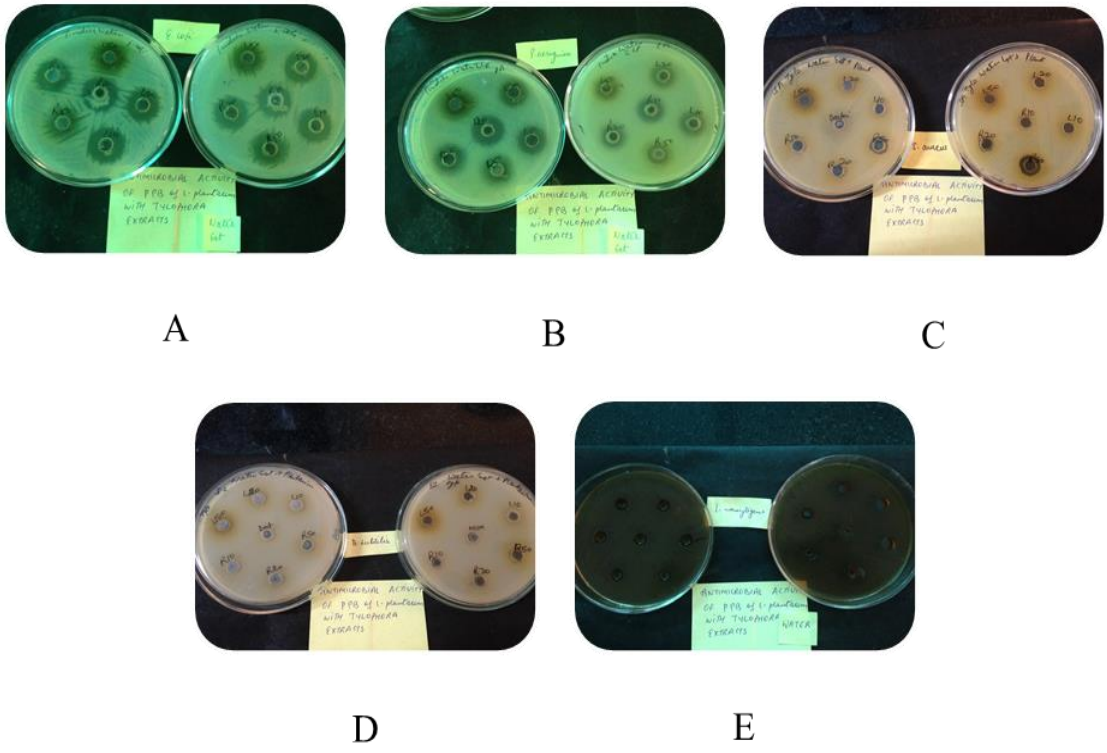
Photograph 4.9: Antimicrobial potential of nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



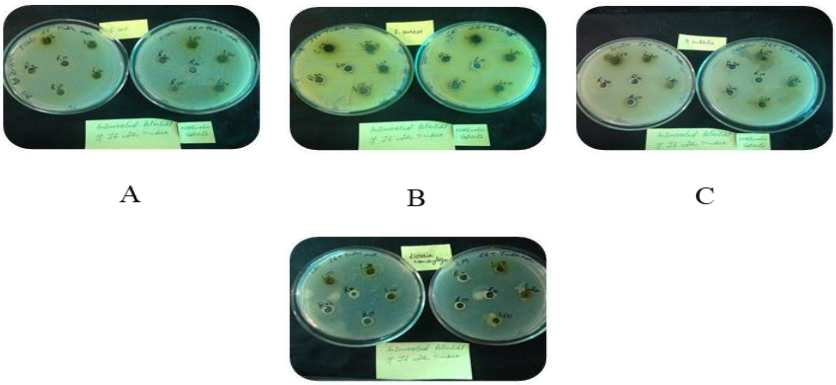
Photograph 4.10: Antimicrobial potential of *T. indica* leaf and roots methanolic extracts with Plantaracin 2083 against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



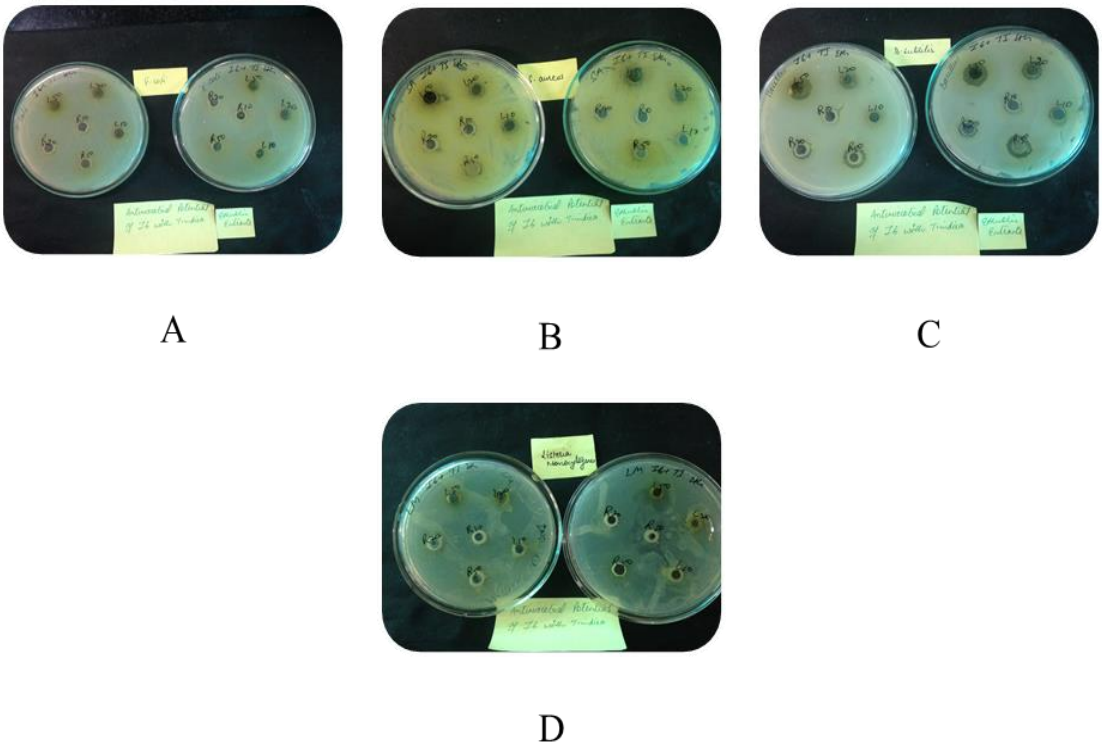
Photograph 4.11: Antimicrobial potential of *T. indica* leaf and roots ethanolic extracts with Plantaracin 2083 against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



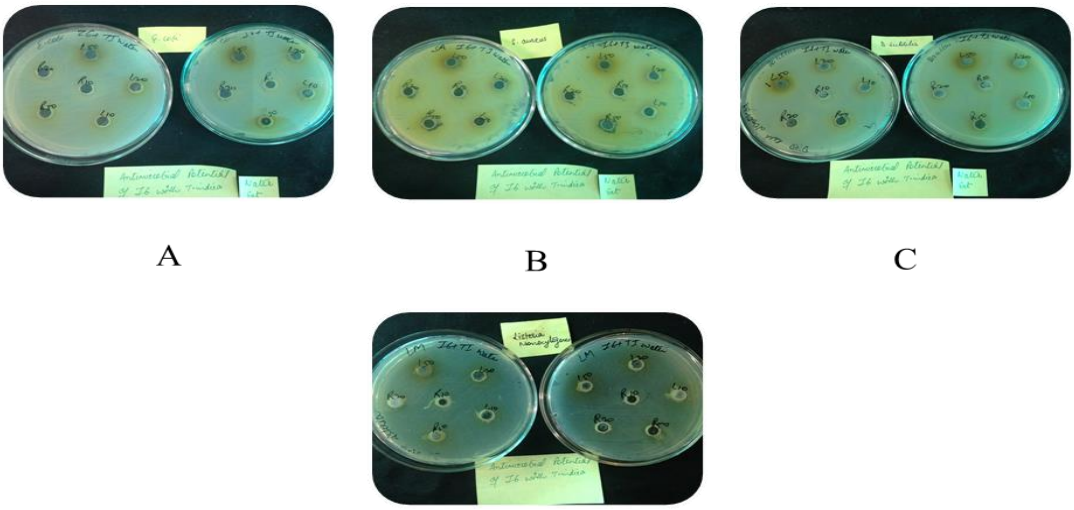
Photograph 4.12: Antimicrobial potential of *T. indica* leaf and roots aqueous extracts with Plantaracin 2083 against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



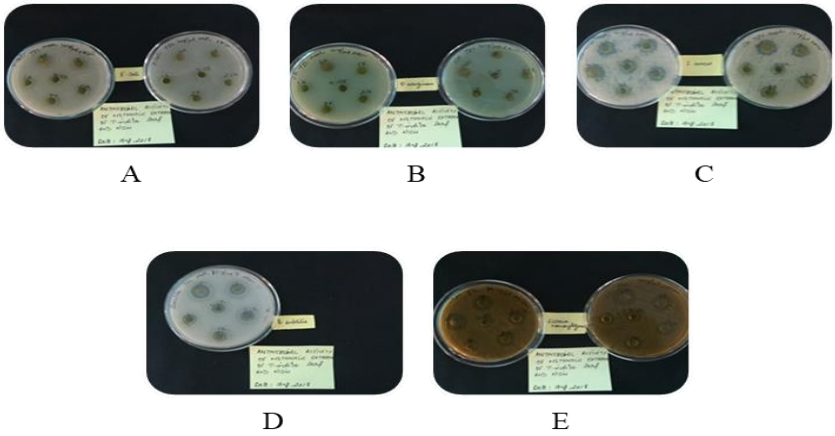
Photograph 4.13: Antimicrobial potential of *T. indica* leaf and roots methanolic extracts with Enterocin YT3 against different food pathogens; A: *E. coli*; B: *S. aureus*; C: *B. subtilis*; D: *L. monocytogenes*



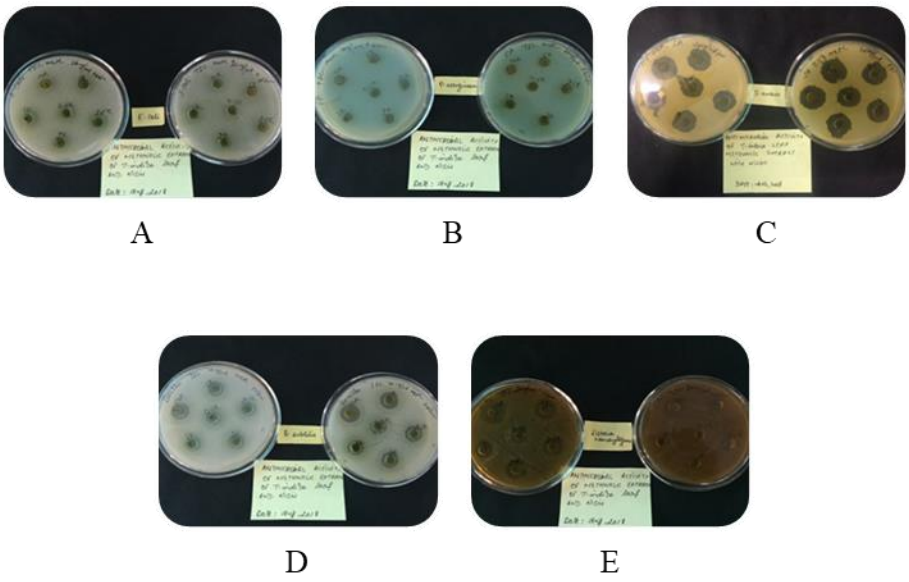
Photograph 4.14: Antimicrobial potential of *T. indica* leaf and roots ethanolic extracts with Enterocin YT3 against different food pathogens; A: *E. coli*; B: *S. aureus*; C: *B. subtilis*; D: *L. monocytogenes*



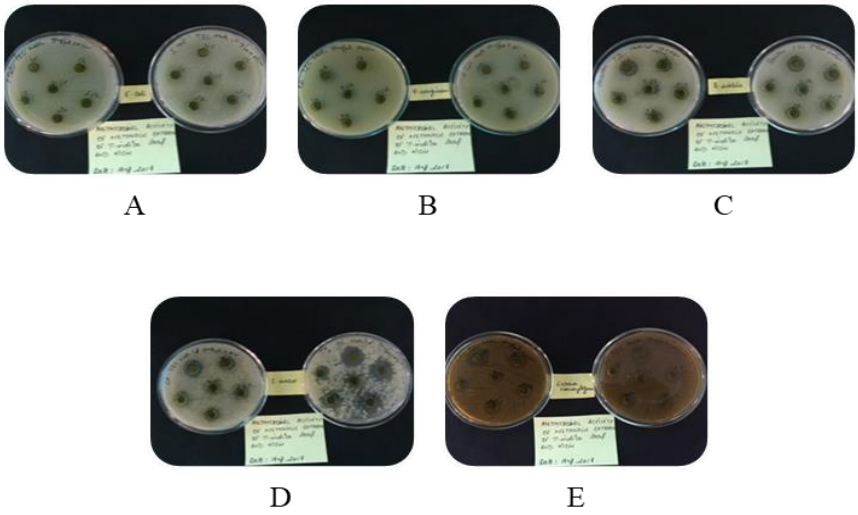
Photograph 4.15: Antimicrobial potential of *T. indica* leaf and roots aqueous extracts with Enterocin YT3 against different food pathogens; A: *E. coli*; B: *S. aureus*; C: *B. subtilis*; D: *L. monocytogenes*



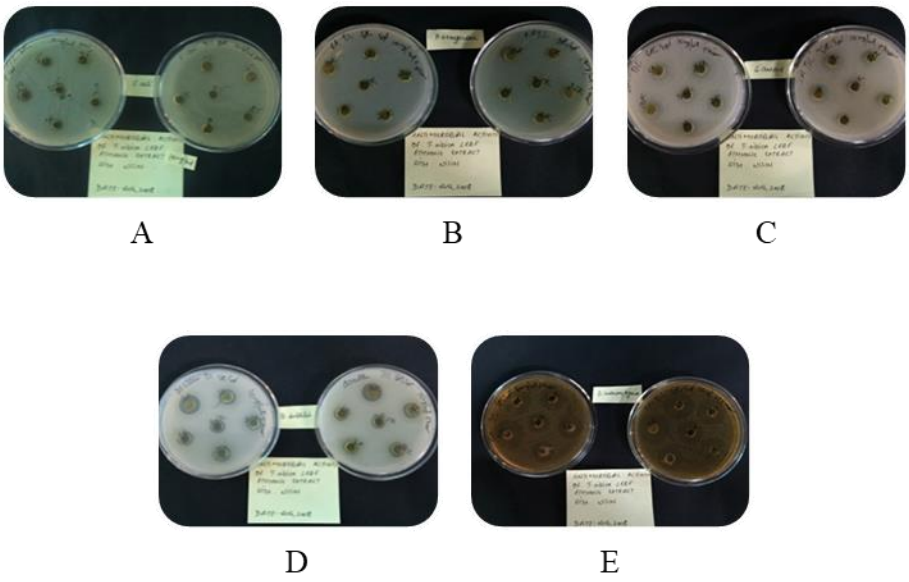
Photograph 4.16: Antimicrobial potential of *T. indica* leaf methanolic extracts (10mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



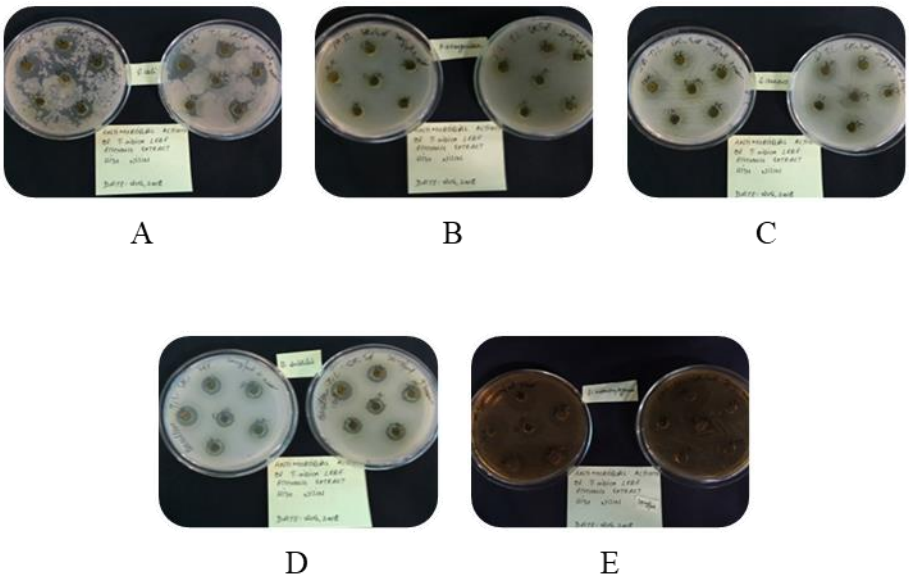
Photograph 4.17: Antimicrobial potential of *T. indica* leaf methanolic extracts (20mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



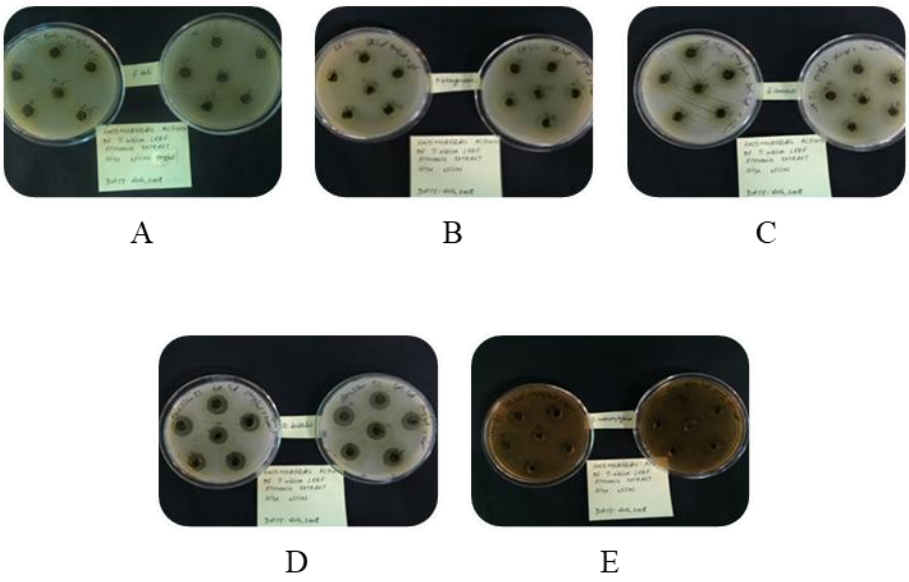
Photograph 4.18: Antimicrobial potential of *T. indica* leaf methanolic extracts (50mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



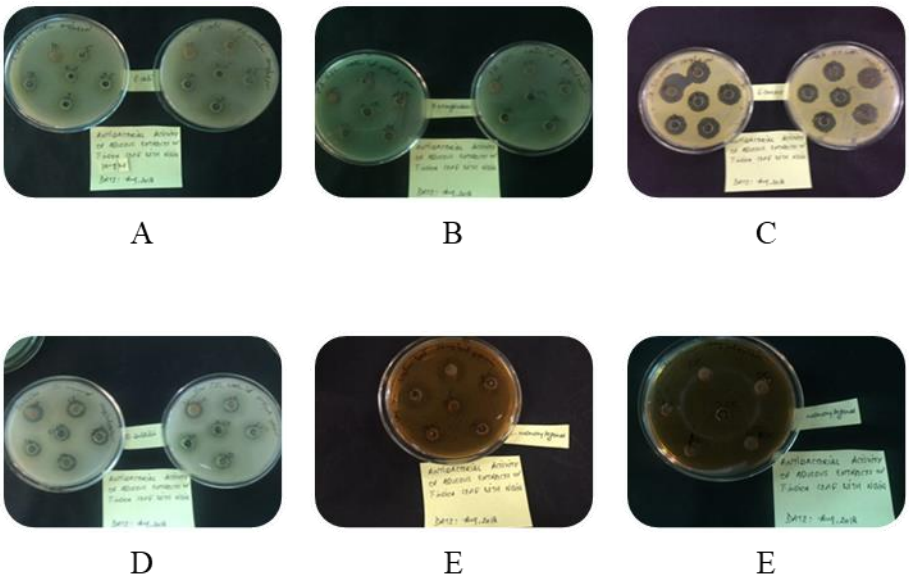
Photograph 4.19: Antimicrobial potential of *T. indica* leaf ethanolic extracts (10mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



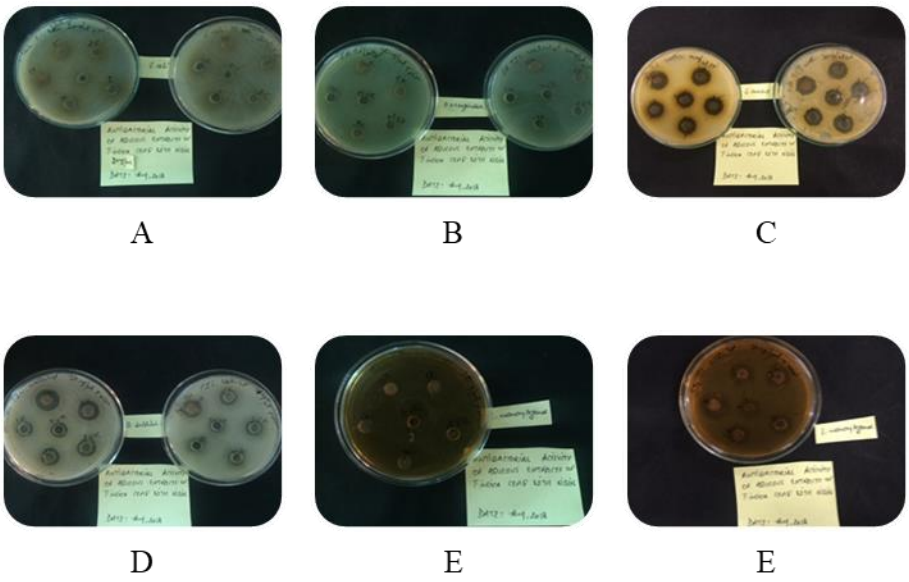
Photograph 4.20: Antimicrobial potential of *T. indica* leaf ethanolic extracts (20mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



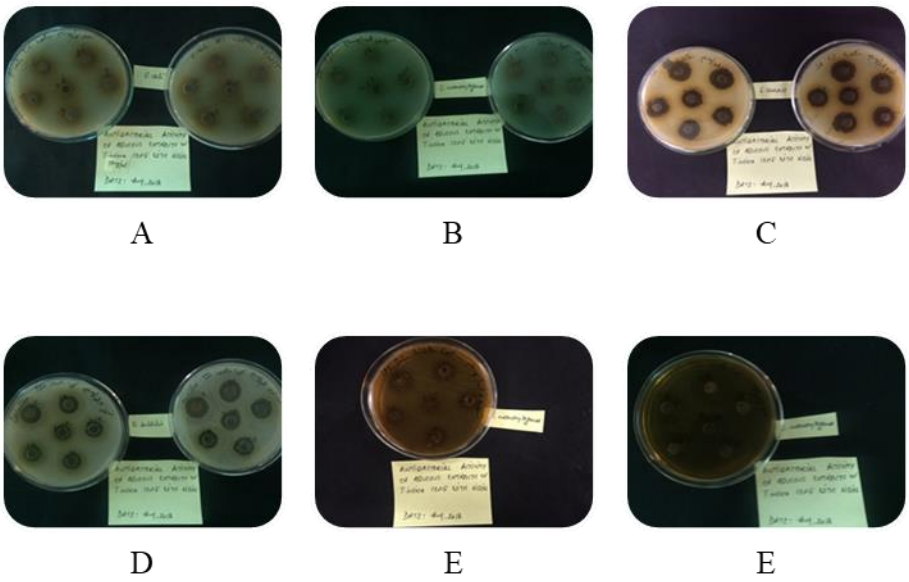
Photograph 4.21: Antimicrobial potential of *T. indica* leaf ethanolic extracts (50mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



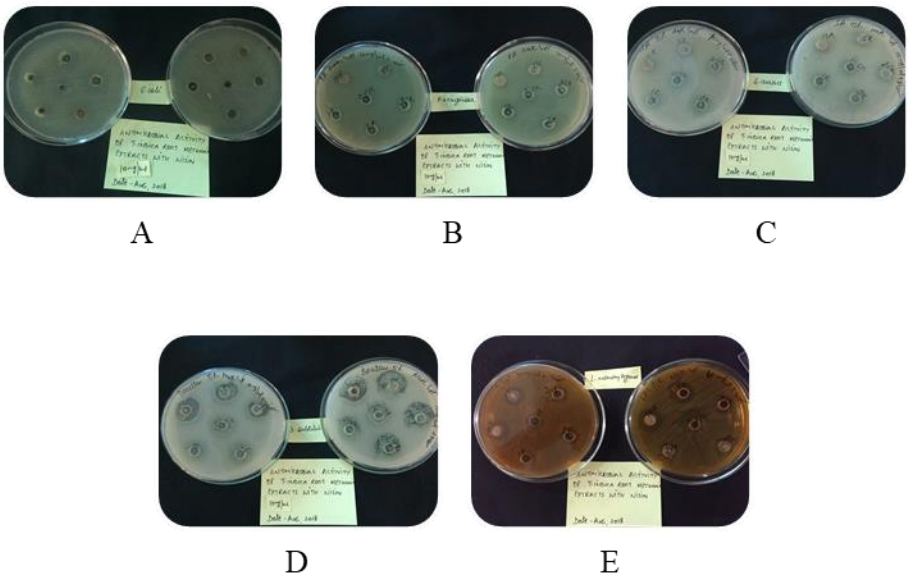
Photograph 4.22: Antimicrobial potential of *T. indica* leaf water extracts (10mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



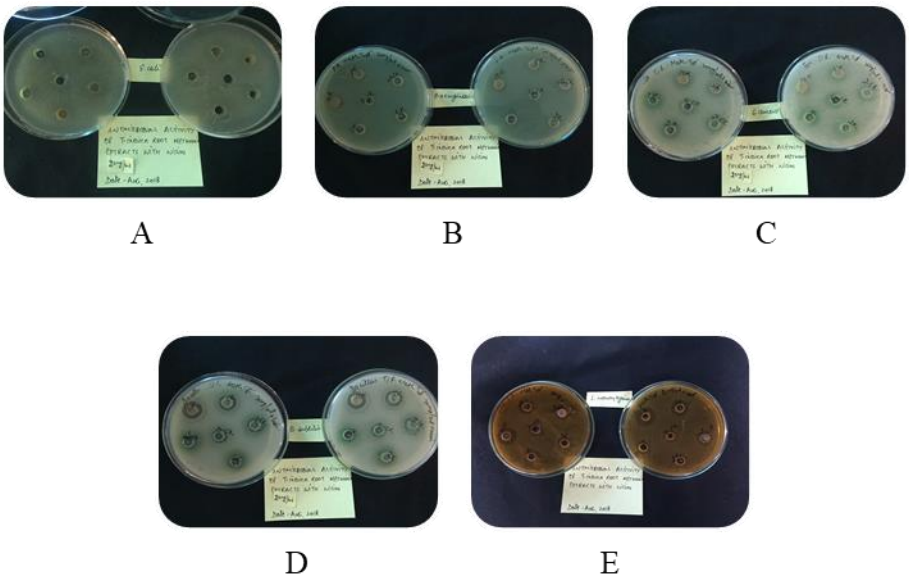
Photograph 4.23: Antimicrobial potential of *T. indica* leaf water extracts (20mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



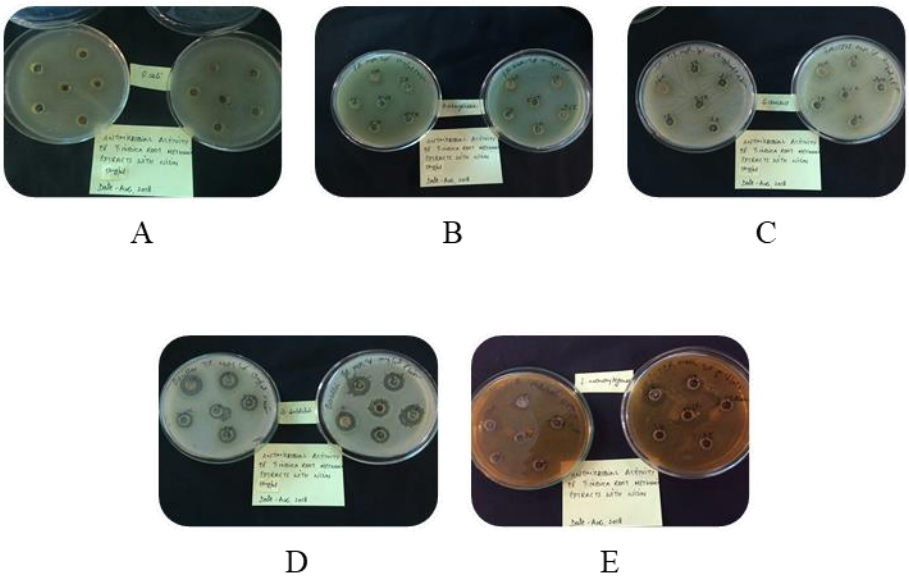
Photograph 4.24: Antimicrobial potential of *T. indica* leaf water extracts (50mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



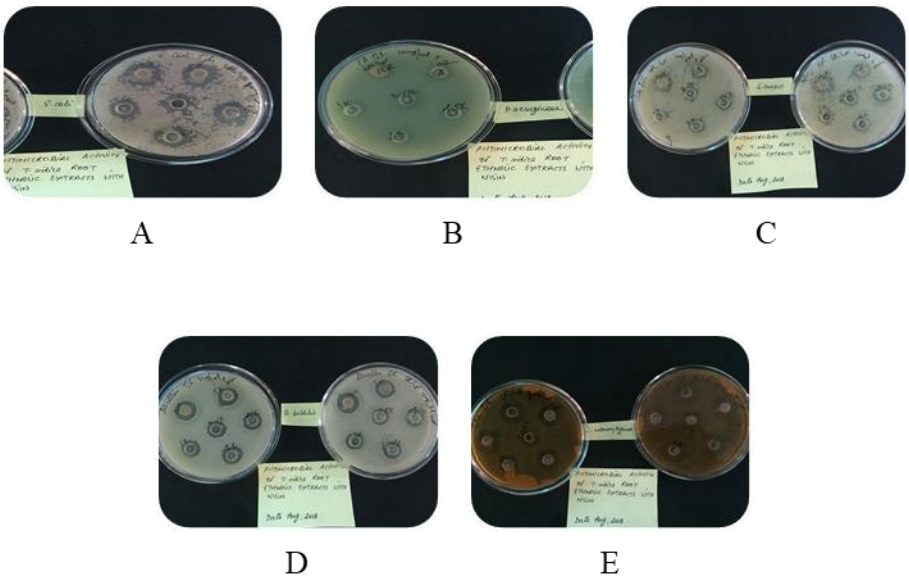
Photograph 4.25: Antimicrobial potential of *T. indica* root methanolic extracts (10mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



Photograph 4.26: Antimicrobial potential of *T. indica* root methanolic extracts (20mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*

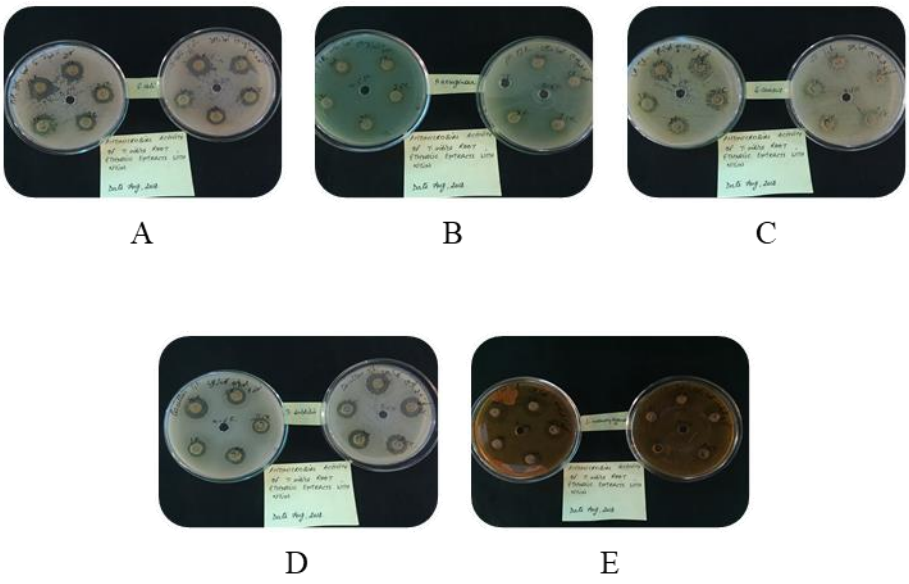


Photograph 4.27: Antimicrobial potential of *T. indica* root methanolic extracts (50mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*

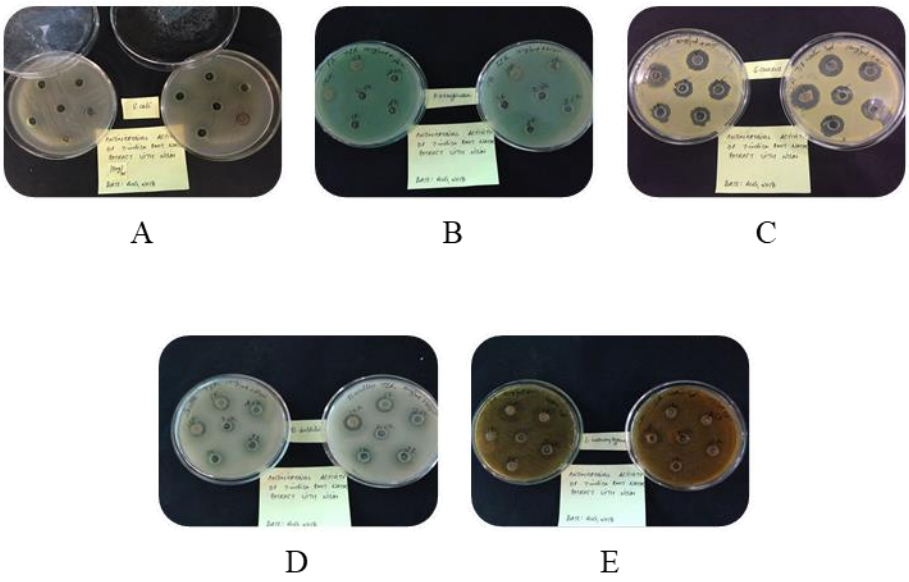


Photograph 4.28: Antimicrobial potential of *T. indica* root ethanolic extracts (10mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*

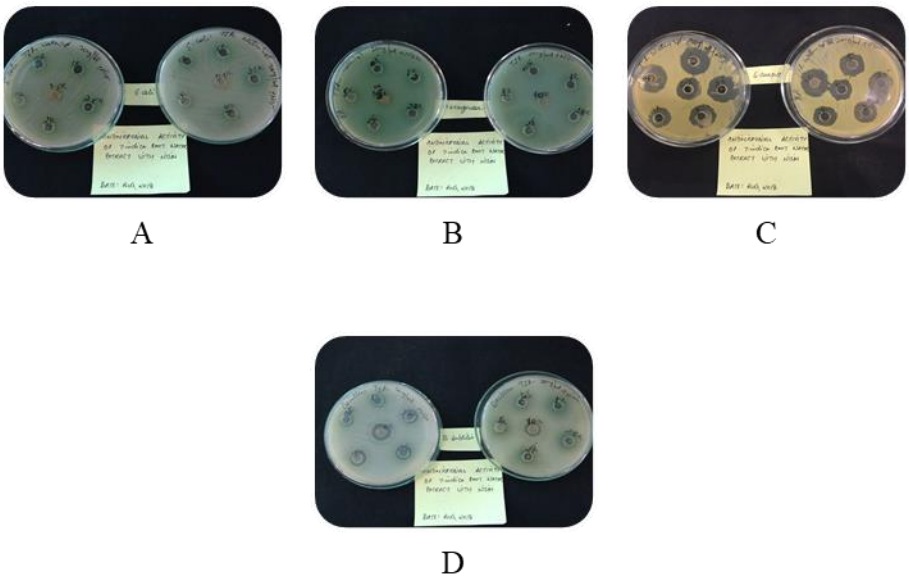
Photograph 4.29: Antimicrobial potential of *T. indica* root ethanolic extracts (20mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



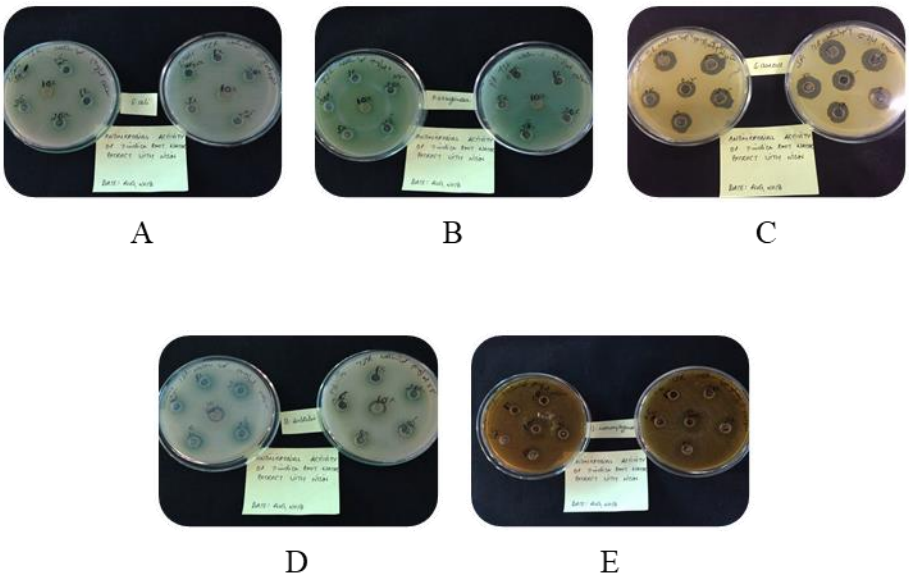
Photograph 4.30: Antimicrobial potential of *T. indica* root ethanolic extracts (50mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*



Photograph 4.31: Antimicrobial potential of *T. indica* root water extracts (10mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*

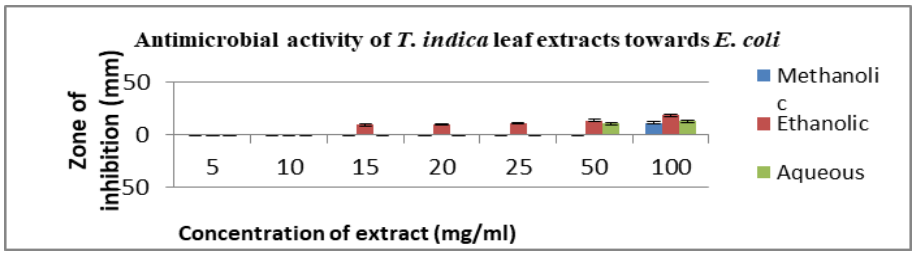


Photograph 4.32: Antimicrobial potential of *T. indica* root water extracts (20mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*

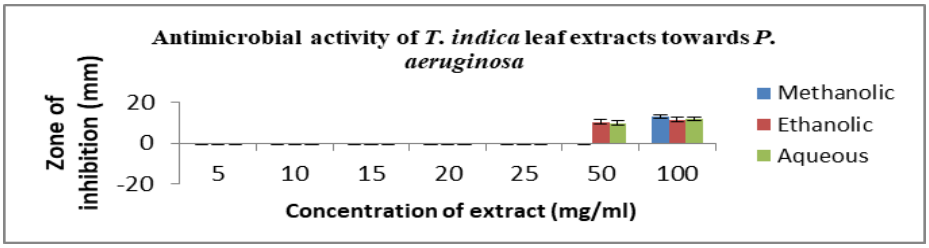


Photograph 4.33: Antimicrobial potential of *T. indica* root water extracts (50mg/ml) and Nisin against different food pathogens; A: *E. coli*; B: *P. aeruginosa*; C: *S. aureus*; D: *B. subtilis*; E: *L. monocytogenes*

Figures:



A



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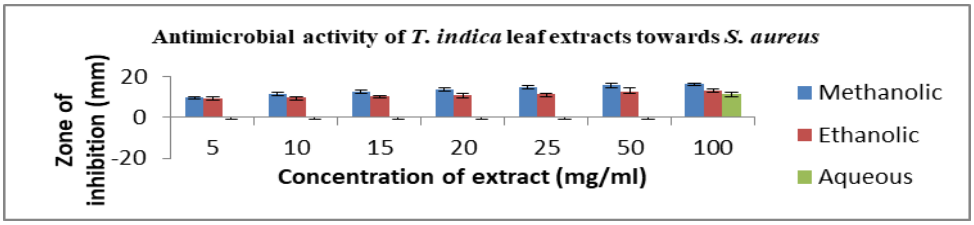

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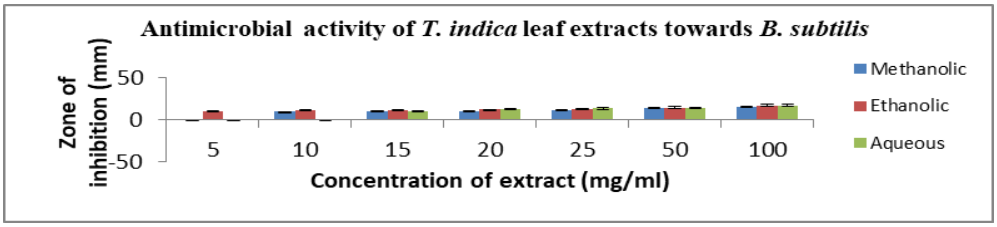

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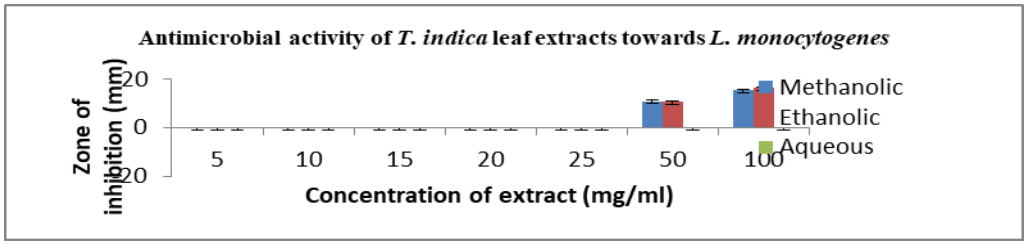
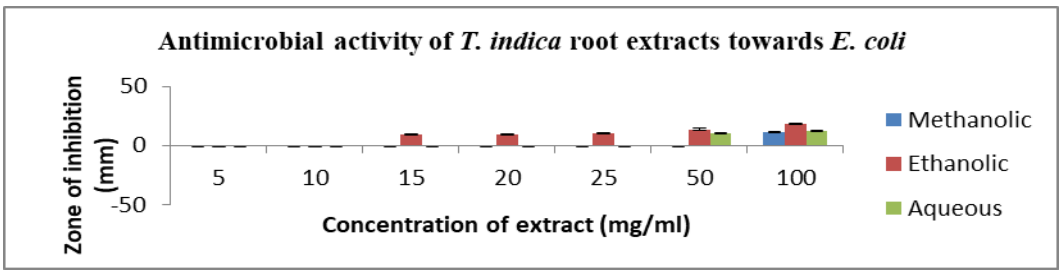
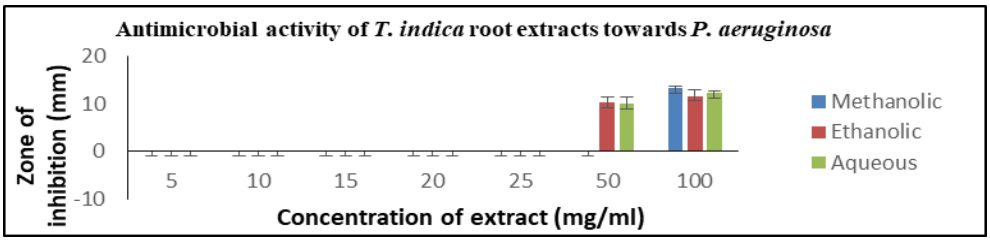


E

Figure 4.1: Antimicrobial potential of leaf extracts of *T. indica* (A-E)



A



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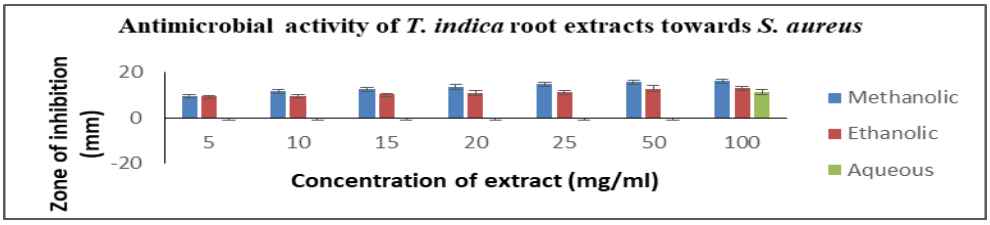

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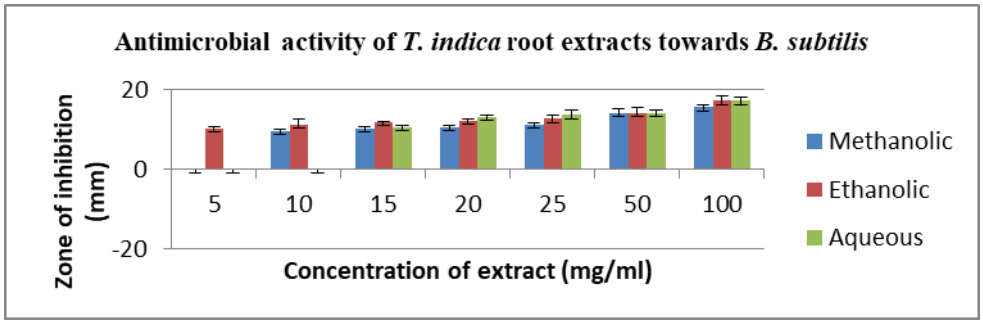

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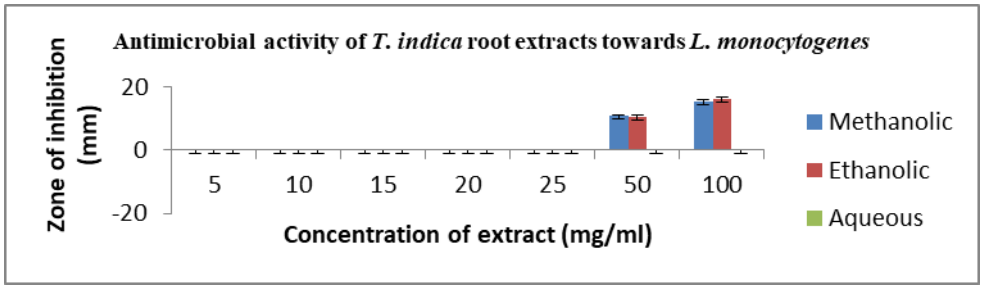
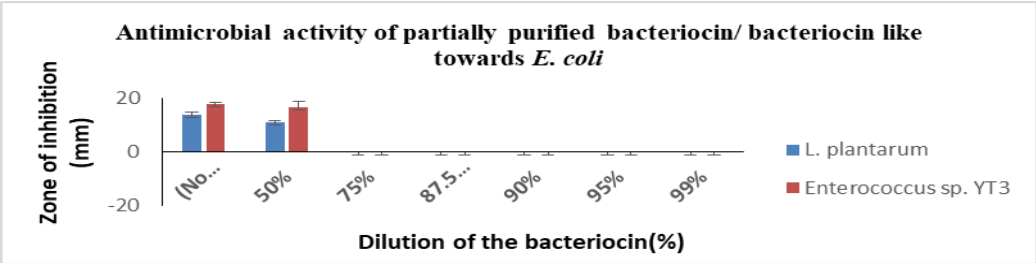
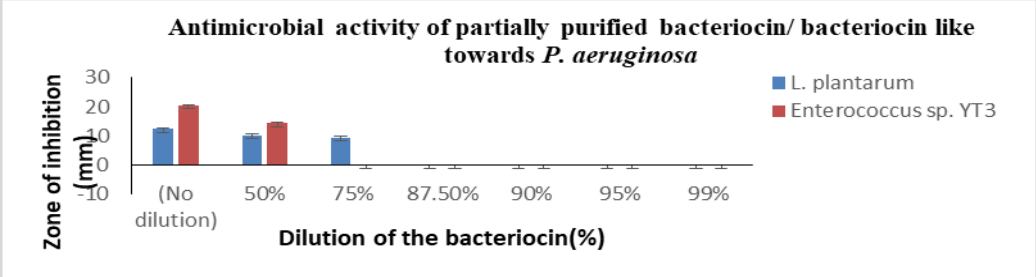


E

Figure 4.2: Antimicrobial potential of root extracts of *T. indica* (A-E)



A



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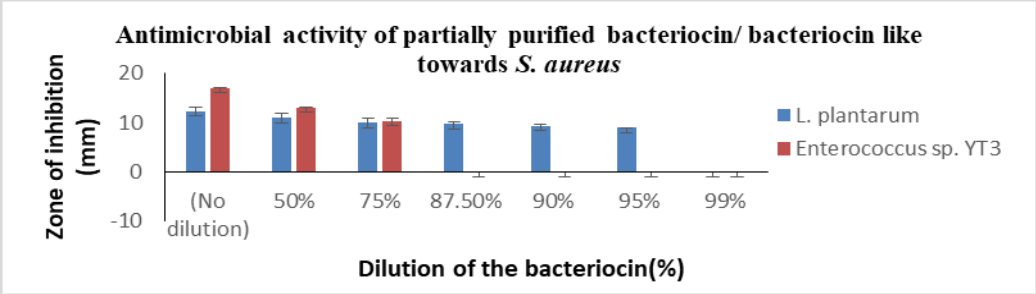

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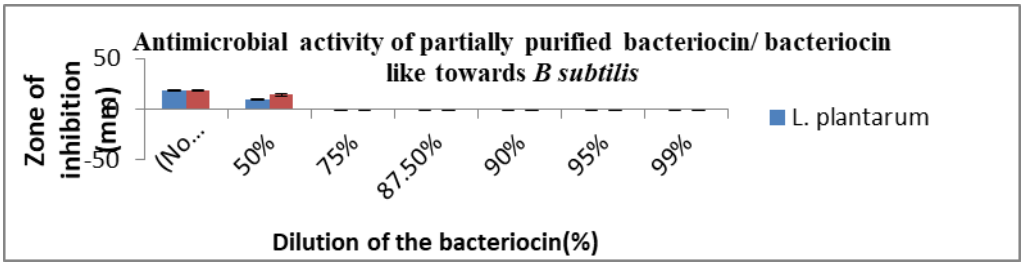

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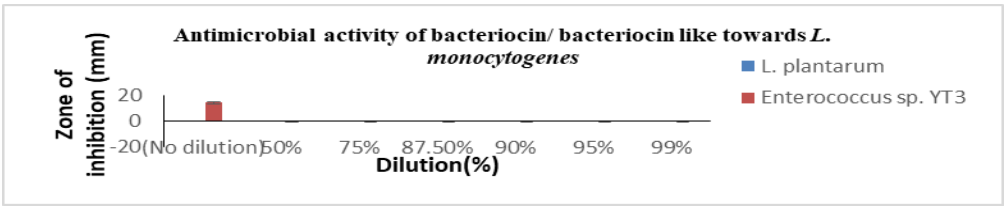
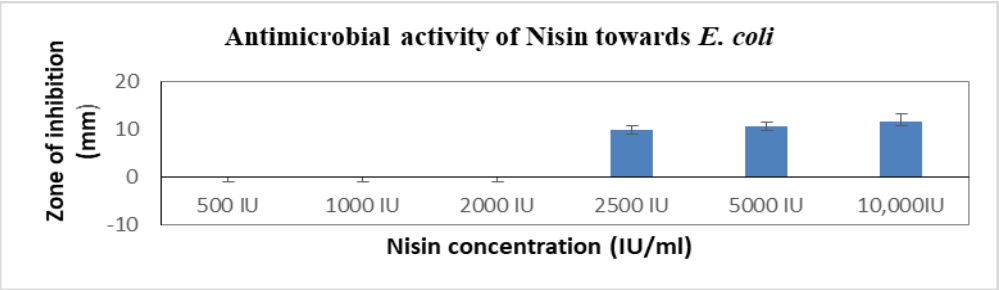


E

Figure 4.3: Antimicrobial activity of Plantaracin 2083 and Enterocin YT3 (A-E)



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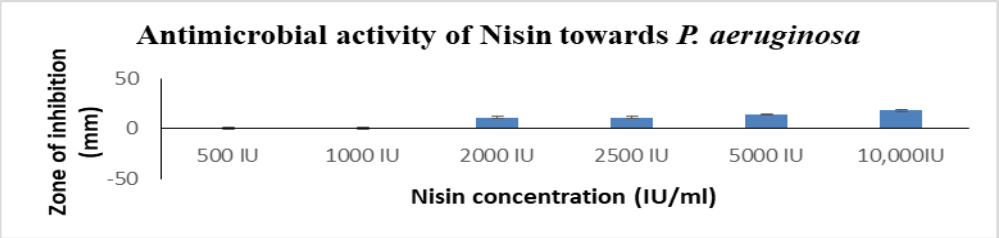

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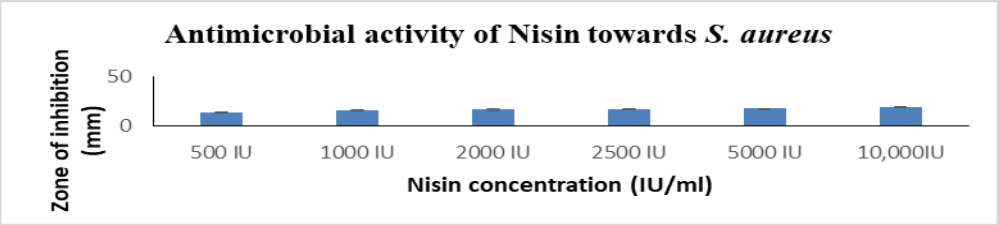

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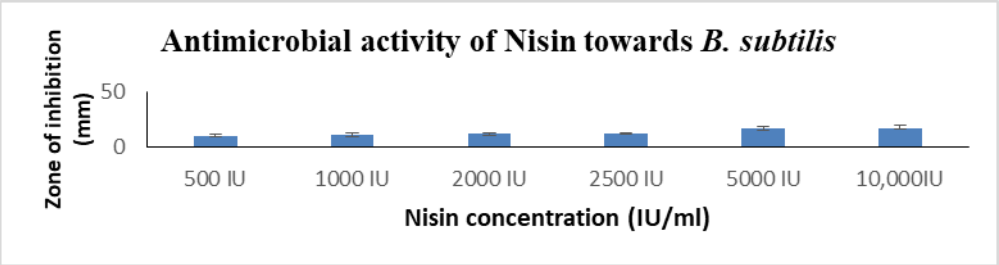

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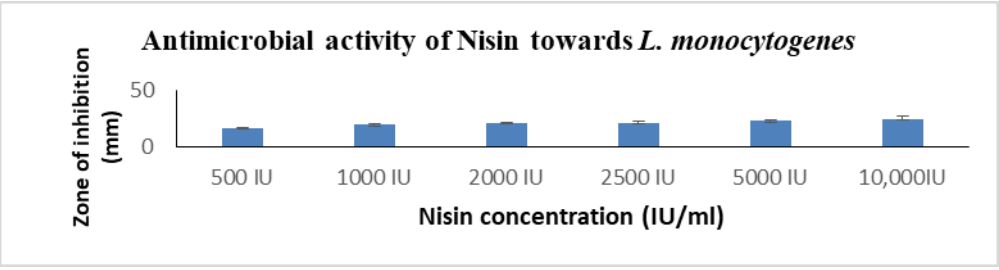
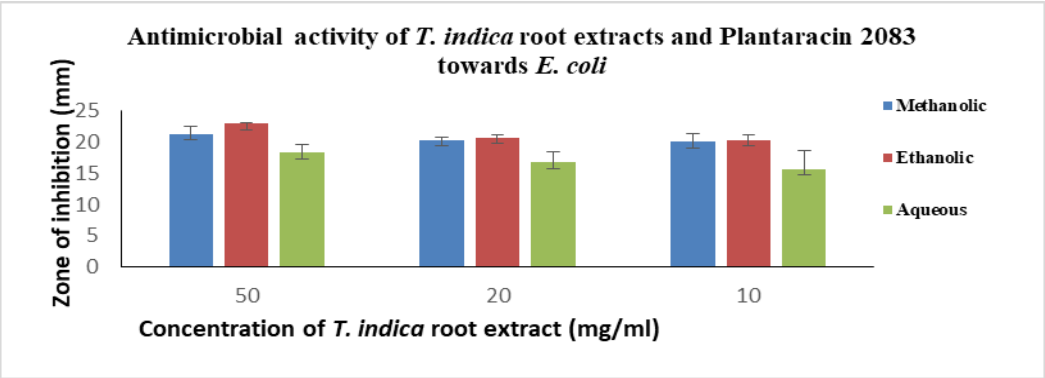


E

Figure 4.4: Antimicrobial activity of Nisin (A-E)



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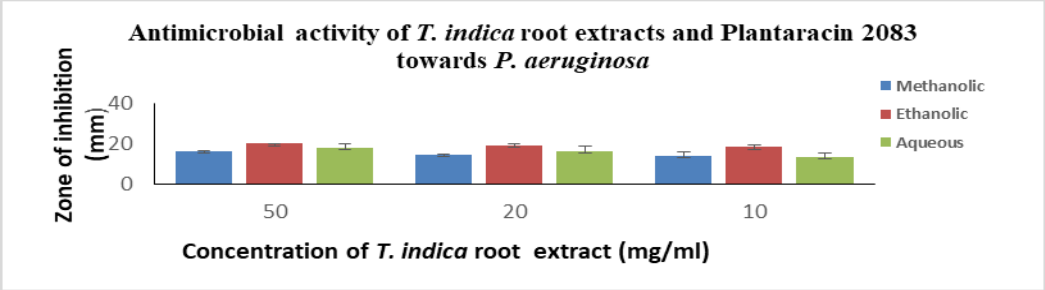

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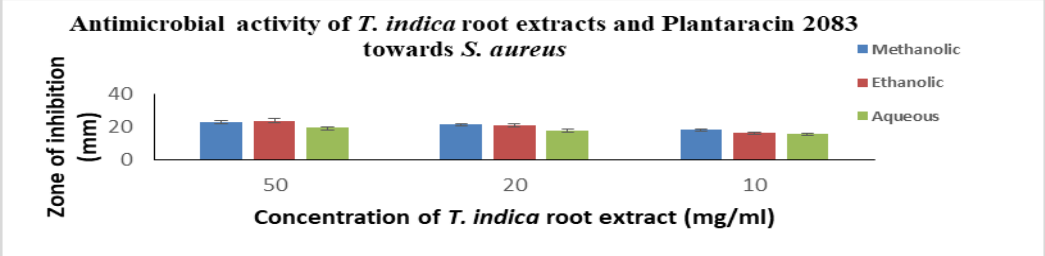

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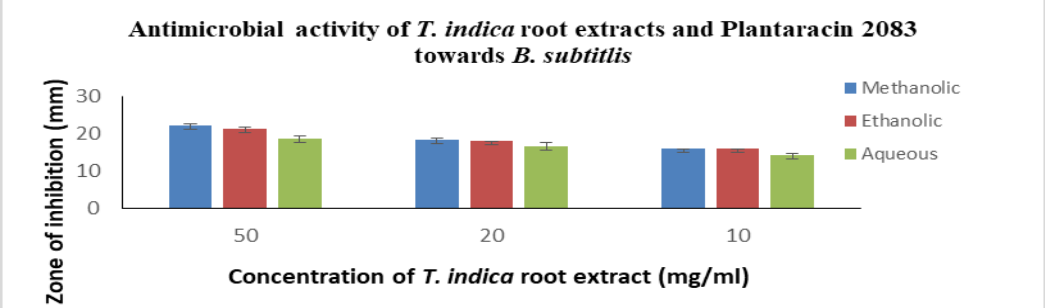

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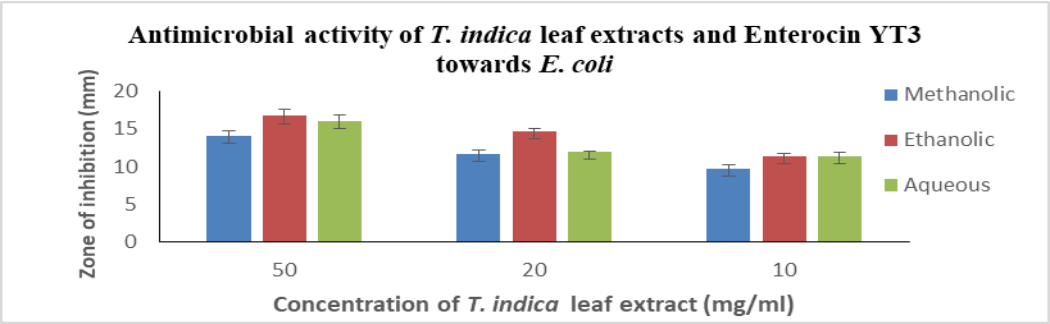
Figure 4.5: Antimicrobial activity of leaf extracts of *T. indica* and Plantaracin 2083 (A-E)



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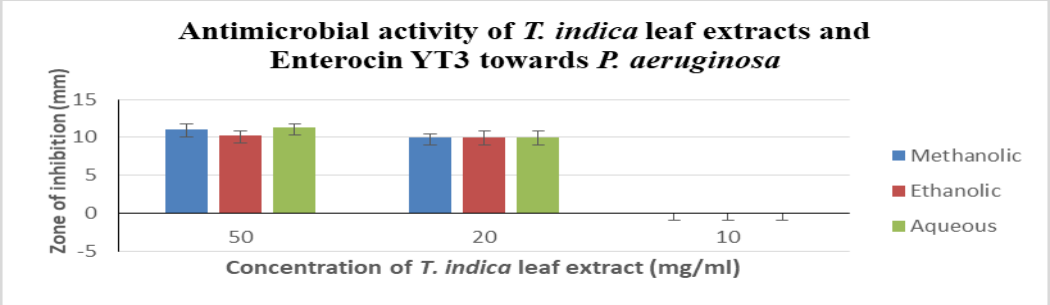

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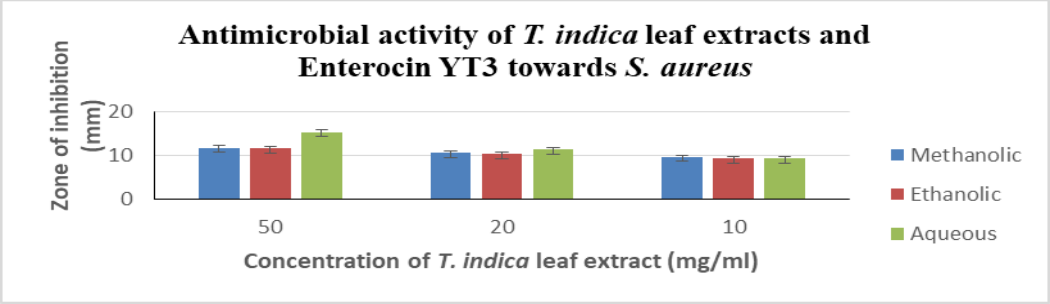

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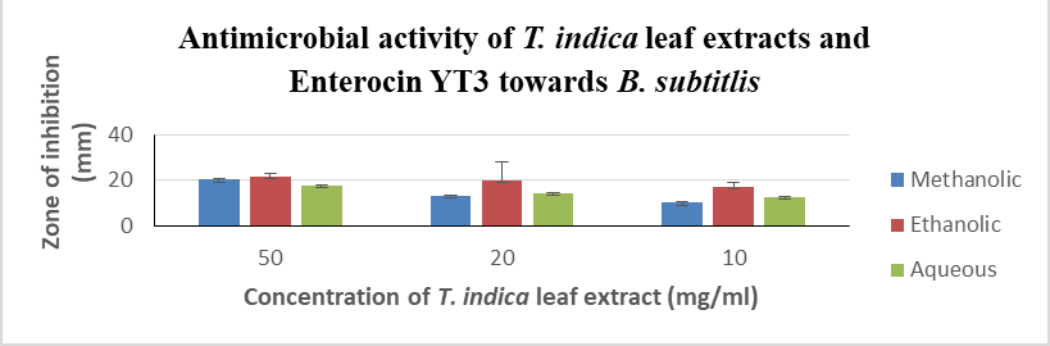
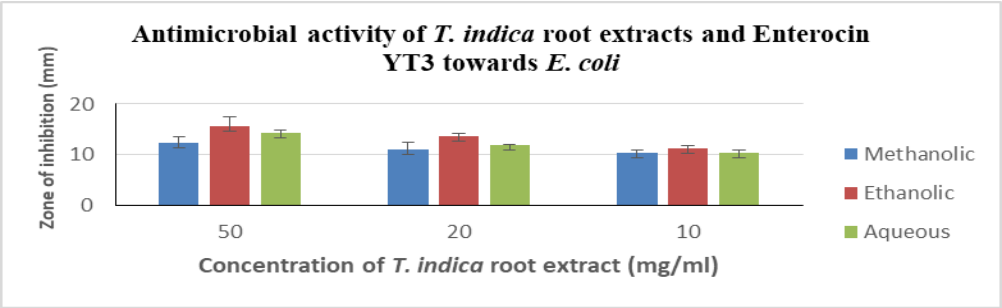


D

Figure 4.6: Antimicrobial activity of leaf extracts of *T. indica* and Enterocin YT3 (A-D)



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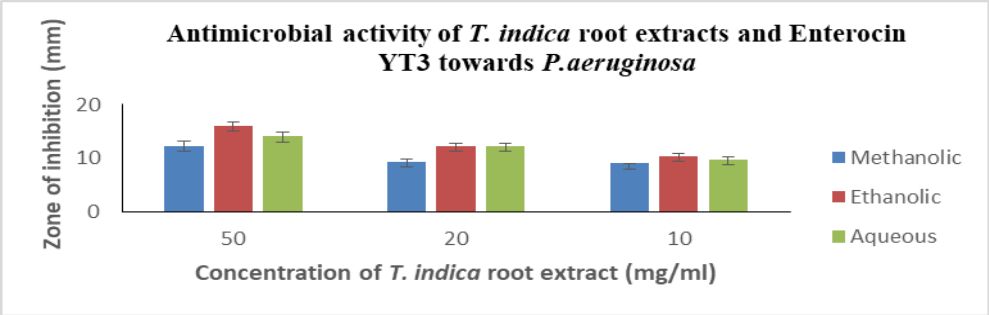

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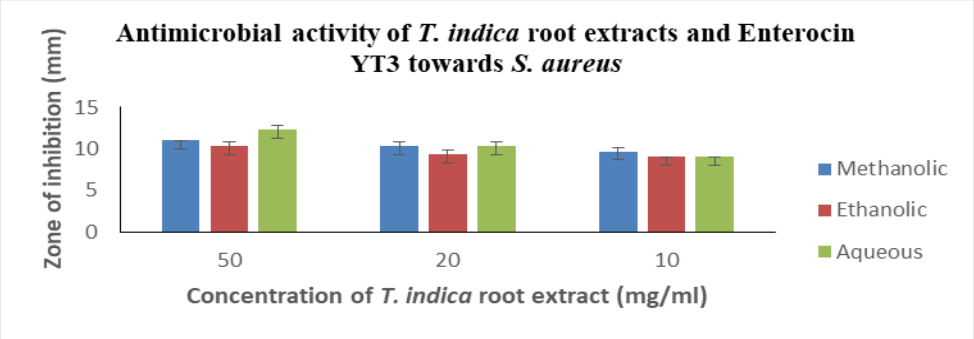

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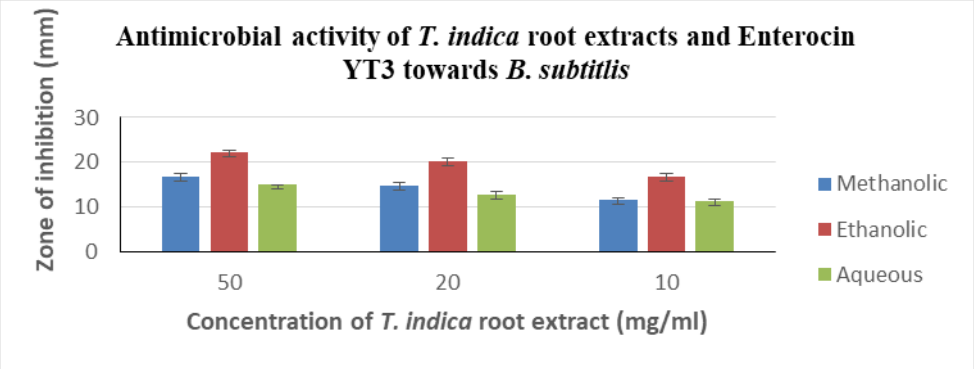
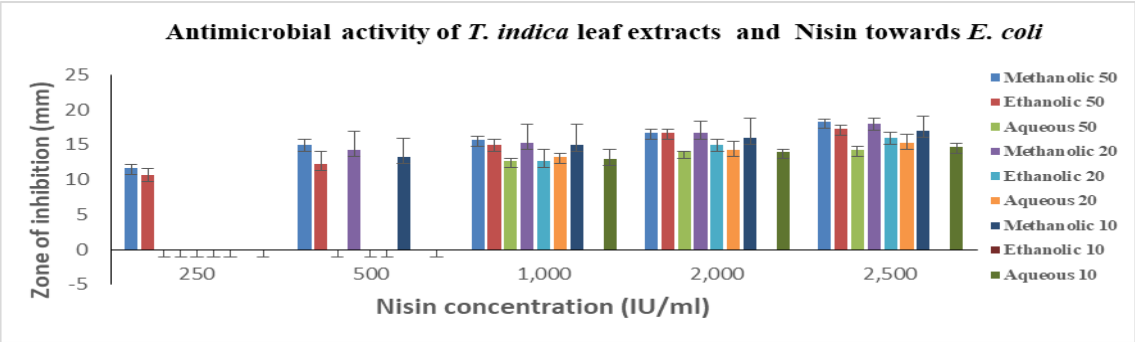
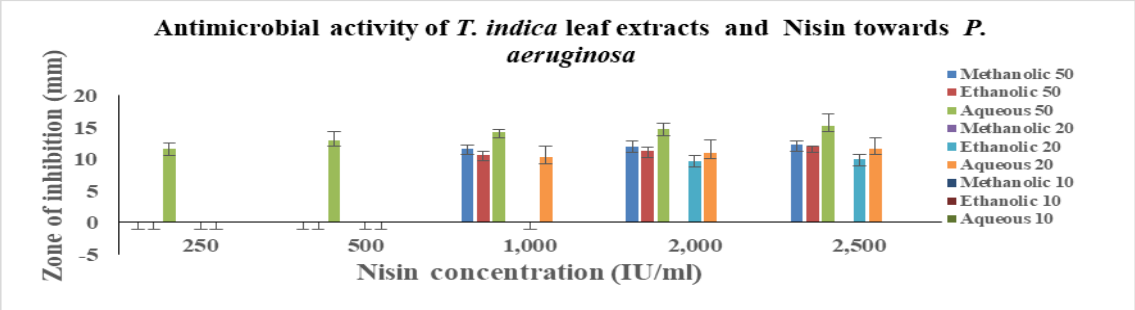


D

Figure 4.7: Antimicrobial activity of root extracts of *T. indica* and Enterocin YT3 (A-D)



A



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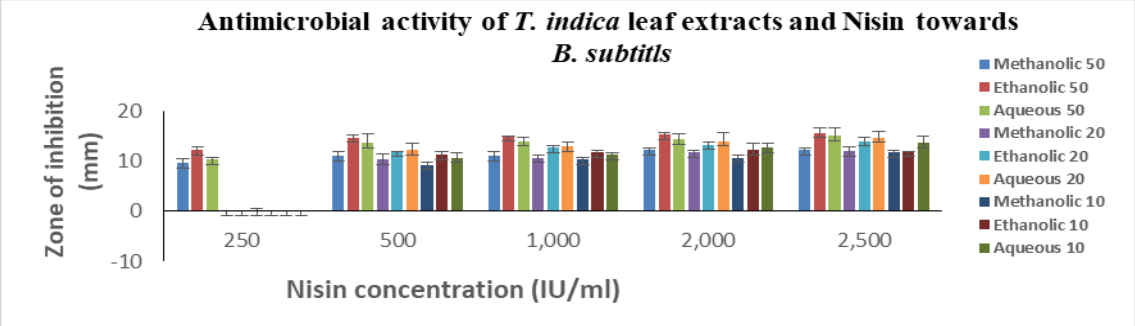

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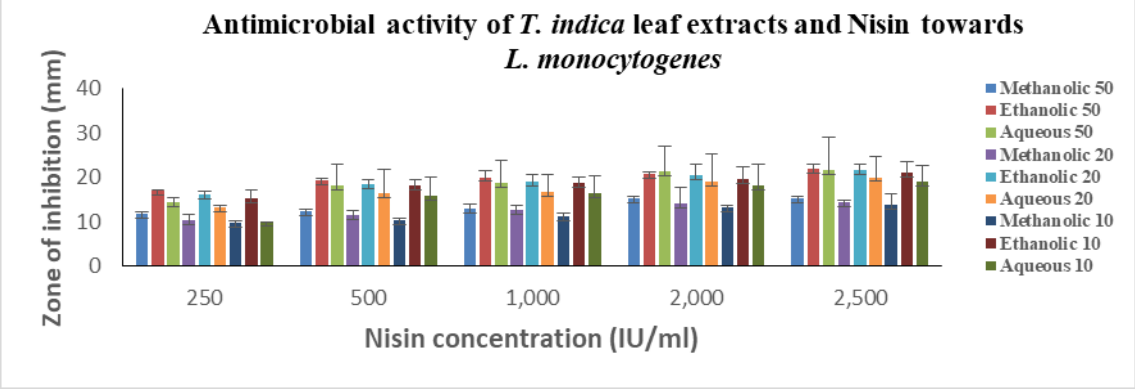
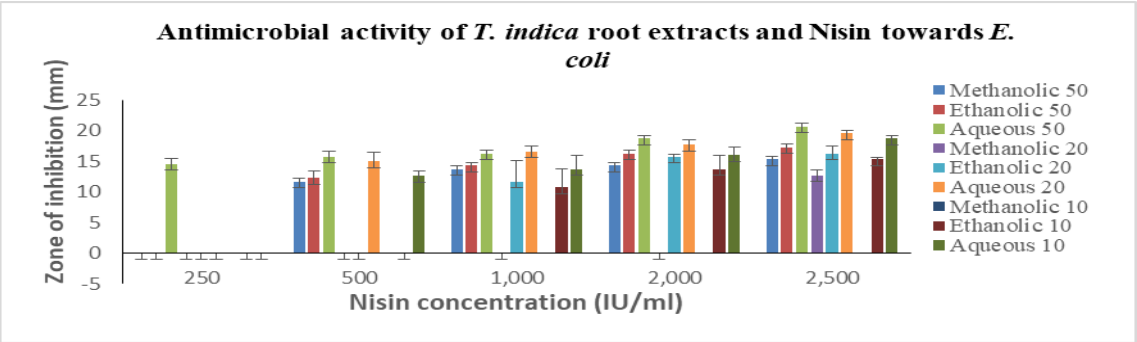
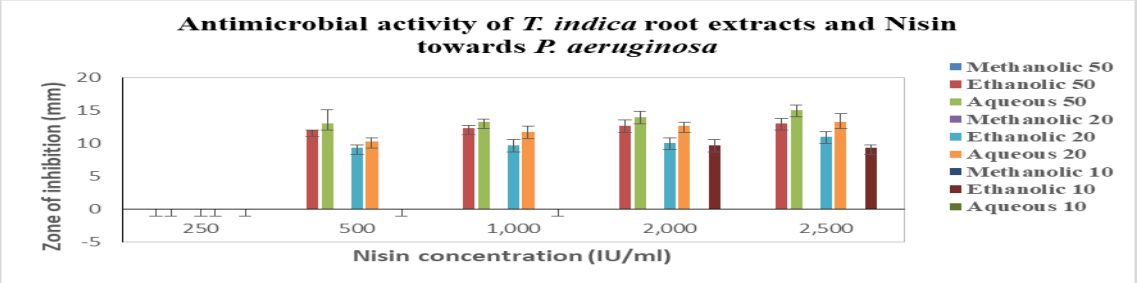


E

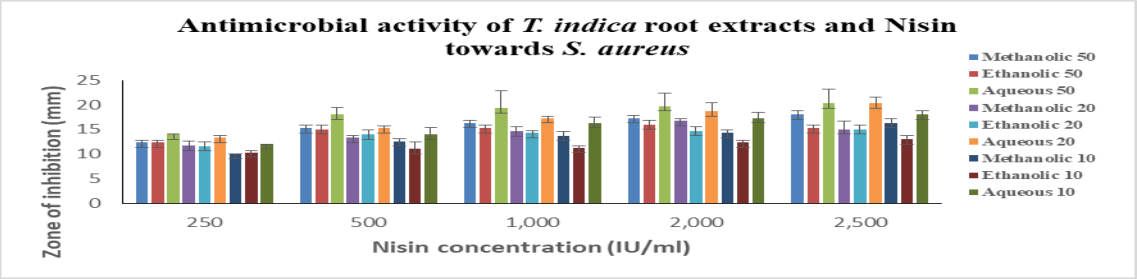
Figure 4.8: Antimicrobial activity of leaf extracts of *T. indica* and Nisin (A-E)


Antimicrobial activity of T. indica root extracts and Nisin towards E. coli
Zone of inhibition (mm)
25
20
15
10
5
0
-5
250
500
1,000
2,000
2,500
Nisin concentration (IU/ml)
Methanolic 50
Ethanolic 50
Aqueous 50
Methanolic 20
Ethanolic 20
Aqueous 20
Methanolic 10
Ethanolic 10
Aqueous 10

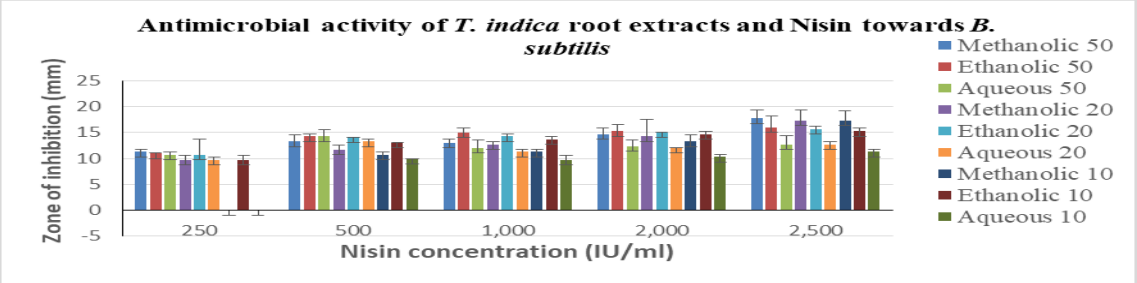
A


Antimicrobial activity of T. indica root extracts and Nisin towards P. aeruginosa
Zone of inhibition (mm)
20
15
10
5
0
-5
250
500
1,000
2,000
2,500
Nisin concentration (IU/ml)
Methanolic 50
Ethanolic 50
Aqueous 50
Methanolic 20
Ethanolic 20
Aqueous 20
Methanolic 10
Ethanolic 10
Aqueous 10

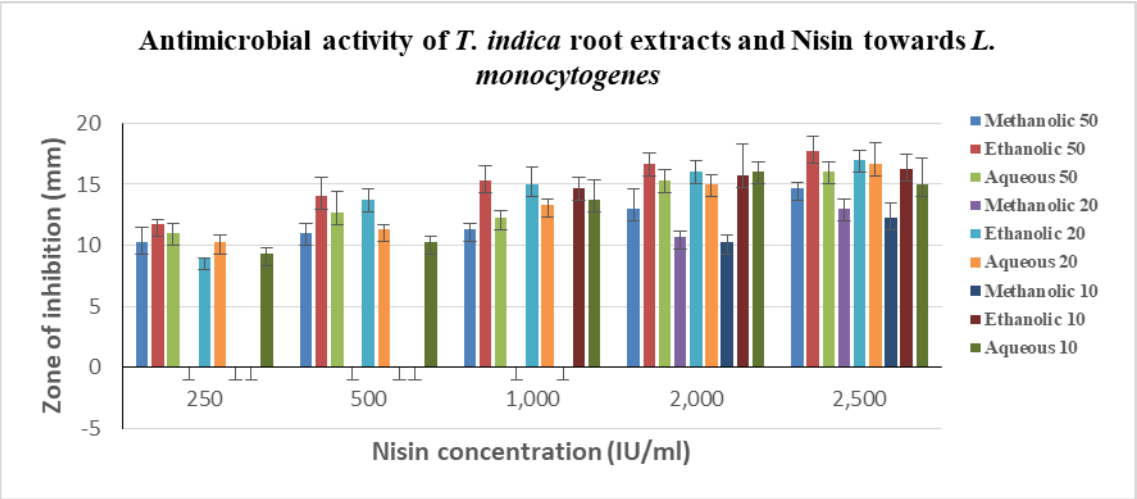
B


Antimicrobial activity of T. indica root extracts and Nisin towards S. aureus
Zone of inhibition (mm)
25
20
15
10
5
0
250
500
1,000
2,000
2,500
Nisin concentration (IU/ml)
Methanolic 50
Ethanolic 50
Aqueous 50
Methanolic 20
Ethanolic 20
Aqueous 20
Methanolic 10
Ethanolic 10
Aqueous 10

C


Antimicrobial activity of T. indica root extracts and Nisin towards B. subtilis
Zone of inhibition (mm)
25
20
15
10
5
0
-5
250
500
1,000
2,000
2,500
Nisin concentration (IU/ml)
Methanolic 50
Ethanolic 50
Aqueous 50
Methanolic 20
Ethanolic 20
Aqueous 20
Methanolic 10
Ethanolic 10
Aqueous 10

D



E

Figure 4.9: Antimicrobial activity of root extracts of *T. indica* and Nisin

Chapter 5

Preparation and characterisation of active packaging film formulations for potential shelf life improvement of meat products

5.1 Introduction and review of literature

Food is the basic source of nourishment of the body and may be constituted by plant or animal origin matter. Lesser number of vegetarian food consumers has been estimated in the western countries (<5%) while in India it has been assumed to be around 5% (Shridhar et al., 2014). This estimation depicts the higher number of non-vegetarian food consumers. With the advancement in the technology, the scope of food industry has also widened to make available the prepared food stuffs to the common consumer. It has been estimated that nearly one-third of the food is wasted or lost from moment of its production up till it is consumed by the humans. This loss and wastage is comparatively higher and even throughout the food supply chain in the developing countries especially with the livestock because of diseases such as pneumonia, parasite infections and digestive infections at the rearing stage (FAO, 2011). The factors contributing the contamination of the meat include and intrinsic and extrinsic conditions during pre-slaughtering and post slaughtering conditions (Table 5.1) (Dave and Ghaly, 2011).

Table 5.1: Conditions affecting spoilage of meat products (Dave and Ghaly, 2011)

Type	Factors
Intrinsic	Species of animal
	Age of animal during slaughtering
	Breed and feeding regimen
	Internal and initial micro flora
	Chemical properties of meat (pH, redox potential, acidity, peroxidase value)
	Processing conditions
	Hygiene (standards of equipment, personnel and cleaning)
Extrinsic	Temperature control
	Packing system
	Storage conditions

This spoliation happens during slaughtering, cutting to smaller pieces, transportation, packaging; hygienic environment and during processing in the slaughter houses or such food units; and the handling and storage at the consumer end (Biswas et al., 2011; Dave and Ghaly, 2011). Since meat provides favorable conditions for the growth of bacteria, fungi and molds, hence the spoilage happens because of physical, chemical and enzymatic activities. This leads to breakdown of the eminent components of meat product such as proteins, fats and carbohydrates resulting to production of off flavors, off odors and slime formation, thereby making such meat products unfavorable for consumption. The basic mechanism responsible for such changes is microbial growth, oxidation and autolysis by the enzymes. The different bacterial contaminants responsible for deterioration of meat includes species belonging to genera *Aeromonas, Acinetobacter, Bacillus, Brochothrix, Campylobacter, Clostridium, Corynebacterium, Enterobacter, Enterococcus, Escherichia, Micro bacterium, Micrococcus, Lactobacillus, Lactococcus, Listeria, Pseudomonas, Salmonella, Staphylococcus* and *Vagococcus* (Adentunji, 2008; Dave and Ghaly, 2011). These microbes cause the changes in pH, degradation of the cellular structures, formation of slime, changes in appearance of meat and off odors. The oxidative deterioration is caused due to autoxidation of lipids along with free radicals production leading to off odors. The lipid hydrolysis in meat is even caused by enzymatic and non-enzymatic processes. The softening and discoloration of the meat tissue happens because of tissue degradation of food components during autolysis process (Dave and Ghaly, 2011). Hence, the food poisoning syndrome along with food loss is a global issue concerned with the meat industry (Biswas et al, 2011). This loss can be controlled by the involvement of preservatives and/or proper packaging technologies (Lipinski et al., 2013). A change in paradigm is observed where the newer concept focuses on the techniques to reduce meat spoilage, extended shelf life, quality preservation, information delivery, eco-friendliness, logistics, safety of packaging along with higher functionality (Lee, 2018).

The antimicrobial agents refer to any of those compounds which possess the capability to supress, block or interfere with the growth of wide range of organisms especially pathogenics such as bacteria, fungi and moulds. Their applications as

preservatives for food items and as medicine can be witnessed since ancient times. With the changing generation, more concern has been witnessed by the general population for the use of chemical and artificial preservatives coming in the food chain. In the recent scenario, ready to eat and packed foods have major hold for the food industry, and packaging of these items holds a high importance. Packaging is a system that aims to provide a wrap or coat to the food items so as to isolate it from the environment bearing pathogenic organisms, pollutants such as dust, moisture, odors and gases. (Sung et al 2013; Joerger, 2007) The contamination of food with these matter lead to its spoilage by causing changes in colour, texture, odor, nutritive amounts and growth of pathogens which renders the food inedible along with its deleterious effects in the body, if eaten. (Malhotra et al, 2015) The preservative, if added in the food during its formulation, gets depleted with time because of its degradation naturally or due to the interaction complexity with the food matrix (Table 5.2).

Table 5.2 Relative merits of active packaging over preservatives directly added in the food matrix (Sung et al, 2013).

S. No	Preservatives in food matrix	Active Packaging
1	Shorter activity due to its direct release in the food matrix	Longer activity duration due to its slow release into the food matrix
2	Food may cause inactivation of antimicrobial activity by neutralization, dilution and hydrolysis	Being attached to film, food may cause limited inactivation of antimicrobial activity
3	May interfere with texture and organoleptic quality of food	Does not interfere with texture and organoleptic quality of food

Thus, the hurdle technology is a supplement for enhancing the shelf life of the packed foods. The strong rise in demand for the use of naturally derived preservatives offering low risk to consumer health led to research involving preparation of films incorporating bio preservatives; either occurring naturally or derived from animals or plants (Nicholson, 1998). The latest trend for food packing system involves the use of

active packaging. This refers to a system in which some modification is targeted in the environment of the packed food leading to alteration of the state of food and the headspace present in it for enhancement of shelf life, improvement for sensory qualities along with retention of microbial safety limits. It incorporates systems such as oxygen or carbon di oxide scavengers, absorption systems for ethylene or moisture, antioxidants and antimicrobial containing or releasing system (Malhotra et al, 2015). Research on the edible and polymeric film packaging system incorporating natural antimicrobials such as bacteriocin and plant extract has been focussed in last decade. The antimicrobial films decrease and prevent the microbial growth on the food surface by the direct contact. Although the efficacy of such films is reduced after certain time due to their degradation, still a control release system provides a better solution. Nisin, a bacteriocin, has been studied in the different packaging formulations alone or along with other antimicrobial agent. Table 5.3 and 5.4 clearly presents the efficacy of different edible and non-edible films incorporating nisin for its antimicrobial potential. Table 5.5 presents the studies which have been conducted to scrutinize the potential of binary combination of nisin along with other antibacterial agents such as EDTA, lysozyme, grape seed extract and green tea extracts. Some research has also been carried to analyze the efficacy of packaging films with bacteriocin other than nisin such as pediocin and lacticin (Table 5.6). Since plants also behave as efficient antimicrobial agents, so few studies analyzing this potential in packaging films has been targeted to understand the effects on shelf life analysis of food products. Table 5.7 presents the various herbal extracts such as turmeric, Neem extract, oregano, cinnamon and rosemary that have been studied in the active packaging films and their potential as preservatives in food products.

Table 5.3: Studies incorporating nisin in the edible films (Adapted from Joerger, 2007)

Type of Film	Test Medium	Duration (Days)	Incubation Temperature (°C)	Test organism	Reduction of log_{10} CFU
Soy	Turkey bologna	21	4	*L. monocytogenes*	Approx. 1
Soy	1% Peptone water	2	22	*L. monocytogenes*	Approx. 1
Soy	Phosphate buffer, pH-7	-	21	*L. monocytogenes*	0
Soy	Phosphate buffer, pH-7	-	21	*Salmonella enterica*	0.2
Zein	0.1% Peptone water	2	23	*L. monocytogenes*	5
Zein	0.1% Peptone water	2	23	*Salmonella enterica Enteridis*	0
Zein/propylene glycol	Chicken	24	4	*L. monocytogenes*	3.2
Zein/ Ethanol	Chicken	24	4	*L. monocytogenes*	1.5
Zein/ Ethanol	Chicken	24	8	*L. monocytogenes*	1.2
Zein/ Propylene glycol	Chicken	24	8	*L. monocytogenes*	1.3
Wheat gluten	Turkey bologna	56	4	*L. monocytogenes*	1
Egg albumin	Phosphate buffered saline	1hr	21	*L. monocytogenes*	1.3
Whey protein isolate	Phosphate buffered saline	1hr	21	*L. monocytogenes*	2.4
Wheat gluten	Phosphate buffered saline	1hr	21	*L. monocytogenes*	1
Soy protein isolate	Phosphate buffered saline	1hr	21	*L. monocytogenes*	2.2
Tapioca starch/ Glycerol	Tryptic soy broth pH-6.5 pH- 7.2 pH- 5.5	28	4	*L. innocua*	 Approx. 2 0 0
Soy protein isolate	Turkey frankfurters	28	4 10	*L. monocytogenes* *L. monocytogenes*	2.1 0.1

Table 5.4: Studies incorporating nisin in the inedible films (Adapted from Joerger, 2007)

Type of Film	Test Medium	Duration (Days)	Incubation Temperature (°C)	Test organism	Reduction of log_{10} CFU/ Zones of inhibition (ZOI)
LDPE with skim milk binders or polyamide	Pot. Phosphate buffer, pH 7, 20mM	7	10	*M. flavus* *L. monocytogenes*	4 approx. 0.5 approx.
LDPE with skim milk binders or polyamide	Agar media	2	-	*E. coli, B. subtilis, B. cereus, L. monocytogenes, L. mesenteroides, P. aeruginosa, S. aureus* *M. flavus*	No zones of inhibition Zones of inhibition found
LDPE	Oyster	25	3	Total aerobic count Coliforms	1.8 approx. 0.3 approx
LDPE	Oyster	15	10	Total aerobic count Coliforms	1 approx. 0.4 approx
LDPE	Ground beef	25	3	Total aerobic count Coliforms	1.5 approx. 0.7 approx
LDPE	Ground beef	15	10	Total aerobic count Coliforms	1.5 1 approx.
LDPE	Tryptic soy broth	2	4	*Micrococcus luteus*	0.8 approx.
LDPE	Raw milk Pasteurised milk	4	4	Total viable count Total viable count	0.3 approx. 0.2 approx.
Polyethylene with methyl cellulose/HPMC coat	Tofu	30	5	*L. monocytogenes*	6.1
Plastic with HPMC/MC	Hot dogs	60	4	*L. monocytogenes*	6.2
Polyethylene	Beef tissue	21	44	*Brochothrix thermosphacta*	1.1
Polyethylene	Beef tissue	21	4	*Brochothrix thermosphacta*	1.2
Polyethylene	Beef tissue	20	12	*Brochothrix thermosphacta*	2.8 approx.
Packaging paper inserts	Cheese under MAP Cheese under MAP Cheese under MAP Cooked, sliced ham Cooked, sliced ham Cooked, sliced ham Cooked, sliced ham	24	4	Non-starter LAB *L. innocua* *S. aureus* LAB Total aerobic count *L. innocua* *S. aureus*	1.8 approx. 2.6 approx. 2.5 approx. 3.9 approx. 1.4 approx. 1.5 approx. 0.9 approx
Paper board with Vinyl acetyl ethylene copolymer	Milk cream Water Nutrient broth Water Water Milk Milk Milk Orange juice Orange juice Orange juice	12 12 12 12 12 16 16 16 16 16 16	10 10 10 10 10 3 10 20 3 10 20	Total aerobic count *M. flavus* *M. flavus* *L. monocytogenes* *E. coli O157:H7* Total aerobic count Total aerobic count Total aerobic count Yeast Yeast Yeast	2 approx. 1.5 approx. 8 approx. 2 approx. 0 6 approx. 9 approx. 0 3 approx. 3 approx. 0
Sodium alginate or k- carrageenan	BHI agar	1	30	*L. innocua, E. coli, S. aureus*	No ZOI

Table 5.5: Studies incorporating nisin along with other antimicrobial agent in the packaging films (adapted from Joerger, 2007)

Type of Film	Additive (Antimicrobial agents)	Test Medium	Duration (Days)	Incubation Temp (°C)	Test organism	Reduction of log_{10} CFU/ Zones of inhibition (ZOI)
Sodium-alginate	Nisin/ EDTA/ Lysozyme	BHI agar	1	30	*E. coli, , L. innocua, Micrococcus luteus, S. aureus, S. enteriditis*	Zone of inhibition found
Polyethylene	Nisin/EDTA	Beef tissue	21	44	*B. thermosphacta*	9.5
Soy	Lauric acid+ nisin	1% peptone water	2	22	*L. monocytogenes*	9.5
	Lauric acid+ nisin	Turkey bologna	21	4	*L. monocytogenes*	1 approx.
	Nisin/ 2.6%citric acid	Phosphate buffer pH-7			*L. monocytogenes*	1.4
	Nisin/2.6% Lactic acid	Phosphate buffer pH-7			*E. coli O157:H7*	0.3
7% gelatin	Lysozyme, Nisin, EDTA	Bologna	28	4	*Brochothrix thermosphacta*	7.6
		Ham			*Brochothrix thermosphacta*	7.7
		Bologna			*E. coli O157:H7*	0.1
		Ham			*E. coli O157:H7*	0
		Bologna			*Lactobacillus sakei*	4.0
		Ham			*Lactobacillus sakei* *L. mesenteroides*	7.9
		Bologna			*L. mesenteroides* *L. monocytogenes*	7.6
		Ham			*L. monocytogenes*	7.7
		Bologna			*S. enterica*	7.7
		Ham			*Typhimurium*	7.4
		Bologna			*S. enterica* *Typhimurium*	4.2
		Ham				0.6
Zein	Nisin/EDTA	0.1% Peptone water	2	23	*L. monocytogenes*	7
	Nisin/EDTA/ Lauric acid				*L. monocytogenes* *S. enterica* *Typhimurium*	7 0.2
	Nisin/ Lauric acid (4%)	0.1% Peptone water	0.25	-	*L. plantarum*	3 approx.
	Nisin/ Lauric acid (8%)					8.5 approx.
Zein/ Propylene glycol	Nisin/ Ca propionate (1%)	Chicken	24	4	*L. monocytogenes*	6.8
				8		1.8
Zein/ Ethanol				4		1.5
				8		2.7
Zein/ Ethanol/ Glycerol	Nisin/ sodium lactate/ sodium diacetate	Turkey Frankfurt	28	4	*L. monocytogenes*	1.4
Zein/ Propylene glycol						3.2
Zein/ Ethanol/ Glycerol	Nisin and Potassium sorbate (0.4%)	Turkey Frankfurt	28	4	*L. monocytogenes*	
					10^6 cfu/g	2.2
					10^4 cfu/g	1.5
Zein/ Propylene glycol					10^6 cfu/g	2.8
					10^4 cfu/g	2.4
Soy protein isolate	Nisin+ Grape seed extract	Turkey frankfurt	4	28	*L. monocytogenes*	3.8
			10			2.7
	Nisin+ Green tea extract		4			3.7
			10			2.8

Table 5.6: Studies incorporating bacteriocin (other than nisin) in the packaging films (Adapted from Joerger, 2007)

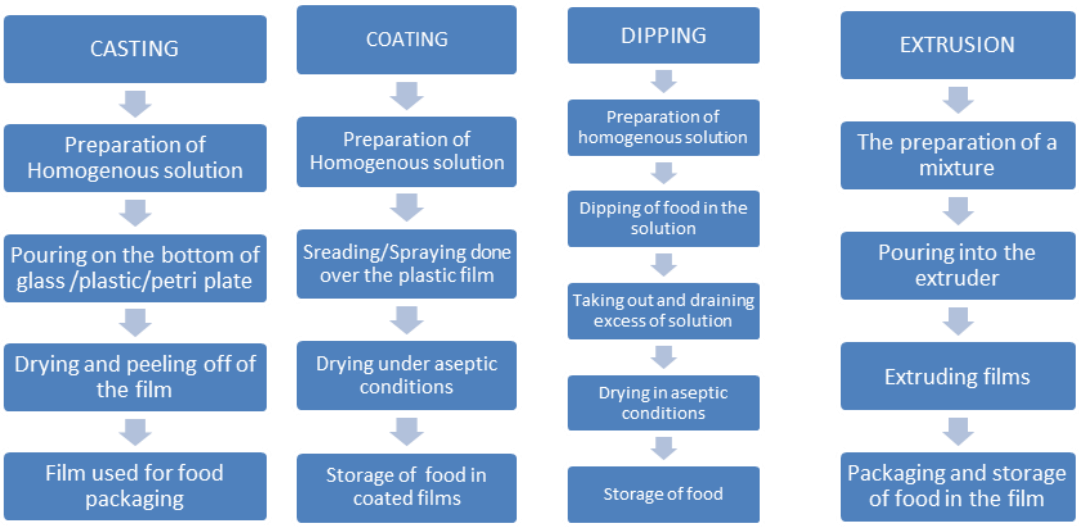
Type of Film	Bacteriocin used	Test Medium	Duration (Days)	Incubation Temp (°C)	Test organism	Reduction of log_{10} CFU/Zones of inhibition (ZOI)
Polythene	Bacteriocin from *L. curvatus*	Phosphate buffered saline	1	21	*L. monocytogenes*	1 approx.
			1	4		1.5 approx.
		Phosphate buffered saline	1	21		0
		Tryptic soy broth	2	4		0
		Tryptic soy broth	2	4		6.5 approx.
			4	22		2.7
		Frankfurter's	3	3		0.2 approx.
		Pork streak				
		Hamburger				
Cellulose	Pediocin powder	Ham	84	4	*L. monocytogenes*	1.6 approx.
		Turkey breast				2.8 approx.
		Beef				1.6 approx.
LDPE with skim milk or Polyacrylamide as binders	Lactocin NK24 or BH5	Agar media	2	-	*E. coli, B. subtilis, B. cereus, L. monocytogenes, L. mesenteroides, P. aeruginosa, S. aureus M. flavus*	No ZOI ZOI found
Polyethylene	Bacteriocin from *L. curvatus*	Cold smoke salmon	4	22	*L. monocytogenes*	2.7
Whey protein	Bacteriocin from *Pediococcus parvulus*	Peptone water	1	10	*L. innocua*	< 0.4
Zein						4.5 approx.

Table 5.7: Studies incorporating plant extracts as antimicrobial agent in the packaging films (Adapted from Joerger, 2007)

Type of Film	Additive (Antimicrobial agents)	Test Medium	Duration (Days)	Incubation Temp (°C)	Test organism	Reduction of log_{10} CFU/ Zones of inhibition (ZOI)
Casein	Turmeric	Carrots	4	-	Total aerobic count Yeast and mould Coliforms	8.3 4.7 6.2
LDPE	Grape fruit seed extract	Curled lettuce	15	5	Total aerobic count LAB *Yeast*	0.1 approx. 0 0
Multi-layered Polyethylene	Grape fruit seed extract (co-extruded) Grape fruit seed extract (coating)	Ground beef	18	3	Total aerobic count Coliforms Total aerobic count Coliforms	0.5 approx. 0.5 approx. 2.5 approx. 0.5 approx.
Starch/ casein	Neem extract	Nutrient agar Listeria selective agar with supplement	1	37	*E. coli, B. cereus, S. aureus, S. enterica Typhimurium*	Zone of inhibition found
Apple puree edible film	Oregano Cinnamon Lemon grass	MacConkey sorbitol	2	37	*E. coli O157:H7*	Zone of inhibition found
Whey protein	Oregano Garlic Rosemary	Brain heart infusion agar	1	37	*E. coli O157:H7, S. aureus, S. enterica Typhimurium, L. plantarum, L. monocytogenes*	Zone of inhibition found No zone of inhibition found
Chitosan	Garlic oil	Muller Hinton agar	1	37	*E. coli, S. aureus, S. enterica Typhimurium, B subtilis*	Zone of inhibition found
Alginate	Garlic oil	Muller Hinton agar	1	37	*E. coli, S. enterica Typhimurium* *S. aureus* *B. cereus*	No zones Zones found
Caseinate	Spice powder (Thyme, sage, rosemary)	Ground beef	10	4	*Coliforms, enterobacteriaceae, Pseudomonas species, Staphylococcus species, Brochothrix species*, LAB	Zone of inhibition found

The above review indicates the potential of different packaging film that may be used as preservatives to enhance the shelf life of food products.

As a function for active anti-microbial food packaging, different strategies have been adopted for the preparation of such films. In a study, films incorporating chitosan-bacteriocin as antimicrobials, different methods were adopted (Figure 5.1). These methods included direct casting, immersing/ dipping, coating, spray coating, spread coating, layer by layer assemble and extrusion (Wang et al., 2018).



(a) (b) (c) (d)

Figure 5.1: Process of packaging film preparation by different techniques: a: Casting; b: Coating; c: Dipping; d: Extrusion

Each method had its own pros and cons, as highlighted in table 5.8. A comparative study of films involving these techniques to analyze the shelf life of beef sticks, the films with coating method was found to be most fruitful illustrating this method to be better (Lago et al., 2014).

Table 5.8: Relative merits of techniques involved in preparation of food packaging materials

Technique	Merits	Demerits
Direct casting of packaging films	• Excellent gas barrier properties • Dense and homogenous films • Green and biodegradable in nature	• Requirement of special equipment • Long drying time • Complicated operated process
Packaging films with coating	• Low cost • Easy process • No need of special equipment • Significant better antimicrobial potential to maintain food quality • Reduce water vapor permeability • Does not significantly affect the physical properties of the film	• Non-biodegradable when plastic film is formed
Direct food coating with antimicrobial coating solution	• Uniform films develop specially over fruits • Alleviate chilling injuries • Lowered color variation • Better quality maintenance • Delayed decay of food product	• Specific storage conditions required • Dependence on wetting capacity of surface of food • Longer processing and draining time
Films prepared with extrusion technique	• Acceptable mechanical properties of some selected films • Good thermal stability of the film • Effective antimicrobial property • Biodegradable	• Poor interaction between polymer and antimicrobial agent may affect mechanical property and hence brittle failure • Temperature maintenance during casting

To study the anti-biological effects of packaging films, different strategies have been adopted and the results conveyed. In an extensive review done by Joerger, 2007, many studies report the efficacy of the films under study with diffusion assays in plates as zone of inhibition, others have reported with changes that happen either in terms of colony counts (CFU/ml or CFU/cm^2) or as log_{10} reduction values (Table 5.3-5.7). The major issue for understanding the real time efficacy of active films remains unanswered, when they are tested against specific organisms only. In real time, enormous and diverse microbes of the environment need to be tackled to enhance the shelf life of the food products (Joerger, 2007). Limited number of studies could be witnessed which reports the effects towards total aerobic counts values (Table 5.3-5.7). Hence, conducting the tests with actual foods should be a required step while

analyzing the efficacy of a film by the researchers. This step determines its importance as the results of antimicrobial potentials in buffers and in solid media may not be reflecting similarly in the actual food stuffs (Joerger, 2007). The functionality of the prepared active packaging films and other antimicrobial food agents are determined by the counts of microbial growth. Any food is assumed to be spoiled when the total microbial counts beyond 10^7 CFU/g. Unpleasant or foul odor may be observed when this count exceeds 10^8 CFU/g. To indicate the shelf life analysis, most researchers refer to this limit of 10^7CFU/g or ml or cm^2 as the standard (Sung et al, 2013).

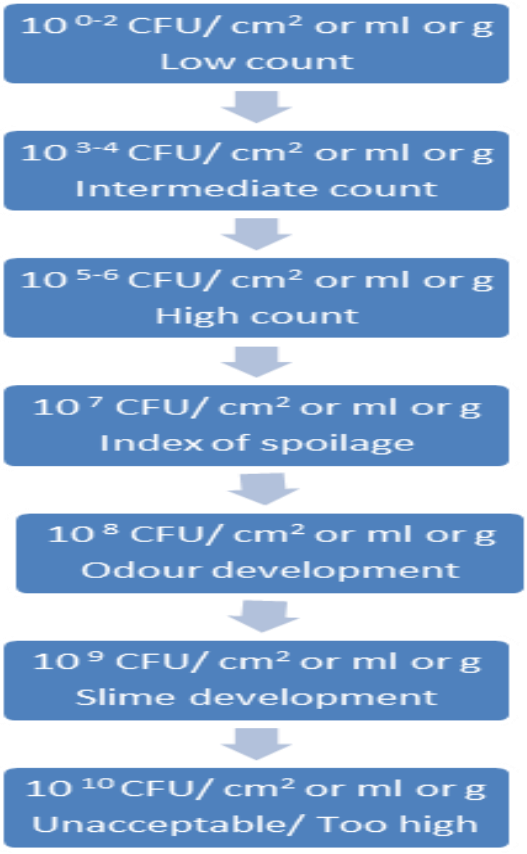


Figure 5.2: Microbial food deterioration index as per the bacterial count (Sung et al., 2013)

Hence, this study has been aimed to prepare different coating solutions involving best selected antimicrobials, as from chapter 4, and incorporate them on the LDPE to form

active packaging film formulation. Thereafter, the prepared films were studied for their role to improve the shelf life analysis in raw chicken meat sample. The film exhibiting best results were characterized for toxicity, and other physical parameters.

5.2 Materials and Methods

5.2.1 Materials

The extracts were prepared using powdered leaves and roots of *T. indica* (as already discussed in Ch. 2). Nisin E234 was obtained from Hangzhou Bioactive Yeast Company Ltd, China. The different growth media used in this study were obtained by Himedia, Mumbai, India. The chemicals used were of analytical grade and were obtained from Loba Chemicals, Mumbai, India. The glassware used was of borosilicate. Stretchable Low Density Polyethylene (LDPE) film was purchased from local shop, Jalandhar, Punjab. Chicken breast was purchased from Easy Day Store, Model Town, Jalandhar, Punjab, India.

5.2.2 Methods

5.2.2.1 Preparation of packaging film using different antimicrobial coating solutions and packaging of meat sample

The cellulose based carrier solution was prepared by dissolving required amount of nisin in 1.25 ml of 0.02M acetic acid (pH 2) in a 100 ml sterilized glass capped bottles. 25 ml of double distilled sterilized water was slowly added in the nisin solution over a magnetic stirrer at 700 rpm. Subsequently, 0.875 g of methylcellulose was slowly added in the above mixture, followed by addition of 0.375g of hydroxyl-propyl methyl cellulose ensuring no clumps were formed. Thereafter, 25 ml ethanol was slowly added by continuous stirring. Finally, 0.75 ml of polyethylene glycol 400 (PEG 400) was added and the solution was stirred at 1700 rpm for 2 minutes for uniform mixing of the constituents. This solution was then allowed to degas for 5 approx. min and was stored at 4°C for further use (Franklin et al., 2004).

For the preparation of coating solutions containing plant extract, some modifications in the above said method was done. For the coating solution containing only *T. indica* leaf extracts as antimicrobial agent, 1000 mg of the methanolic extract was dissolved

in 25 ml ethanol, filter sterilized and was added in the coating solution replacing ethanol to obtain the required concentration. For the coating solution containing only *T. indica* root extract as antimicrobial agent, 2,500 mg of root water extract was dissolved in 25 ml double distilled sterilized water, filter sterilized and was added in the coating solution replacing distilled water after. For coating solution containing only nisin as antimicrobial agent: 1.17 g of nisin powder was used to obtain the solution possessing required concentration. For the film containing nisin and *T. indica* leaf water extract as antimicrobial agents, 470 mg of nisin was added in 1.5 ml acetic acid along with 500 mg of the *T. indica* leaf water extract, dissolved in 25ml double distilled sterilized water and was added in the coating solution replacing distilled water. For the film containing nisin and *T. indica* root water extract as antimicrobial agents, 235 mg of nisin powder was added in 1.5 ml acetic acid along with 1000 mg of the *T. indica* root water extract was dissolved in 25ml double distilled sterilized water, filter sterilized and was added in the coating solution replacing distilled water (Figure 5.3). Coating solution containing no antimicrobial agent served as the control.

Finally, the physical characteristics viz. opacity and color of prepared coating solutions were observed with the naked eye.

The shrinkable LDPE films was cut from the procured roll and taped over the clean and sterilized glass slides (35X14 cm). The surface of each film (490cm^2) was wiped with ethanol and allowed to dry at room environment. 50 ml of each coating solution was poured over the film and was spread evenly with the sterilized brush. This coating was allowed to dry and was then exposed to UV radiations for 5 min in the laminar hood (Franklin et al., 2004; Guo et al., 2014; Wang et al., 2018).

The raw chicken breast was washed with double distilled sterilized water, tapped over absorbing paper (already exposed to UV for 5 min) and kept in the sterilized tray in the sterilized laminar hood. The washed chicken breast was then cut into small cubical pieces of nearly equal size of about 2 cm with nearly 36 cm^2 surface area. Subsequently, the film was cut into square of 7x7 cm^2 with a sterilized blade. The chicken pieces were wrapped with the film tightly so that all the surfaces of the chicken samples were in contact with the coated part of the film without any head

space. The packed raw meat samples were kept in room environment at 37±2 °C, refrigerator at 4±2°C and deep freezer at -20±2°C with 44%, 95% and 95% humidity, respectively.

Unpacked raw chicken sample pieces served as the controls.

5.2.2.2 Shelf life analysis of the chicken meat samples

The shelf life analysis of the packed chicken meat samples were done by swabbing technique (Christopher et al, 1979). Packed/ unpacked chicken samples were taken out aseptically in the laminar hood. Refrigerated and frozen meat samples were thawed. Thereafter, packaging film of the meat samples were opened aseptically. Swabbing was done on all surfaces of each chicken meat piece with a sterile cotton swab. The stick was broken and swab was placed in 10 ml of 0.45% sterilized normal saline solution in test tube, which was used as a stock and the same, was serially diluted using 0.45% sterilized normal saline. 100 µl of the desired diluted samples was plated on the surface of the sterilized nutrient agar media and spread plating done. The plates were incubated at 37°C for 24 h and the plate count was determined on the selected days and colony forming units per ml reported (Christopher et al., 1979)

$$\textbf{CFU/ml} = \frac{\text{CFU counted x Dilution factor}}{\text{Volume of sample mounted (ml)}}$$

Other than the bacteriological investigations, the chicken meat samples were also examined for the changes in the sensory properties such as odor, changes in meat characters such as sliminess and firmness, separation of muscle fibres, water retention and presence of the bubbles around the meat samples.

On the day of procurement (Day 0), washed and unwashed meat samples were swabbed and spread plated on sterilized nutrient agar media and selective and/or differential media including Mannitol salt agar media, Cetrimide agar media, Eosin methylene blue agar media and Listeria selective agar media.

The chicken samples illustrating minimum bacterial contamination were determined and corresponding coating solution and film was selected for further analysis.

5.2.2.3 Characteristics of the packaging film

Selected packaging formulation was characterized for its surface analysis to evaluate uniformity of coating solution on the film using Scanning Electron Microscope **(**SEM). To observe the changes happening over the surface of the film after coating, the LDPE and selected test film were processed and observed under Scanning Electron Microscope **(**SEM) at Sophisticated Analytical Instrumental Laboratory (SAIF), Punjab University, Chandigarh.

The control film was wrapped on the glass plate, followed by coating with the active coating solution as explained earlier. Subsequently, the films were dried, wrapped, packed and were transported to Centre for Skill and Technical Support, Department of Chemicals and Petrochemicals, Ministry of Chemicals and Fertilisers, Government of India, Amritsar, Punjab, India. The films were evaluated for thickness, mechanical properties viz. tensile strength and elongation at break, light barrier properties viz. haze and transparency) and thermal properties viz. shrinkage and melting point (Mulla et al., 2017; Gonzalez and Igarzabal, 2013) The control film roll was also sent for the comparative study.

5.2.2.4 Cytotoxicity analysis of the coating solution

In vitro toxicity assay (MTT assay) was conducted on Human Embryonic Kidney cell lines (HEK-293), at Dabur Research Foundation, Ghaziabad. The selected coating solution was prepared, as discussed earlier, and sent to the test center over an ice box.

5.3 RESULTS AND DISCUSSION

5.3.1 Preparation of packaging film using different antimicrobial coating solutions and packaging of meat sample

Different coating solution were prepared using single antimicrobial or in combination to prepare the antimicrobial active packaging formulation. The antimicrobial agent in coating solution 2 was 20 mg/ml methanolic extract of *T. indica* leaf, coating solution 3 was 50 mg/ml ethanolic extract of *T. indica* root, coating solution 4 was 2500 IU/ml Nisin, coating solution 5 was 1000 IU/ml of Nisin + 20 mg/ml water extract of *T. indica* leaf and coating solution 6 was 500 IU/ml Nisin + 20mg/ml water extract of *T.*

indica root. The coating solution 1, did not possessing any antimicrobial agent and served as the control (Table 5.9; Photograph 5.1).

The base composition in all the coating solutions consisted of methyl cellulose, hydroxyl methyl cellulose and poly ethylene glycol. Methyl cellulose is characterized by its good solubility in water and ethanol, non-toxic, non- allergic, efficient thickening agents with stable viscosities over wide pH range (3-11), possibly prevent settling down of constituents in coating solutions, thereby maintain homogeneity of prepared coating solutions (Nasatto et al, 2015). Hydroxypropyl methyl cellulose (HPMC) has been in use in preparing pharmaceutical products such as tablets and is characterized by solubility in aqueous and organic solvents. It is an effective thickening agent along with potential emulsifier. The safety profile of this polymer for its use in medicines and foods has been ensured since long (Hogan, 1995). Polyethylene glycol acts as a solvent, stabilizer, emulsifier as well as thickening agent in the coating preparation. It also acts as a surfactant and is reported to be non- toxic to animal cells (Jang et al, 2015).

On visual evaluation of coating solutions, coating solution 1 was nearly transparent while coating solution 2, 3 and 4 were opaque. At the same time haziness was observed in coating solution 2 and 4. The color of coating solution 2, 3, 4, 5 and 6 was observed to be light brown, dark green, brownish green, yellowish brown and brown, respectively. The green coloration was possible because of green pigment associated with plant leaves while the brown coloration was possibly because of root contents (Table 5.9). All the solutions were viscous owing to presence of base constituents.

During coating of the packaging films, 50 ml of coating solution was used over 490 cm^2. Hence, each cm^2 was expected to have a coating of antimicrobial strength as enlisted in table 5.10. As per mathematical calculations, each cut film with 49 cm^2 surface may have possessed corresponding strength of coating solution. Any other evaluation to check the concentration of antimicrobial attached to the film surface was not done.

Before packaging of the meat samples, an investigation was done to compare the effects of washing to unwashed meat samples along with study of presence of selected microbes under study in the purchased meat. Hence, standard plate count on nutrient agar media and other selective and differential media was performed. Thereafter, the washed meat samples were tapped over absorbing paper (surface sterilized by pre-exposure to UV for 5 min) to remove extra water and all the samples were preliminary observed for freshness (color-pink, visible sliminess, foul odor, firmness, water retention and separation of fibers). Thereafter, the washed and cut pieces were packed tightly in the films followed by a check for ensuring any film tear, attachment of film to all surfaces of meat pieces and any head space, if left, for air as it may contribute to meat contamination. The packed pieces were stored in the different temperature conditions for analyzing the efficacy of films to extend shelf life of the chicken samples.

5.3.2 Shelf life analysis of the chicken meat samples

On comparison of microbial growth for unwashed and washed meat samples as determined on day 0 by standard plate count (SPC), it was found that the washed samples exhibited lesser bacterial growth in comparison to unwashed samples (Figure 5.5). This indicated that washing of samples with double distilled sterilized water helps in reducing the surface microbial load. Though, distinct bacterial colonies were not formed on any of the tested samples, with clustered growth as well as too-numerous-to-count (TNTC), yet the evaluation was enough to indicate significance of washing in reducing microbial load. In order to determine the microbiological profile of the meat samples, spread plate method was performed which has been used in different studies reporting the shelf life analysis of food products (Ha et al., 2001; Patsias et al., 2006; Seydim et al., 2006). Clustered growth might be due to technical problem related to spreading of the samples, where uneven spreading/ clumping/ overlapping of colonies were observed over the surface of agar plates. Literature suggests that during use of the glass rod while spreading the samples over the solid agar surface, there may be too quick absorbance of the samples which may lead to formation of colonies in the plate center rather even spreading. Hence, in such

attempts, the clumping or overlapping colonies are found which makes the microbial count inaccurate or causing unfeasibility of differentiating the cells/ colonies (Sanders, 2012). Pour plate method was also attempted, but some uncertain results such as no growth in all plates and/ or clumped colonies was observed frequently. The use of hot molten agar may have been the cause of killing of bacteria and improper mixing of samples after pouring of agar is the common reason of clumping of microbial cells (Sanders, 2012). Hence, microbial count could not be assessed and thus it was not possible for determining sample spoilage by quantitative criteria. Still, a qualitative comparative assessment has been reported, where the absence, presence and extent of cluster formation determined.

At the same time, the sample were plated on selective and differential media so as to confirm whether the test organisms, as used for evaluating antimicrobial effect of bacteriocins (isolated from different bacterial species as well as commercially procured, Nisin) and different plant extracts of *T. indica*, as previously determined and explained (Ch 3 and 4), were present on the selected meat samples or not. The colony forming units (CFU/ml) for unwashed and washed samples as found on EMB agar plates was the 9700±13 and 4500±7, respectively, where few colonies showing green metallic sheen indicated *E. coli*. Correspondingly, Cetrimide agar presented green discoloration of media with CFU/ml to be 9100±36 and 3700±16 in unwashed and washed samples, respectively. Similarly, on Mannitol Salt agar, CFU/ml was determined to be 16200±60 and 6800±20 for unwashed and washed samples respectively and few colonies showing yellow discoloration of media was indicative of *Staphylococci* and was higher in unwashed samples. Lastly, CFU/ml on Listeria selective agar was determined to be 18200±42 and 9500±90 in unwashed and washed samples indicating the presence of *Listeria monocytogenes*, although lesser in washed samples. Though shelf life analysis in the current investigation did not target any specific microbial species, and only evaluated the antimicrobial action of different test coating solutions on overall micro-flora present on tested food samples, yet a preliminary indication of presence of test organisms (E. *coli, P. aeruginosa, S. aureus,* and *L. monocytogenes*) was confirmed by this initial evaluation (Table 5.11, Photograph 5.2) The lesser dense growth of total viable count along with lesser

number of specific bacteria reflects the influence of washing where the surface microbial count decreases if washed properly with sterilized water. During the transit of the birds such as chicken to the processing units, the various parts viz. feathers and feet are contaminated with the dust and feces which consequently lead to increase of microbes on the carcass during slaughtering, processing and even handling. Consumption of such contaminated poultry meats may lead to incidences of food borne illnesses such as listeriosis, salmonellosis and campylobacteriosis. Hence, efforts to limit this contamination must be ensured so as to improve the shelf life of the chicken raw meat samples. The hurdles towards hygienic practices for producing better quality poultry meats includes absence of hygienic slaughter facilities, costs of mechanized infrastructure and persistent expenditures (Ruban and Fairoze, 2011).

Subsequently, a study was performed to analyze the potential of prepared film towards shelf life improvement of raw chicken samples when stored in the room conditions. Clustered growth was found by the washed meat samples on day 0 (Table 5.12; Photograph 5.2). After storing for 1 day, the control samples exhibited sliminess with highest in the unpacked samples along with loss of water and reduced firmness from the meat fibers. Slight sliminess was also exhibited by the test samples except from the root extract+ nisin coated film meat samples. While TNTC was observed in all the control samples, test samples exhibited dense clustered growth as compared to light cluster observed in for root extract+ nisin coated film samples. Discoloration was observed in the meat samples being highest in unpacked samples (Buff color) and lowest in *T. indica* root+ nisin meat samples (light pink). After 2 days of storage, water loss, foul odor and bubbles were well observed in the control samples being highest in unpacked samples. Fair or moderate sliminess was also observed in all the test samples, relatively less than controls. Water loss and reduced firmness could also be observed except for root extract+ nisin coated film samples. Microbiological analysis exhibited TNTC growth in all the samples examined. On completion of 4 days of storage in room conditions, maggots could be observed in the unpacked samples. Heavy sliminess, foul odor, loss of water from the meat samples and reduced firmness was characterized in nearly all other samples. Microbiological count study was not performed. The potency of antimicrobial films is dependent on temperature

and is reduced at higher temperatures. The possible reason for such behavior could be the diffusion of biological agents from the film matrix (Cooksey, 2000). The room environmental conditions, as exhibited during the storage, were 37±2 °C along with 44% humidity, which is suitable and may have helped in efficient growth of the microbes in the packed meat samples.

To compare the effects of room to chilling in the refrigerator on the packed meat samples, another set up was experimented, where meat samples, after packaging, were stored in the refrigerators at 4°C (Table 5.13; Photograph 5.3). Clustered growth was again observed in the washed chicken meat samples on day 0 without any change of sensory attribute. After storing for 1 day, controls (unpacked, only film) exhibited TNTC while coated film without antimicrobials sample exhibited $196x10^6$±14 CFU/ml. Amongst the test film meat samples, clustered growth was observed with Nisin coated, and root extract coated film meat samples whereas TNTC was observed with leaf extract coated and $135x10^4$±25 CFU/ml was determined with leaf+ nisin coated film meat samples. No growth was observed root extract + nisin film meat samples. Sliminess was felt with only unpacked meat samples after 1 day of chilled storage. After 2 days of storage, sliminess was found in the control film samples (fairly high in unpacked ones), leaf extract, leaf extract+ nisin and root film samples. Water loss from meat pieces was also observed with unpacked samples. Microbiological analysis exhibited TNTC was observed in all samples except for leaf extract+ nisin film meat samples, where clustered growth was observed. Root extract+ nisin exhibited TFTC. No changes in other sensory features were observed. After 6 days of storage, massive sliminess was observed with all control samples, leaf extract coated and root extract coated film samples. Bubbles and foul odor were observed in unpacked samples only. Standard plate count exhibited TNTC for all samples except for root extract+ nisin coated film samples where limited clustered growth was exhibited. After day 7 of storage, massive sliminess with nearly all samples was observed except for root extract+ nisin coated film in which fair sliminess was observed. Foul odor from most of the packages was observed. To analyze the deterioration in the food samples and performance of active packaging films, 10^7 CFU/ml or cm^2 is normally considered as the threshold, beyond which it is referred to

as spoiled. Presence of foul odor estimates the microbial count to be 10^8 CFU/ml or cm^2 and slime development is estimated at 10^9 CFU/ml or cm^2. 10^{10} CFU/ml or cm^2 and higher values are suggestive to be too high and is recognized as unacceptable foods (Sung et al., 2013). Hence, presence of excessive sliminess and foul odor in our samples on day 7 indicated marked contamination and thus, microbiological examination was not investigated further. As per the public survey analysis, it has been identified that more than 80% of the meat consumers has preference for fresh/ unfrozen chicken to frozen carcass (Khan and Van den Berg, 1967). The shelf life of the poultry is shorter at refrigerator temperature as compared to freezers as the pathogenic and spoilage microbes, present at the rearing, production site, and during transportation, tend to multiply and their metabolic activity is eventually manifested as food spoilage. The initial number of microbes present on/ in the carcass and hygienic controls at different stages of processing is also responsible for the shelf life of the poultry meat (Kozacinski et al., 2012).

Subsequently, the analysis of test and control chicken samples were targeted for their improvement in shelf life in the deep freezer (-20^{o}C), where day 0 exhibited total viable count (TVC) to be $56x10^9$ in the washed control samples (Table 5.14; Photograph 5.4). The analysis of each of all sampled test meat pieces after day 1 storage was determined to be fresh by sensory characters as no sliminess, foul odor or bubbles were observed around the samples although microbial count revealed clustered and/or TNTC in the control samples (Unpacked, with film, film with coating possessing no antimicrobials). After 2 days of storage, TNTC was determined for control samples. Clustered growth was observed for meat sample packed with coating of leaf extract whereas the TFTC was exhibited by nisin film samples. No growth was observed in rest of the samples. No sensory changes were observed by this day. Subsequently, changes were observed, where after 4 days of storage, slight sliminess and separation of fibres were found in control samples along with TNTC as clustered growth over nutrient agar, the observation which was then continuously found with control samples during further storage. Unpacked samples even exhibited reduction in firmness. No microbial growth was observed in test samples except for nisin film sample where 3800± 12 CFU/ml was determined, $136x10^3$± 31 in leaf film samples

and clustered growth in root film samples. Discoloration was observed in control and leaf extract meat samples. On analysis of samples after 7 days of storage, unpacked meat samples were observed with sliminess, loss of firmness along with higher fibre separation as compared to other two controls, where bubbles were not observed. 92 $x10^4$ ± 16 CFU/ml was determined in nisin film samples, clustered growth was found with leaf extract, leaf extract + nisin and root film samples. Sliminess was observed with leaf film samples. Other sensory attributes were not determined for any change. On subjecting the samples freeze storage to 14 days, sliminess was moderately present along with separation of fibres in the control samples. Discoloration was observed in all samples being higher in controls. Firmness was no more observed in all control samples while bubbles were present in the unpacked meat samples. TVC was quite high (TNTC) in these samples. Heavy clustered growth was observed in leaf film samples while light clustering was observed in other test samples. No growth was observed in root+ nisin samples. Sliminess was observed in samples with packaging of nisin, leaf and root extracts. Fibre separation along with reduced firmness was observed with leaf extract film packed samples along with bubbles around the meat pieces. No other sensory change was observed in these samples. Foul odor was still not observed in any of the sample. Subsequently, the samples were analyzed after 20 days of freeze storage. Presence of high sliminess, loss of firmness, separation of fibres and presence of bubbles were observed in the control samples. Dense plate count was suggestive of massive meat contamination especially in unpacked meat samples. All the test samples exhibited sliminess, loss of firmness, loss of water retention, separation of fibers along with TNTC growth on the media plates while clustered growth was exhibited by nisin and $126x10^8$± 42 CFU/ml was determined in root+ nisin film packed meat samples. Foul odor was not observed in any of the samples till day 20 of storage.

High microbial growth in the procured chicken breast sample indicated marked surface contamination of the fresh carcass although sensory changes such as foul odor and sliminess were not felt. The samples were washed, cut in small pieces, packed and immediately kept in deep freezer for quick freezing. The formation of ice in the muscles of stored meat carcass is responsible for disruption of the cells. When the

carcass is thawed, a fluid exudate is observed from the cells called as drip and is responsible for loss of flavor (Mead, 2004). Slow freezing of the poultry is associated with higher loss of drip when thawed, higher proteolysis, higher losses of nucleic acid derivatives and nitrogenous components, reduced water holding capacity of chicken meat along with greater reduction in adenosine-triphosphatase potential of myofibril proteins. Hence, quick freezing provides better quality of stored meat samples (Khan and Van Den Berg, 1967). Control samples revealed sliminess by day 4, illustrating the count of microbes 10^9 CFU/ ml and above. The meat fibers could easily be broken and separated in the unpacked samples along with reduction of firmness. No sensory changes were found in packed meat samples. Microbiological profile indicated higher contamination of leaf extract and root extract coated film samples. By day 7, unpacked meat samples determined more sensory changes in illustrating further deterioration of the samples. Amongst test samples, sliminess was observed in leaf extract coated samples which subsequently increased on 14^{th} and 20^{th} day, indicating the contamination by 7^{th} day. The only film which exhibited potential result was root extract+ nisin coated film sample which exhibited sliminess and clustered growth on 20^{th} day analysis illustrating the acceptable shelf life of meat samples for 14 days. Packaging of foods act as a barrier between food surface and environment, where the physical contamination is hurdled and longer usability of the food item is assured. Economic and better functionality of flexible packaging formulations such as LDPE is responsible for its marked acceptance by the industry as compared to other packages such as glass and metals. This study indicates the potential of root extract+ nisin coated film samples to be best amongst the test films.

The spoilage of stored food items is generally manifested by the presence of surface pathogens. Raw meat foods being organic and highly moisturized in nature provide a potential platform for the flourishment of the microbes, thus are tremendously perishable. Their contamination with the food pathogens occurs quickly, causing physical, chemical and microbial changes, decreasing the storage shelf life and finally rendering such meat foods non-consumable, or be a potent source of infectious diseases to the consumers. Hence, reduction / limitation of surface pathogenic growers may help in improving the quality of meats, leading to its enhanced shelf life.

Active packaging of such food items are being looked upon as a potent solution to this problem. Packaging formulations composing antimicrobial substances when come in contact with the food surface, causes reduction in microbial count and help in improving shelf life, which otherwise gets spoiled earlier (Bailey et al., 2000; Ha et al., 2001; Sung et al., 2013). In past few years, the poultry based chicken meat and its products have gained significant popularity because of its high nutritive quality, easy availability along with low cost. The chicken meat, either fresh or in the pre-cooked form, is generally refrigerated/ frozen while its common products such as meat balls and nuggets are packed and stored in frozen form. Being consumed by large population, their microbial safety is of prime concern to the food and health industry. The spoilage of any such products, if suspected, is generally examined by sensory and/or microbial analysis (Patsias et al., 2006). Hence, this real time study was targeted to determine the efficacy of the prepared packaging films, to reduce/ restrict the growth of food pathogens, and thus to improve the shelf life of the raw chicken meat samples. The packed meat samples were exposed to three different general atmospheric conditions, and sensory along with microbiological analysis of the samples was investigated. Unpacked meat samples served as the control for qualitative comparison of meat samples packed in different active packaging products as prepared earlier.

5.3.3 Characteristics of the packaging film

5.3.3.1 Surface analysis of the film

The coatings, as illustrated in the scanning electron micrographs (Photograph 5.5), presented homogenous and even layering of the coating without any bubbles or some defect along with the presence of crystalline structure on the surface.

Similar observations has been reported by Grower et al., 2004 in which the crystalline white structures were confirmed to be present due to the sodium chloride crystals which are generally present in the nisin powder. In another study, LLDPE film treated with chromic acid exhibited pitting and roughening of the film surface (Mulla et al., 2017)

5.3.3.2 Physical characterization of the packaging film

Physical characterization of food packaging films plays an important role for their acceptance and application in the industry as they are influenced after incorporating the antimicrobials. The packaging films prepared from the polymers determines a range of properties, which enables them to be suitable for foods with characteristic specificity (Table 5.15). Such properties also furnish important information to ensure their application taking into account the processing methods with the food (Bastarrachea et al., 2011).

5.3.3.2.1 Film Thickness

The thickness of control and test film was determined to be 2 and 3 microns, respectively. Hence, there was an increase of 1 micron thickness of the test film indicating the presence of coating layer over the LDPE film. Similar results have been reported for increase in thickness for linear low density polyethylene (LLDPE) film coated with clove oil was determined (Mulla et al., 2017). Similarly, some other studies has reported the same results (Cano et al., 2015; Hauser et al., 2016)

5.3.3.2.2 Mechanical properties

Tensile Strength: The tensile strength of the selected test film in machine direction (MD) was determined to be 121.53 MPa as compared to control which was found to be 109.02 MPa. In transverse direction (TD), the tensile strength for the selected test film was 118.74 MPa which was 18.86 MPa higher than control film.

Elongation at break (EB): The elongation at break for the control film in MD was found to be 159.3% against 293.3% for the control film. Similarly, the EB for TD of the selected test film was determined to be 135.1% against 260.6% for the control sample.

To ensure the quality of the polymeric active packaging films, the understanding of its mechanical properties is highly intended. The tensile strength is the measurement of some substance such as packaging films to withstand the forces, pulling the sample apart, just before breaking. Also renowned as fracture strain, the elongation at break is the ratio of increased length to initial length after breakage of the test sample at a

specified temperature. It may be related to property of a sample to resist shape change without cracking. Such measurements are crucial to understand the behaviour of active polymer packaging film when exposed to varied stresses during handling, processing and storage of the packed food items. It also helps in comparing the brittle and strong materials with soft or flexible ones (Bastarrachea et al., 2011).

The test result clearly signifies that the tensile strength of the active LDPE film has improved with the coating of the selected antimicrobial solution as compared to the control film. The tensile strength improved by 9.52 MPa and 18.86 MPa in MD and LD, respectively. Correspondingly, the elongation in break has been determined to decrease by 135 % and 125.5% MD and TD, respectively. These results suggest that the selected active packaging film has developed more resistance to stress and are lesser elongable.

Similar results has been reported where addition of poly lactic acid layer over soy protein layer resulted in better tensile strength with decreased EB (Gonzalez and Igarzabal, 2013)

5.3.3.2.3 Light barrier properties

The haze and transparency of the films influences the appearance of the packed product. The UV rays in the transmitted light, affects the lipid oxidation of the packed foods and hence, such studies help determining the efficiency of the active packaging film (Singh et al., 2015).

Haze: The haziness of the control and coated film were determined to be 1.41 % and 4.89%, respectively. This increase in haziness or reduction in the clarity of the test film sample may be due to coating solution containing the *T. indica* root extracts and nisin.

Transparency: The transparency of the control and coated film were determined to be 93.8 % and 93.7%, respectively. The decrease of the light transmittance may be due to the presence of plant constituents and nisin in the coating. Similar results illustrating decrease in transparency, has earlier been reported in the literature as well (Mulla et al., 2017).

5.3.3.2.4 Thermal properties

Thermal Shrinkage and Melting point: To analyze the effects of coating on the thermal properties of LDPE films, deviation in thermal shrinkage and melting point were determined. Although no change has been reported for the melting point of test film (114-118^{o} C) when compared to control film (115-118^{o} C), but change in thermal shrinkage has been recognized. Thermal shrinkage at 70^{o} C for 1 h (L x W) for control film has been analyzed to be 0.19x0.44 which has reduced to 0.09x0.27 in the coated film sample.

Some studies has reported a decrease in melting point of the packaging films after the incorporation of the antibacterial substances (Mulla et al., 2017)

5.3.4 Cytotoxicity analysis of the coating solution

To ensure the safety of the coating solution, *In vitro* study has been conducted against the Human Embryonic Kidney cell lines (HEK-293). The aqueous stock of test solution was determined to be non-toxic at all the tested concentration ranges (1 µg/ ml- 10 mg/ ml). The viability of HEK-293 cells was determined to be 90.0% or above at all the test concentrations after 3 days of incubation (Table 5.16).

Similarly, the aqueous: ethanolic stock of test solution was found to be non-cytotoxic at 1 µg/ ml- 100 µg/ ml where viability of HEK-293 cells after 3 days of incubation was determined to be above 92.6%. Subsequently, it was found to be cytotoxic at 500 µg/ ml- 10 mg/ml where viability of HEK-293 cells was less than 61.4%. The control, TritonX-100, was found to be cytotoxic at tested concentration range: 0.1% v/v and 1% v/v.

The MTT cell viability assay is the technique involving the conversion of yellow colored tetrazolium salt, MTT, to purple colored, insoluble formazan crystals. This change happens in the mitochondria of the living cells. Hence, the amount of formazan produced is proportional to the number of metabolically active cells (Pratheesh et al., 2014).

In vitro cytotoxicity analysis by MTT assay for chitosan-alginate polyelectrolyte complex coated film determined low viability (72.70%) of *Mouse fibroblast* cultures

on day 1, which improved to 96.96% by day 4. The film was found to be non-toxic with *Human fibroblast* cytotoxicity study and retained the viability more than 91% over the four day exposure period (Yan et al., 2001). Konjac glucomannan-ethyl cellulose films were found to be non-toxic to L929 cell lines with cell viability greater than 86.6%, resulting to good biocompatibility and determining the potential as the food packaging materials (Li et al., 2015).

The methanolic leaf extract of *T. indica* leaves was found to be cytotoxic with LC_{50} above 30 mg/ml towards *Madin Darby Canine Kidney* (MDCK) cell lines (Chaturvedi and Chowdhary, 2014). 10 µg of crude ethanolic leaf extracts of *T. indica* determined 65% cell viability against HCT-15 human colon adenocarcinoma cell lines as compared to 50 µg which showed 42% and 100 µg determined 28% cell viability (Pratheesh et al., 2014).

5.4 Statistical Analysis

All the experiments have been performed in triplicates. The results have been expressed as mean± standard deviation (SD). The software, Excel 2010 in Microsoft Office programme has been used to calculate the same.

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Tables

Table 5.9: Preparation and characterization of the coating solution

Coating solution	Antimicrobial added	Visible properties
Coating solution 1	Without any antimicrobial agent	Nearly transparent
Coating solution 2	20 mg/ml methanolic extract of *T. indica* leaf	Opaque and dark green
Coating solution 3	50 mg/ml ethanolic extract of *T. indica* root	Yellowish brown
Coating solution 4	2500 IU/ml Nisin	Opaque, hazy and light brown
Coating solution 5	1000 IU/ml of Nisin + 10 mg/ml water extract of *T. indica* leaf	Hazy and brownish green
Coating solution 6	500 IU/ml Nisin + 20mg/ml water extract of *T. indica* root	Opaque and brown
• **Basic components of coating solution (50 ml):** Double distilled sterilized water (25 ml); methylcellulose (0.875 g); hydroxyl-propyl methyl cellulose (0.375g); ethanol (25 ml); polyethylene glycol 400 (0.75 ml)		

Table 5.10: Preparation of the packaging film using prepared coating solutions

Film with coating solution	Concentration on antimicrobial agent per cm^2 of film	Concentration per 49 (7 x 7) cm^2 of film
Coating solution 1	Nil	Nil
Coating solution 2	25 IU of nisin	1,225 IU/ml
Coating solution 3	0.2 mg methanolic extract of *T. indica* leaf	9.8 mg methanolic extract of *T. indica* leaf
Coating solution 4	10 IU of Nisin + 0.2 mg water extract of *T. indica* leaf	490 IU of Nisin + 98 mg water extract of *T. indica* leaf
Coating solution 5	0.5 mg ethanolic extract of *T. indica* root	24.5 mg ethanolic extract of *T. indica* root
Coating solution 6	5 IU of Nisin + 0.2mg/ml water extract of *T. indica* root	245 IU Nisin + 9.8 mg/ml water extract of *T. indica* root
• Film used: LDPE • Cellulose based coating solution is used		

Table 5.11: Shelf life analysis of raw chicken sample

S. No	Media used	Unwashed samples	Washed samples
1	Nutrient Agar	TNTC	TNTC
2	*Enterobacteriaceae* (Eosin Methylene Blue Agar)	9700±13	4500±7
3	*Pseudomonas aeruginosa* (Cetrimide Agar)	9100±36	3700±16
4	*Staphylococcus sp.* (Mannitol Salt Agar)	16200±60	6800±20
5	*Listeria sp.* (Listeria selective agar)	18200±42	9500±90
• Day of evaluation: 0; TNTC: Too numerous to count • **Standard Plate Count**: Technique: Spread Plate method; Incubation temperature:37^{o}C; Incubation period: 24 h			

Table 5.12: Shelf life analysis of raw chicken sample in room environment

Film	Parameters tested	Day 0	Day 1	Day 2	Day 4
Unpacked (Control)	**Sliminess**	—	+++	++++	Maggots present
	Discoloration	+	++	++	
	Water retention/ Firmness	+	—	—	
	Separation of fibres	—	—	—	
	Foul odor	—	—	++	
	Bubbles around the meat sample	—	—	+	
	Microbial Growth	Clustered	TNTC	TNTC	
Only film (Control)	**Sliminess**		++	+++	+++++
	Discoloration		++	++	+++
	Water retention/ Firmness		+	—	—
	Separation of fibres		—	—	Softened sample
	Foul odor		—	++	+++
	Bubbles around the meat sample		—	+	++
	Microbial Growth		TNTC	TNTC	NE
Only coating (Control)	**Sliminess**		++	+++	+++++
	Discoloration		++	++	+++
	Water retention/ Firmness		+	—	—
	Separation of fibres		—	—	Softened sample
	Foul odor		—	++	+++
	Bubbles around the meat sample		—	+	++
	Microbial Growth		TNTC	TNTC	NE
Nisin (Test)	**Sliminess**		—	++	++
	Discoloration		+	++	+++
	Water retention/ Firmness		+	+	—
	Separation of fibres		—	—	Reduced firmness
	Foul odor		—	+	++
	Bubbles around the meat sample		—	—	+++
	Microbial Growth, CFU/mL		Clustered	TNTC	NE
***T. indica* leaf (Test)**	**Sliminess**		+	++	+++
	Discoloration		+	++	+++
	Water retention/ Firmness		+	+	—
	Separation of fibres		—	—	No firmness observed
	Foul odor		—	+	++
	Bubbles around the meat sample		—	—	++
	Microbial Growth		TNTC	TNTC	NE
Nisin+ *T. indica* leaf (Test)	**Sliminess**		+	+	++
	Discoloration		+	++	+++
	Water retention/ Firmness		+	+	—
	Separation of fibres		—	—	No firmness observed
	Foul odor		—	—	++
	Bubbles around the meat sample		—	—	+
	Microbial Growth		Clustered	Clustered	NE
***T. indica* root (Test)**	**Sliminess**		+	+	++
	Discoloration		+	++	+++
	Water retention/ Firmness		+	+	—
	Separation of fibres		—	—	No firmness observed
	Foul odor		—	—	+
	Bubbles around the meat sample		—	—	+
	Microbial Growth		Clustered	TNTC, Clustered	NE
Nisin+ *T. indica* root (Test)	**Sliminess**		—	+	+
	Discoloration		—	+	++
	Water retention/ Firmness		+	+	—
	Separation of fibres		—	—	Marginally firm
	Foul odor		—	—	+
	Bubbles around the meat sample		—	—	+

	Microbial Growth		Clustered	TNTC	NE

- TNTC: Too numerous to be counted; TFTC: Too few to be counted; Clustered: Seemingly not TNTC, but uncountable
- —/ Nil: Not observed; +: Present; ++: Fairly present +++: Moderately present; ++++: Heavily present; NE: Not evaluated

Table 5.13: Shelf life analysis of raw chicken sample in refrigerator environment

Film	Parameters tested	Day 0	Day 1	Day 2	Day 6	Day 7
Unpacked (Control)	**Sliminess**	—	+	++	++++	++++
	Discoloration					
	Water retention/ Firmness	+	+	—	—	—
	Separation of fibres	—	—	—	—	—
	Foul odor	—	—	—	+	+
	Bubbles around the meat sample	—	—	—	—	+
	Microbial growth, CFU/mL	Clustered	TNTC	TNTC	TNTC	NE
Only film (Control)	**Sliminess**		—	+	+++	++++
	Discoloration					
	Water retention/ Firmness		+	—	—	—
	Separation of fibres		—	—	—	—
	Foul odor		—	—	—	+
	Bubbles around the meat sample		—	—	—	+
	Microbial growth, CFU/mL		TNTC	TNTC	TNTC	
Only coating (Control)	**Sliminess**		—	+	+++	++++
	Discoloration					
	Water retention/ Firmness		+	—	—	—
	Separation of fibres		—	—	—	—
	Foul odor		—	—	—	+
	Bubbles around the meat sample		—	—	+	+
	Microbial growth, CFU/mL		$196x10^6 \pm 14$	TNTC	TNTC	NE
Nisin (Test)	**Sliminess**		—	—	++	+++
	Discoloration					
	Water retention/ Firmness		+	+	+	—
	Separation of fibres		—	—	—	—
	Foul odor		—	—	—	+
	Bubbles around the meat sample		—	—	—	+
	Microbial growth, CFU/mL		Clustered	TNTC	TNTC	NE
***T. indica* leaf (Test)**	**Sliminess**		—	+	+++	++++
	Water retention/ Firmness		+	+	+	—
	Separation of fibres		—	—	—	—
	Foul odor		—	—	—	+
	Bubbles around the meat sample		—	—	—	+
	Microbial growth, CFU/mL		TNTC	TNTC	TNTC	TNTC
Nisin+ *T. indica* leaf (Test)	**Sliminess**		—	+	+	+++
	Discoloration					
	Water retention/ Firmness		+	+	—	—
	Separation of fibres		—	—	—	—
	Foul odor		—	—	—	+
	Bubbles around the meat sample		—	—	—	+
	Microbial growth, CFU/mL		$135x10^4 \pm 25$	Clustered	TNTC	NE
***T. indica* root (Test)**	**Sliminess**		—	+	++	+++
	Discoloration					
	Water retention/ Firmness		+	+	—	—
	Separation of fibres		—	—	—	—
	Foul odor		—	—	—	+
	Bubbles around the meat sample		—	—	—	—
	Microbial growth, CFU/mL		Clustered	TNTC	TNTC	NE

Nisin+ *T. indica* root (Test)	**Sliminess**		—	—	+	++
	Discoloration					
	Water retention/ Firmness		+	+	+	—
	Separation of fibres		—	—	—	—
	Foul odor		—	—	—	—
	Bubbles around the meat sample		—	—	—	—
	Microbial growth, CFU/mL		Nil	TFTC	Small clustered	NE

- TNTC: Too numerous to be counted; TFTC: Too few to be counted
- —/ Nil: Not observed; +: Present; ++: Fairly present +++: Moderately present; ++++: Heavily present; NE: Not evaluated

Table 5.14: Shelf life analysis of raw chicken sample in Deep freezer environment

Film	Parameters tested	Day 0	Day 1	Day 2	Day 4	Day 7	Day 14	Day 20
Unpacked (Control)	**Sliminess**	—	—	—	+	++	+++	++++
	Discoloration	—	—	—	+	++	++	++
	Water retention/ Firmness	+	+	+	—	—	—	—
	Separation of fibres	—	—	—	+	++	++	++
	Foul odor	—	—	—	—	—	—	—
	Bubbles around the meat sample	—	—	—	—	—	+	++
	Microbial Growth	$56x10^9$	Clustered	TNTC	TNTC	TNTC	TNTC	TNTC
Only film (Control)	**Sliminess**		—	—	+	+	++	++++
	Discoloration		—	—	+	++	++	++
	Water retention/ Firmness		+	+	+	+	—	—
	Separation of fibres		—	—	—	—	—	+
	Foul odor		—	—	—	—	—	—
	Bubbles around the meat sample		—	—	—	—	—	+
	Microbial Growth		TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
Only coating (Control)	**Sliminess**		—	—	+	+	++	+++
	Discoloration		—	—	+	++	++	++
	Water retention/ Firmness		+	+	+	+	—	—
	Separation of fibres		—	—	—	—	+	+
	Foul odor		—	—	—	—	—	—
	Bubbles around the meat sample		—	—	—	—	—	+
	Microbial Growth		TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
Nisin (Test)	**Sliminess**		—	—	—	—	+	++
	Discoloration		—	—	—	—	+	+
	Water retention/ Firmness		+	+	+	+	+	—
	Separation of fibres		—	—	—	—	—	+
	Foul odor		—	—	—	—	—	—
	Bubbles around the meat sample		—	—	—	—	—	—
	Microbial Growth, CFU/mL		Nil	TFTC	3800± 12	$92 x10^4 \pm 16$	Clustered	Clustered
***T. indica* leaf (Test)**	**Sliminess**		—	—	—	+	++	+++
	Discoloration		—	—	+	+	+	++
	Water retention/ Firmness		+	+	+	—	—	—
	Separation of fibres		—	—	—	—	+	+
	Foul odor		—	—	—	—	—	—
	Bubbles around the meat sample		—	—	—	—	+	+
	Microbial Growth		Nil	Clustered	$136x10^3 \pm 31$	Clustered	Clustered	TNTC
Nisin+ *T. indica* leaf	**Sliminess**		—	—	—	—	—	++
	Discoloration		—	—	—	—	+	++
	Water retention/ Firmness		+	+	+	+	+	—
	Separation of fibres		—	—	—	—	—	++

(Test)	**Foul odor**		—	—	—	—	—	—
	Bubbles around the meat sample		—	—	—	—	—	+
	Microbial Growth		Nil	Nil	Nil	Clustered	Clustered	TNTC
***T. indica* root (Test)**	**Sliminess**		—	—	—	—	+	+
	Discoloration		—	—	—	—	+	+
	Water retention/ Firmness		+	+	+	+	—	—
	Separation of fibres		—	—	—	—	—	+
	Foul odor		—	—	—	—	—	—
	Bubbles around the meat sample		—	—	—	—	+	+
	Microbial Growth		Nil	Nil	Clustered	Clustered	TNTC	TNTC
Nisin+ *T. indica* root (Test)	**Sliminess**		—	—	—	—	—	+
	Discoloration		—	—	—	—	+	+
	Water retention/ Firmness		+	+	+	+	+	—
	Separation of fibres		—	—	—	—	—	—
	Foul odor		—	—	—	+	—	+
	Bubbles around the meat sample		—	—	—	—	—	—
	Microbial Growth		Nil	Nil	Nil	NIL	Clustered	$126x10^8 \pm 42$

- Only coating: coating solution without any antimicrobial agent
- TNTC: Too numerous to be counted; TFTC: Too few to be counted; Clustered: Seemingly not TNTC, but uncountable
- —/ Nil: Not observed or not found; +: Present; ++: Fairly present +++: Moderately present; ++++: Heavily present

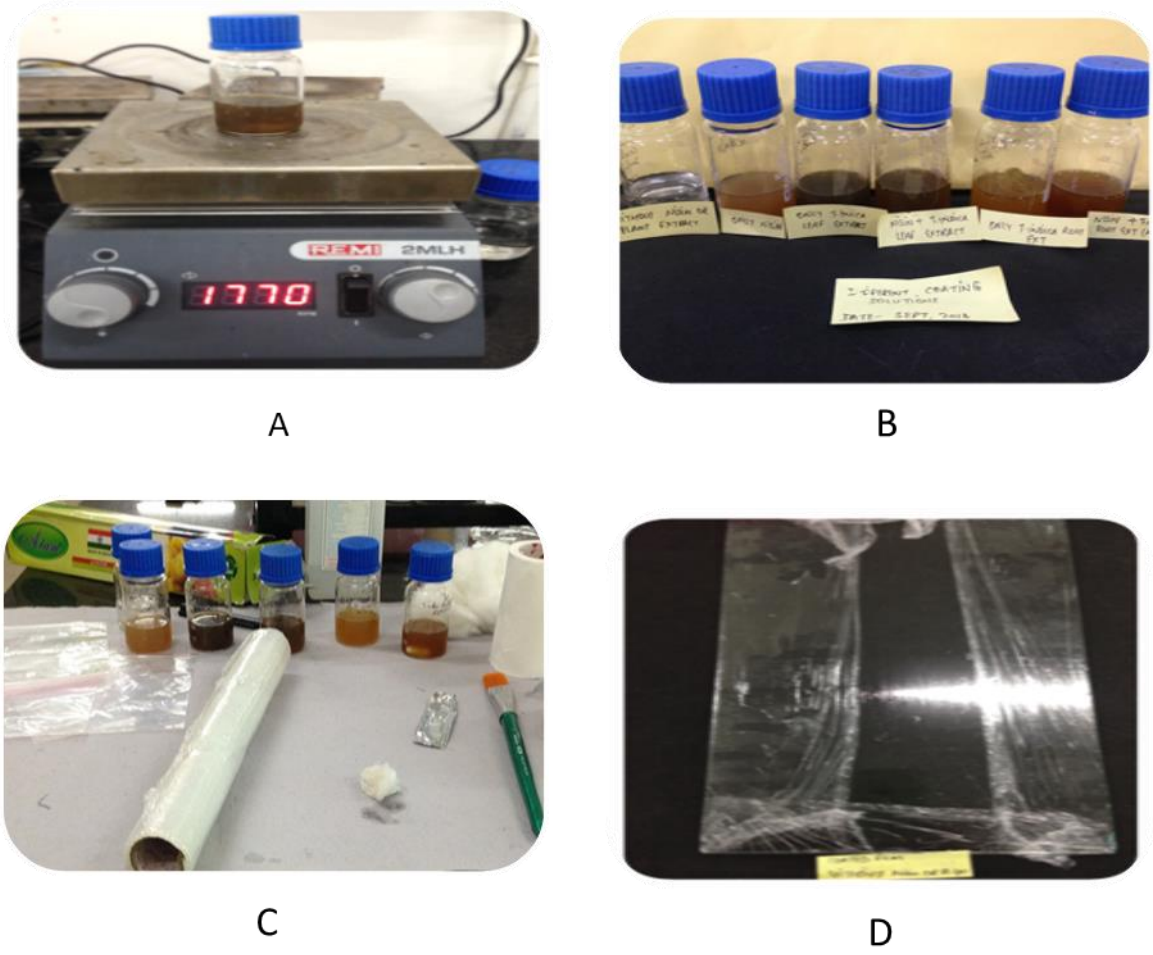
Table 5.15: Physical characterization of the packaging film

S. No	Test			Unit	Test result	
					Control film	**Test Film**
1	Thickness			Microns	2	3
2	Mechanical property	Tensile strength	MD	MPa	109.02	121.53
			TD		99.88	118.74
		Elongation at break	MD	%	293.3	158.3
			TD		260.6	135.1
3	Light barrier property	Haze		%	1.41	4.89
		Transparency		%	93.8	93.7
4	Thermal property	Melting point		^oC	115-118	114- 118
		Thermal shrinkage at 70^oC for 1 h (L x W)		%	0.19x0.44	0.09x0.27
Testing has been conducted at CIPET, Amritsar MD: Machine direction; TD: Transverse direction MPa: Mega Pascal						

Table 5.16: Effect of coating solution on cytotoxicity in HEK-293 cell line

Sample	Concentration	OD1	OD2	OD3	Average	% Cytotoxicity	% Viability
Control		1.613	1.772	1.761	1.715	0.0	100.0
Triton X (% v/v)	0.1	0.097	0.1	0.10	0.101	94.1	5.9
	1	0.091	0.085	0.087	0.088	94.9	5.1
Coating solution: Aqueous Stock (µg/ ml)	1	1.46	1.533	1.64	1.544	10.0	90.0
	10	1.851	1.944	1.967	1.921	-12.0	112.0
	50	1.727	1.794	2.063	1.861	-8.5	108.5
	100	1.678	1.755	1.987	1.807	-5.3	105.3
	500	1.685	1.491	1.68	1.619	5.6	94.4
	1000	1.704	1.499	1.764	1.656	3.5	96.5
	5000	1.165	2.68	2.609	2.151	-25.4	125.4
	10000	0.962	1.754	1.59	1.435	16.3	83.7
Coating solution: Aqueous: Ethanolic Stock (µg/ ml)	1	1.785	1.624	1.376	1.595	7.0	93.0
	10	1.424	2.012	1.893	1.776	-3.6	103.6
	50	1.617	1.644	1.509	1.590	7.3	92.7
	100	1.805	1.633	1.636	1.691	1.4	98.6
	500	0.923	0.963	0.812	0.899	47.6	52.4
	1000	0.893	0.908	0.89	0.897	47.7	52.3
	5000	0.959	1.098	1.097	1.051	38.7	61.3
	10000	0.967	0.772	0.895	0.878	48.8	51.2
Concentrations illustrating cell death or % cytotoxicity ≥ 30%, has been considered as cytotoxic							

Photographs:



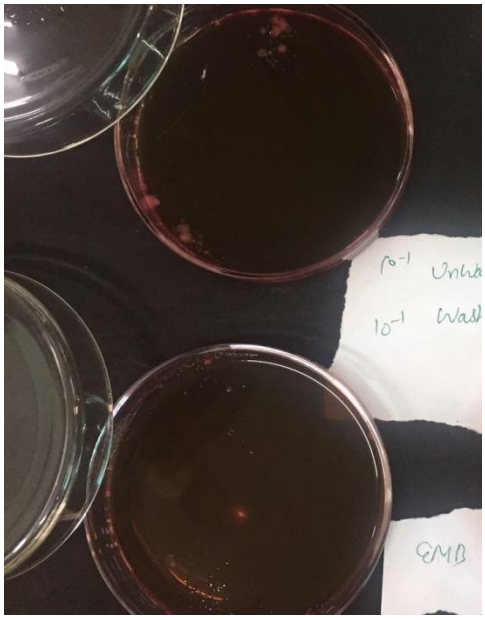
Photograph 5.1: Preparation of the packaging film with coating by brushing technique; A: Preparation of coating solution; B: Different coating solutions; C: Preparation of the film; D: Coated shrinkable LDPE film

A

B


Washed Sample count
Unwashed Sample count
Standard Plate count of Chicken Samples from Easy Day store. Day-0 Sept, 2018

C



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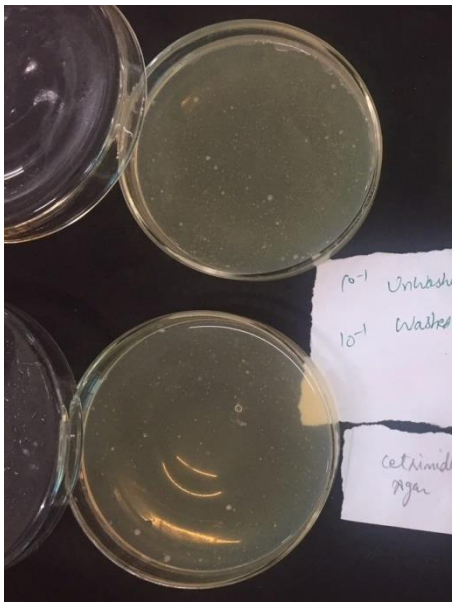

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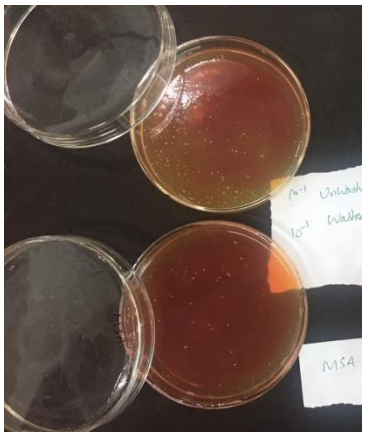

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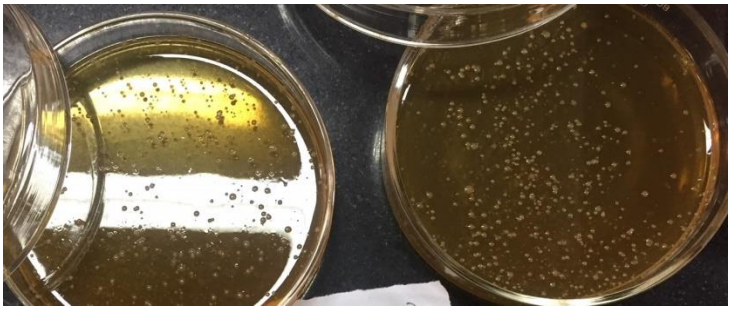
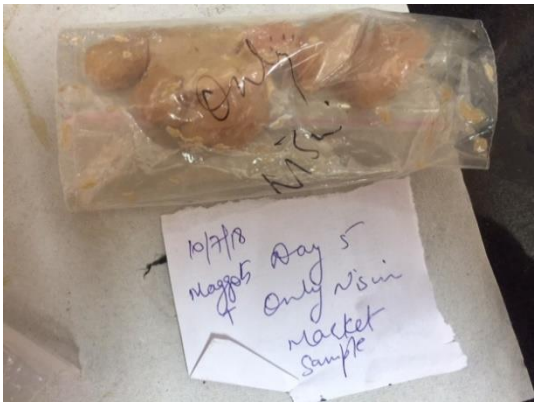


G

H

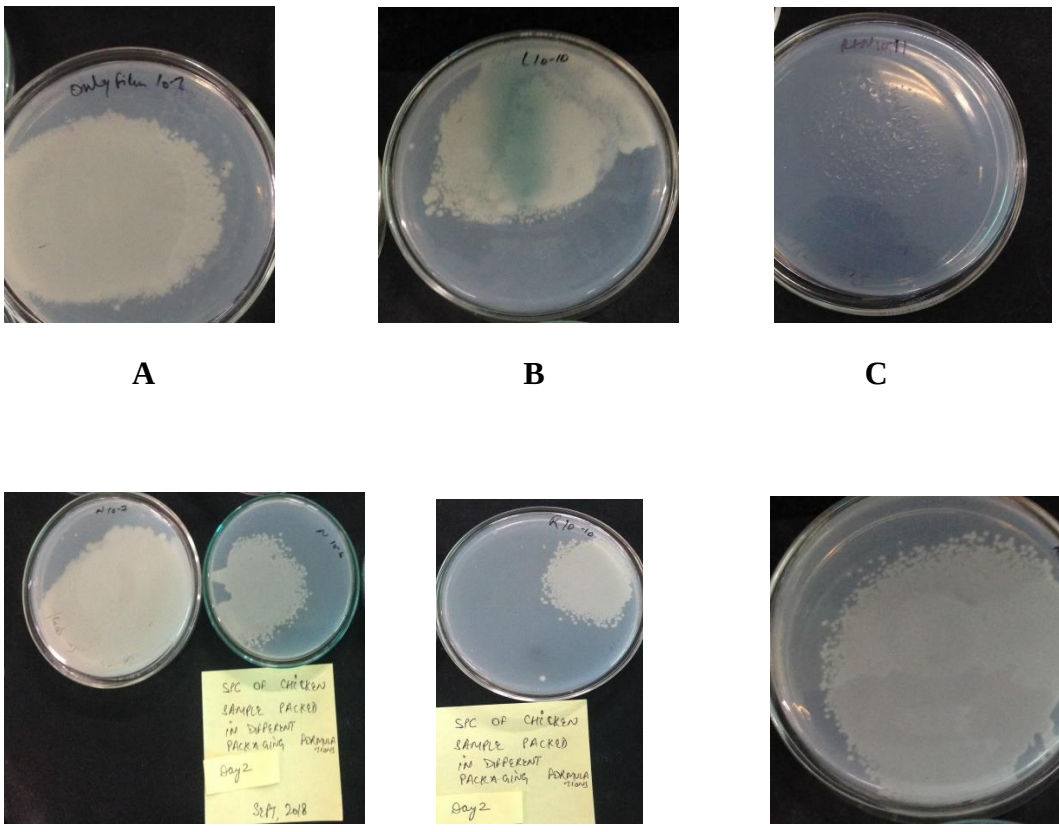
I

J



K

Photograph 5.2: Analysis of chicken samples at room conditions; A: Fresh meat pieces; B: Packed meat pieces; C: SPC of washed and unwashed meat samples on day 0; D-G: SPC of washed and unwashed meat samples on day 0 on EMB agar , Cetrimide agar, MSA and LSA; H: unpacked meat after 1 day of room conditions; I: Conditions of other packed meat samples on day 4; J-K: Presence of maggots in unpacked and nisin film packed samples on day 4 and 5, respectively.



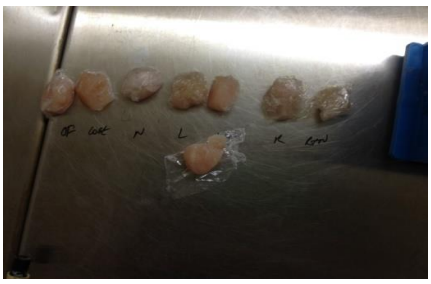
A **B** **C**

D **E** **F**

G **H** **I**

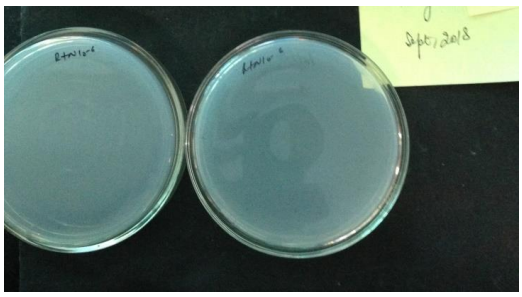
Photograph 5.3: Analysis of chicken samples in refrigerated condition; A-C: SPC of samples with only film, leaf extract film sample and Root and nisin coating samples on day 1, respectively; D-E: SPC of samples with Nisin and with root extract on day 2, respectively; F-G: SPC of unpacked sample and root extract with nisin on day 6; H-I: Condition of all the samples on day 7

A

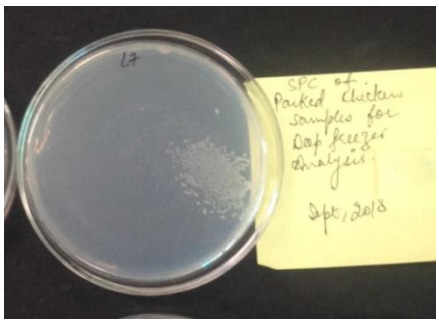


B

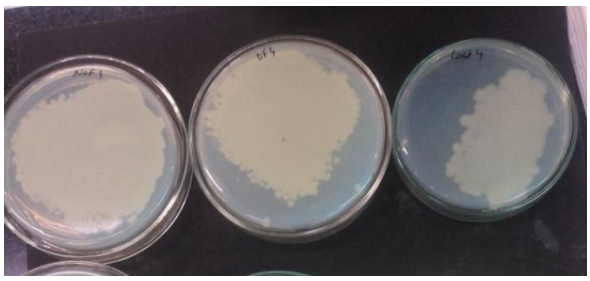
C



D



E

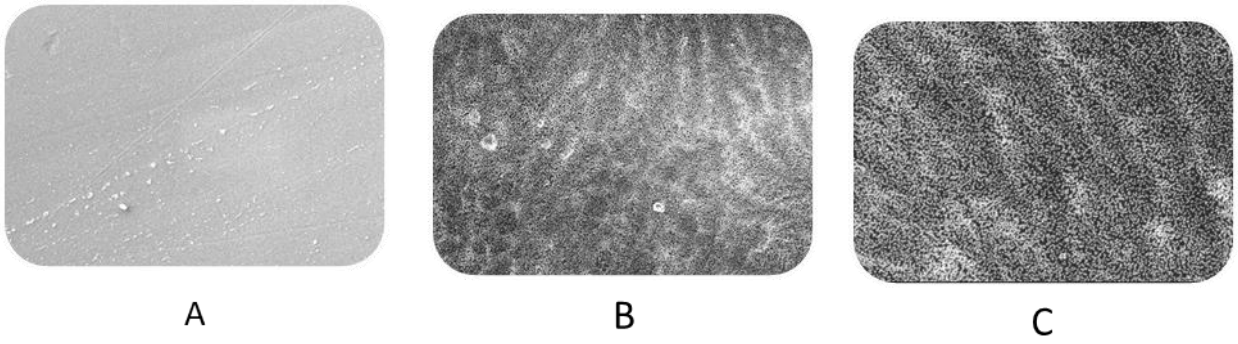


F

G

H

Photograph 5.4: : Analysis of chicken samples at freezer condition; A-B: Packed chicken pieces on day 1 and 4; C-G: SPC of samples without antimicrobial coating on day 4; Root and nisin coating samples on day 4, only leaf extract coating on day 4, Controls on day 7, Root and nisin coating samples on day 20, respectively; H: condition of all meat pieces on day 20



Photograph 5.5: Scanning Electron Micrographs; A: Surface of film without any coating; B, C: Surface of film coated with 500IU/ml Nisin and 20mg/ml *T. indica* root aqueous extract with x 500 and x 1000 magnification respectively.

Figures

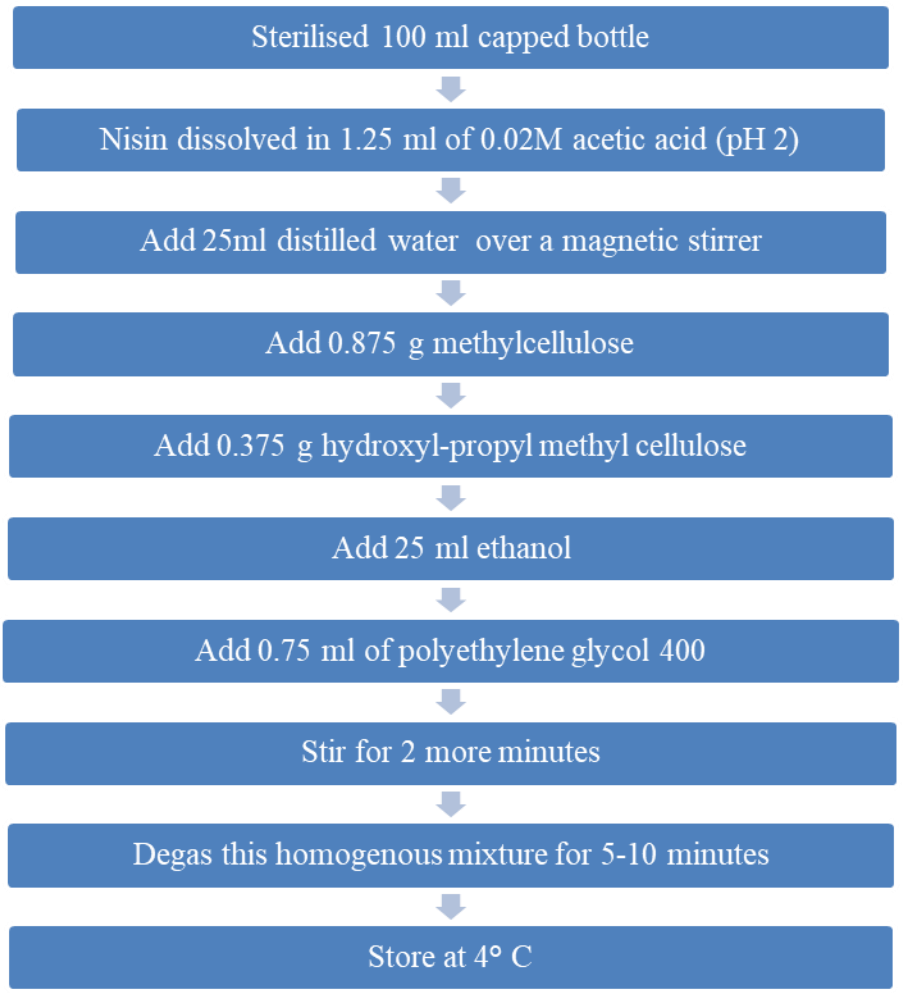


Figure 5.3: Flow chart depicting process of preparation of coating solution

Chapter 6

Summary and Conclusions

Food and health are indispensable issues which are always of major concern with the authorities and organizations globally. Biological contamination of food items not only serve the purpose of its wastage but also becomes the source of infections when consumed, causing the ill health of the consumers. Ruthless use of antibiotics in many such cases, has led to the emergence of antibiotic resistant microbial strains. Hence, such issues are being taken into major consideration at all level of societies, authorities and organizations world-wide. Glance of literature suggests that solutions for the issues related to treating the infectious diseases along with enhancement of shelf life of food items are being perceived by involvement of natural substances. When discussing about natural substances, it includes substances with plant or animal origin. Antimicrobials involving herbals and bacteriocins, regarded as naturals, are being studied extensively as portrayed by the contemporary researches. Hence, *T. indica* and bacteriocins were selected to study for their potential by preparation of low density packaging film and thereafter, analyzing the formed product in extending the shelf life of packed raw chicken meat in lab.

Biological potential of *T. indica* were still not extensively studied, as indicated by the literature review, and hence was considered for this study. The use of authentic and high quality herbals is an integral initial step of GMP concern and therefore, standardization of *T. indica* leaves and roots was determined primarily where the leaves and roots sourced from the Ayushya Vatika, Lovely Professional University, Punjab were subjected to analysis for loss on drying, ash values and extractive values. Results determined the selected plant parts to be of quality raw material when compared to limits as suggested by the authorities. Phytochemical screening of alcoholic and aqueous extracts determined the presence of different classes of chemical constituents such as alkaloids, carbohydrates, saponin, amino acids and

tannins. HPTLC study indicates the presence of tylophorine and XRF values suggested the root to be free of heavy metals. Hence, this study suggests that the leaves and roots collected from this region of Indian subcontinent is of high quality and may be cultivated for its application in pharmaceutical industry. The powder from these selected parts was prepared and kept safely for further study.

Thereafter, two standard Lactic acid bacteria strains, *Enterococcus hirae* MTCC 3612 and *Lactobacillus plantarum* NCIM 2083 along with a bacteriocin producing isolate, *Enterococcus sp.* YT3 were characterized for their bacteriocin. Bacteriocins are the ribosomal produce of some bacteria, generally LAB strains, which are antimicrobial in performance. In this study, *Enterococcus hirae* MTCC 3612 was determined to be catalase positive while other two were potential bacteriocin producers. Optimization of the selected two strains was experimented where maximum bacteriocin production by *Lactobacillus plantarum* NCIM 2083 was determined with MRS broth supplemented with 1% lactose and 0.4% triammonium citrate adjusted to pH 6, incubated for 48 h at 45°C and proteins precipitated with 80% ammonium sulphate. Similarly, maximum bacteriocin production by *Enterococcus sp.* YT3 was determined with MRS broth supplemented with 2% lactose and 4% tryptone adjusted to pH 6, incubated for 48 h at 37°C and proteins precipitated with 80% ammonium sulphate. The bacteriocin was characterised to be of protein origin and maintained antibacterial potential over wide range of temperatures and pH. Hence, 25 ml of semi purified bacteriocins obtained from two selected strains and renamed as Plantaracin 2083 and Enterocin YT3 were prepared and stored safely for their further analysis.

Subsequently, the antimicrobial potential of extracts of standardised leaves and roots of *Tylophora indica* and obtained bacteriocins were targeted towards five prominent food pathogens (*E. coli, P. aeruginosa*, *S. aureus*, *B. subtilis* and *L. monocytogenes*) and analyzed with well diffusion technique. Ethyl acetate extraction of plant samples under study exhibited less than 5% yield, due to which it was not considered for further study. The 5 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml and 25 mg/ml concentration of methanolic, ethanolic and aqueous extracts exhibited antimicrobial potential against some of the selected test food pathogens but methanolic extract of

leaves with 50 mg/ml concentration exhibited biological activity against all test strains. Similarly, ethanolic extract of roots with 50 mg/ml concentration exhibited biological activity against all test strains. The potential of *T. indica* leaves and roots extracts towards *L. monocytogenes* were first time reported in this study. Plantaracin 2083 and Enterocin YT3 exhibited wide spectrum potential by revealing zone of inhibition against test pathogens. Plantaracin 2083 was ineffective towards *L. monocytogenes* while Enterocin YT3 with undiluted form exhibited biological activity against all test strains. Subsequently, an attempt to observe the behaviour of plant extracts with obtained bacteriocin towards the test pathogens was done. The binary combination exhibited the biological potential against all test strains except for *L. monocytogenes* which has been considered as a potent food pathogen especially in refrigerator and freezer conditions. Observing this inefficiency of the combination, Nisin was procured and tested for its antimicrobial potential. The 2,500 IU/ml and above concentrations were effective against all selected indicator organisms. The binary combination of plant extracts and nisin were then studied for biological potential against the five test organisms. Synergism between the two anti-microbial was confirmed at 20mg/ml water extract of *T. indica* leaf with 1000 IU/ml of Nisin and 20mg/ml water extract of *T. indica* root with 500 IU/ml Nisin, where individual concentrations were selectively effective. Hence, five antimicrobials at MIC concentration exhibiting biological potential against all test pathogens, was considered for preparation of film and shelf life analysis of food product.

Cellulose based antimicrobial carrier solution was prepared and was coated over the LDPE film and fastened up to complete dryness. The prepared packaging products were then analyzed for their efficiency to extend the shelf life of raw chicken samples under different atmospheric conditions by standard plate count. On day 0, washed samples exhibited lesser aerobic count in differential media as compared to unwashed samples, illustrating the cleaning effect of washing from the surface of meat samples. In room environment none of film exhibited potential to reduce the growth of aerobic organisms up to CFU 10^7 or lesser, although contamination signs were lesser in the films possessing antimicrobials as compared to the control. When refrigerated, the chicken meat samples packed in *T. indica* root extract+ nisin exhibited best storage

quality up to 6 days, thereafter the samples were deteriorated. In freezer, the best results were again observed with meat samples tested with *T. indica* root extract+ nisin where maximum storage was analyzed to be for 16 days.

Subsequently, the packaging film prepared with *T. indica* root extract+ nisin coating solution was subjected to characterisation. SEM analysis illustrated homogenous and even layering of the coating without any bubbles or some defect along with the presence of crystalline structure on the surface. There was an increase of 1 micron thickness contributed by the coating done over the LDPE film surface. The tensile strength was improved after coating but correspondingly, the elongation of break reduced. Hence, the selected active packaging film has developed more resistance to stress and are lesser elongable. While the increase in haziness reduced the film clarity, slight decrease of transparency was also analyzed. No change in melting point (114-118^{o} C) was observed along with reduction of thermal shrinkage at 70^{o} C was determined.

Subsequently, the toxicity analysis of *T. indica* root extract+ nisin coating solution was determined against *Human Embryonic Kidney* cell lines (*HEK-293*). When dissolved in water, it was found to be non-toxic at all tested concentrations (1 μg/ ml-10 mg/ ml). When dissolved in aqueous: ethanolic stock, the same coating solution was analyzed to be non-cytotoxic at 1 μg/ ml- 100 μg/ ml and cytotoxic at 500 μg/ ml-10 mg/ml.

Lastly, active packaging films coated with cellulose based solution possessing *T. indica* root extracts and nisin at concentrations of 20mg/ml and 500 IU/ml, respectively, was determined to be a potential for improving shelf life of raw chicken meat samples for at least 6 days in the refrigerator conditions and 16 days in deep freezer but could not maintain the quality of chicken in room conditions. Most of the studies conducted with the different active packaging films, have illustrated their efficacy with relation to control of specific food pathogens. However, in real time storage of food items, the number and types of microbial food pathogens are far higher. Hence, real time study of such active packaging formulation products is the

major need of time, to ensure the real time usage to enhance the shelf life of the food products.

Based on the results of this study, it is proposed that the efficacy of prepared active film formulation must be tested against variety of other food items and subsequently, pilot study with some food industry may be done so that it can help serve the purpose to the society.

List of Publications

1. Khanna C, Singh S, Vyas M. "Diverse Pharmacological Potentials of an Indigenous Climber *Tylophora indica* (Burm. F.) Merr.: A Review". Journal of Pharmacy research. 2018; 12 (3): 389-387
2. Khanna C, Singh S, Vyas M. "Antimicrobial potential of *Tylophora indica* and its future considerations in health and food industry". Pharmacognosy Reviews. May 2018, 12 (1): 72-77
3. Khanna C, Vyas M. Singh S. "Physicochemical, qualitative, and high profile thin-layer chromatography study of Tylophora indica (Burm. f) Merr. leaves and roots". International Journal of Green Pharmacy. 2018, 12 (2): 136-141
4. Khanna C, Singh S, Vyas M, Das S. "Biological potential of semi purified Enterocin of *Enterococcus sp*. YT3 against selected food pathogens". Oriental Journal of chemistry. 2019; 35 (5); 1584- 1596

List of Conferences Attended

1. "A Systematic Review of Pharmacognostic and Pharmacological Analysis of an Indigenous Climber-*Tylophora indica":* Poster presentation by Charu Khanna and Shalini Singh at International Conference of Pharmacy at Lovely Professional University, Punjab, 7^{th} -8^{th} April 2017
2. "A Review on the Applications of Nature's Antimicrobial Boon- Bacteriocin" Poster presentation by Charu Khanna, Shalini Singh and Manish Vyas at International Conference on Innovative Strategies for Sustainable Water Management at Lovely Professional University, Punjab, 17^{th}-18^{th} November, 2017
3. "Physico-chemical, Qualitative and HPTLC Profile of Leaves and Root of *Tylophora indica* (Burm. f) Merr.": Oral Presentation by Charu Khanna, Shalini Singh and Manish Vyas at National Conference on Recent trends in Biomedical Sciences at Lovely Professional University, Punjab, March, 16, 2018
4. "Isolation and Characterization of Bacteriocin from *Enterococcus sp. YT3* for its Biological Potential Against Selected Food Pathogens": Oral Presentation by Charu Khanna, Shalini Singh, Manish Vyas and Sujata Das at International Conference of Pharmaceutical Sciences held at Lovely Professional University, Punjab, September, 13-14, 2019

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REVIEW ARTICLE

Antimicrobial Potential of *Tylophora indica* and its Future Considerations in Health and Food Industry

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ABSTRACT

One of the most interesting areas in scientific research has been drug discovery. The discovery of antibiotics led to the improvement of the overall health status of the general population, but in the present scenario, its misuse has caused the development of microbial drug resistance. The focus has shifted toward the use of natural drugs as a source of treatment for various ailments to improve the health of individuals. A climber, identified as *Tylophora indica*, has been in use for a very long time for the treatment of ailments such as asthma and diarrhea. *T. indica* possesses compounds such as phenanthroindolizidine alkaloids and nonalkaloid components which may be responsible for its biological activity. *T. indica* is an endangered species, and its genetic preservation is a matter of concern. Literature reports that the different plant parts of *T. indica*, especially the leaves, possess a wide range of activities against different Gram-positive and Gram-negative bacteria. The experimental observations suggest that the methanolic extracts are more effective as compared to the aqueous extracts. Further studies are needed to explore the potential of *T. indica* extracts for its antimicrobial activities on various microbial pathogens including bacterial species, protozoal parasites, and viruses. This may further open an investigation into healthy and natural food preservation techniques. The article makes an attempt to review the antimicrobial activities of *T. indica*, its potential in the food and health sectors, and the future scope with emphasis on overcoming the limitations such as microbial contamination of food and use of synthetic preservatives.

Key words: Antimool, Asclepiadaceae, phenanthroindolizidine alkaloids, *Tylophora indica*

INTRODUCTION

The use of synthetic and semi-synthetic drugs for the treatment of infectious diseases is on the rise these days. Their misuse has led to the development of drug-resistant microbial species which has further elevated treatment failures in the clinical cases. Looking at this aspect, scientists are now more focused on investigating the use of natural compounds derived from plants. Medicinal plants have always been in use since the ancient era and their commendable and reproducible effects can be observed even today. Ayurveda has discussed the role of herbs to combat various infectious diseases. Chatterji-Wu (2003) reported the role of *Curcuma longa* as an anti-inflammatory drug[1] whereas Samy *et al.* discussed the various bioactive compounds found in plants such as *Azadirachta indica* and *Solanum xanthocarpum*[2] used in the treatment of various pathological conditions of the body.

According to Madhavi *et al.*, *Tylophora indica* (*T. indica*) is known for its role as treatment for asthma.[3] *T. indica* is part of the Asclepiadaceae family and native to India. It can be found in the Himalayas and the sub-Himalayan tract, where it is found growing from Uttar Pradesh to Meghalaya, Orissa, and Bengal, in various plains, forests, and hilly areas of the country. *T. indica* is also known as "Antmool" and is found at altitudes of up to 1260 m as per Harmanjit and Karanveer (2012).[4] Sunita and Priya reported that *T. indica* is a perennial and slender climber and possesses fleshy, long, and knotted roots. It is well branched with a long and twining stem. This semi-shrubby presenting plant inhabits well-drained soil, preferably on sandy soil, and has restricted growth in arid areas. *T. indica* has been included in the Bengal Pharmacopoeia since 1884. The main parts which are used are the roots and the leaves.[5] As stated by Suhas *et al.*, watery latex is found in the plant. The leaves are opposite, elliptic-oblong to ovate-oblong, glabrous and with an acute apex. The leaf has a petiole which is about 6–13 mm long and glabrous and possesses a characteristic pleasant odor. Under the microscope, it has a single-layered epidermis with a thin cuticle. The mesophyll can be differentiated into the palisade and spongy parenchyma with 2–3 layers and 6–8 layers, respectively. Rosettes of calcium oxalate are also found in the spongy parenchyma. The flowers are pale yellow with a purple color within and present in the lateral cymes. The follicle of fruits occurs in pairs and tapers to a fine point in the apex.[6] Anwar *et al.* shared the fact that the wide and careless approach to collecting the plant for its use, the low germinating capability of the seeds, and the lowered potential of vegetative cuttings for propagation has placed *T. indica* in the category of an endangered species.[7] The macroscopic view of *T. indica*, collected from Ayushya Vatika of Lovely Professional University, has been illustrated in Figures 1-5.

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Review Article

Diverse pharmacological potentials of an indigenous climber *Tylophora indica* (Burm. F.) Merr.: A review

Charu Khanna[1], Shalini Singh[1], Manish Vyas[2]

ABSTRACT

In the present day scenario, changes in the natural cycle and its effects over the health in all forms of life have generated an alarming sign all over the world. Attempts are being made to minimize these deleterious effects by creating awareness in general population to implement the maximum use of plant drugs in the daily life. *Tylophora indica* (Burm. f.) Merr. (*T. indica*) is one of the medicinal plants, which have been renowned in the Ayurvedic System of Medicine for the treatment of asthma. The plant possesses various phytoconstituents, of which phenanthroindolizidine alkaloids (tylophorine, tylophorinine, tylophorinidine, etc.) form the main components. The present review has been focused to scan the ethnomedical data available for *T. indica*, analyze against the investigated experimental findings for its multiple pharmacological actions, and discuss the future prospective of the climber. Ethnobotanical surveys have attributed this plant to be antiasthmatic, antiepileptic, antivenom, contraceptive, diuretic, antidysenteric and so on. Some of these potentials (antiasthmatic and antidysenteric) have been supported by the different investigations such as animal model and clinical trials, but still its conventional uses such as contraceptive and anti-venom need further scrutiny. Based on the animal research findings, *T. indica* seems to be a prospective agent as hepatoprotective, anti-inflammatory, antioxidant, myocardial protective, and wound healer when used in the recommended dose of maximum 20mg/kg in humans. This can further be explored by the pharmaceutical industry for newer and effective preparation. The plant and its parts such as leaves stem and root have been studied for the biological activities and found effective against different pathogenic organisms such as *Escherichia coli*, *Staphylococcus aureus*, influenza virus, and so on, but their discussion is beyond the limit of this article. Based on the present review, it may be concluded that *T. indica* may be considered a potential for pharmaceutical and food industry.

KEY WORDS: Antiasthmatic, Hepatoprotective, Pharmacological, Phenanthroindolizidine, *Tylophora*

INTRODUCTION

The National Medicinal Plant Board, AYUSH, states that medicinal plants form an integral part of folklore, traditional, and alternative system of medicines, especially in India. They contribute highly toward the herbal industry, are a source of livelihood of many, and provide health security to large Indian population. The export value of these medicinal plants has registered nearly nine-fold increase in the past decade with about ₹3211 Crore in 2014–15.[1] This rise is highly expected as the common population is becoming aware of the existing environmental health hazards. A much focus has been generated to utilize the plant origin drugs as they are environment-friendly, safer to use with minimal side effects, has better patient tolerance, and is relatively cheaper.[2] Of 6000–7000 medicinal flora available in India,[3] one climber, *Tylophora indica* (Burm. F.) Merr. *(T. indica)*, has been in use and is documented in the treatment of multiple conditions such as asthma, diarrhea and arthritis since classical times.[4] It has been officially included in *Bengal Pharmacopoeia, 1884*, with the name of "Antmool" and the roots have been used as a substitute for ipecac. *T. indica* (Burm. F.) Merr. is also known by "*Tylophora asthmatica*" as the plant, especially leaves have been widely used in asthmatic conditions.[5] It is a perennial, twinning, knotted climber, forms a member of *Asclepiadaceae* family and is indigenous to our country where it is found along the Sub-Himalayan tract with its major flora in southern and eastern parts of India.[6] The species is distributed in various parts of the world and can be spotted in Sri Lanka, Burma, Singapore, Nepal, Laos, St. Vincent,

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RESEARCH ARTICLE

Physicochemical, qualitative, and high profile thin-layer chromatography study of *Tylophora indica* (Burm. f) Merr. leaves and roots

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Abstract

Introduction: India is the land with near estimation of 6000–7000 medicinal floras. Comprehensive ethnobotanical studies of these natural healers highlight their multiple therapeutic applications. Moreover, different parts of a plant reveal different pharmacological activities due to the variation of phytoconstituents. Hence, standards should be available for all parts which are playing an important role in the pharmacological profile of the drug. The whole plant of *Tylophora indica* is used for the different therapeutic attributes, but standards of only leaves are available. Therefore, in the present study roots of *T. indica* were evaluated for establishing the standards of roots. **Materials and Methods:** *T. indica* leaves and roots were collected from the Ayushya Vatika, Lovely Professional University and subjected to physicochemical, qualitative, and high profile thin-layer chromatography (HPTLC) study. **Results:** Results of physicochemical analysis of leaves were complying with the standards and results of roots for loss on drying, total ash, water soluble ash, acid insoluble ash, methanolic extractive values, and water-soluble extractive were 11.3 ± 0.6%, 6.8 ± 1.32%, 4.16 ± 0.98%, 1 ± 1.0%, 30.4 ± 1.75%, and 20 ± 1.6%, respectively. Qualitative analysis revealed that leaves are devoid of steroids, terpenoids, and amino acids. In HPTLC analysis two different solvents were used for both the samples and different numbers of RFs were observed in different samples. X-ray powder diffraction study of root indicates the absence of heavy metals such including mercury, lead, and arsenic. **Conclusion:** The investigated *T. indica* leaf samples comply with the standards. The results of the study revealed entirely different physicochemical, qualitative, and HPTLC profiles of roots. Hence, standards should be developed for the individual plant part which is going to be used as a medicine.

Key words: High profile thin-layer chromatography, physicochemical, phytochemical, qualitative, *Tylophora indica*, X-ray powder diffraction

INTRODUCTION

Recent research has shown its inclination toward the field of pharmaceutics, nutraceuticals, and food industry with the herbal drugs. Nearly 80% of the total world population is still dependent on herbs as the primary health-care treatment.[1] India is one of the countries which possess about 15 agro-climatic zones and has the richest biodiversity in the world with approximately 1178 medicinal species.[2] Nearly 242 medicinal species have an annual consumption level above 100 metric tons which estimate the recognition of these herbs as medicines.[3] Ethnomedicine highlights the therapeutic value of different parts of a plant in different ailments. This has even been investigated with a scientific approach such as *Randia dumetorum* and *Azadirachta indica*. In case of *R. dumetorum*, only the fruits are emetic because of the presence of glycosides such as radianin and radioside A, which is not found in other parts of the plant such as root and bark.[4] The seed oil of *A.*

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Biological Potential of Semi-Purified Enterocin of *Enterococcus* sp. *YT3* Against Selected Food Pathogens

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ABSTRACT

The efforts for prevention of food borne illness and infections draw great attention, worldwide. Different methods, both physical as well as chemical, are commonly used for improving shelf life of food, but limited efficiency of physical methods, and potential health hazards associated with chemical methods, have brought biological processes in the limelight. One such natural, environment friendly, highly effective natural food preservants are, bacteriocins. Thus, there is a continuous need for better bacteriocin producers in the search for more effective bacteriocins than what are already available in the market. In the current study, food samples were collected from local market of Jalandhar, Punjab, and evaluated for bacteriocin producing Lactic acid bacteria. *Enterococcus sp. YT3* was found to be the most efficient bacteriocin producer among the isolates, with higher bacteriocin activity exhibited by the given strain under optimized cultural conditions. The partially purified bacteriocin have molecular weight between 35kDa & 48kDa, possess pH (2-10) and thermal stability (even at 121°C for 20 min), and exhibit antimicrobial activity against different bacteria (*E. coli, P.aeruginosa, S. aureus, B. subtilis and L. monocytogenes*). Future studies will focus on checking different food samples for real time evaluation of shelf life improvement.

Keywords: *Enterococcus sp. YT3*, Bacteriocin, Optimisation, Food pathogens, SDS-PAGE.

INTRODUCTION

Bacteriocins are the ribosomally synthesized anti-microbial peptides produce of some bacteria and are known to act against closely related microbes. Owing to such valuable biological potential, research has been focussed to isolate and characterize efficient bacteriocin producing organisms, which can further be utilized for large scale applications in health and food industry[1]. Lactic acid bacteria (LAB) represent a group of important bacteriocin producers, commonly associated with various food substances. Food sources like, meat and meat products, milk and milk products, are rich in microbial diversity including, Lactic acid bacteria (LAB). The bacteriocins, from LAB strains are considered to be the most eminent and natural alternatives/supplements to some classical food