EFFECT OF SPICE EXTRACTS ON PHYSICOCHEMICAL QUALITY AND STORAGE STABILITY OF BUFFALO MEAT PATTIES

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A dissertation submitted to the Department of Food Technology, Central Campus of Technology, Tribhuvan University, in partial fulfillment of the requirements for the degree of B. Tech. in Food Technology

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Approval Letter

This *dissertation* entitled *Effect of Spice Extracts on Physicochemical Quality and Storage Stability of Buffalo Meat Patties* presented by Muskan Saru Magar has been accepted as a partial fulfillment of the requirements for the B. Tech. degree in Food Technology.

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Abstract

The study was carried out to investigate the effect of spice extracts on quality and storage stability of buffalo meat patties. Three different spices viz. ginger, garlic and turmeric were collected and ethanolic extracts of each of them were incorporated in the patties at concentration of 0.05% of total ground meat mass. Various physicochemical properties were studied. Patties were extracted using methanol to analyze phytochemicals. Also, Storage stability of patties were analyzed at -4°C for 30 days. Analysis of variance (ANOVA) and Tukey's test were performed using GenStat 12th Edition to check the significant relationship at $p<0.05$.

The physicochemical properties (cooking loss, water loss, lipid loss) were found to be the lowest for Ginger extract (GE) incorporated patties (20.24±0.15, 17.64±0.41 and 0.32±0.03 % respectively). Also, GE showed the highest total polyphenol (757.956 mg GAE/100g) and total flavonoid content (189.123±0.61 mg QE/100g), whereas Turmeric extract (TE) showed the highest antioxidant activity (14.3045 % at 5 µM and 71.7848 % at 25 µM) followed by Ginger extract and Garlic extract (GAE). For storage stability, the lowest PV at day30 was encountered for TE treated patties (3.488667 Meq/kg) followed by BHT, GAE, GE and control. GE also contributed to have the highest anti-microbial activity with lowest total plate count (6.88±0.06 log cfu/g) at day 30 followed by GAE, TE, BHT and control. Change in pH was also found to be the lowest for GE treated patties (from 6.18 to 5.95) followed by TE, GAE, BHT and control. Thus, spice extract significantly increased the storage stability of buffalo meat patties as compare to control and can replace synthetic antioxidants.

Contents

Approval Letter ..... **iii**

Acknowledgements ..... **iv**

Abstract ..... **v**

List of tables ..... **ix**

List of figures ..... **x**

List of abbreviations ..... **xi**

1. Introduction **1-3**

1.1 General introduction 1

1.2 Statement of the problem 2

1.3 Objectives 2

1.3.1 General objective 2

1.3.2 Specific objectives 2

1.4 Significance of study 3

1.5 Limitations of the study 3

2. Literature review **4-20**

2.1 Patty 4

2.2 Buffalo patty 4

2.2.1 Buffalo meat 5

2.2.2 Soyabean flour as binder 6

2.2.3 Fat 6

2.2.4 Spices 7

2.3 Physicochemical attributes 9

2.3.1 Cooking loss 9

2.3.2 Emulsion stability 10

2.3.3 Water holding capacity 10

2.4 Lipid oxidation in meat 11

2.4.1 Antioxidants 12

2.5 DPPH radical scavenging assay 14

2.5.1 IC^{50} (Inhibitory concentration) 14

2.6 Phytochemicals 15

2.6.1 Polyphenols 16

2.6.2 Flavonoids 17

2.7 Storage stability 19

2.7.1 Peroxide value 19

2.7.2 Microbial spoilage 20

3. Materials and methods 21-31

3.1 Materials 21

3.1.1 Raw materials 21

3.1.2 Chemical and apparatus required 21

3.2 Methods 21

3.2.1 Preparation of spice powder 21

3.2.2 Ethanolic extraction of Spice 22

3.2.3 Physicochemical analysis 24

3.2.4 Extraction of phytochemicals for analysis 26

3.2.5 Determination of total flavonoid content (TFC) 30

3.2.6 Storage stability study 30

3.3 Statistical analysis 31

4. Results and discussion 32-40

4.1 Chemical analysis of buffalo meat 32

4.2 Physicochemical analysis of patty during preparation 33

4.3 Total polyphenol content 34

4.4 Total flavonoid content 34

4.5 DPPH radical scavenging activity 35

4.6 Storage stability 38

4.6.1 Change in total peroxide value of buff patty over time 38

4.6.2 Estimation of total plate count of buff patty over time 39

4.6.3 Change in pH of buff patty over time 40

5. Conclusion and recommendations 42

5.1 Conclusions 42

5.2 Recommendations 42

Summary 43

References 44-61

Appendices 62-77

List of tables

Table No.	Title	Page No.
2.1	Some important flavonoids and their functions	18
3.1	Formulation of experimental buffalo meat patties (% weights)	23
3.2	Amount of reagents/extract used for the DPPH assay (for extract analysis)	28
3.3	Amounts of reagents/ ascorbic acid used for the DPPH assay (for standard curve)	29
4.1	Proximate analysis of buffalo meat	32
4.2	Physicochemical analysis of different formulated patties	33
4.3	Total polyphenol content of methanolic extracts of herbs and spices	34
4.4	Total flavonoid content of methanolic extract of spices	35
4.5	IC^{50} of methanolic extracts of spices	37

List of figures

Figure No.	Title	Page No.
2.1	Chemical structure of BHT	13
3.1	Preparation of ethanolic extract of spice	22
3.2	Preparation of buffalo meat patty	24
3.3	Flowchart for the determination of total polyphenol content (TPC)	27
4.1	%RSA of methanolic extracts of patties with varying concentration	36
4.2	Change in peroxide value over time for different samples	38
4.3	Change in total plate count over time for different samples	39
4.4	Change in pH over time for different samples	40

List of abbreviations

Abbreviation	Full form
BHA	Butylated hydroxy anisole
BHT	Butylated hydroxyl toluene
CCT	Central Campus of Technology
CFU	Colony-forming unit
EOs	Essential oils
FDA	Food and Drug Administration
GAE	Garlic extract
GDP	Gross domestic product
GE	Ginger extract
PG	Propyl gallate
PV	Peroxide value
RNS	Reactive nitrogen spices
ROS	Reactive oxygen species
TBHQ	Tert-butyl hydroquinone
TE	Turmeric extract
TPC	Total plate count
WHC	Water Holding Capacity

Part I

Introduction

1.1 General introduction

A burger or patty is a handy meat product made from comminuted meat emulsion. The homogenized muscle proteins, fat, water, salt and other non-meat substances were combined to create the meat emulsion (Ismail *et al.*, 2021). In order to produce improved emulsion and gelation qualities, the solubilized intracellular myofibrillar protein, extracted during mixing process on the meat surface, is mixed with the fat, connective tissue, and other non-meat constituents (James G. Brennan, 2011). Grounded meat patties are one of the products that can be easily processed since the sensory qualities and tenderness are influenced by the small size of the meat pieces (Uriyapongson, 2007).

In several developing nations in Asia, buffaloes (*Bubalus bubalis*) are huge ruminants that have an essential place in the lives of millions of people as a source of milk, meat, draught power, transportation, and on-farm manure. In terms of content, quality, and organoleptic qualities, buffalo meat is essentially identical to beef. It also has the benefit of having less fat, cholesterol, and calories. Buffalo meat is ideal for creating meat products with additional value since it has excellent processing qualities (Naveena and Kiran, 2014).

From consumers', producers', and health authorities' points of view, the safety of grounded meat is essential as their shelf life gets shortened due to their nutrient-dense medium for the growth of numerous bacteria (Shan *et al.*, 2009). Numerous research has been conducted to investigate the antioxidative properties of several crude extracts as well as commercially available refined extracts of spices and herbs (Naveena *et al.*, 2013). The essential oils (EOs) and various spice extracts having antimicrobial or antioxidant properties are outstanding alternatives to chemical preservatives such as BHT, which satisfies consumer demands for minimally processed meat products (Pajohi *et al.*, 2011). Many communities have long utilized various spices as preservatives and to improve the flavor and aroma of food (Gupta and Ravishankar, 2005).

Since ancient times, people have utilized garlic (*Allium sativum*) as a spice and as a home medicine. Numerous studies have demonstrated the antimicrobial effects of garlic essential

oil in various media against common infections (Ross *et al.*, 2001). Along with garlic, ginger (*Zingiber officinale*) is a common Asian spice. The antibacterial properties of ginger have been studied by numerous researchers (Ji *et al.*, 1997). Another spice is turmeric (*Curcuma longa*), which belongs to the Zingiberaceae family. In contrast to foodborne pathogens, turmeric extract was proven to have antibacterial activity against a variety of pathogens, including clinical human infections (Ammon and Wahl, 1991).

1.2 Statement of the problem

In fast food restaurants, it is normal for food products such as pre-cooked hamburger patties, chopped veggies, etc. to be prepared ahead of time and stored at ambient temperature, so that the server only needs to take a few steps to complete the food service when the customer arrives (e.g., heating the patty, adding sauce, arranging the dish, etc.) (Hanukov *et al.*, 2019).

Protein oxidation is characterized by free radical chain reactions with initiation, propagation, and termination stages when reactive oxygen species (ROS) are present (Gardner, 1979). Burger patties are also particularly susceptible to oxidation because mincing, chopping, cooking, and salting increase the production of ROS, which in turn intensifies the oxidative processes (Ladikos and Lougovois, 1990). It is becoming more and more appealing to utilize spice extracts as a source of bioactive substances to improve the quality and health-related attributes of fresh meat and meat products (Munekata *et al.*, 2020). But comparative study of the spice extracts incorporated patties were not performed.

Thus, the use of antioxidant rich spices can somehow lower the oxidative effect with better sensory profile. This will eventually increase the chance of stocking the pre-cooked patties for longer duration as the shelf life increases (Fernández-López *et al.*, 2008).

1.3 Objectives

1.3.1 General objective

The general objective was to study the effect of spice extracts on physicochemical quality and storage stability of buffalo meat patties.

1.3.2 Specific objectives

- To prepare ginger, garlic and turmeric extract incorporated buffalo patties.

- To determine cooking loss and emulsion stability during cooking.
- To compare storage stability under refrigerated and vacuum condition.
- To determine antioxidant activity, flavonoid and polyphenol content of ginger, turmeric and garlic.

1.4 Significance of study

Cooked meat products are commonly packaged for commercial use in a modified environment or under vacuum, and it is assumed that they will retain their sensory characteristics throughout extended periods of time in the refrigerator (Chenoll *et al.*, 2007). Plant phenolics are characteristic components of plant materials and are proof of their powerful antioxidant activity with wide variety of pharmacological capabilities, such as anticancer, antioxidant, and platelet aggregation inhibitory actions (Heinonen, 2007). Antioxidants are essential because polyenoic fatty acids rapidly oxidize and produce free radicals, which can be hazardous if present in excessive concentrations. Natural antioxidants are preferred by consumers over synthetic antioxidants, mostly for emotional reasons. Upper safety limits of natural antioxidants are mostly unknown, but they are much safer than synthetic antioxidants (Pokorný, 2007). They are used as food, food additives, flavorings, or to preserve food through their antibacterial characteristics. Many spices contain chemical elements that have medicinal benefits, such as antimicrobial and antioxidant properties, the ability to quench singlet oxygen, the ability to stimulate cytochrome and other enzymes, and the ability to inhibit the growth of cancer cells (Li, 2006a).

1.5 Limitations of the study

- Sensory analysis of the product was not done.
- Shelf life was studied for 30 days only due to time constraints.

Part II

Literature review

2.1 Patty

Burger patties are regarded as one of the widely consumed foods that have contributed significantly to the modern diet's emphasis on a nutrient-dense diet. They are a staple of the ready-to-eat family of foods that are made in restaurants and at home. They are frequently grilled between two heated plates on industrial hot grills. Then, to avoid bacterial contamination, the cooked patties are stored frozen for a bit longer period of time (Özkan *et al.*, 2004).It can also be hand-made from comminuted meat emulsion. The homogenized muscle proteins, fat, water, salt and other non-meat substances were combined to create the meat emulsion (Ismail *et al.*, 2021).

One of the most often consumed meat items for quick meals, particularly in Arab nations, are beef burger patties. Beef is the most popular raw meat ingredient that is widely utilized to make hamburger patties (Abdel-Naeem and Mohamed, 2016). But due to religious taboos, beef is not consumed in Nepal. It is illegal to even sell beef meat (Burris, 2015). Due to a rapid decline in quality parameters and microbiological development, burger patties have a maximum shelf life of 3 days at 4°C (Parafati *et al.*, 2019).

Burger patties that have already been prepared and compressed into discs that are one centimeter thick are readily available in retail stores in Malaysia. The minced meat, chicken, or fish paste used to make these pre-made burger patties is combined with other basic components, including flour, oil, salt, pepper, and a few preservatives. At the retail level, ready-made burger patties are packaged in units, frequently in parts, and marketed as frozen goods (Wong *et al.*, 2012).

2.2 Buffalo patty

Meat and meat products are among the most important protein sources in the daily diet of people. Products from minced meat of buffalo are popular meat products consumed by millions of people from all over the world. The mincing enhances the formation of reactive oxygen species; therefore, the minced meat is highly vulnerable to oxidation. Patties may contain up to 30% fat by weight and retailers usually offer three types of patties, standard,

medium and low fat with a graduating pricing structure with the low-fat product carrying a premium price. As price per pound increases, drip loss decreases. Although volatile cooking losses are greater for highest price product than for lowest price product (Sheridan and Shilton, 2002).

2.2.1 Buffalo meat

Water buffalo is a source of buffalo meat. It is a large bovid, raised for its milk and meat in many countries including India, Nepal, Pakistan, Bangladesh, Philippines, Italy, Russia, Czech Republic, Slovakia and Egypt. Buffalo meat is known by various names in different countries such as buffen or buff in India and Nepal (Ghimire *et al.*, 2022). Indian subcontinent is a home tract of world buffaloes. Because of low cholesterol and calories, buffalo meat is considered healthy meat among the red meat. Besides, buffalo meat have good binding properties and hence is preferred in product manufacturing (Kandeepan *et al.*, 2009b).

Nepal being agriculture-based country with 65% of people are engaged in it, livestock still contributes to national GDP. Also, buffalo meat is known for its cheap price (60% cheaper than mutton) (Neupane *et al.*, 2018). Major attractive features of buffalo meat includes dark red colour, good marbling, low connective tissue, desirable texture, high protein, water holding capacity, myofibrillar fragmentation index and emulsifying capacity (Kandeepan *et al.*, 2013).

Principal components of buffalo meat are proteins with all essential amino acids, fats, substantial amount of vitamins and minerals with a high degree of bioavailability and water. Lean buffalo meat has only 2g of fat per 100g and 24g of protein (Kandeepan *et al.*, 2009b). Buffalo meat is specifically valuable as a source of omega-3 fatty acids, vitamin B12, vitamin B6, niacin and bioavailable iron besides having appreciable amount of Zn and Se (Williams, 2007). Apart from the traditional nutrients which are essentially required for the human body, buffalo meat contains numerous bioactive substances that have been studied for their potential beneficial effects (Arihara, 2006).

2.2.2 Soyabean flour as binder

Binders are protein-rich agents that change water-binding properties, which help bind together different materials in ground meat products. The binders effectively reduce shrinkage during cooking. It is used to enhance the binding of the meat and fat, enhancing the retention of fat and moisture. The organoleptic characteristics of the product significantly enhance with the application of binder. Binders have a macromolecular structure that allows them to catch huge volumes of water generated during heat processing to stop exudation and build matrices to hold onto nutrients and fragrance (Singh *et al.*, 2008).

Soybean is a widely used, inexpensive and nutritional source of dietary protein. Its protein content (40%) is higher and more economical than that of beef (19%), chicken (20%), fish (18%) and groundnut (23%). The distinctive functional properties of soy protein as meat extenders and functional additives are the main reason for its expanding use in meat products. Generally, the term "meat extender" refers to non-meat proteins (often derived from plants) used in meat products to enhance consistency, emulsifying ability, and water holding capacity (Etiosa *et al.*, 2017).

2.2.3 Fat

Fat plays critical functional and sensory roles in meat products. It is involved in the development of flavor compounds and in the perception of tenderness, juiciness and mouthfeel (Berry, 1992). In order to prepare a stable meat emulsion, fat plays a crucial role. Cellular disruption to free the fat, smaller fat globules for optimal stability, and enough solubilized protein to cover the fat surface area (interfacial adsorption) are crucial factors. (Hoogenkamp, 2011). However, recent studies have linked high dietary intake of meat and saturated fat with various health problems, such as coronary heart disease, obesity and diabetes (Webb and O'neill, 2008). Also, lipids are one of the most chemically unstable food components and readily undergo oxidative reactions which are responsible for loss of quality and nutritional value of meat and meat products (Faustman *et al.*, 2010).

Pipek *et al.* (2012) concluded that pork fat (lard) contains more than 60% unsaturated fatty acids and 10% to 16% PUFA using both Gas Chromatography and Iodine value. Double bond present in unsaturated fatty acids, subjected to autoxidation forms free radical followed by the formation of hydroperoxide(Morris, 1954). It is an intermediate of autoxidative

rancidity and may cause several health disorders like UV-induced skin cancer, neurodegeneration, atherosclerosis, etc. (Girotti, 1998).

2.2.4 Spices

Spices, non-leafy flavoring part, are used worldwide. Not only for flavor but also for medicine, stimulants and disinfectants too. Asia is often called 'Land of Spices' where ginger, turmeric, garlic and other 70 more species of spices are grown (Chomchalow, 1996).

The demand for medical herbs, spices, and the products made from them has grown over the past ten years for a range of uses, including herbal medicine, food flavorings, and cosmetics in the forms of tea, pill, capsule, tincture, cream, syrup, and liquid. Many spices contain chemical elements that have medicinal benefits, such as antimicrobial and antioxidant properties, the ability to quench singlet oxygen, the ability to stimulate cytochrome and other enzymes, and the ability to inhibit the growth of cancer cells (Li, 2006b).

Spices with their historical applications contains phytochemicals with their potential medical benefits. There have been reports of large number of bioactive substances, including polyphenols, quinines, organosulfur compounds, flavonoids, alkaloids, polypeptides, etc., that have a variety of pharmacological effects. Specifically, the antibacterial, anti-inflammatory, antioxidant, antidiabetic, anti-hyperlipidemic, hepatoprotective, and antipyretic properties of spices were investigated in spices (Khanal *et al.*, 2021) .

2.2.4.1 Ginger

Ginger (*Zingiber officinale*), originated from South East Asia, then cultivated in Ancient China and India and then to Europe countries. It is an underground rhizome which can be used in various forms like Whole rhizome, Ground rhizome, Ginger candy, Ginger oil, Ginger extract, etc. (Chomchalow, 1996). It is indispensable ingredient due to its refreshing pleasant aroma and carminative property. The functionally significant components of ginger are primarily its aroma and secondarily its pungency. The primary flavoring constituents of the oil include cineol, borneol, geraniol, linalool and farnasene (Vasala, 2012).

Ginger has been used as a spice and as natural additives for more than 2000 years (Bartley and Jacobs, 2000). Also, ginger has many medicinal properties. Studies have shown that, the long term dietary intake of ginger has hypoglycemic and hypolipidemic effect (Ahmed and Sharma, 1997). Ginger has been identified as an herbal medicinal product with pharmacological effect. Ginger suppresses prostaglandin synthesis through inhibition of cyclooxygenase- 1 and cyclooxygenase- 2. In traditional Chinese and Indian medicine, ginger has been used to treat a wide range of ailments including stomach aches, diarrhea, nausea, asthma, respiratory disorders (Grzanna *et al.*, 2005).

2.2.4.2 Garlic

Allium sativum, commonly known as garlic, is a species of the onion family Alliaceae (Saravanan *et al.*, 2010). The native land of garlic is Central Asia (Jancic and Lakušic, 2002). There are a number of beliefs about the exact origin of garlic, such as that it comes from western China, around the Tien Shan mountains, to Kazakhstan and Kyrgyzstan. The Sumerians (2600-2100 BC) actively used the healing properties of garlic and are believed to have brought garlic to China, from where it later spread to Japan and Korea. The spread of garlic probably took place first in the Old World and then in the New World. Even so, some historians still claim that garlic originated in China (Petrovska and Cekovska, 2010).

Garlic consists of very robust organosulfur compounds that function secondary metabolites as defined in the segment entitled chemistry and pharmacology. These compounds are responsible for the very pungent odor and flavor of uncooked garlic (Alam *et al.*, 2016). Fresh garlic contains alliin, allicin and essential oils, when the garlic clove is crushed, the odorless compound alliin is converted to allicin through the enzyme alliinase. Allicin gives garlic its characteristic pungent odor (Williamson and Wren, 2003). It also contains vitamins, minerals and trace elements. Garlic, on the other hand, is a herbal medicine that is used to prevent and treat many illnesses such as cold and flu symptoms by strengthening the immune system and it has anticancer, antioxidant, anti-inflammatory, antimicrobial, antithrombotic, hypocholesterolemia properties, hypoglycemic agents. and 35 hypotensive effects. And it is used to treat diabetes, atherosclerosis, hyperlipidemia, thrombosis, and high blood pressure. It is also effective against strokes, gastrointestinal neoplasms, blood clots (platelet aggregation inhibitors), etc. (Divya *et al.*, 2017).

2.2.4.3 Turmeric

Curcuma longa, commonly known as turmeric, is a tropical perennial monocotyledonous herbaceous plant widely used and cultivated in South and South-eastern Asia (Nwaekpe *et al.*, 2015). It belongs to the Family Zingiberaceae. The term longa refers to the elongated shape of the rhizome, where turmeric is derived from the rhizome of the plant having a characteristic orange-yellow color (Prasad and Aggarwal, 2011). Turmeric contains coloring pigment, curcumin which imparts yellow color to turmeric and possesses potent antioxidant activity (Debjit Bhowmik *et al.*, 2009).

The main bio-active principles of turmeric, the curcuminoids, can be used as anti-inflammatory, anti-oxidant, hypercholesteremic, choleretic, antimicrobial, insect repellent, anti-rheumatic, antivenomous, antiviral, antidiabetic, anti-hepatotoxic, anti-cancerous and anti- helminthic (Singh *et al.*, 2011). Curcuminoids are yellow components comprising of curcumin (96%), desmethoxycurcumin (6%), and bisdesmethoxycurcumin (0.3%) while the volatile oil composes of a number of monoterpenes and sesquiterpenes including zingiberene, curcumene, α- and β-turmerones (Charoenchai *et al.*, 2020). Curcumin (1,7-bis(4-hydrox- y-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is the major active compound with an amount of 2–6% by weight in the rhizome and essential oil comprise of 5.8% (Yadav *et al.*, 2013).

2.3 Physicochemical attributes

2.3.1 Cooking loss

Cooking loss measures the ability of system to bind water and fat after protein denaturation and aggregation (Marchetti and Andrés, 2021). During cooking, material is lost from the meat emulsion. This loss is in the form of vapor, which is almost entirely steam and drip. The mass loss as vapor is independent of patty constituents and depends only on the temperature of heat source. Loss of fluids diminishes the sensory outcome of the cooking process, reduces the weight of the product and will give rise to shrinkage. This may also reduce the acceptability to the consumer and perhaps its consequent sale value (Sheridan and Shilton, 2002).

2.3.2 Emulsion stability

Meat batters, considered as oil-in-water emulsion are heterogeneous composite materials composed of protein-coated fat globules (oil droplets) dispersed in myofibrillar protein gel matrix (Dickinson, 2012). In emulsified meat products, following thermal processing, the dispersed emulsion droplets set in as active filler particles (Theno and Schmidt, 1978). The batter stability and product texture in emulsion-based meat products depend on various factors such as the nature and amount of the lean, fats/oils, added water, additives, other non-meat ingredients used, processing methods, etc. Barbut (1995) reviewed the importance of emulsion theory and physical entrapment theory in meat batter stability which emphasized the importance of fat emulsification and protein matrix in binding the fat respectively. The amount of soluble protein used, the speed of mixing, the final temperature of the emulsion, and the amount of oil initially added, each influence the emulsifying capacity of the soluble protein (Pankey *et al.*, 1965).

2.3.3 Water holding capacity

During meat processing, one common problem is water loss, which is frequently expressed as drip loss, expressible water, cook loss, and cooling loss depending upon the stage during processing in which it was measured (Grau, 1956). The ability of meat or meat products to hold all or part of its own and/or added water is called Water holding capacity (WHC). This ability depends on the method of handling and the state of the system. As the state of meat and its treatment differ considerably the meaning of WHC varies to a large extent (Honikel, 2004). Obviously, any loss of water reduces the weight of the product, which implies financial loss. It also has a great impact on quality attributes such as juiciness and tenderness (Bertram *et al.*, 2000).

According to Guerra *et al.* (2011), WHC increases with increase in fat percentage up to 30% in goat mortadella with pork fat which was due to the increased availability of free molecules or radicals to make links with protein or water. (Gläser *et al.*, 2004).

A number of intrinsic and extrinsic factors affect the WHC of meat and meat products. Among the intrinsic factors, genotype and feeding of animals are the most important ones, which affect muscle characteristics directly. For the extrinsic factors, treatments prior to slaughter such as fasting, epinephrine injection, and stunning, may also affect WHC of meat.

Such treatments are likely to influence WHC through stress, which decreases muscular glycogen reserves, a process which may lead to high ultimate pH and low water content of meat (Micklander *et al.*, 2005). Also, post-slaughter treatments like chilling, ageing, injecting nonmeat ingredients, as well as tumbling have been shown to affect the WHC of meat (Schäfer *et al.*, 2002).

2.4 Lipid oxidation in meat

Lipids are one of the most chemically unstable food components that participate in oxidative reactions induced by several factors through quite complex mechanisms (Min and Ahn, 2005). Major cause of deterioration of fatty tissues in meat is due to lipid oxidation. It is a spontaneous and inevitable process that directly affects meat commercial value and products (de Lima Júnior *et al.*, 2013). Lipid oxidation can be defined as a chain reaction of free radicals that consists of three stages: initiation, propagation, and termination. In the course of the reaction, there is a free radical that reacts with the hydrocarbon chain of the fatty acid forming peroxides, which, in turn, react with other hydrocarbon chains abstracting hydrogens originating hydroperoxides. The carbon chain, from which the hydrogens have been abstracted, will act as new peroxide, perpetuating the cycle (Estévez, 2015).

Natural components found in muscle tissue such as iron, myoglobin (Mb), hydrogen peroxide (H_2O_2) and ascorbic acid can cause lipid oxidation, acting as catalysts or promoting the formation of reactive oxygen species (ROS). Oxidative reactions can also be initiated by physical factors such as radiation and light. Therefore, in biological systems, lipids undergo oxidation via three main reactions: photo-oxidation, enzymatic oxidation, and autoxidation (Wójciak and Dolatowski, 2012).

Free radicals are highly reactive species that have one or more free electrons, which can exist independently for a short period. Some examples of these reactive oxygen molecules are: hydroxyl radical (HO•), organic compound oxygen radicals, peroxyl (ROO•) and alkoxyl (RO•) radicals, superoxide radical (O_2), its radical conjugate hydroperoxide acid ($HO•_2$) and singlet oxygen (O^1_2). These reactive oxygen molecules can be produced intentionally or accidentally. In biological systems, they are produced during the normal aerobic metabolism. Mitochondria consume molecular oxygen reducing it by sequential steps to produce ATP and H_2O. During this process, O^1_2, H_2O_2, HO• are formed as unwanted

by-products. Meanwhile, the cells that protect the body (phagocytes) deliberately generate O^1_2 and H_2O_2 to inactivate bacteria and viruses (de Lima Júnior *et al.*, 2013).

2.4.1 Antioxidants

Any substance that, when present in a low concentration compared to the concentration of the substrate, considerably slows down or inhibits the oxidation of an oxidizable substrate is known as an antioxidant. They may either delay or inhibit the initiation step by reacting with a lipid radical or inhibit the propagation step by reacting with peroxyl or alkoxyl radicals (Madhavi *et al.*, 1995).

$$LOO\bullet + AH \rightarrow LOOH + A\bullet$$

$$LO\bullet + AH \rightarrow LOH + A\bullet$$

The antioxidant free radical may further interfere with chain-propagation reactions by forming peroxyl antioxidant compounds:

$$A\bullet + LOO\bullet \rightarrow LOOA$$

$$A\bullet + LO\bullet \rightarrow LOA$$

The activation energy of the above reaction increases with the increase of the dissociation energy of AH and L-H bonds. Therefore, the effectiveness of antioxidants increases as the binding strength of AH decreases (Antolovich *et al.*, 2002). Antioxidants are substances that generally prevent or delay the onset of rancidity in food products due to the oxidation of unsaturated fatty acids present in food products. The use of antioxidants extends the shelf life of foods, minimizes waste and nutrient loss, and expands the range of uses of different oils/fats (Bhattacharya, 2003).

2.4.1.1 Butylated hydroxytoluene (BHT)

BHT is a synthetic antioxidant that has been a widely used fat-soluble food preservative since 1947, with extensive biological activities. It prevents deterioration by reacting with oxygen, delays development of off-flavors, odors, and color changes caused by oxidation. It protects against animal agonistic radiation and the acute toxicity of various xenobiotics and mutagens. It is a mixture of two isomeric organic compounds, 2-tert-butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole.

BHT prevents spoilage of foods to which it is added. BHT has become very popular among food processors and is now used in a wide variety of products including breakfast cereals, gum, dried potato flakes, fortified rice, French fries, candy, sausage, freeze-dried meat and other foods that contain fat and oil (Manandhar, 2018).

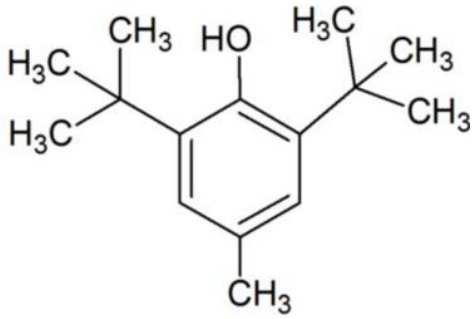

Fig 2.1 Chemical structure of BHT

Source: Karol *et al.* (2017)

2.4.1.2 Natural antioxidant

Natural antioxidants are extracted from medicinal plants and are widely used in foods. These natural antioxidants, particularly polyphenols and carotenoids, have a wide range of biological effects, including anti-inflammatory, anti-aging, anti-atherosclerosis, and anti-cancer. Effective extraction and proper evaluation of antioxidants from foods and medicinal plants are critical to explore the potential sources of antioxidants and promoting their use in functional foods, pharmaceuticals, and food additives. (Xu *et al.*, 2017).

Natural antioxidants can be used in a number of applications even when no choice is left due to company guidelines or food laws and public interest groups. There are many scientific evidences that support the use of natural antioxidants. The antioxidant activity from natural sources has been demonstrated in spices (Chang *et al.*, 1977); Spice extracts and vegetable proteins and their hydrolysates (Manandhar, 2018). The most commonly used natural antioxidants are not exactly natural, but are identical to nature. This means that their structure is identical to that of natural products, but that they were produced synthetically. Like other synthetic antioxidants, they are supplied in a relatively pure state. Tocopherol, Ascorbic Acid, and Citric Acid belong to this group. From the point of view of preparation for use,

they can be viewed as pure synthetic substances that do not require any pre-treatment (Pokorný and Korczak, 2001).

2.5 DPPH radical scavenging assay

DPPH assay measures the ability of a substance to scavenge the DPPH radical, reducing it to hydrazine. When a substance that acts as a donor of hydrogen atoms is added to a solution of DPPH, hydrazine is obtained, with a change in color from violet to pale yellow (Formagio *et al.*, 2014). The DPPH• test is based on the ability of the stable 2, 2-diphenyl1-picrylhydrazyl free radical to react with hydrogen donors. The DPPH• radical displays an intense UV-VIS absorption spectrum. In this test, a solution of radical is decolorized after reduction with an antioxidant (AH) or a radical (R•) in accordance with the following scheme:

DPPH• + AH → DPPH• - H + A•

DPPH• + R• → DPPH• - R

The DPPH• radical is one of the few stable organic nitrogen radicals, which bears a deep purple color. It is commercially available and does not have to be generated before assay. This assay is based on the measurement of the reducing ability of antioxidants toward DPPH•. The DPPH assay is considered to be mainly based on an electron transfer (ET) reaction, and hydrogen-atom abstraction is a marginal reaction pathway. The test is simple and rapid and needs only a UV-vis spectrophotometer to perform, which probably explains its widespread use in antioxidant screening (Prior *et al.*, 2005; Sochor *et al.*, 2010).

2.5.1 IC^{50} (Inhibitory concentration)

In comparing endogenous and exogenous antagonist inhibitors (i.e., small molecules, drugs, or ligands) to a single enzyme, the concept of the half maximal inhibitory concentration (IC^{50}) is extensively used in the pharmaceutical world as a measure of the effectiveness in inhibiting biological or biochemical functions. The IC^{50} value indicates the concentration of the inhibitor which is required to inhibit a given biological or biochemical function by half. In other words, large IC^{50} values denote inhibitors that interact less effectively with an enzyme than inhibitors that have small IC^{50} values (Neubig *et al.*, 2003).

Different mathematical modeling techniques of the raw data (i.e., rate of production of product or velocity) will have a significant effect on the IC^{50} values. The most mathematically accurate manner to fit the raw data is to perform a nonlinear regression analysis (Cornish-Bowden, 1995). It is obvious that the results are dependent upon the correct model being chosen to emulate the experimental data (Segel, 1975).

Sample size that can lower the initial absorbance of DPPH solution by 50% (EC^{50} or IC^{50}) is chosen as the endpoint for measuring antioxidant activity. This change is compared with changed induced by ascorbic acid equivalent. Lower IC^{50}, higher the antioxidant activity. Extract with IC^{50} values in the range 10-50µg/ml can be considered to exhibit strong antioxidant activity. Extract with IC^{50} values in the range 50-100µg/ml and > 100µg/ml can be considered possessing intermediate and weak in antioxidant activity respectively (Phongpaichit *et al.,* 2007).

2.6 Phytochemicals

As the prefix "Phyto" in the name implies, phytochemicals are those chemicals which are produced only by plants. It consists of a large class of natural non-nutritive, biologically active compounds found in plants. Phytochemicals are constitutive metabolites that enable plants to overcome temporary or continuous threats integral to their environment, while also controlling essential functions of growth and reproduction (Molyneux *et al.*, 2007). They serve as defensive barrier against bacteria, fungi and viruses and also provide unique color, aroma and taste to the host plant (Ramanthan *et al.,* 1989). Phytochemicals give hot pepper the burning sensation, onions and garlic the pungent flavor and tomatoes their red color (Lesschaeve and Noble, 2005). Some other good food sources of phytochemicals are cabbage, lettuce, tomatoes, carrots, watermelon, mangoes, papaya, grapes, oranges, apples, cashew apples and nuts, mustard, pears, oats (Obeta, 2015).

Various types of phytochemicals are known and still new compounds are in the process of discovery (Rowland, 1999). Some of the known and important phytochemicals are alkaloids, saponins, flavonoids, tannins, polyphenols, steroids, terpenoids, etc. They not only protect the host plant but also have various physiological benefits to consumers such as cancer prevention, antibacterial, antifungal, antioxidant, hormone action, enzyme stimulation, etc. (Doss and Anand, 2012). According to Birt (2006), phytochemicals work

in synergy and their effects when served together are stronger than the sum of the effects of parts served separately.

2.6.1 Polyphenols

Polyphenols are a family of natural compounds widely distributed in the outer layers of plant that have protective function in the plants. It includes more than 8,000 compounds (Manach *et al.*, 2004). They range from simple molecules such as phenolic acids to highly polymeric compounds such as tannins. Phenolic acid represents about one-third of the total amount of polyphenols in the human diet. These compounds have the ability to reduce free radicals, catalyze metal chelating; activating antioxidant enzymes, reducing α-tocopherol radicals and inhibiting oxidation (Oboh, 2006). They also contribute to unique taste, flavor, and health promoting properties (Ali and Neda, 2011). Polyphenols are the most abundant antioxidants in the diet and are widespread constituents of fruits, vegetables, cereals, dry legumes, chocolate, and beverages, such as tea, coffee, or wine (Scalbert *et al.*, 2005).

Phenolic compounds are good electron donors because their hydroxyl groups can directly contribute to antioxidant action. According to various literature, phenolic compounds exhibit free radical inhibition, peroxide decomposition, metal inactivation or oxygen scavenging in biological systems and prevent oxidative disease burden (Babbar *et al.*, 2015). Structurally, they contain aromatic ring containing one or more hydroxyl groups (O'connell and Fox, 2001). The antioxidant capacity of polyphenols in any diet is much higher than the combined antioxidant effect of beta-carotene, vitamins A and E in the same diet (Gülçin *et al.*, 2004). The total intake of polyphenols in a person's diet is 1 gram per day, and the most common combined dietary intake of β-carotene, vitamin C and vitamin E is about 100 mg per day (King and Young, 1999).

2.6.1.1 Significance of phenolic compound

Phenolic compounds play various role in plants, few of which can be listed below:

1. As antioxidant compounds: The primary and most important function of phenol is as an antioxidant. They act as free radical scavengers, which are formed as a result of excessive UV radiation.

2. As structural polymers: Lignin is the most important and widely distributed phenolic compound that serves as a plant structural unit.
3. As defensive compounds: Plants develop an astringent taste due to the presence of tannins. Tannins interact with and precipitate proteins, resulting in the bitter taste of plants. As a result, in the majority of cases, they act as a feed deterrent.
4. As signal molecules: Phenolic compounds serve as signal molecules in many biochemical metabolic pathways. In the salicylic acid pathway, for example, methyl salicylate (a phenolic compound) acts as a signaling compound. Dehydrodiconiferyl alcohol glucosidase has also been identified as a phenolic signaling compound.
5. As pollinator attractants: Simple phenolic acids with low molecular weight are responsible for flower aroma and attractive coloration, which attract pollinators.
6. As a UV screen: The phenolics present in the plant cuticle play an important role in reducing the amount of UV radiation that reaches the earth through the ozone layer.

Source: Manandhar (2018)

2.6.2 Flavonoids

Flavonoids are a large family of hydroxylated polyphenolic compounds having a benzo-γ-pyrone structure and are present in plants (Kumar and Pandey, 2013; Pande, 2019). Some of the most widely studied of these compounds include flavanols, quercetin, anthocyanin, myricetin and kaempferol. It is estimated that even in an industrial society, the intake of flavonoid may be as high as 1 gram per day (Gee and Johnson, 2001). Flavonoid polymer is also called pro-anthocyanidin. Flavonoids exist as secondary plant metabolites and acts as pigmentation agents, antioxidants, antibacterial agents, anti-stress agents and UV protectants (Vaya and Aviram, 2001). So far, more than 4000 flavonoids have been described, of which many are found in plant parts commonly eaten by humans (Ghasemzadeh *et al.*, 2010). Antioxidant activity of flavonoids is believed to be due to their ability to act as free radical acceptor and to complex metal ions (Hertog *et al.*, 1992). They are biologically active against liver toxins, tumors, viruses and other microbes, allergies and inflammation (De *et al.*, 1999).

Table 2.1: Some important flavonoids and their functions

S. N.	Flavonoids	Functions
1	Hesperidin	Raises blood level of the "good cholesterol and lowers blood level of the "bad" cholesterol
		Prevents inflammation and relieves pains
		Can prevent incidence of head and neck cancers
2	Quercetin	Protects the lungs from harmful effects of pollutants and cigarette smoke
3	Tangeretin	Induces cell death in cancer cells (Leukemia) but promotes the life of normal healthy cells
4	Resveratrol	May reduce the risks of heart diseases, stroke and blood clots.
5	Flavanols (Anthocyanins)	Act as potent antioxidant
		Helps to improve balanced coordination and short- term memory in the elderly
6	Anthocyanins	Helps to prevent urinary tract infection.

Source: Hertog *et al.* (1992)

2.6.2.1 Biological activity of flavonoids

Flavonoids have recently attracted people's attention due to their extensive biological and pharmacological activities. According to reports, they have a variety of biological properties, including antibacterial, cytotoxic, anti-inflammatory and anti-tumor activities, but the best descriptive property of almost every group of flavonoids is their ability to act as powerful antioxidants, protecting the human body from foreign materials (Ramos, 2008). The ability of flavonoids as antioxidants depends on their molecular structure such that the position and other characteristics of the hydroxyl group in the chemical structure of flavonoids are

important for their antioxidant and free radical scavenging activities (Heim *et al.*, 2002). The β ring hydroxyl configuration is the most significant determinant of scavenging of ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species) because it donates hydrogen and an electron to hydroxyl, peroxyl, and peroxy-nitrite radicals, stabilizing them and giving rise to a relatively stable flavonoids radical (Kumari and Jain, 2015).

2.6.2.2 Significance of flavonoids

The actions done by flavonoids may include:

1. Suppression of ROS formation either by inhibition of enzymes, namely H. Microsomal monooxygenase, glutathione transferase, mitochondrial succinate oxidase, NADH oxidase, etc. or by chelating trace elements involved in free radical generation
2. Scavenging ROS
3. Regulation or protection of antioxidant defenses.

Some of the effects mediated by them may be the combined result of free radical scavenging activity and interaction with enzyme functions (Lewandowska *et al.*, 2016).

2.7 Storage stability

Consumers are continuously expecting new and high-quality food products that remain in good quality with their corresponding sensory and nutritional properties during the period between of their purchase and consumption. Manufactures have different methods to predict and calculate the end point of storage life under a given set of storage conditions. Criteria based on the measured numbers of spoilage and pathogenic microorganisms and their growth pattern are capable of relatively clear definition. Non-microbiological criteria are more difficult to define, although criteria based on well-defined chemical composition, such as pH, peroxide value, acid value, vitamin content, are addressable (Kilcast and Subramaniam, 2000).

2.7.1 Peroxide value

Peroxide serves as a useful indicator for the degree of oxidation of lipids, fats, and oils. The peroxide value shows the extinct of peroxidation and measures the amount of total peroxides in the substance (Kouba and Mourot, 2011). The double bonds found in fats and oils play a

role in autoxidation. Oils with a high degree of unsaturation are most susceptible to autoxidation. The best test for autoxidation (oxidative rancidity) is determination of the peroxide value. Peroxides are intermediates in the autoxidation reaction. Autoxidation is a free radical reaction involving oxygen that leads to deterioration of fats and oils which form off-flavors and off-odors. Peroxide value, concentration of peroxide in an oil or fat, is useful for assessing the extent to which spoilage has advanced (Chakrabarty, 2003).

2.7.2 Microbial spoilage

Usually, meat gets contaminated because of improper handling, open transportation practice, unhygienic cutting tools, and can cause various food–borne illnesses (Koirala *et al.*, 2020). The shelf-life of products is strongly related to their deterioration, creating a line between an acceptable and unacceptable bacterial concentration, which determines off-odors, off-flavors, and an undesirable appearance. These sensorial changes are related to the number and type of microorganisms that are initially present, as well as their subsequent growth (Ray and Bhunia, 2001). The total viable count and coliform count of ready to eat meat products should not be $\geq 10^7$ cfu/g (FSSAI) and ≥100 cfu/g (ICMSF), respectively (Ockerman and Pellegrino, 1988)

There may be variation in microbiological level due to sanitation, processing, storage, moisture and temperature conditions. The large number of thermophilic bacteria remaining in the product after processing would not be expected to increase during storage of the 16% moisture product, but may become inoculums when mixed with other foods of higher moisture content (Ockerman and Pellegrino, 1988).

Part III

Materials and methods

3.1 Materials

3.1.1 Raw materials

Deboned round muscle meat of buffalo and other ingredients such as soyabean flour, sugar, salt, pork fat, monosodium glutamate (MSG), white pepper powder, garlic powder, nutmeg, ginger and turmeric powder were purchased from the local market of Dharan sub-metropolitan city, Sunsari, Nepal.

3.1.2 Chemical and apparatus required

All the chemicals, laboratory glassware and equipment used for study were lab grade quality and collected form CCT laboratory. The major apparatus and chemicals required are listed in Appendix A.

3.2 Methods

3.2.1 Preparation of spice powder

Ginger were washed, peeled, cut into small pieces and are dried on cabinet dryer at 60℃ until moisture content is less than 10%. After drying, ginger cuts were crushed into powder for extraction. Turmeric powder and Garlic powder were brought and subjected for extraction.

3.2.2 Ethanolic extraction of Spice

The extraction was carried out according to the method described by Fatope *et al.* (1993) with slight modifications:

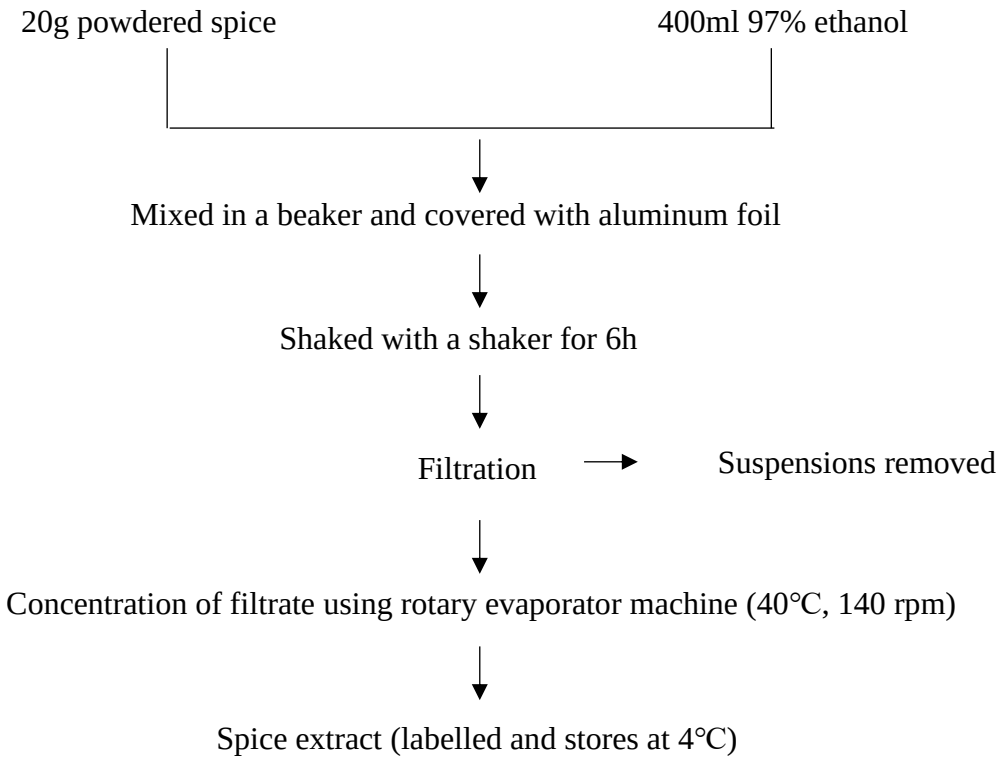

Fig 3.1: Preparation of ethanolic extract of spice

Source: Fatope *et al.* (1993)

Table 3.1: Formulation of experimental buffalo meat patties (% weights)

Ingredients (%)	Treatment				
	T1	T2	T3	T4	T5
Round muscle	65	65	65	65	65
Pork fat	10	10	10	10	10
Binder (Soyabean flour)	7	7	7	7	7
Water/ice	10	10	10	10	10
Sugar (powder)	1	1	1	1	1
Salt	2	2	2	2	2
*Spices	3.5	3.5	3.5	3.5	3.5
Monosodium glutamate	1.5	1.5	1.5	1.5	1.5
Garlic extract	-	0.05	-	-	-
Ginger extract	-	-	0.05	-	-
Turmeric extract	-	-	-	0.05	-
BHT	-	-	-	-	0.05
Total	100	100	100	100	100

*Spices= 50% white pepper powder + 30% garlic powder + 20% powdered nutmeg

Source: Moretto *et al.* (2020)

Process flow diagram

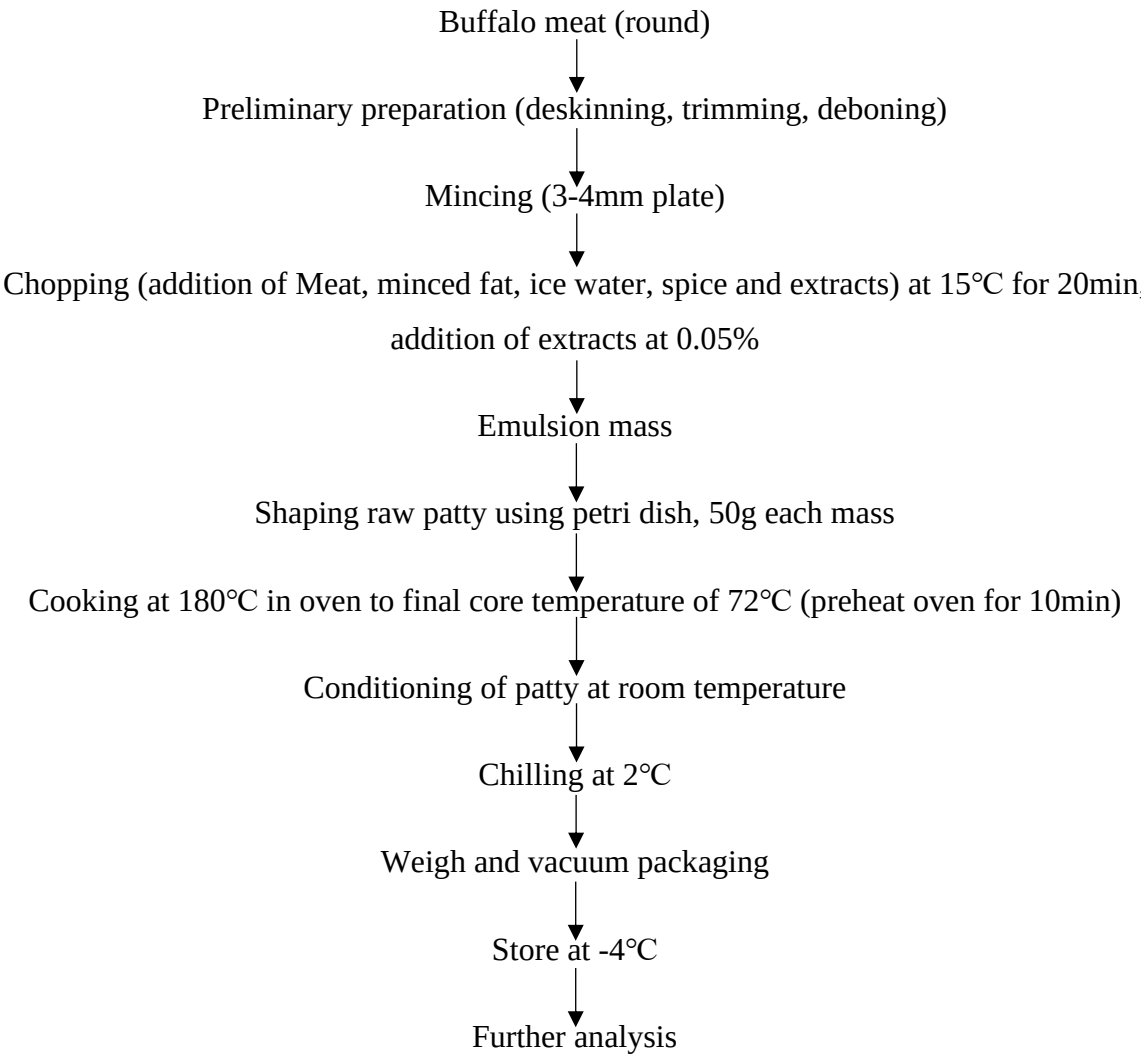

Fig 3.2: Preparation of buffalo meat patty

Source: Moretto *et al.* (2020)

3.2.3 Physicochemical analysis

3.2.3.1 Emulsion stability

The emulsion stability of meat batter was determined by assessing water and lipid loss using method given by Choi *et al.* (2009) with slight modifications. Absorbent cotton was placed at the bottom of a tube to absorb water and oil loss from the meat batter during cooking. Batter (3±0.5g) was stuffed onto the mesh. The tube was loosely closed to avoid the possible effect of vapor pressure, then cooked to a core temperature of 73℃ for 60s using water bath

at 85℃. Subsequently, the tube was cooled to room temperature (20±2℃) for 12 h. The weight of cotton- absorbing water and lipid loss were then determined. To determine the water content, cotton was dried in a drying oven at 105℃ for 3hr. The water and lipid loss from the batter was calculated as percentage using the following equations:

$$\text{Water loss (\%)} = \frac{\text{A-C}}{\text{Weight of batter (g)}} \times 100$$

$$\text{Lipid loss (\%)} = \frac{\text{A-B}}{\text{Weight of batter (g)}} \times 100\text{-water loss (\%)}$$

Where, A is the weight of the cotton after cooking, B is the weight of cotton before cooking and C is the weight of cotton after drying

3.2.3.2 Determination of cooking loss

For determination of cooking loss, a meat sample of 1 cm^3 was cut from different areas of round meat of buffalo. After weighing, the sample was heat treated in a water bath at 85℃ for 10 min resulting in a core temperature of approximately 75℃. Subsequently the meat sample was lightly dabbed and weighed after cooking. The cooking loss was calculated according to Bertram *et al.* (2003) as:

$$\text{\% Cooking loss} = \frac{\text{a-b}}{\text{a}} \times 100$$

Where, a = Weight of meat sample before cooking and b = Weight of meat sample after cooking

3.2.3.3 Procedure for WHC determination

300mg of cooked meat was weighed on Whatman no. 1 filter paper and covered with another filter paper and pressed between two Plexiglas plates and left for 5 min. The sample was then oven dried at 65℃ for 48h to determine for moisture content. The amount of water released from the sample was measured indirectly by measuring the area of the filter paper wetted relative to the area of pressed sample (Grau and Ludorff, 1944).

$$\text{WHC} = \left(\frac{A_m}{A_t}\right) \times 100$$

Where, A_m = Area of meat sample (cm^2) and A_t = Total area covered by meat sample and water released from meat (cm^2)

3.2.3.4 pH

10gm meat sample was taken and 90ml of distilled water was added, it was grinded and mixed to make homogeneous solution. After calibrating pH meter with buffer of pH 4 and pH 7, pH of sample was measured and recorded after stable reading appeared in pH meter (Korkeala *et al.*, 1986).

3.2.4 Extraction of phytochemicals for analysis

The extraction was performed in 15ml test tube by adding 5ml of methanol to 0.25g of patty sample and vigorously agitated in a vortex mixer for 20min at room temperature. Afterwards the mixture was centrifuged at 3000rpm for 10min to separate the supernatant from the solid residuals. Two more washing were done by resuspending the residue in 5ml methanol and centrifuging for 3min (for additional washing). The methanol extracts were pooled and filtered through Buchner funnel with fritted glass (16-40µm pore size) and volume made up in 25ml graduated measuring cylinder with methanol. The extracts were used for the determination of TPC, TFC and DPPH radicals scavenging activity (Khajehei *et al.*, 2018).

3.2.4.1 Total phenolic content (TPC)

3.2.4.1.1 Preparation of gallic acid (3,5,7-trihydroxybenzoic acid) standard

Gallic acid stock (100ml) was prepared according to the guideline given by (Waterhouse, 2005) in 10% ethanolic distilled water to give gallic acid concentration 5g/L (0.5%). Gallic acid was first dissolved in 10ml of ethanol before adding water to make 100ml. This was thought necessary because gallic acid is more soluble in ethanol compared to that of water (Daneshfar *et al.*, 2008).

3.2.4.1.2 Preparation of reaction mixture

Before preparing reaction of standard, it is usual to prepare appropriate dilutions separately so that same volume (for example, 1ml) of standard can be used in the final mixture. Alternatively, a back calculation can also be performed (without performing separate

dilutions) so that volume of the stock solution can be varied to get the same amount of standard in the final mixture. Accordingly, following scheme was used to prepare series containing 0.25, 0.5, 1.25 and 2.5mg of gallic acid in a constant volume of 50ml. The rows in the first column also indicate the sequence/order of reagent addition.

3.2.4.1.3 Spectrophotometric determination

First the spectrophotometer was left turned on for stabilization. Spectrophotometric measurement (absorbance at 765nm) of gallic acid standard and the sample extracts were carried out after setting the absorbance of the blank to zero. A standard curve was drawn using a gallic acid solution as the reference standard. TPC was calculated using the regression line and result expressed as milligram gallic acid equivalent per 100g of dry weight (mg GAE/100g, dw).

Determination of total polyphenol content (TPC)

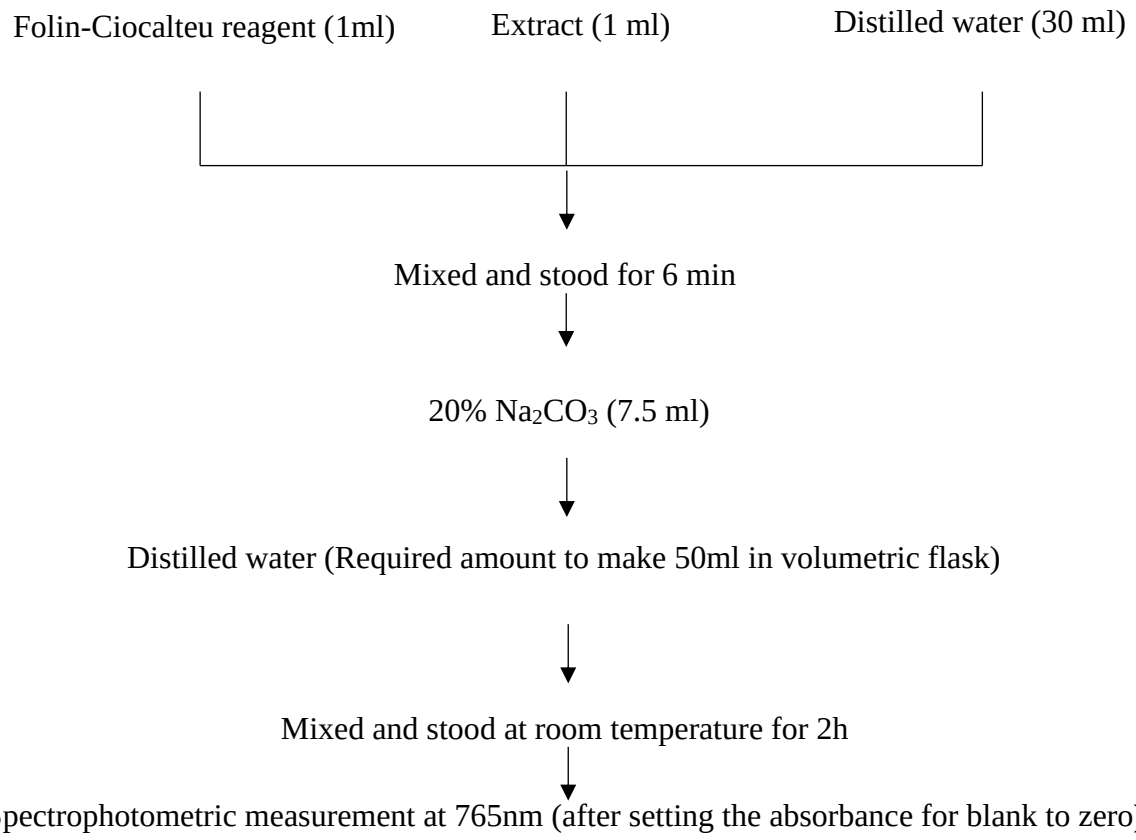

Fig 3.3: Flowchart for the determination of total polyphenol content (TPC)

Source: Singleton *et al.* (1999)

3.2.4.2 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacity

Briefly, 2ml of the methanolic extract (or the standard) as prepared above was mixed with 2ml of DPPH (0.004%) in methanol, corresponding to 100µM in test tube and incubated at 37°C in dark (wrapped with aluminum foil) for 20min (for completion of reaction) before spectrophotometric analysis. Absorbance was measured at 517nm for sample as well as the standard after setting the absorbance to zero for the blank. Ascorbic acid (50µM stock) was used as the standard, the absorbance data of which were used for drawing the reference curve for calculating the IC^{50} and RSA. The RSA was expressed as mg ascorbic acid equivalent per 100g dry weight (mg AAE/100g, dw) (Blois, 1958). Detail of amounts of reagents/standard/extracts used before incubation are given in table below:

Table 3.2: Amount of reagents used for the DPPH assay (for extract analysis)

Reagent	Distribution in cuvette						
	(-) ve control	Blank 1	Extract				
DPPH, ml	2	0	2	2	2	2	2
Extract, ml	0	2	0.4	0.8	1.2	1.6	2
Methanol, ml	2	2	1.6	1.2	0.8	0.4	0
Ascorbic acid, ml	0	0	0	0	0	0	0
Total, ml	4	4	4	4	4	4	4

Source: Blois (1958)

3.2.4.2.1 Preparation of ascorbic acid stock solution

8.8mg ascorbic acid was dissolved in 100ml distilled water to give 500µM stock solution and diluted 10 times (10 ml stock+90ml distilled water) to get a working solution of 50µM.

Table 3.3: Amounts of reagents used for the DPPH assay (for standard curve)

Reagent	Distribution in cuvette				
	Blank 2	(+) ve control/standard			
DPPH, ml	0	2	2	2	2
Extract, ml	0	0	0	0	0
Methanol, ml	2	1.5	1	0.5	0
Ascorbic acid, ml	2	0.5	1	1.5	2
Total, ml	4	4	4	4	4

Source: Blois (1958)

The (-) ve control contains DPPH and solvent only, the (+) ve control contains both ascorbic acid (in graded concentration) and DPPH, Blank 1 and Blank 2 imply blanks for extract and ascorbic acid standard respectively.

3.2.4.2.2 Calculation of DPPH radical scavenging capacity

The DPPH radical scavenging activity (RSA) (also termed inhibition ratio, DPPH inhibition) was calculated using the equation (Kamleshiya *et al.*, 2012):

$$\text{RSA } (\%) = \frac{A_c - A_s}{A_c} \times 100$$

Where, A_c = Absorbance of the negative control (DPPH without sample), A_s = Absorbance of the test sample (test sample + DPPH) and A_1 = Absorbance of the blank (Sample without DPPH)

3.2.4.2.3 Determination of DPPH radical scavenging capacity in terms of IC^{50}

According to Van De Velde *et al.* (2013), a series of dilutions of extract were used and absorbance recorded for IC^{50} calculation. The IC^{50} of ascorbic acid was similarly determined

and ascorbic acid equivalent antioxidant capacity (AEAC) of the extract was calculated. The calculation can be done in a computer (using Microsoft Excel). Inhibition ratio (y) were plotted against the sample concentrations (x) and a regression line was drawn.

The IC^{50} of ascorbic acid can be easily calculated using the trend line equation by solving for x after putting the value of y = 50.

3.2.5 Determination of total flavonoid content (TFC)

According to Pękal and Pyrzynska (2014), 1ml of the methanolic extract was mixed with 0.3ml of $NaNO_3$ (3% v/v) and after 5min, 0.5ml of $AlCl_3$ (2%, w/v) was added. The sample was mixed and 6 min later, neutralized with 0.5ml of 1M NaOH solution. The mixture was left for 10min at room temperature and then absorbance was taken at 510nm against a blank where the sample was substituted by distilled water. Quercetin prepared in methanol at a concentration of 100µg/ml was used as the standard.

3.2.5.1 Preparation of standard solution

10mg of quercetin was accurately weighed into a 10ml volumetric flask and dissolved in 10ml methanol and solution was made up to 10ml with same solvent.

1ml of standard or extract solution (20, 40, 60, 80, 100 mg/l) was taken into 10ml volumetric flask containing 4 ml of distilled water. 0.3 ml of 5% $NaNO_2$ added to the flask. After 5 min, 0.3ml 10% $AlCl_3$ was added to the mixture. At the 6^{th} min, 2ml of 1M NaOH was added and volume made up to 10ml with distilled water. The absorbance was noted at 510 nm using UV-Visible spectrophotometer (Ribarova *et al.,* 2005).

3.2.6 Storage stability study

The finished product was packed in polypropylene (PP) by vacuum packaging machine. Storage stability of vacuum-packed finished product was conducted for 1 month at -4℃.

3.2.6.1 Peroxide Value

Measurement of peroxide value (PV) was done according to the Sallam *et al.* (2004). The PV was calculated and expressed as milli-equivalent peroxide per kg of sample:

$$PV(Meq/kg) = \frac{S \times N \times 100}{W}$$

Where, S = Volume of titration (ml), N = Normality of sodium thiosulfate solution and W = Weight of sample (kg).

3.2.6.2 pH

10gm patty sample was taken and 90ml of distilled water was added, it was grinded and mixed to make homogeneous solution. After calibrating pH meter, pH was measured and recorded after stable reading appeared in pH meter (Korkeala *et al.*, 1986).

3.2.6.3 Microbial load evaluation

Microbial analysis was performed and analyzed to know the exact viable count of microorganisms in the stored patties prepared aseptically.

3.2.6.3.1 Total Plate Count

Determination of total plate count was performed according to the method prescribed by (Harrigan and McCance, 1976) using plate count agar and distilled water as diluent.

i. 1 ml of sample from 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} dilution was withdrawn with the help of sterile pipette and transferred to each sterile petri-plate.
ii. To each plate, 10 ml of molten Plate Count Agar (PCA) at 45°C was added and the medium and inoculums were mixed immediately by a combination of to-and-fro shaking and circular movements lasting 5-10 s.
iii. The plates were allowed to set and were incubated at 37°C for 24-48 h.

3.3 Statistical analysis

Experiment was conducted in three replications. ANOVA at 5% level of significance was carried out on the obtained data using GenStat 12th Edition. Tukey test was used for the post hoc test. All the referencing of citations was done using Endnote X9 in CDFT format.

Part IV

Results and discussion

Buffalo meat and various ingredients were brought from Dharan market. Dried ginger powder, turmeric powder and garlic powder were used in the extraction of phytochemicals. Effect of phytochemicals on different physicochemical properties (cooking loss, emulsion stability and WHC) and storage stability of the prepared patties were studied for 1 month at -4°C. Patties prepared without addition of spice extracts were used as control and addition of BHT as standard. Also, DPPH radical scavenging capacity, total phenols and total flavonoids of all spices were calculated. Results and discussion of the overall study are described in the following headings.

4.1 Chemical analysis of buffalo meat

Chemical analysis was carried out for freshly obtained buffalo meat and the results found are tabulated in the table 4.1.

Table 4.1 Chemical analysis of buffalo meat

Constituents	Value
Moisture content (%)	75.87±0.51
Crude fat (%, wb)	1.64±0.55
Crude protein (%, wb)	21.61±0.62
Total ash (%, wb)	1.32±0.38
pH	5.66±0.13
WHC (%)	12.1±0.45

*Values are means of triplicate with standard deviation.

Values thus obtained were slightly different from the result obtained as per DFTQC (2017) which were moisture 78.7%, fat 0.9%, protein 19.4% and ash 1%. Value of pH is in normal range (5.63 to 5.76) according to Rasuli *et al.* (2021).Water holding capacity (WHC) of

Murrah buffaloes analyzed by Sahoo and Anjaneyulu (2000) was found to be 16.67 % which is slightly near to the value obtained. Differences in result may be due to different breed, age, feed and slaughter practices (Contò *et al.*, 2022; Kandeepan *et al.*, 2009a).

4.2 Physicochemical analysis of patty during preparation

Table 4.2: Physicochemical analysis of patties during preparation

Ethanolic extract	Cooking loss (%)	Emulsion stability (%)	
		Water loss	Lipid loss
GE	20.24^{a}±0.15	17.64^{a}±0.41	0.32^{a}±0.03
GAE	22.75^{d}±0.07	20.26^{b}±0.17	1.26^{b}±0.1
TE	21.07^{b}±0.08	19.36^{b}±0.19	1.2^{b}±0.05
BHT	22.08^{c}±0.2	18.38^{a}±0.38	1.68^{c}±0.03
Control	25.22^{e}±0.32	19.57^{b}±0.11	2.17^{d}±0.04

*Values are the means of triplicate. Different superscript in column represent significant difference ($P<0.05$) from each other (Appendix C)

Statistical analysis at 5% level of significance shows that the cooking loss is significantly different with the treatments. Significantly highest value was obtained for control patties (25.22^{e}±0.2 %) and lowest was found in ginger extract (GE) incorporated patties (20.24^{a}±0.15). GE incorporated patties also shows highest emulsion stability with lowest water loss (17.64^{a}±0.41 %) and lowest lipid loss (0.32^{a}±0.03 %) among others. The present findings are in consistent with the study, which reported positive effects of plant ingredients on reduction of cooking loss in emulsified meat batters (Kamani *et al.*, 2019). Zahid *et al.* (2020) found slightly similar cooking loss with the use of artificial antioxidant BHT (19.82 %) . Pawar *et al.* (2009) also reported that the 5% ginger rhizome extract (GRE) was found to have significantly ($P < 0.05$) lower cooking loss at every interval of time period and almost all the cooking characteristics of the patties were found to be improved in GRE-treated samples over the untreated one (control).

4.3 Total polyphenol content

Total polyphenol content of methanolic extracts of patties are shown in Table 4.3. The total phenol contents of spice extracts incorporated patties were found to be significantly different ($p < 0.05$). Gallic acid standard curve was used for the determination of phenol (Appendix B).

Table 4.3: Total polyphenol content of methanolic extracts of patties

Methanolic extract of patties	Total Phenolic content (mg GAE/100g)
GE	757.956^{b}±15.295
GAE	743.998^{b}±24.177
TE	618.37^{a}±24.177

*Values are the means of triplicate. Different alphabets in the superscript represent significant difference ($P<0.05$) from each other (Appendix C)

Statistical analysis at 5% level of significance shows that the total phenol content is significantly different with varieties. Significantly highest value was obtained for GE (757.956^{b} mg GAE/100g) and lowest was found in Turmeric extract (TE) (618.37^{a}±24.177 mg GAE/100g). Offei-Oknye *et al.* (2015) found that the total phenolic content for oven dried ginger was 796.46 ± 8.16 mg GAE/100g which was somewhat similar to our findings. Malik (2015) reported phenol content to be 136.82 mg GAE/100g which was slightly lower than the value obtained. Wojdyło *et al.* (2007) reported that content of TPC in turmeric powder was 825.58 mg GAE/100g which is higher than the value obtained. Several studies reported that the differences in polyphenol content could be attributable to biological factors (genotype, cultivars), as well as environmental (temperature, salinity, water stress and light intensity) conditions. Moreover, the extraction of phenolic compounds depends on the type of solvent used, the degree of polymerization of phenolics, and their interaction (Mamun *et al.*, 2016).

4.4 Total flavonoid content

The flavonoid content of methanolic extract of patties are shown in Table 4.4:

Table 4.4: Total flavonoid content of methanolic extract of patties

Methanolic extract of patties	Total flavonoid content (mg QE/100g)
GE	189.123^{c}±0.61
TE	73.469^{b}
GAE	59.184^{a}

*Values are the means of triplicate. Different alphabets in the superscript represent significant difference (P<0.05) from each other (Appendix C)

Statistical analysis at 5% level of significance shows that the total flavonoid content is significantly different with spices. Highest value and lowest value of flavonoid content were obtained for GE (189.123^{c}±0.61 mg QE/100g) and garlic extract (GAE) (59.184^{a} mg QE/100g) respectively. Awan *et al.* (2019) showed similar result of flavonoid (58.45±1.24 mg QE/100g) as the values obtained (59.184^{a} mg QE/100g) for methanolic GAE. Offei-Oknye *et al.* (2015) found higher value of flavonoid for ginger (302.18 ± 6.12 mg QE/100g). Total flavonoid content in methanolic extract of processed turmeric was found to be 37.98 mg/100 g of dry extract according to Chavhan (2017) . Mushtaq *et al.* (2019) also reported even lower (3.88 ± 0.25 mg QE/100g) flavonoid content in turmeric extract. The differences in the result may be due to various factors which need to be considered such as extraction, temperature, extraction time, solid to solvent ratio (Duy *et al.*, 2019).

4.5 DPPH radical scavenging activity

DPPH radical scavenging activity (% RSA) of methanolic extracts of patties are shown below in the scatter diagram fig. 4.1. Ascorbic acid was used as standard for DPPH standard curve.

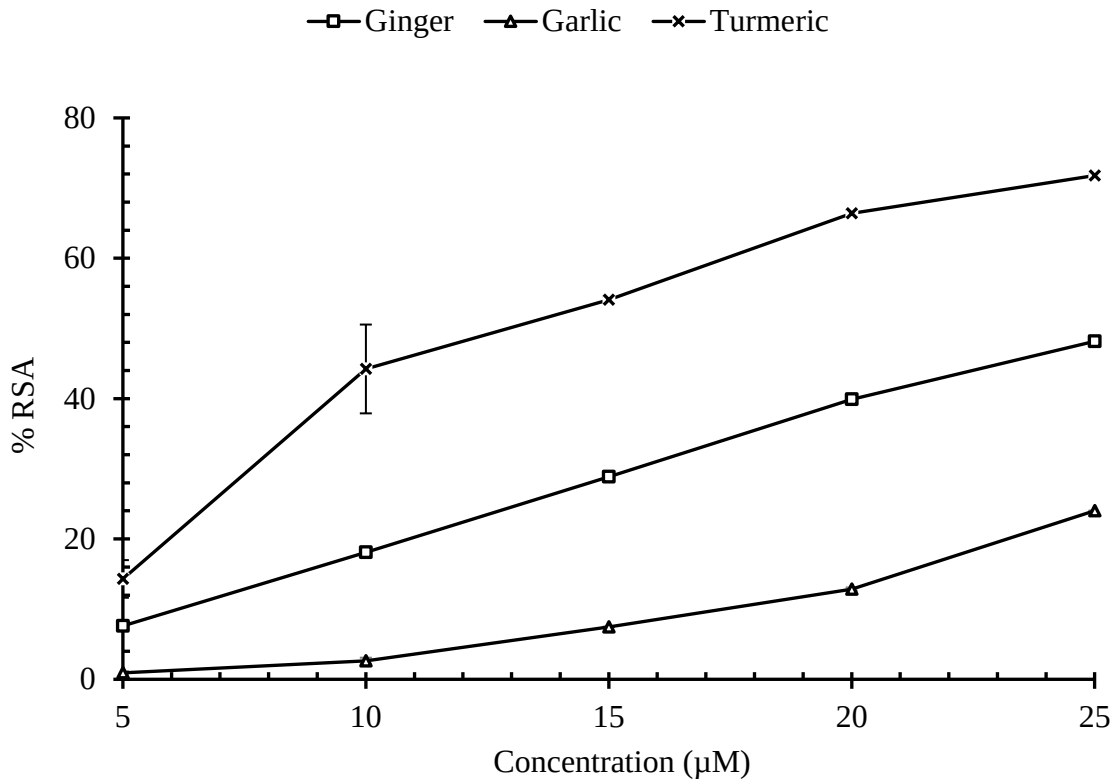

Fig. **4.1** %RSA of methanolic extracts of patties with varying concentration.

The data points are the mean values of triplicate observations. Error bars represent standard deviation from the mean. Significance testing results are presented in Appendix C.

Both extracts addition and its concentration had significant effects on antioxidant activity. The highest value was observed in TE treated patties (14.3045 % at 5 µM and 71.7848 % at 25 µM) whereas the lowest result was found in GAE treated patties (0.9186 % at 5 µM and 24.0157 % at 25 µM).

Statistical analysis at 5% level of significance shows that the DPPH radical scavenging capacity is significantly different with varieties. Antioxidant activity increases with the increase in concentration. This result is supported by the result of Zandi and Gordon (1999), in which old tea leaves extract activity was increasing with concentration in the range 0.02 to 0.25%. Antioxidant activity (%RSA) is in the order of TE>GE>GAE with varying concentrations which are consistent with the result by Odukoya *et al.* (2005); Shobana and Naidu (2000). Offei-Oknye *et al.* (2015) reported (69.09 ± 0.58) % DPPH for oven dried

ginger. Kumar *et al.* (2018) observed that turmeric and ginger have more antioxidant activity than garlic.

IC^{50} of methanolic extracts of patties are shown in Table 4.5. The activity of spices extracts was found to be significantly different ($p < 0.05$).

Table 4.5: IC^{50} of methanolic extracts of patties

Methanolic extract of patties	IC^{50} (µg/ml)
Garlic	50.8144^{c}
Ginger	25.434^{b}
Turmeric	14.9425^{a}

*Values are the means of triplicate. Different alphabets in the superscript represent significant difference ($P<0.05$) from each other (Appendix C)

Statistical analysis at 5% level of significance shows that the total antioxidant content is significantly different with spices. Lowest amount of antioxidant activity (IC^{50}) was found for GAE (50.8144^{c} µg/ml) and highest antioxidant activity (IC^{50}) for TE (14.9425^{a} µg/ml). Kebede *et al.* (2021) found 23.05 µg/ml to be the lowest IC^{50} value among turmeric varieties in Ethiopia and Sabir *et al.* (2020) found the IC^{50} value of ethanolic TE to be 27.2 ± 1.1 µg/ml which were both slightly higher than our findings. The extracts of *Zingiber officinale* and *Zingiber ligulatum* showed moderate antioxidant activity with IC^{50} values of 15.10 ± 2.50 and 15.89 ± 2.92 µg/ml respectively (Phuaklee *et al.*, 2010), which are lower than the value obtained (25.434^{b} µg/ml). IC^{50} of GAE was found to be 50.8144^{c} µg/ml which was slightly lower than the result (64.033 µg/ml) obtained by (Thampi and Jeyadoss, 2015). This is due to plant antioxidant activity varies by spices and differences within the same spices have been discovered depending on the solvent extraction, the physical condition of the spices (fresh or dried), or environmental factors (Kratchanova *et al.*, 2010).

4.6 Storage stability

4.6.1 Change in total peroxide value of buff patty over time

In the five samples of buff patty incorporated with 0.05% BHT, GE, GAE, TE and control (without addition of extracts and BHT), peroxide value was recorded over time. The obtained values are tabulated in Appendix D. The trend of change in peroxide value is presented in fig 4.2

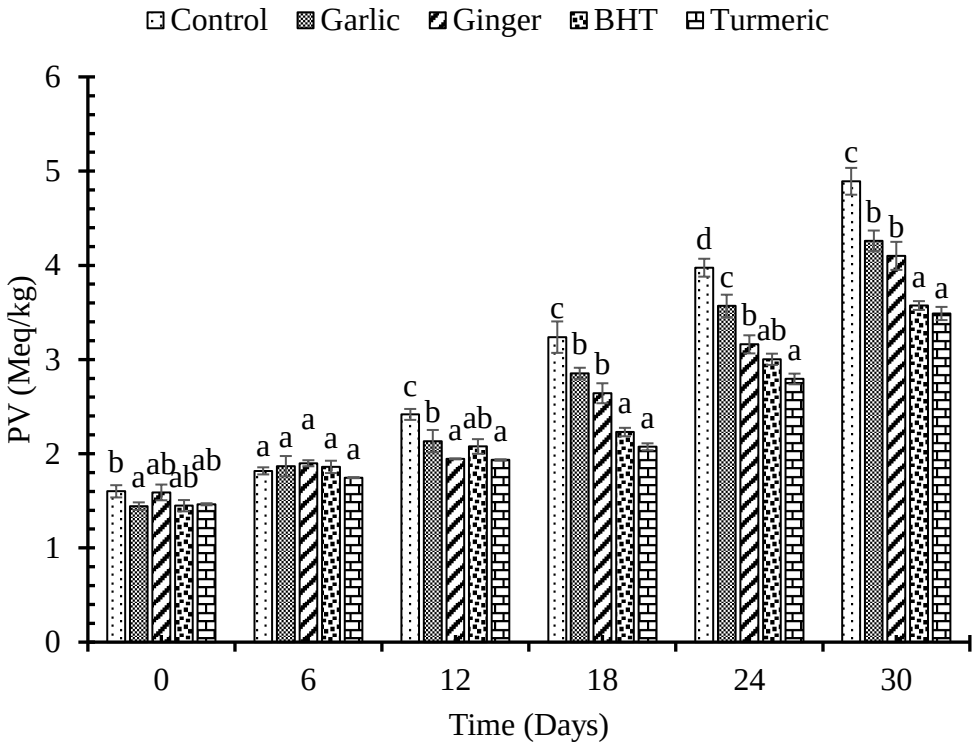

Fig 4.2 Change in peroxide value over time for different samples

The data points are the mean values of triplicate observations. Error bars represent standard deviation from the mean.

At 5% level of significance, there are significant differences between the PV at different days for 30 days for control, GAE, GE, BHT and TE. Peroxide value increased linearly with time (Devatkal and Naveena, 2010). At day 30, control sample showed highest peroxide value (4.892 Meq/kg) followed by GAE (4.261 Meq/kg), GE (4.10033 Meq/kg), BHT (3.573133 Meq/kg) and TE (3.488667 Meq/kg) treated patties. Higher antioxidant effect on peroxide value was observed here. The result obtained was agreed with Disha *et al.* (2020)

findings in which the presence of bioactive compounds exert an antioxidant effect may have caused less increase of peroxide value.

The codex maximum level of acceptability for peroxide value in fatty foods is 10 Meq/kg (Alimentarius, 1999). Thus, it was confirmed that one month is not enough for lipid to oxidize in the spice extract incorporated patty at low temperature due to the inhibiting effect of spices on lipid peroxidation as reported by El-Alim *et al.* (1999).

4.6.2 Estimation of total plate count of buff patty over time

In the five samples of buff patty incorporated with 0.05% BHT, GE, GAE, TE and control (without addition of extracts and BHT), total plate count was recorded over time. The obtained values are tabulated in Appendix E. The trend of change in total plate count is presented in fig 4.3

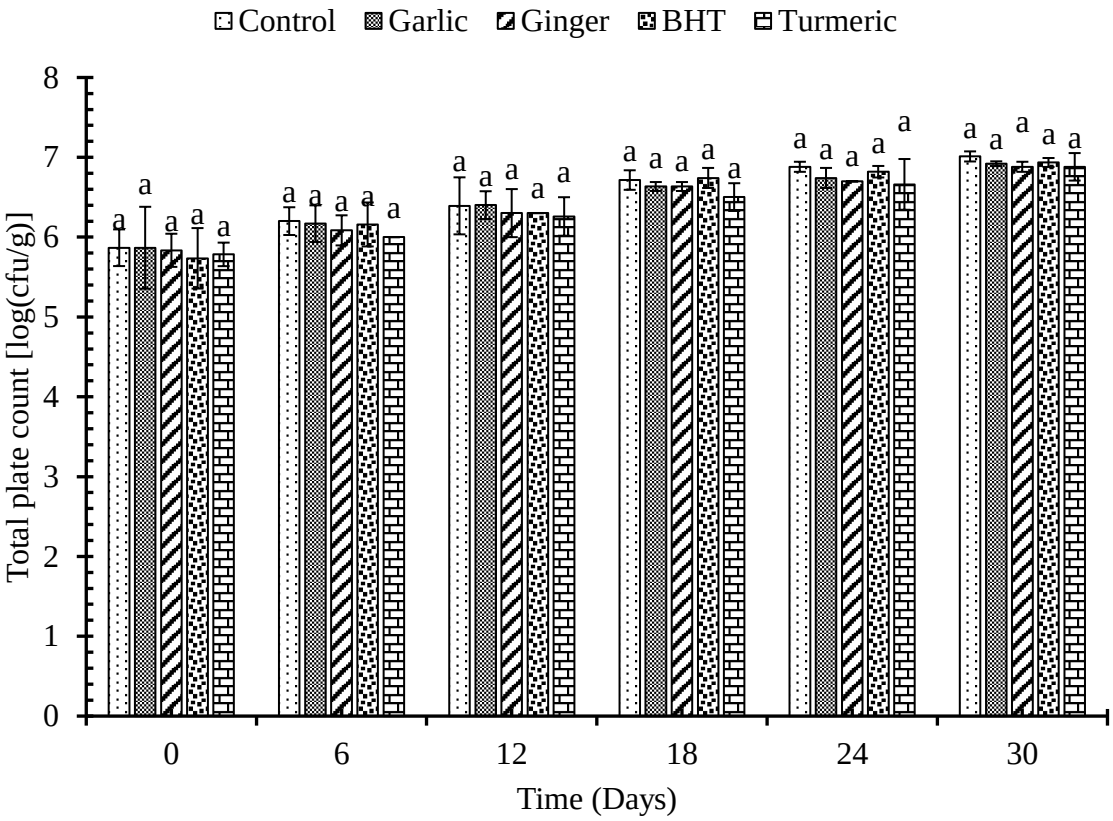

Fig. 4.3 Change in total plate count over time for different samples

The data points are the mean values of triplicate observations. Error bars represent standard deviation from the mean.

At 5% level of significance, both sample and the storage time significantly affected TPC values of ground meat. At day 30, highest and lowest total plate count values were obtained from Control (7.011±0.06 log cfu/g) and TE (6.88±0.17 log cfu/g) treated patties respectively. Only the control sample exceeded 7.0 log cfu/g which is the minimum level for spoilage as described by Osburn and Keeton (1994) whereas other extract treated were under the threshold limit .Thus, use of spice extract increases the shelf-life significantly. These findings were coherent with the result by Kenawi and Petrovic (2012) on spices extract on stability of frozen buffalo meat product.

4.6.3 Change in pH of buff patty over time

In the five samples of buff patty incorporated with 0.05% BHT, GE, GAE, TE and control, pH was recorded over time. The obtained values are tabulated in Appendix F. The trend of change in total plate count is presented in fig 4.4.

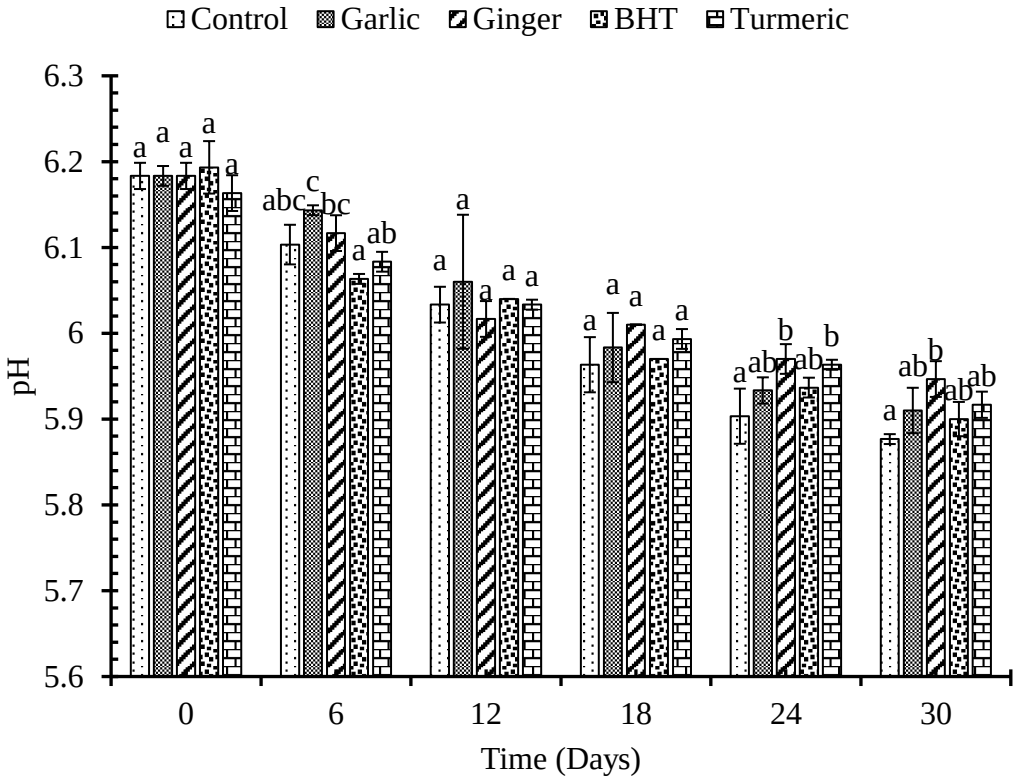

Fig. 4.4 Change in pH over time for different samples

At 5% level of significance, both sample and the storage time significantly affected pH values of buffalo patties. pH values were slightly lowered from 6.18 to 5.88, 6.18 to 5.91, 6.18 to 5.95, 6.19 to 5.9 and 6.16 to 5.92 for control, GAE, GE, BHT and TE treated patties up to day 30. Increase of free fatty acids over time might be the cause of decrease in the pH value. Less decrease of pH in treated samples than untreated samples might be due to the effect of natural antioxidants which retarded the formation of free fatty acids as stated by Serdaroğlu *et al.* (2018) concluded that the acidity and alkalinity of the raw material incorporated in the meat product formulation is crucial for ultimate pH value and thereby functional characteristics of the product.

Part V

Conclusion and recommendations

5.1 Conclusions

Based on the results and discussion, following conclusions can be drawn:

1. Ginger extract treated patties were found to have lowest cooking loss and highest emulsion stability with lowest water and lipid loss.
2. The phenolic content of spice extract incorporated patties was found to be in the order as Ginger>Garlic>Turmeric.
3. The flavonoid content of spice extract incorporated patties was found to be in the order as Ginger>Turmeric> Garlic.
4. It was found that turmeric exhibits the highest antioxidant activity followed by ginger and garlic.
5. Lipid oxidation in terms of peroxide value was found to be the lowest in turmeric extract treated meat patties up to one month in refrigerated storage.
6. Antimicrobial activities were significantly similar to each other with slightly highest in turmeric followed by ginger, garlic, BHT and control meat patties.
7. All spice extract treated patties were found to have less decrease in pH than untreated control sample up to one month with the lowest in ginger followed by turmeric, garlic, BHT and control.

5.2 Recommendations

1. Use of ethanolic extracts of ginger, garlic and turmeric significantly increases phenolic content.
2. Patties can be stored up to one month safely using 0.05 % treated spice extract (ginger, garlic and turmeric).

Summary

Nowadays, Ready-made burger patties are gaining popularity due to the busy schedule of consumers and relying more on fast foods. Modern civilization and imitating western culture can also be the cause. Patties are better source of protein as they are meat products. However, there is a problem of lipid oxidation and microbial spoilage in stored patties. In order to overcome these problems, synthetic antioxidants like BHT, TBHQ, etc. are widely used. But health concerned consumers do not prefer artificial substances and rather consume healthy natural foods such as, spices with natural antioxidant and anti-microbial agent.

Spices viz. ginger, garlic and turmeric were collected and extraction were carried out. 0.05% of each extract were used to prepare different meat patties. During preparation, cooking loss, water loss and lipid loss were analyzed and found to be the lowest in GE incorporated patties (20.24±0.15, 17.64±0.41 and 0.32±0.03 % respectively). The highest amount of total phenol was found in GE treated patties (757.956 mg GAE/100g) and lowest was found in TE treated patties (618.37±24.177 mg GAE/100g). Highest value and lowest value of flavonoid content were obtained for GE treated patties (189.123±0.61 mg QE/100g) and GAE treated patties (59.184 mg QE/100g) respectively. The highest value of antioxidant activity (%RSA) was observed in TE treated patties (14.3045 % at 5 µM and 71.7848 % at 25 µM) whereas the lowest result was found in GAE treated patties (0.9186 % at 5 µM and 24.0157 % at 25 µM) with IC^{50} value of 14.9425 µg/ml and 50.8144 µg/ml respectively.

At day 30, control sample showed highest peroxide value (4.892 Meq/kg) followed by GAE (4.261 Meq/kg), GE (4.10033 Meq/kg), BHT (3.573133 Meq/kg) and TE (3.488667 Meq/kg). At day 30, highest and lowest total plate count values were obtained from control patties (7.011±0.06 log cfu/g) and TE treated patties (6.88±0.17 log cfu/g) respectively. Only the control sample exceeded threshold limit (7.0 log cfu/g). pH values were slightly lowered from 6.18 to 5.88, 6.18 to 5.91, 6.18 to 5.95, 6.19 to 5.9 and 6.16 to 5.92 for control, GAE, GE, BHT and TE treated patties up to day 30.

These results showed that the use of spice extracts could be effective in preventing burger patties against lipid oxidation as well as inhibitory activity towards microorganisms at refrigerated storage. Also, their performance was significantly higher compared to the synthetic antioxidants.

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Appendices

Appendix A

Chemicals:

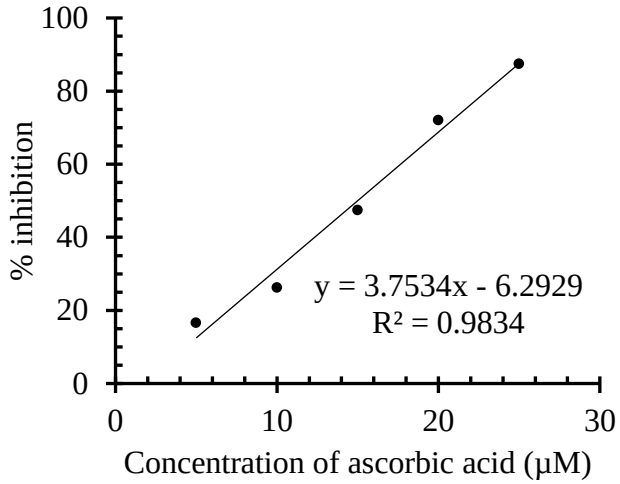
1. Methanol
2. 97% Ethanol
3. Gallic acid
4. Folin-Denis Ciocalteu reagent
5. Sodium carbonate
6. Sulphuric acid
7. Ascorbic acid
8. Aluminum chloride
9. Sodium nitrate
10. Sodium hydroxide
11. Quercetin
12. Acetic acid
13. Chloroform
14. Potassium Iodide
15. Plate count agar

Apparatus:

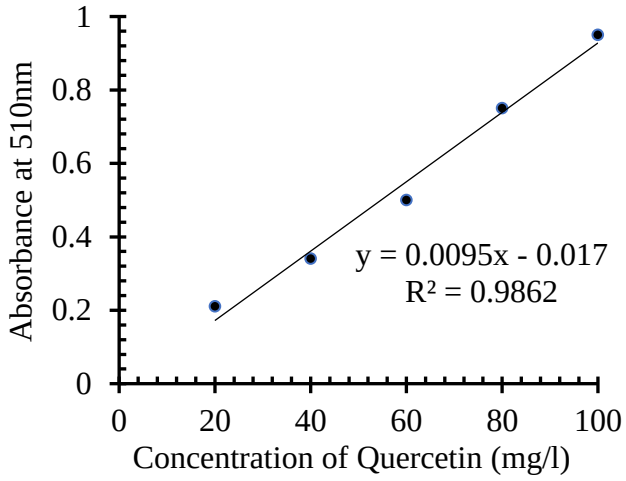
1. Refrigerator
2. Spectrophotometer
3. Glassware
4. Water bath
5. Weighing Balance
6. Mincer
7. Meat chopper
8. Hot air oven
9. Incubator
10. Centrifuge machine
11. Vacuum evaporator
12. Shaker

Appendix B

B.1 Standard curve of Gallic acid



B.2 Standard curve of Quercetin



Appendix C

C.1 Significance test for cooking loss

Dependent variable: cooking loss

Table C.1 Test of between subject effects

Source	DF	Sum of Squares	Mean Square	VR	Prob>F
Sample	4	43.55476	10.88869	204.62	<.001
Residual	10	0.53213	0.05321		
Total	14	44.08689			

Connecting Letters Report

Level						Mean
Ginger	a					20.24
Turmeric		b				21.07
BHT			c			22.08
Garlic				d		22.75
Control					e	25.22

*Levels not connected by same letter are significantly different.

C.2 Significance test for lipid loss

Dependent variable: lipid loss

Table C.2 Test of between subject effects

Source	DF	Sum of Squares	Mean Square	VR	Prob>F
Sample	4	5.637027	1.409257	281.10	<.001
Residual	10	0.050133	0.005013		
Total	14	5.687160			

Connecting Letters Report

Level					Mean
Ginger	a				0.317
Turmeric		b			1.200
Garlic		b			1.260
BHT			c		1.683
Control				d	2.170

*Levels not connected by same letter are significantly different.

C.3 Significance test for water loss

Dependent variable: water loss

Table C.3 Test of between subject effects

Source	DF	Sum of Squares	Mean Square	VR	Prob>F
Sample	4	12.8010	3.2002	27.48	<.001
Residual	10	1.1647	0.1165		
Total	14	13.9656			

Connecting Letters Report

Level			Mean
Ginger	a		17.64
BHT	a		18.38
Turmeric		b	19.36
Control		b	19.57
Garlic		b	20.26

*Levels not connected by same letter are significantly different.

C.4 Significance test for phenol

Dependent variable: phenol

Table C.4 Test of between subject effects

Source	DF	Sum of Squares	Mean Square	VR	Prob>F
Sample	2	35461.7	17730.9	45.50	<.001
Residual	6	2338.1	389.7		
Total	8	37799.9			

Connecting Letters Report

Level			Mean
Turmeric	a		618.4
Garlic		b	744.0
Ginger		b	758.0

*Levels not connected by same letter are significantly different.

C.5 Significance test for flavonoid

Dependent variable: flavonoid

Table C.5 Test of between subject effects

Source	DF	Sum of Squares	Mean Square	VR	Prob>F
Sample	2	30463.9762	15231.9881	1.237E+05	<.001
Residual	6	0.7386	0.1231		
Total	8	30464.7149			

Connecting Letters Report

Level				Mean
Garlic	a			59.2
Turmeric		b		73.5
Ginger			c	189.1

*Levels not connected by same letter are significantly different.

C.6 Significance test for DPPH Radical Scavenging Activity

Dependent variable: DPPH Radical Scavenging Activity

Table C.6 Test of between subject effects

Source	DF	Sum of Squares	Mean Square	VR	Prob>F
Sample	3	17134.174	5711.391	2018.61	<.001
Conc.	4	17608.616	4402.154	1555.88	<.001
Sample Conc.	12	3562.023	296.835	104.91	<.001
Residual	40	113.175	2.829		
Total	59	38417.987			

Connecting Letters Report

Post hoc test by sample

Level					Mean
Garlic	a				9.58
Ginger		b			28.53
Ascorbic acid			c		50.01
Turmeric			c		50.16

*Levels not connected by same letter are significantly different.

Post hoc test by conc.

Level						Mean
5	a					9.86
10		b				22.81
15			c			34.48
20				d		47.82
25					e	57.87

*Levels not connected by same letter are significantly different.

Appendix D

D. Significance test for effect of various samples of extract to Peroxide Value with time

D.1 Within samples

Table no. D.1 One-way analysis of Day 0 by sample

Source	DF	Sum of Squares	Mean Square	VR	Prob>F
Day 0	4	0.074764	0.018691	5.52	0.013
Residual	10	0.033869	0.003387		
Total	14	0.108632			

Connecting Letters Report

Level				Mean
Garlic	a			1.444
BHT		ab		1.448
Turmeric		ab		1.464
Ginger		ab		1.589
Control			b	1.601

*Levels not connected by same letter are significantly different.

Table no. D.2 One-way analysis of Day 12 by sample

Source	DF	Sum of Squares	Mean Square	VR	Prob>F
Day 12	4	0.461487	0.115372	24.62	<.001
Residual	10	0.046858	0.004686		
Total	14	0.508346			

Connecting Letters Report

Level					Mean
Turmeric	a				1.934
Ginger	a				1.945
BHT		ab			2.077
Garlic			b		2.134
Control				c	2.417

*Levels not connected by same letter are significantly different.

Table no. D.3 One-way analysis of Day 18 by sample

Source	DF	Sum of Squares	Mean Square	VR	Prob>F
Day 18	4	2.659155	0.664789	72.11	<.001
Residual	10	0.092190	0.009219		
Total	14	2.751345			

Connecting Letters Report

Level				Mean
Turmeric	a			2.074
BHT	a			2.230
Ginger		b		2.642
Garlic		b		2.854
Control			c	3.238

*Levels not connected by same letter are significantly different.

Table no. D.4 One-way analysis of Day 24 by sample

Source	DF	Sum of Squares	Mean Square	VR	Prob>F
Day 24	4	2.674692	0.668673	85.03	<.001
Residual	10	0.078635	0.007864		
Total	14	2.753328			

Connecting Letters Report

Level						Mean
Turmeric	a					2.794
BHT		ab				3.002
Ginger			b			3.161
Garlic				c		3.569
Control					d	3.975

*Levels not connected by same letter are significantly different.

Table no. D.5 One-way analysis of Day 30 by sample

Source	DF	Sum of Squares	Mean Square	VR	Prob>F
Day 30	4	3.89300	0.97325	79.45	<.001
Residual	10	0.12249	0.01225		
Total	14	4.01549			

Connecting Letters Report

Level				Mean
Turmeric	a			3.489
BHT	a			3.573
Ginger		b		4.1
Garlic		b		4.261
Control			c	4.892

*Levels not connected by same letter are significantly different.

Appendix E

E. Significance test for effect of various samples of extract to pH with time

E.1 Within samples

Table no. E.1 One-way analysis of Day 6 by sample

Source	DF	Sum of Squares	Mean Square	VR	Prob>F
Day 6	4	0.0113067	0.0028267	12.11	<.001
Residual	10	0.0023333	0.0002333		
Total	14	0.0136400			

Connecting Letters Report

Level						Mean
BHT	a					6.063
Turmeric		ab				6.083
Control			abc			6.103
Ginger				bc		6.117
Garlic					c	6.143

*Levels not connected by same letter are significantly different.

Table no. E.2 One-way analysis of Day 24 by sample

Source	DF	Sum of Squares	Mean Square	VR	Prob>F
Day 24	4	0.0085067	0.0021267	6.13	0.009
Residual	10	0.0034667	0.0003467		
Total	14	0.0119733			

Connecting Letters Report

Level				Mean
Control	a			5.903
Garlic		ab		5.933
BHT		ab		5.937
Turmeric			b	5.963
Ginger			b	5.970

*Levels not connected by same letter are significantly different.

Table no. E.3 One-way analysis of Day 30 by sample

Source	DF	Sum of Squares	Mean Square	VR	Prob>F
Day 30	4	0.0078000	0.0019500	5.42	0.014
Residual	10	0.0036000	0.0003600		
Total	14	0.0114000			

Connecting Letters Report

Level				Mean
Control	a			5.877
BHT		ab		5.900
Garlic		ab		5.910
Turmeric		ab		5.917
Ginger			b	5.947

*Levels not connected by same letter are significantly different.

Appendix G

Color Plates

Plate 1 Ethanolic extraction process of spices using rotatory evaporator

Plate 2 Ethanolic extracts of spices to incorporate in patty

Plate 3 Preparation of buffalo patties

Plate 4 Methanolic extracts of patties for analysis

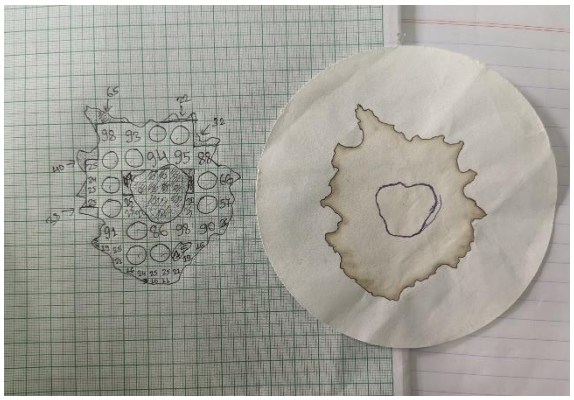


Plate 5 Calculation of WHC

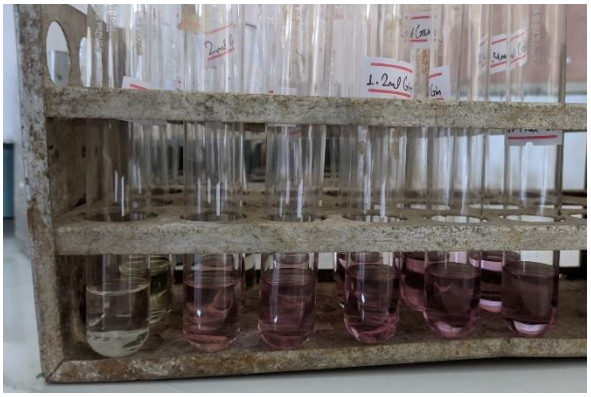


Plate 6 Analysis of DPPH