PHYTOCHEMICAL SCREENING OF ETHNOMEDICINAL HERBAL EXTRACTS AND THEIR EFFECT ON MICROBIAL QUALITY OF *SUKUTI*

by

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April, 2022

Phytochemical Screening of Ethnomedicinal Herbal Extracts and their Effect on Microbial Quality of *Sukuti*

A dissertation submitted to the Central Department of Food Technology, Institute of Science and Technology, Tribhuvan University, in partial fulfillment of the requirements for the degree of M. Tech. in Food Technology.

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April, 2022

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Approval Letter

This *dissertation* entitled *Phytochemical Screening of Ethnomedicinal Herbal Extracts and their Effect on Microbial Quality of Sukuti* presented by Sangen Ruma Rai has been accepted as the partial fulfillment of the requirement for the M. Tech. degree in Food Technology.

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April 25, 2022

Acknowledgments

It is a genuine pleasure to express my deepest sense of thanks and gratitude to my mentor and guide teacher Prof. Basanta Kumar Rai (Department Chief, Central Department of Food Technology). It is only because of his overwhelming attitude to help his students that work of this complexity could be accomplished. His valuable guidance, meticulous scrutiny, and unwavering support have helped me to the greatest extent in accomplishing this task.

I owe a debt of gratitude to Prof. Dr. Dhan Bahadur Karki, Managing Director of Dharan Multiple Campus for providing research facilities and motivation throughout the work.

I am also grateful to Mr. Devraj Acharya, Asst. Prof. of Central Department of Food Technology for his valuable suggestions and encouragement for this work.

Very special thanks to Prof. Pashupati Mishra (Central Campus of Technology) for his constant support and motivation throughout my research period.

Above all, I am very much indebted to my parents and family for their constant encouragement, love, inspiration, and moral support throughout the work. They made countless sacrifices to provide me with the best opportunities in my life. Words are incapable to express my appreciation for them.

Last but not the least, I take this opportunity to acknowledge the entire staff of the Central Department of Food Technology and Dharan Multiple Campus for their kind cooperation and for lending their helping hand in this work.

Date of submission: April, 2022

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(Sangen Ruma Rai)

Abstract

The main objective of the study was to screen phytochemical constituents of ethnomedicinal herbs and to determine their effect on the microbial quality of *sukuti*. Specific objectives include preparation of herbal extracts, isolation, and identification of microorganisms from *sukuti*, determination of antimicrobial property of herbal extracts, and microbial analysis of herbal *sukuti*. Four ethnomedicinal herbs (*Zanthoxylum armatum*, *Litsea cubeba*, *Heracleum nepalense*, and *Evodia fraxinifolia*) having culinary importance were selected for the study. For the preparation of herbal extract, herbs were ground (particle size < 250 µ), treated with 50% ethanol (v/v), and concentrated in a rotary vacuum evaporator at 50^{o}C. Four of the spoilage and pathogenic microorganism viz. *Salmonella*, *Staphylococcus*, *E. coli*, and Lactic acid bacteria were isolated and identified from the *sukuti* samples for the study. Herbal extracts at the concentration of 40, 20, 10, and 2 mg/ml were tested against the isolates to determine the antimicrobial property of the extracts. The best herb with optimum concentration was selected based on its antimicrobial activity.

Zanthoxylum armatum at 40 mg/ml concentration showed the highest zone of inhibition against the test organisms and therefore selected for final product development. Meat strips (1 cm × 1 cm × 25 cm) were marinated with *Zanthoxylum armatum* (40 mg/ml) extract at the rate of 2%, aged (24 h at 4 ± 2°C), and dried at cabinet drier for 2 days at 55°C. Total Plate Counts of control (untreated) and herbal *sukuti* (treated) were carried out for 20 days within an interval of 10 days to determine the microbial stability of the final product. The total plate count for treated sample was significantly lower ($p<0.05$) than that of untreated sample.

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List of Abbreviations

Abbreviation	Full form
ANOVA	Analysis of variance
CDC	Centers for Disease Control
CFU	Colony Forming Unit
EO	Essential oil
EOs	Essential oils
FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
ICMFS	International Commission on Microbiological Specifications for Foods
IMViC test	Indole, Methyl Red, Vogues Proskauer, Citrate test
LAB	Lactic Acid Bacteria
MR-VP	Methyl Red - Vogues Proskauer
NMDH	New Mexico Department of Health
PCA	Plate Count Agar
RTE	Ready to Eat
SS Agar	Sallmonella-Shigella Agar
TPC	Total Plate Count
USDA	United States Department of Agriculture

Part I

Introduction

1.1 General introduction

Dried meat and meat products can be defined as whole muscle or ground and formed meat products which have been subjected to dehydration, resulting in unique sensory properties and enhanced stability (Skandamis and Gounadaki, 2009). Dried, cured meat has existed for hundreds of years. Over time different types of meat, including beef, chicken, fish and game have been adapted into a number of different dried meat forms. Beef jerky, which is smoked, seasoned and cut into strips is much different from biltong, which is cured, air dried and can come in strips, shredded or even in powdered form. But there are a number of other dried meat forms as well (Anon., 2016). Dried meats are microbiologically stable at ambient temperature because of their low water activity value. Dried meats are prepared for use in formulated foods (e.g. soup mixes), for campers and the military and as a snack food (ICMSF, 2006).

Sukuti is the Nepali word for dry meat. *Sukuti* is an indigenous dried meat product especially from buffalo lean meat. In the traditional method, lean meat is cut into strips and hung over the fire place in kitchen, and subjected to heat and smoke of the burning woods till the strips become adequately dry and hard. It can also be prepared by air drying or sun drying but drying and smoking over fire place imparts more characteristics, pleasant taste and texture than the sun dried or air dried counterpart. Most of the *sukuti* available in the local market are air-dried, sun-dried or smoke-dried, which should be cooked prior to consumption (Acharya, 2014).

The microbiological profile of meat products presented to the consumers is the sum of the total of the slaughtered animal health, conditions under which it was reared, quality of slaughtering, processing, packaging and conditions under which the meat was stored. Major meat associated pathogenic bacteria include *Clostridium perfringens*, *Staphylococcus aureus*, *Salmonella* spp, pathogenic strains of *Escherichia coli*, *Campylobacter* spp, *Yersinia enterocolitica*, *Listeria monocytogenes* and *Aeromonas hydrophila* (Douglas and Farid, 2001).

Spoilage of dried meats is unusual and is mainly due to insufficiencies or defects in the manufacturing processes. The main spoilage organisms associated with low-moisture dried meats are molds, and in particular those capable of growing at low water activity (e.g. *Eurotium* spp). Spoiled intermediate moisture meats are often dominated by molds, yeasts, lactobacilli, as well as by staphylococci. Studies on the microflora of biltong have shown that xerophilic molds are the main spoilage species of this type of dried meat (Hocking, 1991). In addition, several yeasts including *D. hansenii, C. zeylanoidese,* and *Trichosporon cutaneum* are also frequently isolated from intermediate moisture meats such as biltong, and cabanossi (Hocking, 1991; Wolter *et al.*, 2000). Other studies have shown that staphylococci and micrococci are often present in intermediate moisture meats such as *pastirma* and *cecina* (Garcia *et al.,* 1995; Kotzekidou, 1992; Vilar *et al.,* 2000).

Biopreservatives could be defined as compounds, from natural sources or formed in food, able to prevent or retard spoilage related with chemical or biological deterioration that prolong product shelf life. Currently, biopreservatives are becoming important due to increasing public demand for healthy and quality foods. Biopreservatives can be obtained from plants, animals, or from microorganisms commonly used in food fermentation. Most of the proposed natural alternatives to substitute synthetic or conventional preservatives are biopreservatives; however, this term is rarely used. Essential oils (EOs) are the most common and studied biopreservatives obtained from plants, mainly from herbs and spices (Mani-López *et al.*, 2018).

Herbs, spices, aromatic plants, berries, and their extracts and essential oils have been studied as potential natural antimicrobial in meat and meat products (Aminzare *et al.,* 2016). Many studies have been conducted on the aromatic chemical compounds extracted from plants and effective on spoilage and pathogenic microorganisms present in meat and meat products. Many of these tests have demonstrated the positive effects of these compounds on meat and meat products, particularly the effect that they have as a natural agent on spoilage and pathogenic bacteria (Bajpai *et al.*, 2012; Samojlik *et al.*, 2010).

1.2 Statement of the problem

In recent years, manufactures of meat products have encountered a growing demand in consumers for better quality and healthier meat products, especially for fully natural, less processed and ready-to-use products with precise labeling of additives (Mariutti *et al.,* 2011). In general meat and meat products are highly nutritious and high moisture food with pH. All of these features combined together make it a good culture medium for most microorganisms and thus contamination with spoilage organisms is almost inevitable. This has made meat preservation more challenging than other foods (Ikeme, 1990). The improper storage and processing of these products can therefore cause a quality deterioration and public health risks (Fratianni *et al.,* 2010; Solomakos *et al.,* 2008).

The application of synthetic additives to extend shelf life and overall quality through the inhibition of microbial spoilage is limited by food legislation and consumers are concerned about the safety of synthetic food additives; therefore, the demand for the use of natural ingredients in foodstuffs has increased lately (Devatkal *et al*., 2014). Even though chemical alternatives for food preservation are available, they are likely to exhibit carcinogenic and teratogenic properties. Therefore, with growing concern of residual toxicity towards chemical preservatives, investigation on the use of other novel antimicrobial agents and exploration of natural alternatives for antimicrobial needs more attention (Pandey and Singh, 2011). According to Mitscher (1975), plants may be a poorly exploited source of antimicrobial agents because the structures and modes of action are not known (Nychas, 1995).

Various meat and meat product spoilage epidemics with different degrees of severity occur every year in different parts of the production chain, such as in the preparation, storage and distribution of products. In addition to lipid oxidation and autolytic enzymatic spoilage, microbial deterioration also plays a major role in the process of quality deterioration; this process can then lead to substantial economic and ecological damage (Aminzare *et al.*, 2016).

The safety of meat and meat products, which is delineated by a series of challenges associated with either microbial pathogens or other (biological or not) issues, has been one of the major societal concerns. Various events have been identified as potential explanations for

the rising meat safety concerns of recent years including: changes in animal production, product processing, and distribution; increased international trade; increased worldwide meat consumption; changing consumer needs and consumption patterns (e.g., preference for minimally processed foods); higher numbers of consumers at risk for infection; and increased interest, awareness, and scrutiny by consumers. Although various non-biological concerns have been, related to meat safety, microbial pathogens are traditionally associated with the most serious meat safety issues in terms of both foodborne illness and product recalls (Sofos, 2008).

Several studies shows that dried meat contains fairly high levels of many spoilage organisms and pathogenic organisms capable of causing food poisoning including *Salmonella*, *E. coli*, and toxin producing Staphylococci and molds (Garcia *et al*., 1995; Wolter *et al.*, 2000; Hui *et al*., 2001; Burfoot *et al.*, 2010; Mhlambi *et al.*, 2010). Many documented outbreaks worldwide due to the consumption of dried meat has increased the concern over pathogen growth and survival in dried meats (Tompkin, 1986; Eidson *et al.*, 2000; NMHD, 2004; Nummer *et al.*, 2004).

Review of literature reveals that researches on *sukuti* so far are limited to survey, *sukuti* preparation and process optimization. Microbial aspects of the *sukuti* which is very important in the preservation of *sukuti* and prevention of health hazards, appears to have been ignored.

1.3 Significance of the study

Microbial spoilage has a negative effect on the quality of meat and meat products, causing changes in their sensory and nutritional properties. Herbs contain biologically active compounds with antimicrobial properties. This study therefore was an attempt to use herbal extracts as substitutes for commercial synthetic antimicrobials to prevent spoilage without violation of food legislation.

Among the rising meat safety concerns in the recent years, microbial pathogens are traditionally associated with the most serious meat safety issues in terms of foodborne illness. Also the growing health concern and awareness has led public interest towards search of food that are natural, less processed and safe. Therefore, one of the aims of this study was to

develop a novel food that is natural and microbiologically safe. Many studies have demonstrated the efficacy of plant derived products as potential antimicrobials. Therefore, the present study was an effort towards value addition along with shelf-life enhancement of *sukuti*. Since herbs are also known for their aromatic principles, herbs can be used not only as a preservative but also as a flavor component. Therefore, incorporation of herbal extracts in *sukuti* can enhance the flavor of the product. Also this study was an attempt of value addition of neglected or underutilized medicinal herbs of Nepal.

1.4 Objectives

1.4.1 General objective

The general objective of the present work is the phytochemical screening of ethnomedicinal herbal extracts and their effect on microbial quality of *sukuti*.

1.4.2 Specific objectives

i. To prepare herbal extracts.
ii. To isolate and identify microorganisms from *sukuti*.
iii. To determine antimicrobial property of herbal extract.
iv. To prepare herbal extract incorporated *sukuti*.
v. To carry out microbial analysis of prepared *sukuti*.

1.5 Limitations

The limitations of the study are:

i. Specific bio-active compound of herbs were not identified.
ii. Only few of the principal pathogenic and spoilage micro flora associated with meat were investigated.

Part II

Literature review

2.1 Dried meat

Dried meat and meat products can be defined as whole muscle or ground and formed meat products which have been subjected to dehydration, resulting in unique sensory properties and enhanced stability (Skandamis and Gounadaki, 2009). Depending on the degree of dehydration, dried meats can be conveniently classified as

(i) low moisture meats - products that contain less than 25% moisture and have a water activity (a_w) level of <0.60

(ii) intermediate moisture meats - products that contain less than 50% moisture and have a a_w level of 0.60 to 0.85

As the production of dried meats is often combined with other processing technologies such as curing (i.e. addition of salt or curing salts) and/or fermentation, several different types of meat products can be classified as dried meats. Moreover, variations in the type of meat used, as well as in the processing conditions applied give rise to a large diversity of products (Skandamis and Gounadaki, 2009).

2.2 Types of dried meats

There exists a broad range of traditional dried or intermediate moisture meats originating from different developing states, such as the Brazilian *carne do sol* and *charque* and the Sahelian *kilishi*. The same is true for industrialized countries where traditional dried meats, such as bacon, ham and Grisons meat, enjoy a good popularity among consumers. Yet, there is scarcity of statistical data on the world production of dried meat products. This could partially be explained by the fact that a large number of traditional products, especially from developing countries, are generally meant for the domestic market (Santchurn *et al.*, 2012).

2.2.1 Biltong

Biltong is an uncooked, air-dried meat product consumed mainly as a snack. The product is similar to the American dried meat product known as Jerky, but is indigenous to South Africa. It is often made from beef or game meat. Most muscles in the carcass may be used but the large ones are most suitable. The product was developed by the early Dutch settlers as way of preserving meat. Biltong can be consumed in its raw as a light snack. Over the years the preparation of biltong has evolved according to different cultural practices different. The South African traditional method of preparation involves soaking the meat pieces in apple cider before spicing with coriander, black pepper, salt and brown sugar. This is followed by air dying. Unlike other semi-dried meat products such as salami and cured sausages, microorganisms do not seem to play a role in the development of biltong flavor. The divergence in production is in the spicing, which is used to achieve the unique distinct flavor of the various varieties. Biltong is flavored with various spices and salt and many of the spice formulations are passed on through generations, in home-made biltong or special formulations derived by industries (Burfoot *et al.*, 2010; Matsheka *et al.*, 2014)

2.2.2 Jerky

Jerky is ready-to-eat and today it is a popular snack, particularly in North America. It can be made from various species with beef being the most common type of meat used, various forms of meat (thick or thin slices, ground), various marination techniques (ingredients, volume, time, temperature) and various drying processes. Although it is similar to biltong in being a dried meat it is different in being dried at a higher temperature or smoked/cooked before drying (Burfoot *et al.*, 2010; Calicioglu *et al.*, 2003a).

2.2.3 *Pastirma*

Pastirma is a meat product made of salted and dried beef, highly esteemed in Turkey and Egypt as well as other Muslim countries. It is also popular in some parts of the Soviet Union. In Turkey it is produced from September to November when conditions are more favourable (lower temperature, humidity, absence of flies). Meat from 5 to 6 years old beef cattle is used, taken from the hind-quarter within 6 to12 h of slaughter. The meat is cut into long strips (500 to 600 mm) with a diameter not more than 50 mm. The strips are rubbed and covered with salt

containing potassium nitrate and several slits are made in the meat to aid salt penetration. The strips are piled 1 m high and kept one day at room temperature. The process is repeated, turning the pile from top to bottom. The strips are then washed and air dried for 2 to 3 days in summer or 15 to 20 days in winter. After drying, the strips are piled up to 300 mm high and pressed with heavy weights for 12 h. They are dried for a further 2 to 3 days and pressed again for 12 h. Finally the meat is air dried for 5 to10 days. After salting and drying, the surface of the meat is covered with a 3 to 5 mm thick layer of a paste called cemen (containing freshly ground garlic, helba, hot red paprika, kammon, mustard and water). Helba is used as a binder and the other ingredients are for flavour. The paste covered meat strips are stored in piles and dried for 5 to12 days in a well-ventilated room. Approximately 80 kg beef gives 50 kg pastirma and the end product has 30.35% moisture and can be stored at room temperature for 9 months (Burfoot *et al.*, 2010).

2.2.4 *Pemmican*

In Central America, pemmican, a form of chopped, dried meat, is prepared. *Pemmican* was originally developed by North American Indians to preserve game, and it traditionally included the dried fruits of wild berries, which added a sweet-sour flavor to the product (Redhead, 1990).

2.2.5 *Sambal daging*

In Indonesia and Malaysia, highly-seasoned dried beef products, such as *sambal daging*, are prepared from lean meat which is prepared from lean meat which is pounded until it disintegrates. The fibrous strands are heated in a sauce containing salt, chilly and selected spices before drying. When required for use, the spiced dried meat is often reconstituted by heating in coconut milk (Redhead, 1990).

2.2.6 *Kilishi*

Kilishi is a tropical intermediate moisture meat product produced in African countries. It is prepared essentially from beef slices, infused in slurry of defatted groundnut paste and spices and sundried. *Kilishi* can be stored without refrigeration for several months (Ogunsola and Omojola, 2008).

2.2.7 *Tasajo*

Tasajo is a salted meat based product made in Cuba as a version of charqui. Traditionally, the meat is salted then sun dried a process that takes at least three weeks. Industrially, it is made by wet salting in a saturated salt brine (1%) for 8 h, dry salted, and finally hot air dried at 60°C until a 50% weight loss is achieved (Burfoot *et al.*, 2010).

2.2.8 *Nikku*

Nikku is a dried product eaten in the Canadian Artic, particularly by the Inuit population. It is one of a range of raw or partially cooked locally prepared traditional or "country" foods derived from wild game meat. Traditionally, *nikku* was made by cutting caribou meat into strips and hanging them in the sun until dried. Seal meat has also been used (Burfoot *et al.*, 2010).

2.2.9 *Sou gan*

Sou gan are Chinese dried meat products of which at least 30 different variants are known. Consumption is large and their popularity is growing. They are valued for their flavour, storage (no refrigeration) and transport properties (light) as well their nutritive value. Products vary according to the species of meat, the type of technology and the spices used. Water activity can lie between 0.6 to 0.9 (Intermediate Moisture Food) or be less than 0.6 (Low Moisture Food). Three basic processes are used to achieve either dried meat slices, dried meat cubes or strips, or shredded dried m*e*at (Burfoot *et al.*, 2010).

2.2.10 *Charqui*

Charqui comes from South America, with much produced in Brazil, and differs from biltong in that it is a fatty product (Thomas, 1975). A traditional approach to making charqui has many similarities to that used in the dry curing of bacon. A fresh side of beef is cut into three pieces that are butchered open and cut into strips similar to biltong and then hung to cool at ambient temperature for about an hour. The strips are immersed in brine for a further hour, drained, dipped in coarse dry salt, stacked 1-1.5 m high, covered in salt and left overnight. The piles are turned daily for 4 days with strips from the top going to the bottom and vice versa and the piles re-covered with salt. Drying begins on the 5th day when meat is hung over

drying racks and exposed to the sun for no longer than 1 to 2 h. It is removed from the racks and piled about 1m high under a tarpaulin for 2-3 days to "cure". This drying and curing is repeated 5-7 times until the meat has lost 40% of its fresh weight (Burfoot *et al.*, 2010).

2.2.11 *Sukuti*

Suka ko masu (meaning dried meat) is an ethnic meat product of Eastern Himalayas (Rai *et al.*, 2010). *Sukuti* is a dried meat product native to Nepal usually made from buffalo, and sometimes from lamb or goat. It is also consumed in Himalayan part of India and Tibet. Its Limbu term is *sakhekya*. It was used as a source of protein and also to add variety to diet during winter seasons in the mountain areas. Today food is generally available almost all year around among mountain communities so *sukuti* has become a delicacy to be eaten at times (Wikipedia, 2022).

For as long as Nepali people have been drying meat, *sukuti* has been prepared in the kitchens. *sukuti* is versatile and can be prepared in different ways. It can be deep fried or smoked or roasted, and then pounded along with spices and served as a pickle or it can be fried and eaten as the snack. Also it can be cooked along with the vegetables of choices and served as stew (Subedi, 2019). Table 2.1 shows the typical composition of *sukuti*.

Table 2.1 Typical composition of *sukuti*

Parameter	Value (%), wet basis
Moisture	8-10
Protein	75-77
Ash	5-6
Fat	4-5

Source: Kharel *et al.* (2010)

2.2.11.1 Preparation of *sukuti* in Nepal

Dried meat products have a history of more than thousand years in China. During the Sung dynasty already 200 types of dried meat products, based on red meat, poultry and fish were known. Despite the lack of such meat and fish products documented record, *sukuti* preparation in rural area has marked popularity in Nepal. Commercial *sukuti* is made almost exclusively from buffalo meat. In Nepal, buffalo meat is abundantly found in the market. It is also cheaper than any other meats. Most of the portion is lean and is therefore suitable for *sukuti* preparation (Acharya, 2014).

2.2.11.2 Process of *sukuti* making

Local butchers slaughter the buffaloes in open ground by traditional method. In general the buffaloes are stunned by direct blow in the skull using a pole axe, then bleeded with sticking the major arteries of the neck immediately. Jhatka (deheading with heavy knife) method is rarely used. The most objectionable thing is that there is lack of slaughter hygiene and no provision of modern slaughtering. So meat often becomes contaminated with dust, mud, etc., leading to entry of different types of spoilage and pathogenic microorganisms in the meat. The buffaloes themselves may be suffering from various zoonotic diseases, which are very dangerous for human health.

Preferentially hindquarter portion is taken for making *sukuti*. However, forequarter, sirloin can also be used. Bones, fat and other undesirable portions are removed. The next step consists of cutting the muscles into thin strips. There is no any fixed standard dimension for meat strips. In general, they are stripped in the dimensions of approximately 250 mm × 20 mm × 20 mm.

There are some process variations but generally the meat strips are dried by sun, air or over smokeless fire to 10 to 20% moisture content. In traditional method meat strips are hung over the fireplace in kitchen where the strips dried due to the mild heat and smoke produced during cooking the meal. A typical sun dried product requires a drying time of 3-10 days. In the rural areas, some people still used *perunga* (a bag prepared by weaving bamboo strips) as a packaging material. Perungo along with the product is often hung in the andiron for storage. The traditional process of making *sukuti* was given in Fig. 2.1 (Acharya, 2014).

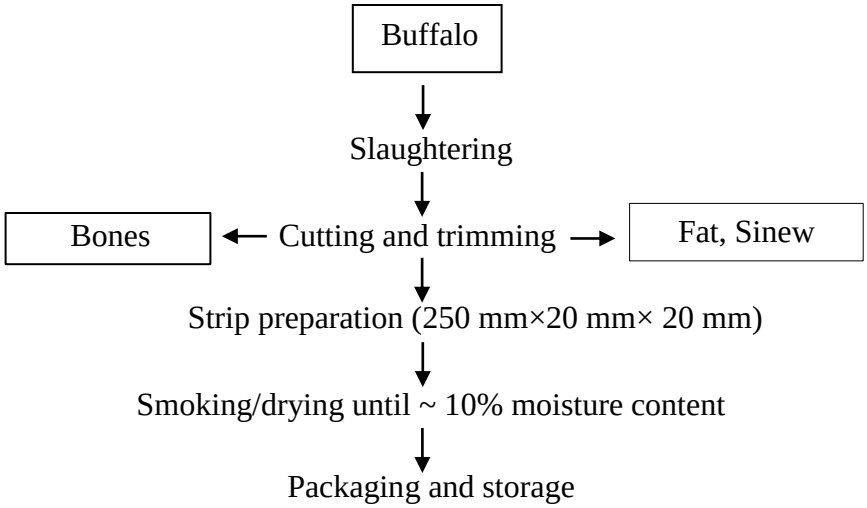

Fig. 2.1 Traditional process of making *sukuti*

2.3 Meat microbiology

Meat products are perishable and unless processed, packaged, distributed and stored appropriately can spoil in relatively short time. Over-growth of incidental pathogenic bacteria like *Listeria monocytogenes, Salmonella sp*. and diarrhea-genic *Escherichia coli* followed by under-cooking or inadequate preparation may cause a potential hazard for the consumer. Despite the proliferation of food safety regulations and the introduction of safety management systems, such as hazard analysis critical control point (HACCP), risk assessment studies show that food-borne disease has remained a main concern in the last decade (Sofos, 2005).

The prevalence and levels of bacteria on meat carcasses depend on a number of factors including the origin of the animal, sanitation procedures and hygienic practices employed during handling and processing and conditions of storage. Extremely high numbers of microorganisms are found in the animal's intestinal content, and it is expected that some will find their way to the surface of the carcass during the dressing operations. Microorganisms reach the carcass via butcher's hands, tools, clothing, water etc. The number can be proliferated during cutting and distribution. Raw meat quality is often judged by the size of its microbial population able to grow at 30-37^{o}C. However, this count most appropriately used to monitor hygiene, not quality (Brown, 1982). Microbial contamination can be divided into two sorts: that caused by pathogenic bacteria and that by microbes that cause spoilage (Warriss, 2000).

2.3.1 Spoilage microorganisms

Meat spoilage is, according to Pennacchia *et al.* (2011), most frequently caused by the following groups of bacteria: *Pseudomonas* spp., Enterobacteriaceae, *Brochothrix thermosphacta* and Lactic acid bacteria. It is common knowledge that meat can be spoiled quickly under aerobic conditions (in the presence of an atmosphere comprised of air). This is caused by the rapid growth of pseudomonades. *Pseudomonas* species are associated with spoilage of meat causing off-odors, off-flavors, discoloration and gas production (Bantawa *et al.*, 2018). *P. fluorescens* occurs more frequently on fresh meat, though during longer periods of storage *P. fragi* becomes dominant. A population of pseudomonades of 10^7 – 10^8 cfu/g causes slime to form on meat and a bad smell to appear (Kameník, 2013).

Vacuum packaging and MAP favour facultative anaerobic bacteria, including lactic acid bacteria and *Brochothrix thermosphacta*. Lactic acid bacteria, in particular, are highly competitive in a modified atmosphere. The genera *Lactobacillus, Carnobacterium* and *Leuconostoc* may be associated with the spoilage of chilled meat. The psychrotrophic lactic acid bacteria occurring most frequently on meat include *Lactobacillus sakei*, which is considered an agent of spoilage of vacuum-packed meat. In the case of cured meat, an entirely different flora comes into play. Most commonly the following occur: *Staphylococcus*, *Micrococcus*, *Lactobacillus*, *Microbacterium*, *Pediococcus*, *Streptococcus*, *Clostridium* and *Bacillus* species (Doulgeraki *et al.*, 2012).

Numerous species of the Enterobacteriaceae family have been found on beef, lamb, pork and chicken meat and offals. The genera *Serratia, Enterobacter, Pantoea, Klebsiella, Proteus* and *Hafnia* all contribute to spoilage. In view of its potential to spoil meat, the most important in this regard are *Serratia liquefaciens, Hafnia alvei* and *Enterobacter* (*Pantoea*) *agglomerans*. The genus *Serratia* is the most frequent genus of the Enterobacteriaceae family to be found on meat. *S. liquefaciens*, in particular, is isolated from meat stored in various atmospheres. *S. grimesii* and *S. proteamaculans* are other such species. The species *Citrobacter freundii* and *Proteus vulgaris* have been detected in minced beef stored in an aerobic or modified atmosphere. *Hafnia alvei* is often found in minced meat in a modified atmosphere (MAP) or in a vacuum (Kameník, 2013).

2.3.2 Pathogenic microorganisms

Besides causing spoilage of meat, microorganisms may also be responsible for illness in humans (Cassens, 1994). The most important foodborne bacterial pathogens associated with meat are *Salmonella* spp., *Staphylococcus aureus*, *Escherichia coli*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Clostridium perfringes* and *Yersinia enterocolitica*. Among them, *Salmonella* species, *Campylobacter jejuni*, *Listeria monocytogenes* and verocytotoxin producing *E. coli* O157 are of major public health concern. Similarly, Vibrio species are the leading cause of gastroenteritis, wound infection and septicemia in human (Bantawa *et al.*, 2018).

Salmonella in red and white meat is a worldwide problem. Food borne salmonella infection results from the ingestion of large numbers of the organism, which then multiply within the small intestine (Roberts, 1982). It is probably widespread, and some large outbreaks have been documented. There are numerous serotypes of *the Salmonella* organism, and it occurs commonly in the intestines of domestic animals and birds. This provides a high possibility for contamination of the meat by contact with intestinal contents either during slaughter or by contact with feces clinging to the hide. Estimates of contamination range up to 30% for poultry meat and up to 15% for other meats. The organism is quite heat sensitive so adequate cooking inactivates it (Cassens, 1994).

Listeria monocytogenes is amongst the dangerous bacterial food-borne pathogens in the world, which cause severe human diseases. Listeriosis is a zoonotic disease that is mainly acquired through consumption of contaminated food by *L. monocytogenes*. Many food-borne listeriosis outbreaks have been linked to diverse food products, but different types of meat have been implicated in major human listeriosis outbreaks worldwide. This disease occurs in specific segments of the population, which are the elderly, pregnant women, unborn babies and immunocompromised people such as those suffering from acquired immune deficiency syndrome (AIDS) or cancer or those who have undergone organ transplants. Listeriosis may be asymptomatic or may be characterized by septicemia, fever, malaise and brain infection (Matle *et al*., 2020).

Staphylococcal food poisoning arises from consumption of an enterotoxin produced by *Staphylococcus aureus*. The result is a severe gastroenteritis with an onset usually in 2 to 4 h, and recovery in 24 to 48 h. The organism is tolerant to salt and nitrite and therefore a concern in cured meat. During growth and production of the heat stable toxin, there is no change in sensory attributes of the food. The organism may be present in animals and is often found in humans in discharge from the nose and throat, and on hair, skin and fingers. Proper hygiene is important, and the organism can be controlled by adequate refrigeration (Cassens, 1994).

Bacillus cereus is a spore-forming organism capable of developing at a wide range of temperatures, pH and water activity values. This bacterium, frequently associated with food borne diseases can be found in the natural environment and isolated from various foods, including meat and meat products. Some of the enterotoxins produced by *B. cereus* cause food borne diarrhea, characterized as the "diarrheic syndrome". The symptoms last for a period of from 12 to 24 h and consist of abdominal pain, watery diarrhea and nausea. It is believed that the enterotoxins are mostly produced in the small intestine of the individual. Foods associated with outbreaks of *B. cereus* diarrhea frequently include protein-rich foods and researchers have emphasized the importance of the presence of the microorganism or its spores in meat and meat products for the occurrence of such outbreaks (Soares *et al*., 2012).

Enter-pathogenic *Escherichia coli* is commonly known as travelers' diarrhea. It is usually contracted from untreated water, and is associated with fecal contamination and poor sanitation. Of recent significance in meat is the illness caused by *E. coli* O157:H7. One manifestation may be a life-threatening illness characterized by severe abdominal cramps and bloody diarrhea. Animals appear to be a reservoir for the organism and the primary foodstuff implicated is inadequately cooked hamburger (Cassens, 1994).

Clostridium perfringens bacteria are one of the most common causes of foodborne illness (food poisoning). *C. perfringens* can be found on raw meat and poultry, in the intestines of animals, and in the environment. These bacteria make spores, which act like protective coatings that help the bacteria survive. Under certain conditions, such as when food is kept at an unsafe temperature (between 40°and 140°F), *C. perfringens* can grow and multiply. After someone swallows the bacteria, it can produce a toxin that causes diarrhea (CDC, 2021).

Campylobacter jejuni is a prominent bacterial cause of human gastroenteritis. It is well recognized that *C. jejuni* is one of the main causes of gastroenteritis in humans, and poultry meat is reported to be the main source (Mohamed-Yousif, 2021).

While the foregoing organisms present the most serious threats regarding microbiological safety of meat, there are three other possibilities to mention. *Shigella* has been of decreasing significance and is associated primarily with seafood. Since transmission from human reservoirs to foods is through improper sanitation, control deals more with food handling than production and processing practices. *Yersinia enterocolitica* may be of a concern since pigs serve as a reservoir, and the organism can grow at refrigerated temperatures. *Vibrio* is responsible not only for cholera, but for gastroenteritis. *Vibrio vulnijicus* is a marine bacteria, often associated with oysters and clams, and it is particularly virulent in humans (Cassens, 1994).

2.4 Spoilage and safety of dried meats

Microorganisms on the dried product are those that have survived the initial cooking and those were acquired during mincing and placing in the dryer. Microbial numbers will increase if drying is not controlled properly. Microbial stability depends on water activity of the products. Mold and yeasts may grow during storage, particularly when moisture is absorbed from the environment (ICMSF, 2006).

Spoilage of dried meats is unusual and is mainly due to insufficiencies or defects in the manufacturing processes. For example, prolonged drying of large pieces of meat may result in the formation of wet spots in which microbial growth of Enterobacteriaceae and staphylococci is likely to occur. Moreover, undercooking of the meat decreases the rate of dehydration, thus resulting in the possible occurrence of wet spots. In addition, packaging of dried meat in high-permeability films will result in the uptake of water and the subsequent growth of molds and yeasts during storage (Pestka, 1986). Hence, the microbiological quality and stability of the dried meat depend entirely on the removal of moisture during the drying step, and on the water activity (a_w) of the final product and the relative humidity (RH) at which the dried meat is stored. It is therefore essential to ensure that drying of meat is done at a constant rate, and that

the moisture content of dried meat at any stage during processing and storage is below the minimum levels for microbial growth (Skandamis and Gounadaki, 2009).

As with other dried foods, the shelf life of dried meat products is dictated by their water activity. There is an added factor in spoilage due to fat rancidity, which may be induced by yeast or mold growth during drying and continue in storage. The most common spoilage species on dried meat are *Eurotium* species, *A. candidus*, other *Aspergillus*, and *Penicillium* species. The salt-tolerant yeast *Debaryomyces hansenii* and the lipolytic species *Y. lipolytica* are among the most common yeasts isolated from dried meat products (Hocking, 2014). Other studies have shown that Staphylococci and Micrococci are often present in intermediate moisture meats such as *pastrima* and *cecina* (Garcia *et al*., 1995; Hui *et al*., 2001).

Indeed, it has been well established that a moisture content of 20% inhibits most bacteria, yeasts, and molds, but a level of 15% is needed to inhibit xerotolerant species of fungi. Moreover, meat with a high fat content (40%) must be dried to about 9% moisture in order to ensure inhibition of mold growth, since higher levels of moisture (12%) may result in the growth of mold after several weeks. It has also been shown that storage (at 20, 28 and 37°C) of dried meats at high RH (90%) results in the faster growth (5 - 6 days) of mold compared to storage at 75% RH (36 - 40 days) (Pestka, 1986).

Production of low and/or intermediate moisture dried meats of high microbiological quality should be formulated correctly in order for deficiencies in processing to be avoided. It is also essential that the preservative system and temperature control should remain stable during storage, as moisture migration could lead to areas of localized high water activity levels, which in turn result in a heterogeneous structure (Skandamis and Gounadaki, 2009).

During the manufacture of dried meats, the rate at which the water content of meat is reduced, as well as the water activity of the dried meat, will significantly affect the survival and proliferation of foodborne microorganisms. Many pathogenic bacteria, such as *Salmonella*, *Staph. aureus*, clostridia etc., that originate from raw meat or are contaminants during preparation may survive the drying process. A recent report indicated that for the period of 1990 - 1999, the cumulative prevalence of *Salmonella* and *L. monocytogenes* in

dried meats (jerky) produced in federally inspected plants in the United States of America (USA) was 0.31 and 0.52%, respectively (Eidson *et al.,* 2000). In addition, documented outbreaks due to the consumption of dried meats including jerky, *pastirma*, and *biltong* increases the concern over pathogen growth and survival in dried meats (Tompkin, 1986; Eidson *et al.,* 2000; NMHD, 2004).

Several surveys of microorganisms on commercial biltong have been carried out. Total viable counts up to 7 log cfu/g; Enterobacteriaceae and coliforms up to 4 log cfu/g; yeasts up to 7 log cfu/g; molds up to 5 log cfu/g; lactic acid bacteria up to 8 log cfu/g; and Staphylococci up to 8.5 log cfu/g, have been found in recent surveys (Wolter *et al.,* 2000; Mhlambi *et al.,* 2010; Naidoo and Lindsay, 2010a; Naidoo and Lindsay 2010b). Pathogens have occasionally been found in biltong samples. Raw meat may contain pathogens and these can survive for long periods in biltong. *Salmonella* dublin was isolated in 6 month old biltong (Burfoot *et. al*., 2010). Many studies have investigated the microbiological issues associated with producing jerky. At least eight gastroenteritis outbreaks occurred in New Mexico between 1966 and 1995 from ingestion of meat jerky. Two outbreaks were due to contamination with *Staphylococcus aureus* and six were due to contamination with *Salmonella*. Over 250 illnesses were reported. Some traditional home recipes and drying processes were shown to be inadequate to destroy *Escherichia coli* O157, *Salmonella*, *Staphylococcus aureus*, and *Listeria monocytogenes* in both whole-muscle and ground-meat jerky (Nummer *et al.*, 2004).

The most frequent and significant outbreaks have arisen from enteric bacteria coming from the raw meat and from cross-contamination and poor handling. The use of contaminated spices may also pose a risk (Burfoot *et. al*., 2010). Several published reports on foodborne outbreaks involving dried meats in the USA and around the world are indicative of the survival potential of Salmonella in dried meats (Eidson *et al.,* 2000). The danger in dehydrating meat and poultry without cooking it to a safe temperature first is that the appliance will not heat the meat to 71°C and poultry to 74°C (temperatures at which bacteria are destroyed) before the dehydrating process. After drying, bacteria become much more heat resistant. Within a dehydrator or low-temperature oven, evaporating moisture absorbs most of the heat. Thus, the meat itself does not begin to rise in temperature until most of the moisture

has evaporated. Therefore, when the dried meat temperature finally begins to rise, the bacteria have become more heat resistant and are more likely to survive. If these surviving bacteria are pathogenic, they can cause foodborne illness to those consuming the jerky (USDA, 2016).

Clostridia, bacilli, *Staph. aureus*, Salmonellae and other pathogenic bacteria, originally present on raw or as contaminant during preparation, may survive the drying process (Skandamis and Gounadaki, 2009). During storage the number of vegetative cells will decline. According to ICMSF (2006), commercially prepared beef jerky have caused salmonellosis. Home-made vension jerky has caused type F botulism. Surviving pathogens will grow in re-hydrated product if held at temperatures permitting their development.

According to Matsheka *et al.* (2014), earlier studies have revealed that biltong can serve as a vector for foodborne pathogens such as *Listeria*, *Salmonella*, enterotoxigenic Staphylococci spp. and *E. coli* O157:H7. This concern has been substantiated by outbreaks of foodborne illnesses associated with the consumption of this product. Mewa *et al.* (2018) reported that the Total Viable Counts (TVC) of beef dried at 30, 40 and 50^{o}C were higher (4.46 to 6.04 log cfu/g) than the fresh beef (4.89 log cfu/g). Also Staphylococci count were highest (7.08 log cfu/g) for beef samples dried at 30^{o}C. *Staphylococcus aureus* is one of the most common meat pathogens and the minimum number of cells to produce sufficient enterotoxin and causing food poisoning is about 7 log cfu/g. Similarly, Rai *et al.* (2010) reported that the total viable count, total mold count and total yeast count of ethnic dried meat products collected from Eastern Himalayas were up to 8 log cfu/g, 1 log cfu/g and 5 log cfu/g respectively.

2.5 Sources of contamination

The microbiology of carcass meat is highly dependent on the conditions under which animals are reared, slaughtered and processed. The initial contamination of meat occurs during slaughtering. Hygiene deficiencies cannot be compensated for even by the most rigorous hygiene measures during later production process (Podpecan *et al.*, 2007). The extent to which contamination occurs and the composition of the flora that results reflects the standard of hygiene in the slaughterhouse. The hide or skin of the particular animal and others being

dressed in its close proximity is probably the major source of saprophytic species. In addition to skin, the gastro-intestinal and respiratory tracts, urine and milk are other important animal sources of infection. Generally, Escherichia coli comprise a greater proportion of the total aerobic flora of the intestine than of the hide or fleece (Brown, 1982). The contamination comes from external sources during bleeding, handling and processing. Knives, tools, cloths, hands and air serve as the sources of contaminants. The microbial contamination in meat has important consequences in public health, storage life and spoilage of meat (Gracey and Collins, 1994).

In many developing countries, lack of appropriate slaughtering facilities and unsatisfactory slaughtering techniques are causing unnecessary losses of meat as well as invaluable by-products from animal carcasses. Slaughtering places are frequently contaminated and not protected against dogs, rodents and insects. Meat products coming from such conditions are often deteriorated due to bacterial infection or contaminated, which may cause food poisoning or disease in consumer (Joshi *et al*., 2003).

Contamination of sterile animal muscle used as food is a direct consequence of slaughtering and dressing of animal carcasses. Wide ranges of microorganisms from different sources are introduced onto moist muscle surfaces that are rich in nutrients. It is argued that only a small portion (10%) of these microorganisms is capable of survival and proliferation during storage, distribution, and retail sales of meats (Hui *et al*., 2001).

Spoilage microorganisms can come either from outside the animal, or from the gut, or by being introduced at slaughter on the bolt of the captive bolt-stunning pistol or on the blade of the sticking knife. In living, healthy animals, bacteria in the gut are usually prevented from invading the surrounding tissues and the blood system, or their growth is controlled, by the mucous lining of the gut wall, by antibodies in the blood and by phagocytosis by reticule-endothelial cells especially in the lymph nodes. Normally therefore, the tissues of healthy animals are sterile. Some organisms, such as *Salmonella enteritidis*, can nevertheless sometimes pass into the bloodstream and tissues and cause systemic infection (Warriss, 2000).

Raw meat and other meat products can act as vehicles of various hazards that may have serious impact on human health. There are various types of hazards, which may be chemical, biological or physical. Biological hazards are of concern because the microorganisms or pathogens are found naturally in the environment or even on live animals. Therefore, the occurrence of pathogens on raw meat can be due to different factors, which include poor farm animal management, improper slaughter practices, processing, storage conditions and lack of meat safety knowledge among meat handlers. The consumer needs to be provided with safe and wholesome meat, which will not cause any health problem. This can be achieved by practicing better farm animal management, good personal hygiene and providing adequate knowledge on food safety to all the meat handlers in the production chain (Pal *et al*., 2018).

2.6 Processing and its effects on the microflora

Dried meats are prepared using traditional methods, modern methods and combined processing technologies.

2.6.1 Traditional methods

Sun-drying is the traditional method of preserving meats and is characterized by direct solar radiation and natural air circulation on the product. This type of preservation was probably developed in dry climates to produce products such as *charqui* in South America, and *biltong* in South Africa, where dried meats were the major source of animal protein. Large quantities of meat are still sun-dried in many areas (of Africa) using traditional techniques or improved approaches such as solar-drying. Solar-drying is the improved version of the sun drying technique and is based on indirect solar radiation (Skandamis and Gounadaki, 2009). Specifically, solar-drying is performed in closed systems (consisting of a solar collector and a meat drying chamber) with a specific micro-climate (controlled air circulation; temperature of 30 to 55°C; air humidity of 20 to 60%) that favors the fast evaporation of moisture from meat (Ratti and Mujumdar, 1997; Kok and Kwendakwema, 1985).

Typically, for the production of dried meats using these traditional methods (sun-drying and solar-drying), the raw meat is usually cut into narrow strips or flat pieces that are then either suspended in the open air (for sun-drying) or spread on drying trays and placed in

drying chambers (for solar-drying). The duration of the drying process ranges from 2 days for solar-drying to up to 10 days for meats drying in the open air; it is highly dependent on temperature, humidity and the size of the pieces of meat. In particular, the use of thin and well separated pieces of meat accelerates the drying process, whereas the use of large or closely packed meats increases the duration of drying and increases the likelihood of formation of wet spots in which microbial growth may occur. By the end of drying process the meat has a typical moisture content of 12 to 18% (a_w 0.5 - 0.6) and is ready for consumption (Skandamis and Gounadaki, 2009).

2.6.2 Modern methods

In industrialized countries, substantial quantities of dried meat are produced with the use of specifically designed dehydrators that operate under well controlled conditions. Cabinet dryers, otherwise called air-dryers and freeze-dryers are among the most common dehydrators used for the production of dried meat (Lewicki, 2004). Each process uses a different technique: air-drying is based on evaporation, whereas freeze-drying is based on sublimation. More specifically, during evaporation the water content of raw meat is reduced as the heat applied converts water from its liquid phase to the gaseous phase (vapour). During sublimation, reduction of water content occurs on frozen meat by the direct transfer of the solid phase of water (ice) to vapour. The differences in the principles of the applied techniques obviously result in differences in the production processes. Regardless of the drying technology, the first step in the production process of dried meats is to defat the meat. In most industrialized countries, the meat is then cooked in order to minimize case hardening, thus improving quality (Varnam and Sutherland, 1995). The extent of cooking is important, since overcooking degrades the connective tissue while undercooking decreases the rate of dehydration. Moreover, the United States Department of Agriculture (USDA) has proposed cooking/heating as a lethal performance standard for the production of certain ready-to-eat (RTE) products, including dried meats such as jerky (USDA, 2001; USDA 2007). Such standards require that the time and temperature used in making jerky ensure the safety of the product (USDA, 2001; USDA 2004; USDA, 2007).

In the case of air-drying, the cooked meat can either be minced into small cubes and transferred direct to the air-dryer, or cooled and refrigerated before drying. Mincing of meat in small pieces provides a large surface area to volume ratio that results in the reduction of the drying duration. Drying temperature is known to significantly influence the quality of dried products (Varnam and Sutherland, 1995). Drying of meat in systems that operate under low temperatures (80°C) drying systems are generally more conventional, as the drying time is reduced to approximately 2 h. Before packaging, air-dried meats with a final moisture content of 4 to 8% can sometimes be compressed in order to reduce volume and facilitate handling. This is usually achieved by pressing the meat into cans or moulds to form blocks. Dried meats must be then packaged with moisture-impermeable films, in order to prevent moisture uptake or high humidity during storage (Skandamis and Gounadaki, 2009).

During the production of freeze-dried meats, the pre-cooked meat is drained and cooled prior to freezing in a wind tunnel or a blast freezer. Freezing of meat is usually achieved at -20 °C in order to ensure that the majority of water is converted to ice. Frozen meat is then placed into a chamber under vacuum where the temperature is raised (>60°C) prior to extraction of frozen water. Residual water (unfrozen) is removed either by desorption or secondary drying. This step completes the separation of water from the meat and results in drying. This process can take several hours (even days) to complete as gradual drying is desired to maintain the structure of the meat. After drying, the pressure is equalized with nitrogen and the meat is conditioned to achieve an even distribution of the residual water. During freeze-drying, sub-zero temperatures must be maintained until the reduction of water content. As this process is not lethal (Jay *et al.*, 2005), the microbiological quality of the final product is highly dependent on the initial microbial flora of the meat and on any bacterial post-process (after drying) contamination. As with air-dried meats, the final products must be packaged with moisture-impermeable films to avoid moisture uptake during extended storage.

2.6.3 Production of dried meats with combined processing technologies

Apart from air-dried and freeze-dried meats, a large variety of dried meats are produced with combined or modified processing technologies. Such modifications have been made either to improve the quality and safety as well as the nutritional status of the product, or to increase

consumer acceptance (Harrison *et al.,* 2001). Modifications include (i) the pre-treatment (i.e. seasoning, dry-curing, and marinating) of meat and (ii) the addition of humectants (i.e. salt, sugar, glycerol etc.) in combination with a decrease in drying time (intermediate-moisture meats). For example, for the production of biltong, a well-known RTE dried meat originating from Africa, the raw meat is cured for several hours (< 12 h) in a salt/pepper mixture, and then dipped in a vinegar solution prior to drying. Production of *pastirma*, another RTE dried meat product originating from Asia, is characterized by a series and repeated lengthy (2-3 days) applications of salting, drying and spicing, prior to final drying. Moreover, processing of jerky is known to include marinating (salt, sugar and flavoring ingredients), heating, and drying; however, some interventions, mainly heat and antimicrobial, have been shown to increase safety (Harrison and Harrison, 1996; Faith *et al.,* 1998; Harrison *et al.,* 2001; Calicioglu *et al.,* 2002; Calicioglu *et al.,* 2003b; Calicioglu *et al.,* 2003c; Nummer *et al.,* 2004)

2.7 Plant based additives used in meat and meat products

With the population surge and the globalization of food supply, the demand for large-scale meat production is growing. This has consequently given rise to challenges for the meat industry to maintain quality and safety throughout the supply chain. Food additives and preservatives have been used in perishable commodities, including meat, to maintain product consistency, quality, and safety (Nair *et al.*, 2020).

2.7.1 Flavoring agents

Plant-derived compounds are traditionally used in foods as flavoring agents (Burt, 2004). These compounds are classified into four groups: herbs, botanicals, essential oils, and oleoresins, based on their biological origin, formulation, chemical description, and purity Spices are an integral part of food preparation in many world cuisines and used to add flavors to the meat. The spices are derived from plant parts, and the FDA has approved a list of spices that can be used in foods. Spices contain flavor compounds such as cinnamaldehyde from cinnamon, eugenol and eugenyl acetate from clove, gingerol, shogaol, neral, and geranial from ginger, 1-8-cineole from bay leaves, piperine and beta-caryophyllene from black pepper, and capsaicin and dihydrocapsaicin from chilli pepper (Nair *et al.*, 2020).

2.7.2 Coloring agents

A variety of coloring agents are permitted in meat products. The compounds such as carotene, annatto, chlorophyll, saffron, and turmeric are used to impart color to casings or rendered fats (Nair *et al.*, 2020).

2.7.3 Preservatives

Extensive research has been done to evaluate efficacy of plant based compounds (herbs, botanicals, essential oils, and oleoresins) as preservatives in meat products to extend shelf life (Fernandes *et al.*, 2016; Shan *et al.*, 2009; Wang *et al.*, 2015) . More detail is given in Section 2.13.

2.8 Ethno medicinal herbs of Nepal

Medicinal plants are abundant source of antimicrobial molecules. A wide range of medicinal plants extracts are used to treat several infections as they have potential antimicrobial activity. Some of these bioactive molecules are screened and traded in market as raw material for many herbal industries. Experts turned their concentration back towards obtaining advantages from medicinal plants after observing more side effects of synthetic drugs compared to their benefits. It is estimated that about 35,000 to 70,000 plants species are used as medicinal plants out of 422,127 reported worldwide plant species (Javid *et al*., 2015). A list of medicinal herbs showing antimicrobial properties is given in Table 2.2.

Traditional beliefs about the diverse uses of plants are deeply rooted in Nepalese culture. The various ethnic groups of the country have developed their own knowledge systems for the use of plants in food, clothing, shelter, medicine and their spiritual needs. From time immemorial many medicinal plants are well known in Nepal for various ailments. Therefore, country was mentioned as a sacred heaven of medicinal and aromatic plants in *Vidic* and *Pauranic* literature, which founded the base of the Ayurvedic system of medicine (Bhattarai, 2018).

Table 2.2 A list of medicinal herbs showing antimicrobial properties

Herbs	Parts of plant	Target microorganisms	References
Aerva lanata	Leaves	*Aspergillus niger, Candida neoformans*	(Vijayan *et al*., 2010)
Aloe secundiflora	Leaves	*Streptococcus pneumonia*	(Amadi *et al*., 2016)
Annona reticulata	Leaves	*Proteus vulgaris, Salmonella typhi,*	(Chandra, 2013)
Bergenia ciliate	Roots	*Bacillus subtilis, E. coli, Staphylococcus aureus*	(Khan *et al.,* 2013)
Carissa edulis	Leaves	*Streptococcus pneumonia*	(Amadi *et al*., 2016)
Chebulic myrobalan	Fruits	*E. coli, Staphylococcus aureus*	(Bag *et al.,* 2009)
Curcuma longa	Rhizome	*Aeromonas hydrophila*	(Ramasamy and Chellam, 2005)
Heracleum nepalense	Roots	*Bacillus cereus, E. coli, Staphylococcus aureus*	(Koirala and Singh, 2017)
Lagerstroemia indica	Leaves	*Staphylococcus aureus, Salmonella typhi*	(Chandra, 2013)
Litsea cubeba	Fruits	*Botrytis cinerea, E. coli*	(Li *et al.,* 2014; Wang *et al*., 2019)
Ocimum sanctum	Leaves	*Enterococcus faecalis*	(Chandrappa *et al*., 2015)
Rosmarinus officinalis	Foilage	*Salmonella, Staphylococcus aureus*	(Manilal *et al.,* 2021)
Syzygium aromaticum	Fruits	*Listeria monocytogenes, E.coli*	(Kim *et al*., 2011)
Terminalia brownii	Leaves	*Streptococcus pneumonia*	(Amadi *et al*., 2016)
Vernonia brachycalyx	Stems and Leaves	*Streptococcus pneumonia*	(Amadi *et al*., 2016)
Zanthoxylum armatum	Fruit	*Staphylococcus aureus, Enterococcus faecalis*	(Phuyal *et al.*, 2020)
Zingiber officinale	Rhizome	*Salmonella, Staphylococcus aureus, Klebsiella*	(Islam *et al.,* 2014)

Nepal is an excellent repository of cultural heritage and the use of plants as folklore medicines has been practiced since the beginning of human civilization. Local plant-based therapy is a common practice in the rural communities of Nepal. While this is not only medical options available, most communities lack direct access to modern medicine and it is estimated that about 80% of the population depends upon herbal medicines. Approximately 1700 species of flowering plants are currently being utilized as medicines in Nepal and the number is expected to grow as infrastructure allows greater access to unexplored parts of the country (Shrestha *et al.*, 2016). The following paragraphs describe four of the ethno medicinal plants of culinary importance and used in this study.

2.8.1 *Zanthoxylum armatum*

Zanthoxylum armatum, locally known as *Boke Timur* and *Aankhe Timur*, is a deciduous shrub or a small tree, around six meters tall or higher, with dense foliage, armed branched flattened prickles, yellow flowers and red seeds. Belonging to Rutaceae, it naturally occurs in Nepalese forests and on open sites at altitudes ranging from 1000 to 2100 meters and can be found in different management systems. As suggested by the peoples who are cultivating this plant, certain environments are better for grow, such as sites with deep, well-drained moist soils in full sun or semi-shade (Dhakal and Sharma, 2020). It is regarded as an important medicinal plant due to its immense therapeutic efficacy. The bark, fruits and seeds of *Zanthoxylum armatum* are extensively used in indigenous system of medicine as a carminative, stomachic and anthelmintic drug. The fruit and seeds are employed as an aromatic tonic in fever and dyspepsia. The volatile oil is employed as antidiarrheal, antiseptic, deodorant and anticataerhal. An extract of the fruits is reported to be effective in expelling round worms. Because of their deodorant, disinfectant and antiseptic properties, the fruits are used in oral hygienic preparations, and their lotion for scabies (Bhattacharjee *et al.*, 2019). The seeds taste great on addition in pickles making it a household preference in Nepalese kitchen (Dhakal and Sharma, 2020).

Various pharmacological studies have been demonstrated that a broad group of chemical compounds containing alkaloids, flavonoids, lignins, coumarins, phenols, terpenoids, etc. have been found in this plant which are responsible for various biological activities like

antioxidative, antimicrobial, antiviral, hepato-protective, insecticidal and anticancerous (Kale *et al.*, 2010). Gautam *et al.* (2021) have compared antimicrobial and synergistic potential of extracts of leaves of *Zanthoxylum armatum* DC. from different geographical regions of Himachal Pradesh, India. Gas chromatography-mass spectrometry analysis of the essential oil resulted in the identification of 38 components, of which linalool (62%) and limonene (18.1%) were the major components. Therapeutic potential of *Zanthoxylum armatum* extract is directly related to total phenolic and flavonoids contents.

Many studies suggested that various factors such as extraction method, extraction solvent, harvesting time, parts of plants used, etc., affects the phytochemicals content in the extract (Alam *et al.*, 2019; Dhakal and Sharma, 2020; Phuyal *et al.*, 2020). According to Ullah *et al.* (2012) total phenolic content (TPC) of ethanolic extracts of *Zanthoxylum armatum* fruit and bark were found to be 21.68 ± 0.44 mg of GAE/g and 16.48 ± 1.33 mg of GAE/g respectively. Similarly, in another study, the TPC value of methanol extract of fruits was as high as 366.3 mg of GAE/g (Guleria *et al.*, 2013).

2.8.2 *Heracleum nepalense*

Heracleum nepalense is a flowering plant in the carrot family Apiaceae. The species is called Nepal cowparsnip in the book on Traditional Chinese Medicine (Wikipedia, 2019). Polunin and Stainton (1984) stated that, it is distributed from Himachal Pradesh to Bhutan. However, Flora of China (2005) stated that it is found in Bhutan, North and West of Yunnan – China, North East India, Sikkim, Myanmar and Nepal. Its Nepali vernacular name is *Chimphing* (Shrestha *et al.*, 2016). It has white flowers and starts flowering from May. Fruiting begins in June-July and starts maturing from August onwards. The seeds are feathery (has weak wing) when crush, it gives strong smell almost similar to the smell of cumin seeds (Mukhia, 2012).

The fruits of *H. nepalense* are used as pickles, as anti-typhoid, nausea and vomiting. The plant is used in veterinary medicine as it shows stimulant property that increases the rate of respiration and blood pressure in goats. The roots are used in folk medicine as digestive, an aphrodisiac, and carminative and anti-diarrheal. The roots of the plant was reported to have coumarins and steroids. *H. nepalense* has future prospective for new therapeutic applications

(Mukhia, 2012). Antibacterial activity of methanol extract of roots of *Heracleum nepalense* D. Don against bacteria causing diarrhea has been investigated by Bose *et al.* (2007) . The crude methanol extract of *H. nepalense* root was found to be active against both Gram-positive and Gram-negative organisms (Dash *et al.*, 2005).

2.8.3 *Litsea cubeba*

Litsea cubeba, commonly known as Mountain Pepper, is a deciduous shrub or small evergreen tree, unisexual with aromatic leaves, branches and flowers. It belongs to a Lauraceae family. The plant is found in China, India, Taiwan, Burma, Indonesia, Malaysia, Japan, Java, Nepal, Bhutan at altitudes of 300-3200 m. The leaves are lance shaped, oblong, or elliptic. Flowers are borne in solitary or clustered. Fruits are nearly spherical, pepper-like black at maturity. Seeds are spherical, white. Its Nepali vernacular name is *Sil timur* (Mitra *et al.*, 2020).

Litsea cubeba is a pioneer herb traditionally utilized in medicine. Different extracts from its plant parts, such as bark, leaf, root, and fruits, have been utilized in traditional Chinese medicines for curing various diseases. The fresh green fruit is used for culinary purposes like salad preparation, chutneys, pickles, etc. The *L. cubeba* essential oil (LEO) extracted from fresh fruits contains about 60–90% citral content, and is essential oil with volatile compounds having an intense lemon-like, fresh, sweet aroma, and insoluble in water. LEO is also used as an antifungal agent and bio-insecticides in the storage of grains, foods, archival documents, and/or clothing. The dried fruits are used for several medicinal purposes such as carminative (relieves flatulence), diuretic (aids urine passage), expectorant (aids secretion of sputum), stimulant, stomach ache, antiasthmatic, sedative, antidysentric, and antiseptic. The major groups of compounds found in *L. cubeba* include alkaloids, monoterpenes, sesquiterpenes, diterpenes, flavonoids, amides, lignans, steroids, and fatty acids. These compounds have anticancer, anti-inflammatory, antimicrobial, antioxidant, antidiabetic, and anti-HIV properties, and therefore have immense potential for treating various diseases (Kamle *et al.*, 2019).

2.8.4 *Evodia fraxinifolia*

Evodia fraxinifolia is a species of flowering plant in the citrus family, Rutaceae. It is deciduous tree growing to 15 m. Its fruit is used in chutney. The red fruits are about 12 mm in diameter. Another report says that it is the seeds that are used to add flavor and taste to lentil soups, fresh pickles, vegetables and curries. Its range include from E. Asia including Himalayas from Nepal to Sikkim. Its Nepali vernacular name is *Khanakpa*. Seed paste is mixed with water and consumed during indigestion and stomach disorder (Shrestha *et al.*, 2016). It is found at altitude up to 2100 m. It is also used in fever and headache. The plant is used as an antipyretic and is used in the treatment of typhoid. The major groups of compounds found in *Evodia fraxinifolia* include alkaloids, antraquinones, sesquiterpenes, triterpenes and glycosides (Ghosal, 2015).

2.9 Herb extraction methods

For isolation of biological components, extraction from plant is one of the more sustainable approaches (Jadhav *et al.*, 2009). Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Extraction (as the term is pharmaceutically used) is the separation of medicinally active portions of plant (and animal) tissues using selective solvents through standard procedures. The products so obtained from plants are relatively complex mixtures of metabolites, in liquid or semisolid state or (after removing the solvent) in dry powder form, and are intended for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts or powdered extracts. Such preparations have been popularly called galenicals, named after Galen, the second century Greek physician. The general techniques of medicinal plant extraction include maceration, infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, countercurrent extraction, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction, and phytonic extraction (with hydrofluorocarbon solvents) (Tiwari *et al*., 2011).

2.9.1 Cold extraction

The different plants parts dried in an artificial environment at low temperature (50-60 °C) and dried powder then further used for extraction purpose using various solvents. Weighed dried powder with respective solvents is kept in room temperature for 30 min shaking after each 24 h for seven days. Finally the extract is filtered using whatman filter paper (Ingle *et al.*, 2017).

2.9.2 Maceration

In this process, the whole or coarsely powdered crude drug is placed in a stoppered container with the solvent and allowed to stand at room temperature for a period of at least 3 days with frequent agitation until the soluble matter has dissolved. The mixture then is strained, the marc (the damp solid material) is pressed, and the combined liquids are clarified by filtration or decantation after standing (Singh, 2008).

2.9.3 Infusion

Fresh infusions are prepared by macerating the crude drug for a short period of time with cold or boiling water. These are dilute solutions of the readily soluble constituents of crude drugs (Singh, 2008).

2.9.4 Solvent extraction

Universal Extraction System (Buchi) is recently used for solvent extraction. The dried powder of various plant parts placed in glass thimble for extraction purpose using various solvents. The procedure is carried out for 10 cycles for each extract and adjusts the temperature just below the boiling point of the respective solvents. The resulting solvent extract is filtered and concentrated in vacuum concentrator (Ingle *et al.*, 2017).

2.9.5 Microwave assisted extraction (MAE)

MAE is a simple, environment friendly and economical technique for the extraction of biologically active compounds from different plant materials. Upon absorption by a material, electromagnetic energy of microwaves is converted to heat energy. 2450 MHz (2.5 GHz) is the most commonly used frequency for commercial microwave instruments (in order to avoid

Interference with radio communications), which has an energy output of 600-700 W. When plant material is immersed inside a microwave transparent solvent, the heat of microwave radiation directly reaches to the solid without being absorbed by the solvent, resulting in instantaneous heating of the residual moisture in the solid. Heating causes the moisture to evaporate and creates a high vapour pressure, which breaks the cell wall of substrate and releases the content into solvent (Kothari *et al.*, 2012).

2.9.6 Ultrasonication assisted extraction (UAE)

UAE involves application of high-intensity, high-frequency sound waves and their interaction with materials. UAE is a potentially useful technology as it does not require complex instruments and is relatively low-cost. It can be used both on small and large scale. UAE involves ultrasonic effects of acoustic cavitations. Under ultrasonic action solid and liquid particles are vibrated and accelerated and, because of that solute quickly diffuses out from solid phase to solvent. Several probable mechanisms for ultrasonic enhancement of extraction, such as cell disruption, improved penetration, and enhanced swelling, capillary effect, and hydration process have been proposed (Kothari *et al.*, 2012).

2.9.7 Supercritical fluid extraction (SFE)

Supercritical Fluid Extraction (SFE) involves use of gases, usually CO_2, and compressing them into a dense liquid. This liquid is then pumped through a cylinder containing the material to be extracted. From there, the extract-laden liquid is pumped into a separation chamber where the extract is separated from the gas and the gas is recovered for re-use. Solvent properties of CO_2 can be manipulated and adjusted by varying the pressure and temperature. The advantages of SFE are, no solvent residues left in it as CO_2 evaporates completely (Ingle *et al.*, 2017).

2.10 Extraction solvents

Extraction from the plant is an empirical exercise since different solvents are utilized at varying conditions such as time and temperature of extraction (Ingle *et al.*, 2017). Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant

extractions includes, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate. The factors affecting the choice of solvent are quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process, potential health hazard of the extractants (Eloff, 1998). The choice of solvent is influenced by what is intended with the extract. Since the end product will contain traces of residual solvent, the solvent should be nontoxic and should not interfere with the bioassay. The choice will also depend on the targeted compounds to be extracted (Ncube *et al.*, 2007). An illustration of the solvents used for active component extraction is shown in the Table 2.3.

The various solvents that are used in the extraction procedures are:

1. Water: Water is universal solvent, used to extract plant products with antimicrobial activity. Though traditional healers use primarily water but plant extracts from organic solvents have been found to give more consistent antimicrobial activity compared to water extract.

2. Acetone: Acetone dissolves many hydrophilic and lipophilic components from the two plants used, is miscible with water, is volatile and has a low toxicity to the bioassay used, it is a very useful extractant, especially for antimicrobial studies where more phenolic compounds are required to be extracted. A study reported that extraction of tannins and other phenolics was better in aqueous acetone than in aqueous methanol. Both acetone and methanol were found to extract saponins which have antimicrobial activity.

3. Alcohol: The higher activity of the ethanolic extracts as compared to the aqueous extract can be attributed to the presence of higher amounts of polyphenols as compared to aqueous extracts. It means that they are more efficient in cell walls and seeds degradation which have unpolar character and cause polyphenols to be released from cells. According to Gramatina *et al*. (2017), the higher concentration of total phenolic compounds were detected in samples extracted with 50% ethanol due to its higher polarity than pure

ethanol. By adding water to the pure ethanol up to 50% for preparing 50% ethanol the polarity of solvent was increased. Additionally, ethanol was found easier to penetrate the cellular membrane to extract the intracellular ingredients from the plant material. Methanol is more polar than ethanol but due to its cytotoxic nature, it is unsuitable for extraction in certain kind of studies as it may lead to incorrect results.

4. Chloroform: Terpenoid lactones have been obtained by successive extractions of dried barks with hexane, chloroform and methanol with activity concentrating in chloroform fraction. Occasionally tannins and terpenoids will be found in the aqueous phase, but they are more often obtained by treatment with less polar solvents.

5. Ether: Ether is commonly used selectively for the extraction of coumarins and fatty acids.

6. Dichloromethanol: It is another solvent used for carrying out the extraction procedures. It is specially used for the selective extraction of only terpenoid (Tiwari *et al*., 2011).

Table 2.3 Solvents used for active component extraction

Solvent	Components extracted
Water	Anthocyanins, Starches, Tannins, Saponins, Terpenoids, Polypeptides, Lectins
Ethanol	Tannins, Polyphenols, Polyacetylenes, Flavonol, Terpenoids, Sterols, Alkaloids
Methanol	Anthocyanins, Terpenoids, Saponins, Tannins, Xanthoxyllines, Totarol, Quassinoids, Lactones, Flavones, Phenones, Polyphenols
Chloroform	Terpenoids, Flavonoids
Ether	Alkaloids, Terpenoids, Coumarins, Fatty acids
Acetone	Phenol, Flavonols

Source: Tiwari *et al*. (2011)

2.11 Major groups of antimicrobial compounds from plants

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. Most are secondary metabolites, of which at least 12,000 have been isolated, a number estimated to be less than 10% of the total. In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores. Some, such as terpenoids, give plants their odors; others (quinones and tannins) are responsible for plant pigment. Many compounds are responsible for plant flavor (e.g., the terpenoid capsaicin from chili peppers), and some of the same herbs and spices used by humans to season food yield useful medicinal compounds (Cowan, 1999). Useful antimicrobial phytochemicals can be divided into several categories which are discussed in Section 2.11.1- 2.11.9.

2.11.1 Simple phenols and phenolic acids

Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-derived compounds which are in the highest oxidation state. The common herbs tarragon and thyme both contain caffeic acid, which is effective against viruses (Wild, 1994), bacteria (Brantner *et al.,*1996) and fungi (Duke, 1985). Catechol and pyrogallol both are hydroxylated phenols, shown to be toxic to microorganisms (Geissman, 1963). In addition, some authors have found that more highly oxidized phenols are more inhibitory. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Mason and Wasserman, 1987). Phenolic compounds possessing a C_3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oils and often cited as antimicrobial as well. Eugenol is a well-characterized representative found in clove oil. Eugenol is considered bacteriostatic against both fungi (Duke, 1985) and bacteria (Brantner *et al.,* 1996).

2.11.2 Quinones

Quinones are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive. These compounds, being colored, are responsible for the browning reaction in cut or injured fruits and vegetables and are an intermediate in the melanin synthesis pathway in human skin (Schmidt, 1988). In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in protein, often leading to inactivation of the protein and loss of function (Stern *et al.*, 1996). For that reason, the potential range of quinone antimicrobial effects is great. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism. As with all plant-derived antimicrobials, the possible toxic effects of quinones must be thoroughly examined (Cowan, 1999). Kazmi *et al.* (1994) described an anthraquinone from *Cassia italica*, a Pakistani tree, which was bacteriostatic for *Bacillus anthracis*, *Corynebacterium pseudodiphthericum*, and *Pseudomonas aeruginosa* and bactericidal for *Pseudomonas pseudomalliae*.

2.11.3 Flavones, flavonoids, and flavonols

Flavones are phenolic structures containing one carbonyl group. The addition of a 3-hydroxyl group yields a flavonol. Flavonoids are also hydroxylated phenolic substances but occur as a C_6-C_3 unit linked to an aromatic ring. Since they are known to be synthesized by plants in response to microbial infection (Dixon *et al.*, 1983), it should not be surprising that they have been found in vitro to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, as described above for quinones. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al.*, 1996).

Catechins, the most reduced form of the C_3 unit in flavonoid compounds, deserve special mention. These flavonoids have been extensively researched due to their occurrence in oolong green teas. It was noticed some time ago that teas exerted antimicrobial activity (Toda *et al.*, 1989) and that they contain a mixture of catechin compounds. These compounds inhibited in

vitro *Vibrio cholerae* O1 (Borris, 1996), *Streptococcus mutans* (Batista *et al.*, 1994), *Shigella* (Vijaya *et al.*, 1995), and other bacteria and microorganisms (Sakanaka *et al.*, 1992).

2.11.4 Tannins

"Tannin" is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin from solution, a property known as astringency. Their molecular weights range from 500 to 3,000 (Haslam, 1996) and they are found in almost every plant part: bark, wood, leaves, fruits, and roots (Scalbert, 1992). They are divided into two groups, hydrolyzable and condensed tannins. This group of compounds has received a great deal of attention in recent years, since it was suggested that the consumption of tannin-containing beverages, especially green teas and red wines, can cure or prevent a variety of ills (Serafini *et al.,* 1994). Many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumor activity, and a wide range of anti-infective actions, have been assigned to tannins (Haslam, 1996). One of their molecular actions is to complex with proteins through so-called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Haslam, 1996; Stern *et al.,* 1996). Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, etc.

2.11.5 Coumarins

Coumarins are phenolic substances made of fused benzene and a-pyrone rings (O'Kennedy and Thornes, 1997). They are responsible for the characteristic odor of hay. As of 1996, at least 1,300 had been identified (Hoult and Payá, 1996). Several coumarins have antimicrobial properties. Coumarin was found in vitro to inhibit *Candida albicans* (Cowan, 1999). Hydroxycinnamic acids, related to coumarins, seem to be inhibitory to gram-positive bacteria (Fernández *et al.,* 1996). Also, phytoalexins, which are hydroxylated derivatives of coumarins, are produced in carrots in response to fungal infection and can be presumed to have antifungal activity (Hoult and Payá, 1996).

2.11.6 Terpenoids and essential oils

The fragrance of plants is carried in the so called quinta essentia, or essential oil fraction. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure. They are called terpenes, their general chemical structure is $C_{10}H_{16}$, and they occur as diterpenes, triterpenes, and tetraterpenes as well as hemiterpenes and sesquiterpenes. When the compounds contain additional elements, usually oxygen, they are termed terpenoids (Cowan, 1999). Terpenoids are synthesized from acetate units, and as such they share their origins with fatty acids. They differ from fatty acids in that they contain extensive branching and are cyclized. Terpenenes or terpenoids are active against bacteria (Habtemariam *et al.*, 1993; Mendoza *et al.*, 1997) and fungi (Kubo *et al.*, 1993; Suresh *et al.*, 1997). In 1977, it was reported that 60% of essential oil derivatives examined to date were inhibitory to fungi while 30% inhibited bacteria (Chaurasia and Vyas, 1997). The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds. Food scientists have found the terpenoids present in essential oils of plants to be useful in the control of *Listeria monocytogenes* (Aureli *et al.*, 1992).

2.11.7 Alkaloids

Heterocyclic nitrogen compounds are called alkaloids. The first medically useful example of an alkaloid was morphine, isolated in 1805 from the opium poppy *Papaver somniferum* (Fessenden *et al.*, 1998); the name morphine comes from the Greek Morpheus, god of dreams. Codeine and heroin are both derivatives of morphine. Diterpenoid alkaloids, commonly isolated from the plants of the Ranunculaceae, or buttercup family, are commonly found to have antimicrobial properties (Omulokoli *et al.*, 1997). Berberine is an important representative of the alkaloid group. It is potentially effective against trypanosomes and plasmodia. The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine and harmane is attributed to their ability to intercalate with DNA (Cowan, 1999).

2.11.8 Lectins and polypeptides

Peptides which are inhibitory to microorganisms were first reported in 1942 (Balls *et al.*, 1942). They are often positively charged and contain disulfide bonds (Zhang and Lewis, 1997). Their mechanism of action may be the formation of ion channels in the microbial

membrane or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors (Terras *et al.,* 1993; Zhang and Lewis, 1997). Thionins are peptides commonly found in barley and wheat and consist of 47 amino acid residues (Colilla *et al.*, 1990; Mendez *et al.*, 1990). They are toxic to yeasts and gram-negative and gram-positive bacteria (Fernandez de Caleya *et al.,* 1972). Fabatin, a newly identified 47 residue peptide from fava beans, appears to be structurally related to g-thionins from grains and inhibits *E. coli*, *P. aeruginosa*, and *Enterococcus hirae* but not *Candida* or *Saccharomyces* (Zhang and Lewis, 1997).

2.11.9 Other compounds

Many phytochemicals not mentioned above have been found to exert antimicrobial properties. This review has attempted to focus on reports of chemicals which are found in multiple instances to be active. It should be mentioned, however, that there are reports of antimicrobial properties associated with polyamines (in particular spermidine), isothiocyanates, thiosulfinates, and glucosides (Cowan, 1999).

2.12 Mechanisms of the antibacterial effects of herbal extracts and essential oils

Essential oils (EOs) and plant extracts obtained from aromatic medicinal plants have been reported to show singularly good antimicrobial effects against bacteria, filamentous fungi, yeasts, and viruses. These are very complex natural mixtures including hydrocarbons (mainly terpenoids) and oxygenated compounds (alcohols, ethers, esters, ketones, aldehydes, lactones, phenols and phenol ethers (Stefanakisa *et al.*, 2013).

Various studies have investigated the mechanisms of the antibacterial effects of the phenolic compounds present in herbal extracts and essential oils; however, given the large number of chemical compounds in these extracts, their antibacterial effects cannot be attributed to a specific mechanism; rather, these extracts and essential oils serve different purposes in bacterial cells. These mechanisms do not act in isolation; instead, some are affected by the others (Burt, 2004).

The effect of phenolic compounds can be concentration dependent. At low concentration, phenols affect enzyme activity, particularly those associated with energy production, while at

high concentrations, they cause protein denaturation. The antimicrobial effect of phenolic compounds may be due to their ability to alter microbial cell permeability, thereby permitting the loss of macromolecules from the interior (for example ribose and Na glutamate). They could also interfere with membrane function (electron transport, nutrient uptake, protein, nucleic acid synthesis, and enzyme activity) and interact with membrane proteins, causing deformation in structure and functionality. The high antibacterial activity of phenolic components can be further explained in terms of alkyl substitution into the phenol nucleus. The formation of phenoxyl radicals that interact with alkyl substituents does not occur with more stable molecules such as the ethers myristicin or anethole, which was related to the relative lack of antimicrobial activity of fennel, nutmeg, or parsley EOs (Tiwari *et al.*, 2009).

Ward *et al.* (1998) reported that the antimicrobial activity of isothiocynates derived from onion and garlic is related to the inactivation of extracellular enzymes through oxidative cleavage of disulfide bonds and that the formation of the reactive thiocyanate radical was proposed to mediate the antimicrobial effect. carvacrol, β-carvone, thymol, and trans-cinnamaldehyde are reported to decrease the intracellular ATP (adenosine triphosphate) content of *E. coli* O157:H7 cells while simultaneously increasing extracellular ATP, indicating the disruptive action of these compounds on the plasma membrane. Inactivation of yeasts can be attributed to the disturbance of several enzymatic systems, such as energy production and structural component synthesis (Tiwari *et al.*, 2009).

The antimicrobial activity of essential oils and plant extracts would be related to the respective composition and structural configuration of the plant volatile oils, their functional groups and possible synergistic actions and reactions between components. Phenolic compounds comprise the main antimicrobial components in spices and their derived essential oils and extracts, and include, for instance, cinnamic aldehyde from cinnamon; thymol from thyme and oregano; eugenol from clove, allspice and cinnamon; carvacrol from oregano and anethole from anise. As a result, natural antimicrobials are receiving a good deal of attention for a number of microorganism-control issues (Pezeshk *et al.,* 2015).

2.13 Factors affecting the antimicrobial properties of herbal extracts

Antimicrobial activity of EOs and plant extract is influenced by a number of factors including botanical source, time of harvesting, stage of development, and method of extraction (Janssen *et al.*, 1987). In general, EOs produced from herbs harvested during or immediately after flowering possesses the strongest antimicrobial activity. The composition of EOs from different parts of the same plant can also differ widely. For example, EO obtained from the seeds of coriander (*Coriandrum sativum* L.) has a quite different composition to EO of cilantro, which is obtained from the immature leaves of the same plant. In general, the higher amount of phenolic compounds in an essential oil, the higher will be its antibacterial properties against food pathogens. The chemical structure of extracts or essential oils also affects their mechanisms of effect. The presence of the hydroxyl group is vital to phenolic compounds such as carvacrol and thymol. The relative position of the hydroxyl group in the phenolic ring has little effect on its antibacterial properties. For instance, thymol has the same antibacterial effect on *Bacillus cereus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* as that of carvacrol. In one study, however, carvacrol and thymol were found to have different effects on gram positive and gram negative species of bacteria. The composition of the essential oils also affects the proteins in the cell membrane (Burt, 2004).

Most studies on the antimicrobial effects of essential oils and extracts have been limited to In vitro bacteria culture media and very few have focused on their application in the food industry. Furthermore, due to their interaction with the compounds in foods, the effectiveness of essential oils and extracts is higher in culture media than in actual food products. Factors such as pH, fat and protein, water activity (a_w), temperature, the presence of other preservatives, and even physical properties and type of packaging affect the effectiveness of the essential oil in the food model. In meat products, the amount of fat contained in the product has a huge effect on the effectiveness of the extract, and high levels of fat reduce the antimicrobial effects of essential oils against most microorganisms. Overall, the concentration of extract used in food products is higher than the concentration used for samples under in vitro conditions; for instance, it is 10 times higher in the case of pork liver sausage, 50 times in soups and 25-100 times in soft cheeses. On the other hand, using high concentrations of

extracts and essential oils in meat and meat products leads to adverse organoleptic changes. To avoid this issue, combination methods (combination of the extract and essential oil with other antimicrobial compounds or non-thermal storage methods), encapsulation of essential oils and the addition of the extracts and essential oils to edible antimicrobial films are highly preferred these days (Aminzare *et al.*, 2016).

The majority of studies conducted on the effect of herbal extracts and essential oils on spoilage organisms and pathogens in food products support the claim that these substances have a slightly greater effect on gram positive than on gram negative bacteria. The lower sensitivity of gram negative bacteria to the essential oils' antibacterial effects may be due to the external membrane of the cell wall in this group of bacteria, which limits the permeation of the hydrophobic components of extracts into the bacterial cell (Burt, 2004).

Each component of an extract and essential oil acts in different degrees against gram positive or gram negative bacteria. The chemical compounds in extracts and essential oils obtained from a specific plant species may be different depending on the geographical region or stage of harvest from which they have been taken. The difference in the effects of these essential oils and extracts on gram negative and gram positive bacteria may be due to the changes in their chemical compounds (Aminzare *et al.*, 2016).

2.14 Effect of herbal extracts on the microbial quality of meat and meat products

Only a few food preservatives currently used contain herbal extracts and essential oils. Many studies have been conducted on the aromatic chemical compounds extracted from plants and effective on spoilage and pathogenic microorganisms present in meat and meat products. Many of these tests have demonstrated the positive effects of these compounds on meat and meat products particularly the effect that they have as a natural agent on spoilage and pathogenic bacteria (Bajpai *et al.*, 2012; Samojlik *et al.*, 2010). The incorporation of spice and herb extracts inhibit major pathogenic organisms such as *Salmonella enteritidis, Listeria monocytogenes,* and *Staphylococcus aureus* in different meat products (Hygreeva *et al.*, 2014).

Fernandes *et al.* (2016) evaluated the influence of oregano extract on the level of microorganisms in sheep burgers stored in MAP. Oregano extract slightly improved the microbial quality of the meat products. Burgers containing oregano showed significantly lower LAB levels in comparison to the control. The extract also improved the color stability of the meat. Similarly, EO of *Salvia officinalis* at low concentrations (≤0.1%) inhibited the growth of *Salmonella anatum* and *S. enteritidis* in minced beef meat stored at 4–7°C for 15 days. Under the same conditions, concentrations ≥1.5% of EO reduced *Salmonella* counts up to 2.8 log cfu/g (Hayouni *et al.,* 2008).

Shan *et al.* (2009) investigated the use of cinnamon stick, oregano, cloves, pomegranate peel and grape seed extracts as natural preservatives for pork meat packed aerobically and stored at 20^{o}C. Their antibacterial activity was tested against foodborne pathogens (*L. monocytogenes*, *S. enterica*, *S. aureus*). All the extracts inhibited the growth of the tested bacteria. Gram-positive strains were more susceptible than Gram-negative. Similarly, Radha krishnan *et al.* (2014) studied the effect of dried cloves, cinnamon, oregano and mustard, individually and in combinations, on the shelf life of raw chicken meat. The spice extracts significantly affected the microbial quality of the raw chicken meat and inhibited bacteria growth. The total viable counts (TVC), LAB, Enterobacteriaceae and *Pseudomonas* sp. counts were lower in meat samples treated with the extracts compared to the controls. However, mixed spice extracts had stronger antibacterial effects than individual spices.

Wang *et al.* (2015) investigated the effect of green tea and grape seed polyphenols on the quality of dry-cured bacon during ripening and storage. After 3 weeks of storage, the TVC values in samples treated with grape tea and grape tea seed extracts were 5.37 and 5.72 log cfu/g, respectively, which means that they were not considered spoilt. The amount of Enterobacteriacea was significantly lower in the bacon treated with plant extracts.

Jaworska *et al.* (2021) studied the effect of antimicrobial activity of oregano (*Origanum vulgare*) and black pepper (*Piper nigrum*) at 0.3% of dry herbal or 0.003% as industrial extract on the minced meat. The microbiological, chemical and sensory tests were performed at specified times and storage temperature. Based on the studied criteria, products maintained constant and adequate quality by up to 10 days while stored in 4°C. The preservative role of

herbs and extracts in meat products during processing and storage was observed. Oregano and black pepper in both forms maintained good microbial quality and showed their inhibitory effects on the growth of psychrotrophic bacteria.

Part III

Materials and methods

3.1 Materials

Raw materials, analytical grade chemicals, calibrated glassware and equipment were used in the study and are described in the following section.

3.1.1 Raw materials and sources

To carry out the study, fresh male buffalo (*Bubalus bubalis*) meat and *sukuti* were purchased from the local market of Dharan. Meat from the round was purchased. According to butcher, age of buffalo was 2.5 years and reared in Bishnupaduka, Dharan. Similarly, the dried herbs were also collected from the local market of Dharan, which were from Sankhuwasabha, a district in eastern region of Nepal. Four types of ethno medicinal herbs viz., *Zanthoxylum armatum* (*Bokey Timur*), *Heracleum nepalense* (*Chimphing*), *Litsea cubeba* (*Sil timur*) and *Evodia fraxinifolia* (*Khanakpa*) (Plate P.1, Appendix G) were purchased for the proposed study.

3.1.2 Equipment and chemicals

The following chemicals and equipment were used from Dharan Multiple Campus. The list of chemicals used for the analysis is shown in Table 3.1 and the list of equipment is shown in Table 3.2.

Table 3.1 List of chemicals used

Chemicals	Manufacturer/Supplier	Specification
Ethanol	Changshu Hongsheng Fine Chem Co. Ltd.	99.9%
Hydrochloric acid	Thermo Electron LLS India Pvt. Ltd.	36%
Sulfuric acid	Thermo fisher Scientific India Pvt. Ltd.	97%
Folin-Ciocalteu reagent	Fischer Scientific, India	2N
Ferric Chloride	Thermo Fischer scientific India, Pvt. Ltd	96%

Continued ….

Table 3.1 List of chemicals used (continued)

Chemicals	Manufacturer/Supplier	Specification
Gallic acid	HiMedia Laboratories Pvt. Ltd.	98 %
Simmon Citrate Agar	HiMedia Laboratories Pvt. Ltd.	M099
MR-VP Medium	HiMedia Laboratories Pvt. Ltd.	M070S
Mannitol Salt Agar	HiMedia Laboratories Pvt. Ltd.	M118
Nutrient Broth	HiMedia Laboratories Pvt. Ltd.	M002
Nutrient Agar	HiMedia Laboratories Pvt. Ltd.	M001
Sodium Carbonate	Qualigens fine chemicals	99.5%
Man Rogosa and Sharpe Agar	HiMedia Laboratories Pvt. Ltd.	M641I
Standard plate count agar	HiMedia Laboratories Pvt. Ltd.	M091
Eosine Methylene Blue Agar	HiMedia Laboratories Pvt. Ltd.	M317
Hydrogen Peroxide	Fischer Scientific	100%
Salmonella Shigella Agar	HiMedia Laboratories Pvt. Ltd.	M108
Selenite F Broth	HiMedia Laboratories Pvt. Ltd.	M052
Triple Sugar Iron Agar	HiMedia Laboratories Pvt. Ltd.	M021
Tryptone Broth	HiMedia Laboratories Pvt. Ltd.	M463

Table 3.2 List of equipment used

Equipment	Specification
Electronic balance	Model: HZT-A500, India
UV-Vis spectrophotometer	Model: 2371, Wavelength range: 200-1000 nm
Vacuum rotary evaporator	Model: SARE-T43
Refrigerator	Toshiba, Japan
Digital caliper	Aerospace, scale: 0-150 mm
Digital Incubator	Model: DH5000BII, Faithful
Trinocular microscope	Model:Prime 200, Ycoo
Centrifuge	Model: RM-12C, India

Continued…….

Table 3.2 List of equipment used (continued)

Equipment	Specification
Autoclave	-
Electronic grinder	Yasuda®, Japan
Hot air oven	Navyug Udyog, 30 Haryana, India
Tray dryer	VR® Dhiman, India

3.1.3 Glassware

Glassware like conical flasks, measuring cylinders, volumetric flasks, beakers, petri-plates, sample tubes, ampules, round bottom flasks, pipette, test-tubes, glass-slides, etc. were taken from Dharan Multiple Campus.

3.2 Preparation of herbal extracts

According to Gramatina *et al.* (2017), the higher concentration of total phenolic compounds were detected in samples extracted with 50% ethanol. Therefore, for the current investigation herbal extracts were prepared from dry herbal material (*Bokey timur*, *Sil timur*, *Khanakpa* and *Chimphing*) treated in 50% ethanol/water proportion. To prepare the extracts, the herbs were ground to the particle size of < 250 µ with the help of grinder. Then the 50 g of powdered herbs were added to the 400 ml of 50% (v/v) ethanol for 12 h in enclosed flasks with constant shaking (100 rpm). After filtration with Whatman No. 2 filter paper, the residue was re-extracted with an additional 200 ml of 50% ethanol for an additional 12 h and then filtered. The combined filtrates were concentrated in a rotary evaporator at 50^{o}C with a vacuum pump and dried. Dried extracts were placed in sealed vials and stored at 4^{o}C until analysis as shown in Plate P.6, Appendix G. (Zhang *et al.*, 2016). The yields of the extracts were calculated using the following equation:

$$\%\text{Yield} = \frac{\text{Weight of extract}}{\text{Weight of herb}} \times 100$$

3.3 Phytochemical qualitative analysis

To detect the various phytochemicals present, preliminary phytochemical screening was carried out for all four herb extract; *Zanthoxylum armatum, Litsea cubeba, Heracleum nepalense* and *Evodia fraxinifolia.*

Herbs extract were subjected to standard phytochemical analysis to find the presence of different phytoconsituents such as tannins, phenolic compounds, steroid, saponin, alkaloid and flavonoids (Jaradat *et al.*, 2015; Sofowora, 1993).

3.3.1 Test for tannins and phenolic compounds

Two milliliter of 2% solution of $FeCl_3$ mixed with crude extract. Black or bluegreen color indicated the presence of tannins and phenols (Jaradat *et al.*, 2015).

3.3.2 Test for steroids

Two milliliter of chloroform and concentrated H_2SO_4 were mixed with the entire plant crude extract. In the lower chloroform layer produced red color that indicated the presence of steroids. Another test was performed by mixing 2 ml of each of acetic acid with H_2SO_4 concentrated and crude extract with 2 ml of chloroform. Green color indicated the entity of steroids (Jaradat *et al.*, 2015).

3.3.3 Test for saponins

Five milliliter of distilled water was added to crude plant extract in a test tube and it was shaken vigorously. The foam formation indicated the presence of saponins (Jaradat *et al.*, 2015).

3.3.4 Test for flavonoids

Two milliliter of 2% NaOH solution was mixed with plant crude extract, intensive yellow color was formed, which turned into colorless when added 2 drops of diluted acid to solution, this result indicated the presence of flavonoids (Jaradat *et al.*, 2015).

3.3.5 Test for alkaloids

To 0.5 g of plant material, 5 ml of 1% aqueous hydrochloric acid was added and stirred on a water bath and the water content was filtered. To 1 ml of filtrate Mayer's reagent was added. Appearance of cream colored precipitate indicates presence of alkaloid (Sofowora, 1993).

3.4 Determination of total phenolic content in herbal extracts

The total phenolic contents of the ethanol extract of herbs were estimated using the Folin Ciocalteau reagent as described by Singleton and Rossi (1965). The calibration curve (Appendix A) was plotted by mixing 1 ml aliquots of 50, 100, 150, 200, 250, 300, 350, 400 and 450 µg/ml Gallic acid solutions with 5.0 ml of Folin Ciocalteu reagent (diluted tenfold) and 4.0 ml of sodium carbonate solution (75 g/L). The absorbance was measured after 30 min at 765 nm. For the ethanolic extracts (1 g /100 ml), 1 ml was mixed separately with the same reagents, as performed for constructing the calibration curve. After 1 h, the absorbance was measured to determine the total phenolic contents in the extracts using the formula,

$C = C_1 \times V/m$

where, C = total phenolic content in mg/g, in GAE (Gallic acid equivalent),

C_1 = concentration of Gallic acid established from the calibration curve in mg/ml,

V = volume of extract in ml, and

m = the weight of the plant extract in g

3.5 Threshold study

EOs and herbal extracts are characterized for their intense odor and flavor, so a great impact on food sensory properties is to be expected. In addition, high concentrations of EOs and herbal extracts are required when added to foods as antimicrobials; therefore, application of EOs and herbal extracts is limited by organoleptic changes. Panelists in general reject foods treated with EOs due to their acidic taste, intense chemical aromas, and/or herbal aromas Mani-López *et al.*, 2018). In order to reduce sensory impact of EOs on foods, they can be used not only as a biopreservative but also as a flavor component, thus flavor compatibility between food and biopreservative does not affect food flavor but improves it. This approach has been tested on dry fermented sausage and good sensory acceptance was reported when 0.005% of

garlic, oregano, or rosemary EOs were added to inactivate 10^3 cfu/g of *L. monocytogenes* or 0.005% of thyme EO to inactivate 10^3 cfu/g of *Salmonella* spp. After 12 days of drying (García-Díez *et al*., 2016). Novel attempts like incorporating herbs in traditional foods like *sukuti* can be daunting as these can mar the characteristic sensory quality of the product, resulting in a reduced acceptability, or even rejection of the novel product. Ultimately, it's the consumer acceptability that counts for any product to sustain in this competitive market. Threshold testing measures the limits of sensory perception of individuals or groups of individuals (ASTM, 2004a; ASTM, 2004b). Developers are often interested in knowing the point at which certain substances affect the acceptability of food. Therefore, threshold study were carried out by running trial experiments. Different batches of *sukuti* were prepared by marinating meat in different concentrations of herbal extracts. The maximum and minimum ranges at which herbs start to give off too strong a flavor and at which herb flavor just gets detected (respectively) were noted. Threshold concentrations were ultimately selected from sensory analysis (ASTM, 2004a; ASTM, 2004b).

3.6 Screening of microorganisms from *sukuti*

Certain microorganisms such as *Salmonella*, *Shigella*, *E.coli*, *Staphylococcus* and lactic acid bacteria were isolated from *sukuti*.

3.6.1 *E. coli*

E. coli were isolated on Eosine Methylene Blue Agar (EMBA) medium by pour plate method as described by Harrigan and McCance (1976) using sterile water as diluent with slight modification. For identification the biochemical tests such as gram staining, IMViC test, coagulase and catalase test were carried out on the basis of methods proposed by Gordon *et al.* (1973).

Twenty five gram of ground sample was mixed with 225 ml of sterile distilled water and serial dilution was carried out. 1 ml each of the required dilutions was transferred into sterile petri plates. To each plate approximately 20 ml of previously melted and cooled (45°C) sterilized agar medium was added. The contents were mixed thoroughly by gently rotating the plates into 8 shape. Agar was then allowed to cool and incubated at 37°C for 24 - 48 h.

3.6.2 *Salmonella*

Salmonella were isolated according to the Varadaraj (1993) with some modifications. The flow chart for *Salmonella* isolation is given in Fig. 3.1. Observation of colonies morphology and biochemical tests like gram staining, IMViC test, coagulase test, H_2S test and catalase test were done for confirmation.

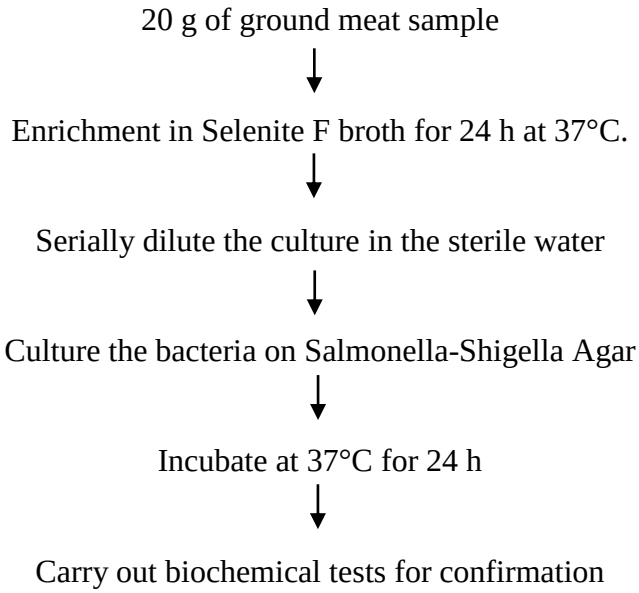

Fig. 3.1 Flow chart for the isolation of *Salmonella* species

3.6.3 *Staphylococcus*

Staphylococcus was isolated on Mannitol Salt Agar by pour plate method carried out according to Harrigan and McCance (1976). The bacteria were characterized by microscopic morphological examination and conventional biochemical test.

3.6.4 Lactic acid bacteria (LAB)

Lactic acid bacteria were isolated by pour plate method on MRS agar carried out according to Harrigan and McCance (1976). The bacteria were characterized by microscopic morphological examination and conventional biochemical test.

3.7 Preparation of bacterial inoculum/ suspension

Required colonies of freshly cultured test organisms were inoculated aseptically to glass vials containing sterile nutrient broth and incubated at 32^{o}C for 24 h.

3.8 Determination of antimicrobial activity of herbal extracts

The antimicrobial activity of herbal extracts was examined in triplicate using the agar well diffusion method to detect the growth inhibition of *Salmonella*, LAB, *E.coli* and *Staphylococcus* (Balouiri *et al*., 2016). Tests for individual antimicrobials were carried out at the following diluted herbal extract concentrations in 50% ethanol: 40, 20, 10 and 2 mg/ml (concentration range selected from trial experiment). The 50% ethanol was used as a control. The sterile nutrient agar plates are used for the test. Carpet culture of the prepared cell suspension was done aseptically with the help of a cotton swabs over the dry surface of agar plates. Sterile cork-borer (5.2 mm dia.) was used to punch a hole aseptically on the agar plates, and 0.1 ml of herb extracts at each dilution was then aseptically transferred into the well. The control was 0.1 ml of 50% ethanol alone. With the lid on, the plates were incubated at 35 ± 2^{o}C for 24 h. The inhibitory effect was assessed by measuring the disc diameter of the inhibition zone (clear zone) in millimeter around the extract with the help of ruler or vernier caliper.

3.9 Preparation of herbal extract incorporated *sukuti*

Sukuti was prepared using cabinet dryer as described by Thapa (2017) with slight modification. Buffalo meat was cut into strips of 1 cm thick and 25 cm long. Herbal extract having best antimicrobial activity was selected and dissolved in distilled water for application on meat strips. Herbal extract was applied at the rate of 2% on meat strips. The marinated meat strips were kept in the refrigerator for 24 h at 4 ± 2^{o}C. Then they were placed on trays and dried in cabinet dryer at 55^{o}C for 2 days. The process flow chart used for herbal extract incorporated *sukuti* making is presented in the Fig. 3.2. The yield of the *sukuti* was calculated using the following equation:

$$\%\text{Yield} = \frac{\text{Weight of sukuti}}{\text{Weight of meat}} \times 100$$

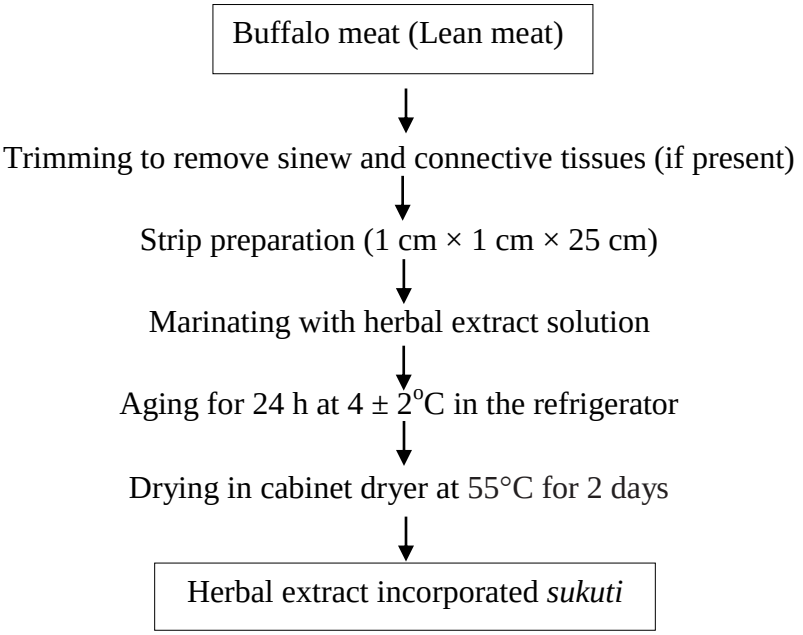

Fig. 3.2 Preparation of herbal extract incorporated *sukuti*

3.10 Microbiological analysis of prepared *sukuti*

Total plate count (TPC) of untreated *sukuti* and herbal *sukuti* were carried out by pour plate method as described by Harrigan and McCane (1976). The TPC of sample was expressed in terms of log colony forming units (cfu) per gram and the results obtained were statistically processed.

3.11 Statistical analysis

The work were carried out in duplicate while the analyses were carried out in triplicate. All the calculations were performed in Microsoft Office Excel (2010). The results were expressed as mean values ± the standard deviation (SD). For significance analysis, data were analyzed by one way and two- way ANOVA using Genstat Release v12 and means were separated using Fischer's Least Significant Difference (LSD) test at 5% level of significance.

Part IV

Results and discussion

For the study, four ethno medicinal herbs namely were *Zanthoxylum armatum, Litsea cubeba, Heracleum nepalense* and *Evodia Fraxinifolia* were used. The herbal extract was prepared using 50% ethanol as solvent for the determination of antimicrobial property against meat spoilage and pathogenic bacteria isolated from *sukuti*. The different herbal extract concentrations selected for investigation were 2 mg/ml, 10 mg/ml, 20 mg/ml and 40 mg/ml which was determined by threshold study. Finally, herbal extract with the best antimicrobial activity was selected for the incorporation to *sukuti*.

4.1 Percentage yield of herbal extract

Fifty gram each of dry ground herbs having particle size of <250 µ were treated with 400 ml of 50% ethanol and concentrated in a rotary vacuum evaporator at 50^{o}C (80 rpm) and dried. The yield of the extracts were calculated based on dried weight of powdered samples of herbs. The % yield of *Zanthoxylum armatum, Litsea cubeba, Heracleum nepalense* D. Don and *Evodia fraxinifolia* were found to be 20%, 14%, 15% and 8% respectively. According to Guleria *et al.* (2013), % yield of methanol extract of *Zanthoxylum armatum* was found to be 20.14% which is in accordance with the current result. This result showed that extraction yield for different samples vary even when same extraction method and solvent are used. This may be due to difference in chemical composition, physical structure and cell matrices of individual herbs.

4.2 Qualitative phytochemical analysis of herbal extracts

The analysis of phytochemical constituents of ethanol extract of *Zanthoxylum armatum, Litsea cubeba, Heracleum nepalense* D. Don and *Evodia Fraxinifolia* revealed the presence of various phytoconstituents. The results are presented in Table 4.1.

Table 4.1 Qualitative phytochemical analysis of herbs

Herbs	Phenol	Flavonoid	Steroid	Saponin	Alkaloid	Tannin
Z. armatum	+	+	+	−	+	+
L. cubeba	+	+	+	+	+	+
H. nepalense	+	−	−	+	−	+
E. fraxinifolia	+	−	−	−	+	+

'+' indicates present, '−'indicates absent

The phytochemicals screening of ethanol extract of the *Z. armatum* and *L. cubeba* showed that both were rich in phenol, tannins, flavonoids, steroids, alkaloids. *H. nepalense* was found to be lacking flavonoid, steroid and alkaloid whereas *E. fraxinifolia* was found to be lacking flavonoid, steroid and saponin. Several studied showed the similar result for phytochemicals screening of these aforementioned herbs (Ghosal, 2015; Koirala and Singh, 2017; Kamle *et al.*, 2019; Dhakal and Sharma, 2020).

4.3 Total phenolic content of herbal extract

The total phenolic content of herbal extracts are shown in the Table 4.2. It was found that the ethanolic extract of *Zanthoxylum armatum* had higher total phenol content followed by *Litsea cubeba*, *Heracleum nepalense* and *Evodia fraxinifolia*.

Table 4.2 Total phenol content of herbal extract

Ethanolic extract	Total phenolic content (mg GAE/g)
Zanthoxylum armatum	21.414 ± 0.204^{a}
Litsea cubeba	16.9220 ± 0.1508^{b}
Heracleum nepalense	8.860 ± 0.264^{c}
Evodia fraxinifolia	5.851 ± 0.180^{d}

All the results are the average values of the three replicates. Values are presented in the form of Mean ± Standard deviation. Figures in the row bearing different alphabet in superscript are significantly different at $p<0.05$. ANOVA table for total phenol content is given in Appendix B.

Table 4.2 shows *Zanthoxylum armatum* contains highest total phenol content whereas *E. fraxinifolia* showed lowest total phenol content. The total phenol content of *Zanthoxylum armatum* was found to be 21.414 mg GAE/g. This is in accordance with the study of Ullah *et al.* (2012). Similarly, the value obtained for *Heracleum nepalense* total phenol content was similar to the result obtained by Koirala and Singh (2017).

4.4 Screening of microorganisms

E. coli, *Salmonella*, *Shigella*, *Staphylococcus* and Lactic acid bacteria were successfully screened from *sukuti* by using a modification of the screening techniques given by Harrigan and McCance (1976) and Varadaraj (1993). The screened isolates were identified by morphological examination and conventional biochemical tests and the observed results for the same are shown in Appendix C.

4.5 Antimicrobial activity of herbal extracts

Antimicrobial activity of four herbs (*Zanthoxylum armatum*, *Heracleum nepalense*, *Litsea cubeba* and *Evodia fraxinifolia*) were determined by well diffusion method (Section 3.7) and zone of inhibition shown by herbal extracts against the four test organisms (*Salmonella*, *E. coli*, LAB and *Staphylococcus*) at four different levels of concentration (2 mg/ml, 20 mg/ml, 30 mg/ml and 40 mg/ml) were measured. The statistical data and the graphical representation for the same is given in Table 4.3 and Fig. D.1 (Appendix D) respectively.

Table 4.3 Summary of zone of inhibition showed by herbal extracts for test organisms at different level of concentration

Conc.	Herbs	*E. coli*	LAB	*Salmonella*	*Staphylococcus*
2 mg/ml	*E. fraxinifolia*	ND	ND	ND	ND
	H. nepalense	ND	ND	ND	ND
	L. cubeba	ND	5.53±0.11	ND	5.87±0.06
	Z. armatum	ND	6.33±0.15	ND	6.43±0.06
10 mg/ml	*E. fraxinifolia*	ND	ND	ND	ND
	H. nepalense	ND	6.37±0.21	ND	6.6±0.1
	L. cubeba	5.57±0.15	8.4±0.1	5.8±0.1	8.23±0.25
	Z. armatum	6.2±0.1	8.27±0.31	6.27±0.21	9.13±0.15
20 mg/ml	*E. fraxinifolia*	ND	ND	ND	ND
	H. nepalense	5.67±0.15	8.17±0.15	6.2±0.1	8.1±0.1
	L. cubeba	7.07±0.21	11±0.1	8.23±0.25	11.1±0.1
	Z. armatum	8.43±0.21	12.63±0.15	9.5±0.2	13.2±0.26
40 mg/ml	*E. fraxinifolia*	ND	ND	ND	ND
	H. nepalense	8±0.1	12.63±0.15	9.2±0.2	12.3±0.2
	L. cubeba	10.5±0.2	15.17±0.15	11.1±0.2	15.37±0.15
	Z. armatum	11.47±0.25	16.27±0.25	13.4±0.17	17.07±0.23

ND = Not Detected. Values represent means± standard deviation.

Table 4.4 shows ANOVA table of zone of inhibition for the treatments (concentration, herbs, isolate) and their interactions. The concentrations of the extract, the type of herbs and the bacterial isolates, all yielded significantly different ($p<0.05$) zones of inhibition.

Table 4.4 ANOVA of zone of inhibition for the treatments (concentration, herbs, isolate) and their interactions.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Concentration	3	1686.96625	562.32208	33017.08	<.001
Herbs	3	2375.06125	791.68708	46484.38	<.001
Isolate	3	397.15958	132.38653	7773.15	<.001
Concentration × Herbs	9	574.06583	63.78509	3745.18	<.001
Concentration×Isolate	9	8.37583	0.93065	54.64	<.001
Herbs×Isolate	9	141.73583	15.74843	924.68	<.001
Concentration× Herbs × Isolate	27	107.70208	3.98897	234.21	<.001
Residual	128	2.18	0.01703		
Total	191	5293.24667			

Multiple comparisons using post hoc test (Fischer's LSD method) at 5% level of significance are shown in Table 4.5, 4.6 and 4.7. Table 4.5 shows highest zone of inhibition at the 40 mg/ml concentration (9.54±6.05 mm) whereas 2 mg/ml concentration showed least zone of inhibition. The result showed positive correlation between concentration and zone of inhibition. It has been reported that the antibacterial effect of the plant extract increases with increase in its concentration (Haghgoo *et al.*, 2017). This may be due to the presence of higher amount of active antimicrobial principles at higher concentration.

Table 4.5 Summary of effect of concentration on zone of inhibition

Concentration	2 mg/ml	10 mg/ml	20 mg/ml	40 mg/ml	LSD (5%)
Zone of inhibition (mm)	1.51 ± 2.65^{a}	4.43 ± 3.6^{b}	6.85 ± 4.48^{c}	9.54 ± 6.05^{d}	1.764

Values represent means± standard deviation. Means with different alphabets in different column are significantly different ($p<0.05$).

Among the herbs selected for the study, *Z. armatum* and *L. cubeba* were found to be most potent ($p<0.05$) against the test organisms (*Salmonella*, LAB, *Staphylococcus* and *E. coli*), followed by *H. nepalense*. The statistical data for the same is shown in Table 4.6. Unlike rest of the herbs, *E. fraxinifolia* showed no zone of inhibition against any test organism and thus can be considered ineffective. The reason behind inferiority of *E. fraxinifolia* and high efficacy of *Z. armatum* against test organisms may be due to their phenolic content (Table 4.2): the higher the amount of phenolic compounds in the herb extract, higher will be its antibacterial properties against food pathogens (Burt, 2004).

Table 4.6 Summary of effect of herb type on zone of inhibition

Herb	*E. fraxinifolia*	*H. nepalense*	*L. cubeba*	*Z. armatum*	LSD (5%)
Zone of inhibition (mm)	0^{a}	5.21 ± 4.48^{b}	8.08 ± 4.31^{c}	9.04 ± 4.84^{c}	1.586

Values represent means± standard deviation. Means with different alphabets in different column are significantly different ($p<0.05$).

Table 4.7 shows that *Staphylococcus* was more susceptible to be herbal extract among the test organisms. Similarly, LAB was second most susceptible after *Staphylococcus*. However, *E. coli* showed the least zone of inhibition followed by *Salmonella.* This may due to the difference in their composition of cell wall, which affects the antimicrobial properties of herbal extracts. It has been reported that herbal extract have a slightly greater effect on Gram positive than on Gram negative bacteria (Burt, 2004). This may be attributed to the single layer of cell wall structure in Gram positive bacteria as compared to multilayered cell wall structure of Gram negative bacteria, which limits the permeation of extracts into the bacterial cell.

Table 4.7 Summary of effect of herbal extracts on zone of inhibition for different isolate type

Isolate	*E. coli*	*Salmonella*	LAB	*Staphylococcus*	LSD (5%)
Zone of inhibition (mm)	3.93 ± 4.24^{a}	4.38 ± 4.77^{a}	6.92 ± 5.56^{b}	7.1 ± 5.7^{b}	2.055

Values represent means± standard deviation. Means with different alphabets in different column are significantly different ($p<0.05$).

4.6 Preparation of *sukuti* using extract of the selected herb

Herb showing the highest zone of inhibition for test organisms was selected. Section 4.6 shows the superiority of *Z. armatum* extract among herbs under investigation in terms of antimicrobial property. At 40 mg/ml concentration, *Z. armatum* extract showed the highest zone of inhibition for the test organisms where *Staphylococcus* being highly susceptible. Using *Z. armatum* extract at 40 mg/ml concentration, herbal *sukuti* was prepared as per the techniques described in PART III, Section 3.9 which is shown in Fig. 3.2. The yield of *sukuti* was found to be 27.6 %.

4.7 Microbiological analysis of prepared herbal *sukuti*

Total Plate Count of treated *sukuti* (herbal *sukuti*) and untreated *sukuti* (control) was carried out for 20 days within the interval of 10 days. Table 4.8 shows the comparison between total plate count of treated and untreated samples.

Table 4.8 Comparison between total plate count of treated and untreated samples

Treatment	Total Plate Count (log cfu/g)		
	Day 0	Day 10	Day 20
Control (untreated)	3.46 ± 0.151^{a}	3.86 ± 0.035^{a}	4.378 ± 0.068^{a}
Herbal *sukuti* (treated)	2.36 ± 0.104^{b}	2.507 ± 0.18^{b}	2.66 ± 0.794^{b}

Values represent means± standard deviation. Means with different alphabets in same column are significantly different ($p<0.05$).

As is evident from Table 4.8, that the total plate count for treated sample was significantly lower ($p<0.05$) than that of untreated sample. Overall, the results demonstrate the effectiveness of the tested herb for inhibiting microbial growth. The incorporation of herbal extract inhibits major pathogenic microorganisms in different meat products (Hygreeva *et al.*, 2014). This may be due to the presence of active principles in the herbal extract that exerted antimicrobial effect in *sukuti*. Herbs have antimicrobial ability mainly due to their phenol compounds (Cowen, 1999). The possible mechanisms for the antimicrobial effect of phenol compounds include: altering microbial cell permeability, interfering with membrane function (electron transport, nutrient uptake, protein, nucleic acid synthesis, and enzyme activity) and interacting with membrane proteins, causing deformation in structure and functionality (Tiwari *et al.*, 2009).

Table 4.9 shows ANOVA table of Total Plate Count (log cfu/g) for the treatments (days, treatment) and their interactions. The treatments on the *sukuti* and the days, all yielded significantly different ($p<0.05$) Total Plate Count (log cfu/g).

Table 4.9 ANOVA of zone of inhibition for the treatments (concentration, herbs, isolate) and their interactions.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days	2	1.11401	0.55701	42.76	<.001
Treatment	1	8.69445	8.69445	667.38	<.001
Days×Treatment	2	0.28823	0.14412	11.06	0.002
Residual	12	0.15633	0.01303		
Total	17	10.25303			

4.8 Cost of herbal *sukuti*

The cost of herbal *sukuti* and untreated *sukuti* (Appendix E) was calculated to be NRs. 2152.512/kg and 1776/kg respectively. The cost calculation was done taking into account the cost of raw materials, transportation cost and processing cost incurred in *sukuti* preparation (with 20% overhead cost of the final product).

Part V

Conclusions and recommendations

The present study was aimed at preparing *sukuti* in the laboratory by using extract of ethno medicinal herb having best antimicrobial properties against few pathogens and spoilage bacteria isolated from *sukuti*. Based on the study, following conclusions can be drawn:

1. The antimicrobial activity of an herbal extract is contingent upon factors like the herb type, concentration of the antimicrobial principle(s), and the types of target microorganisms.
2. Dried meat (*sukuti*) can harbor some of the important foodborne pathogens.
3. Higher the amounts of phytochemicals, higher the antimicrobial activity.
4. Herbal extract can improve the microbial stability of *sukuti*.

Based on the present work, following recommendations can be made:

1. *Zanthoxylum armatum* extract at the concentration of 40 mg/ml can be incorporated in *sukuti* to improve microbial stability of the product.
2. Specific bio active compounds responsible for the antimicrobial property of the herb can be identified and their antimicrobial activity against wide range of pathogenic and spoilage microorganisms can be studied.

Part VI

Summary

Sukuti is an indigenous dried meat product especially from buffalo lean meat. Various meat and meat product spoilage epidemics with different degrees of severity occur every year in different parts of the production chain. Microbial pathogens are traditionally associated with the most serious meat safety issues in terms of both foodborne illness and product recalls. Researches on *sukuti* so far are limited to survey, *sukuti* preparation and process optimization. Microbial aspects of the *sukuti* which is very important in the preservation of *sukuti* and prevention of health hazards, appears to have been ignored.

The main objective of the study was to determine the effect of extracts of ethnomedicinal herbs on microbial quality of *sukuti*. Specific objectives include preparation of herbal extracts, isolation and identification of microorganisms from *sukuti*, determination of antimicrobial property of herbal extracts and microbial analysis of herbal *sukuti*. Four ethnomedicinal herbs (*Zanthoxylum armatum*, *Litsea cubeba*, *Heracleum nepalense* and *Evodia fraxinifolia*) having culinary importance were selected for the study. For the preparation of herbal extract, herbs were treated with ethanol (50%) and concentrated in a rotary vacuum evaporator at 50^{o}C. Four of the spoilage and pathogenic microorganism viz. *Salmonella*, *Staphylococcus*, *E. coli* and Lactic acid bacteria were screened (in selective agar), identified (microscopic examination and biochemical tests) from the *sukuti* samples for the study.

Herbal extract at the concentration of 40, 20, 10 and 2 mg/ml (concentration range selected from trial experiment) were tested against the isolates to determine the antimicrobial property of the extracts. The best herb and optimum concentration was selected on the basis of its antimicrobial activity (zone of inhibition). *Zanthoxylum armatum* showed highest zone of inhibition against the test organisms followed by *Litsea cubeba* and *Heracleum nepalense* whereas *Evodia fraxinifolia* showed no zone of inhibition against any of the isolates. Among the test organisms, *Staphylococcus* was found to be highly susceptible to herbal extract followed by LAB, *Salmonella* and *E.coli*.

Zanthoxylum armatum (40 mg/ml) was found to possess greater antimicrobial property and selected for final product development. *Zanthoxylum armatum* (40 mg/ml) extract was applied at the rate of 2% on meat strips (1 cm thick and 25 cm long), aged (24 h at 4 ± 2°C) and dried at cabinet drier for 2 days at 55°C. Total Plate Count of control (untreated) and herbal *sukuti* (treated) were carried out for 20 days within interval of 10 days to determine the microbial stability of final product. The total plate count for treated sample was significantly lower ($p<0.05$) than that of untreated sample. This may be due to the presence of active principles in the herbal extract that exerted antimicrobial effect in *sukuti*. On the basis of this finding, it can be concluded that herbal extract can improve the microbial stability of *sukuti*.

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Appendices

Appendix A

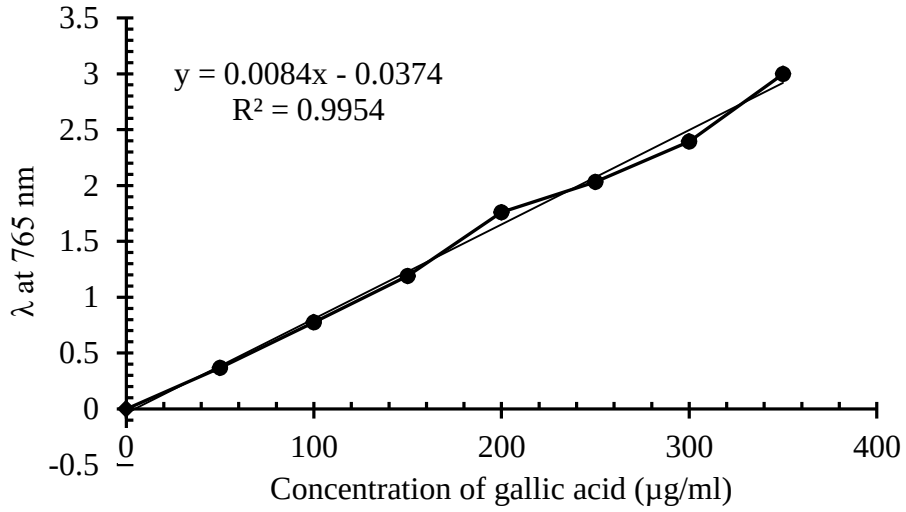


Fig. A.1 Calibration curve for total phenol content

Appendix B

Table B.1 ANOVA of total phenol content

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Sample	3	349.295	116.432	2804.23	0.000
Error	8	0.332	0.042		
Total	11	349.628			

Appendix C

Table C.1 Morphological and biochemical characteristics of isolated bacteria

Isolates	Morphological characteristics	Gram staining	IMViC test		Catalase test	Coagulase test	H_2S test
E. coli	Small and pink colonies with green metallic sheen on EMB agar.	Gram negative	Indole	+	+	−	−
			MR	+			
			VP	−			
			Citrate	−			
Staphylococcus	Small yellow colonies on mannitol salt agar, cocci shaped arranged in grape-like clusters	Gram positive	Indole	−	+	+	−
			MR	+			
			VP	−			
			Citrate	+			
Salmonella	Colorless, transparent colonies with jet black center	Gram negative	Indole	−	+	−	+
			MR	+			
			VP	−			
			Citrate	+			
LAB	Opaque creamy white colonies, rod shaped arranged in chain	Gram positive	Indole	−	−	−	−
			MR	−			
			VP	−			
			Citrate	−			

'+' indicates positive, '−' indicates negative

Appendix D

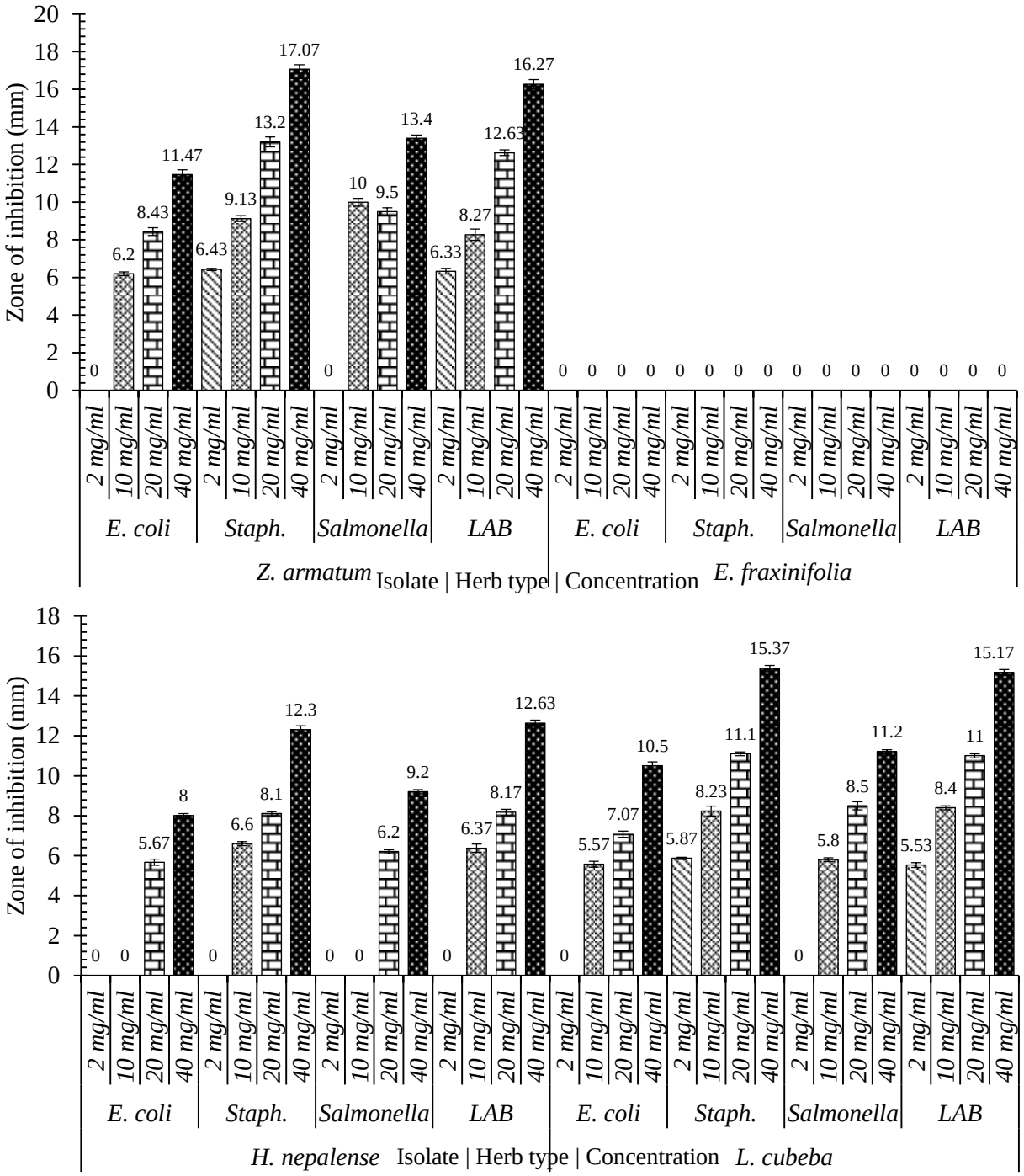


Fig. D.1 Graphical representation of zone of inhibition showed by herbal extracts for the test organisms at different level of concentration

Appendix E

Table E.1 ANOVA of effect of herb type on zone of inhibition

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Herbs	3	2375.06	791.69	51.00	<.001
Residual	188	2918.19	15.52		
Total	191	5293.25			

Table E.2 ANOVA of effect of concentration on zone of inhibition

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Concentration	3	1686.97	562.32	29.31	<.001
Residual	188	3606.28	19.18		
Total	191	5293.25			

Table E.3 ANOVA of effect of isolate on zone of inhibition

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	3	397.16	132.39	5.08	0.002
Residual	188	4896.09	26.04		
Total	191	5293.25			

Appendix F

Table F.1 Cost calculation for 1 kg of *sukuti* and herbal extract incorporated *sukuti*

Ingredients	Rate	Quantity	Cost (NRs)
Buffalo meat	400/kg	3.7 kg	1480
Total cost of *sukuti* with 20% overhead			1776/kg
Zanthoxylum armatum	1200/kg		
Zanthoxylum armatum extract	6000/kg	800 mg/kg of meat	$4.8 \times 3.7 = 17.76$
Total cost of herbal extract incorporated *sukuti*			1793.76
Final cost of herbal extract incorporated *sukuti* with 20% overhead			2152.512/kg

Appendix G

Color Plates

(a) *Bokey Timur*

(b) *Chimphing*

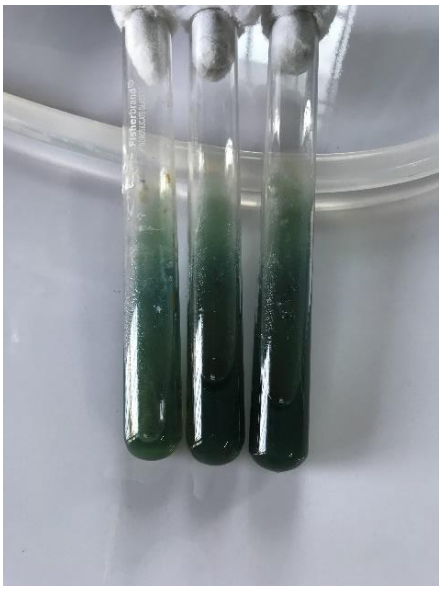
(c) *Sil Timur*

(d) *Khanakpa*

P.1 Collected herbs

a) Methyl Red Test

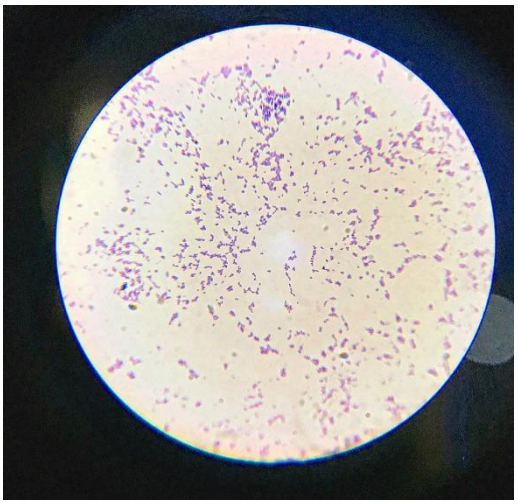
b) MR-VP test



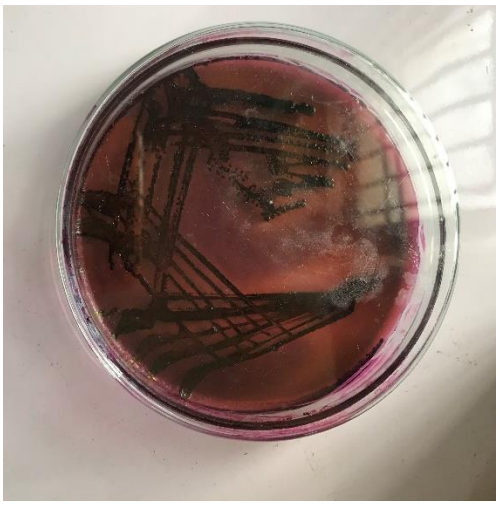
c) Simmons'Citrate Agar Test

d) Coagulase test

P.2 Biochemical tests for identification of isolates



P.3 *Staphylococcus* under (100 ×) microscope

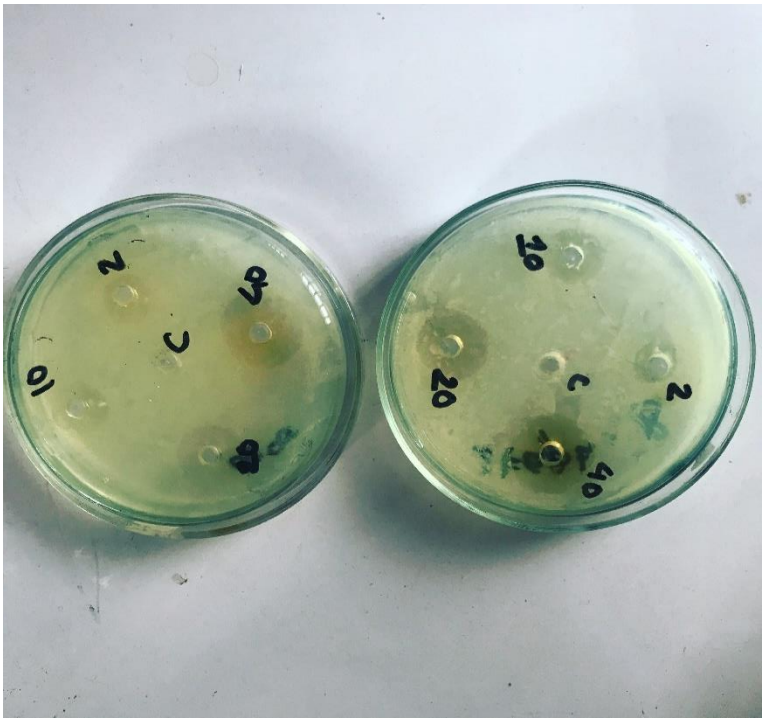


P.4 *E. coli* on EMB Agar

P.5 *Salmonella* in SS Agar

P.6 Herbal extract

P.7 Rotary vacuum evaporator



P.8 Herbal extract showing zone of inhibition

P.9 Performing microbiological analysis in the lab

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