OPTIMIZATION OF THE MIXTURE OF SELECTED NEPALEASE HERBS IN TEA (*Camellia sinensis*)

by

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Approval Letter

This *dissertation* entitled *Optimization of The Mixture of Selected Nepalese Herbs in* Tea (*Camellia sinensis*) presented by *Durga Niraula* has been accepted as the partial fulfillment of the requirement for the Bachelor degree in Nutrition and Dietetics.

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Abstract

Total phenol content (TPC), total flavonoid content (TFC), and tannins along with the DPPH redical scavenging activity and antimicrobial activity of six different herbal tea sample were observed. The samples were rolled, fermented (180 min) and dried in a cabinet dryer. The Thearbigin and Theaflavin were evaluated from dried sample while the phytochemical value was observed from the methanolic extract.

The highest mean value of total phenol (TPC), total flavonoid content (TFC), and tannins were found to be 349.5$\pm$11.8mgGAE/g, 43.84$\pm$3.2mgQE/g, and 79.5$\pm$66.6mgGAE/g for sample B.Theaflavins (TF) and Thearubigins (TR) are responsible chemicals for the formation of tea liquor color and brightness and the estimation of quality of orthodox black tea. The value of TF&TR are expressed in percentage in which the highest mean value of %TF was found for sample E which was 0.1643$\pm$0.001%. Similarly, the highest mean value for %TR was found for sample B which was 7.258$\pm$0.25% ($p \leq 0.05$). The sensory analysis was carried out for dry tea appearance, brew aroma, brew liquid color, brew taste and texture of tea infusion and from the result sample B was found to be superior to other samples.Hence, it can be concluded that among several combinations the sample B with the combination of215g tea, 30g mint, 94.5g lemongrass, 30g asuro and 30.6g curry leaves had a significantly higher acceptability.

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List of abbreviations

Abbreviation	Full form
CTC	Crushing, tearing, curling
DPPH	2,2-Diphenyl 1-picrylhydrazyl
EC	Epicatechin
ECG	Epicatechin gallate
EGCG	Epigallocatechin gallate
GAE	Gallic Acid Equivalent
TPC	Total Phenol Content
TFC	Total Flavonoid Content
TF	Theaflavin
TR	Thearubigin
NaOH	Sodium Hydroxide
HCl	Hydrochloric acid
Na_2Co_3	Sodium Carbonate
$AlCl_3$	Aluminium Chloride
$NaNo_2$	Sodium Nitrate
$FeCl_3$	Ferric Chloride
H_2So_4	Sulphuric acid

Part I

Introduction

1.1 General Introduction

Tea, the most popular beverage consumed by two-thirds of the world's population is made from the processed leaf of *Camellia sinensis*. Tea types, based on processing or harvested leaf development are black (fermented), green (non-fermented) and oolong (semi-fermented). These major tea types differ in how tea is produced and processed according to the different processes of drying and fermentation that determine its chemical composition. Green tea is best studied for its health benefits, including cancer chemo preventive and chemotherapeutic effects but emerging data is showing that black tea may possess similar health promoting attributes (Naghma Khan and Mukhtar, 2013).

Tea is an infusion of the leaves of the *Camellia sinensis* plant and is the most widely consumed beverage in the world after water. It is increasingly appreciated that tea contains polyphenols and other components that may reduce the risk of developing chronic diseases such as cancer, cardiovascular diseases, arthritis and diabetes. More recently, the beneficial properties associated with daily consumption of green tea are getting better recognized. Particularly interesting are the studies which report that green tea reduces the risk of cancer, which is the major cause of mortality throughout the world. It has become increasingly clear that tea acts as a chemo preventive agent against a wide range of cancers (Qiao *et al.*, 2014).

More than 80% of world tea production is black tea. The steps involved in the processing of black tea include withering, leaf disruption, fermentation, drying, and grading. All steps are designed to achieve optimal oxidation of tea catechins and produce tea products with good flavor and color. Oolong tea is prepared by frying the leaves after rolling to terminate the oxidation process. It is only partially oxidized and retains a considerable amount of the original polyphenols (Hollman and Katan, 1999).

Like tea (*Camellia sinensis*), infusion of other plant materials such as lemongrass and mint are also being consumed in different parts of the world. Lemongrass (*Cympogon citratus*) and mint (*Mentha piperita*) has been widely used in herbal medicine and they have significant antimicrobial and antiviral activities, strong antioxidant and antitumor actions, and some antiallergenic potential. They also has immunomodulating actions and chemopreventive potential (McKay and Blumberg, 2006).

1.2 Statement of the problem

Non-communicable diseases (NCDs), such as cardiovascular diseases, cancer, diabetes and chronic respiratory diseases, are the leading global cause of death and are responsible for 70% of deaths worldwide (WHO, 2017). NCDs account death of 15 million women and men between the ages of 30 and 70 each year. In Nepal, 65% of death occurs from NCDs where as 22% of population has risk of premature death from NCDs (WHO 2017).

Studies have suggested that several diseases are involved with the production of free radical or reactive oxygen species (ROS) in the cells, and these results in oxidative stress, a pathological situation related to various age-related diseases, cancer, and aging (Upadhyay *et al.*, 2013). Researchers suggest that ROS, by-products of cellular respiration, play a role in normal aging by causing random deleterious oxidative damage to a variety of tissues.

An excess of free redicals in the body leads to oxidative stress which plays an important role in the pathogenesis of several human diseases. These diseases include atherosclerosis, cancer, inflammatory joint disease, asthma, diabetes, senile dementia and degenerative eye disease. Most free redical damage to cells involves oxygen free redicals or, more generally, activated oxygen species (Aoshima *et al.*). The AOS can damage genetic material, cause lipid peroxidation in cell membranes, and inactivate membrane-bound enzymes. Humans are well endowed with antioxidant defenses against AOS; these antioxidants, or free redical scavengers, include ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), beta-carotene, coenzyme Q10, enzymes

such as catalase and superoxide dismutase, and trace elements including selenium and zinc (Florence, 1995).

Phytochemicals includes a variety of plant ingredients with different structures that are capable of health-promoting effects. They occur only in low concentrations and usually have a pharmacological effect. Since antiquity, these effects have been used in naturopathy in the form of medicinal herbs, spices, teas, and foods. A high dietary intake of phytochemicals with vegetables, fruits, nuts, legumes, and whole grain is associated with a reduced risk for various non-communicable diseases (Leitzmann, 2016).

Natural antioxidants present in foods protect against these free redicals and are therefore important in maintaining and preserving good health. Therefore, the present study takes into consideration the phytochemical analysis and antioxidant activities of black tea *(Camellia sinensis)* combined with other four locally available herbs, namely curry leaf (*Murraya koenigii*)**,** mint (*Mentha piperita*), lemongrass (*Cymbopogon citratus*) and asuro (*Justicia adhatoda*) since to promote as functional beverage.

1.3 Objective of the research

1.3.1 General objectives

The general objectives of this work was to prepare herbal black tea by substituting green tea leaves with four herbs namely curry leaf (*Murraya koenigii*), lemongrass (*Cymbopogon citratus*), asuro *(Justicia adhatoda)* and mint (*Mentha piperita).*

1.3.2 Specific objectives

i. To optimize the proportion of tea leaves with curry leaf (*Murraya koenigii*), lemongrass (*Cymbopogon citratus*), asuro *(Justicia adhatoda)* and mint (*Mentha piperita)* and fermentation time.
ii. To determine total phenolic content, antioxidant activity and DPPH redical scavenging activity.
iii. To evaluate the DPPH redical scavenging activity of all tea samples.
iv. To determine antimicrobial activity (zone of inhibition) and sensory analysis.

1.4 Significance of the work

The efforts of World Health Organization (WHO) in compiling a global inventory of medicinal plants are not worthy and if adopted by the primary health care (PHC) as strategy, it could provide the people of all nations especially in the developing countries, with comprehensive health care (Vedavathy, 2003).

The popularity of herbal tea consumption has increased significantly during the past two decades. Hundreds of different teas made up of varied mixtures of roots, leaves, seeds, barks, or other parts of shrubs, vines, or trees are sold in health food stores (Manteiga *et al.*, 1997). Herbal teas are derived from an herb, fruit seed, or root. Their exact chemical compositions vary depending on the type of tea, but these drinks are usually full of antioxidants and other medicinal properties that promote health(Serafini *et al.*, 2011). The health benefits ascribed to the consumption of teas may be related to the high content of bioactive ingredients such as polyphenols. Polyphenols have been reported to possess antioxidant, antiviral, and anti-inflammatory activities; modulate detoxification enzymes; stimulate immune function and decrease platelet aggregation (Lampe, 2003). Among all tea polyphenols, epigallocatechin gallate (EGCG) has been found to be responsible for much of the health-promoting ability of green tea (N. Khan *et al.*, 2006). In general, green tea has been found to be superior to black tea in terms of health effects, owing to the higher content of EGCG, although the role of thearubigins and theaflavins contained in black tea have not been properly investigated. Over the last few years, clinical studies have revealed several physiological responses to tea that may be relevant to the promotion of health and the prevention or treatment of some chronic diseases (Cabrera *et al.*, 2006).

An Asian pacific country produces different varieties of tea and other herbs. The north hilly region of India and Nepal has been famous for the varieties of tea and other herbs. Introduction of tea along with Nepalese herbs can be very effective as people of our country mostly rely on herbal medicine rather than modern drugs due to their higher cost and limited access. Thus, this study shows the synergic and antagonist effect on mixing the different four locally available herbs (mint, lemongrass, asuro and curry leaf) with the mostly consumed variety of tea (Takdah) and also to find out the

bioactive components present. This dissertation will prove to be beneficial in contributing to the social, economic and health development of Nepal and the people.

1.5 Limitation of the study

- Single variety of plants were studied
- Single extraction technique was used to prepare extract solution

Part II

Literature Review

2.1 Tea (*Camellia sinensis*)

2.1.1 History of Tea

Tea (*Camellia sinensis*) is popular all over the world. Tea has a very long history. It is said to have been originated in China in 2737 BC when the emperor Shen Nung used tea as medicine. In the 18th century, tea entered India from China through the activities of the East India Company. Tea cultivation started in Darjeeling in 1835, but a commercial nursery was established a decade later (Greathead, 1997).About 1873 Colonel Gajraj Singh Thapa, made to implement two plantations: Ilam and Soktim Tea Estate of 52 hectares each and thus began the Nepali tea industry. In 1985 the Government declared five districts of Eastern Nepal, Jhapa, Ilam, Panchtar, Terhathum and Dhankuta 'Tea Zone'. Today tea garden covers an area of 16000 hectares approximately. The production also strongly increased by slightly more than 2 tons in 1920 to 140000 tons approximately today. Most of the tea plants are hybrids of the variety Chinese *"Camellia sinensis"*. The tea gardens are located at an altitude that varies between 1,000 and 2,000 meters above the sea level and produce one of the finest teas of the world (Poudel, 2010).

Nepal produces two types of tea, CTC (Cut, Tear and Curl) and Orthodox tea. Orthodox tea is grown in high altitudes, whereas the CTC tea is grown in low altitudes or plain areas. Historically, the term ‘Orthodox’ refers to a method of producing tea in India, by which the leaves are partially dried and then allowed to ferment to produce black tea as opposed to green tea which is not fermented. But in a broader sense, ‘Orthodox’ also refers to ‘traditional’ or ‘hand-processed’ tea. Orthodox tea is produced by a special process in which only the top two leaves and bud from each branch ‘dui pat ek suiro’ are picked at the precise moment when they are budding (Rana, 2007). Orthodox tea is also known as ‘hill tea’ or ‘leaf tea’, and its major production area is East Nepal (the four hill districts of Ilam, Panchthar, Dhankuta and Terathum. When prepared, it has a light color, unique aroma and subtle, slightly fruity flavor. Due to its

fine quality and high price, orthodox tea is in high demand among overseas consumers (Poudel, 2010).

2.1.2 Botany of tea (*Camellia sinensis*)

Camellia sinensis is an evergreen tree or shrub. It has yellow-white flowers and long, serrated leaves. Flowers are axillary, solitary or up to three in a cluster. They are 2.5-3.5cm in diameter and have six to eight petals. The outer petals are sepaloid and the inner petals are obovate to broadly obovate. There are numerous stamens 0.8-1.3 cm in length. Young leaves have short white hairs on their underside and young branches are grayish yellow and glabrous. Terminal buds are silvery gray and sericeous. Petioles are 4-7 mm in length, pubescent and glabrescent. Leaf blades are elliptic, oblong-elliptic or oblong. Seeds are brown, sub globose and 1-1.4 cm in diameter. Flowering of *Camellia sinensis* occurs from October through February and fruiting occurs from August to October (W. Han *et al.*, 2018; Mondal, 2013).

Scientific Classification:
Kingdom: Plant
Family: Theaceae
Genus: *Camellia*
Species: *C. sinensis*
Binomial name: *Camellia sinensis* (L.) Kuntze
(Mondal, 2013)

Fig. 2.1 Figure of tea plant

2.1.3 Chemical constituents

Tea is reported to contain nearly 4000 bioactive compounds of which one third is contributed by polyphenols. Other compounds are alkaloids (caffeine, theophylline and theobromine), amino acids, carbohydrate, proteins, chlorophyll, and volatile organic compounds. Polyphenols found in tea are mostly flavonoids. The polyphenols a large group of plant chemicals that includes the catechins, are thought to be responsible for the health benefits that have traditionally been attributed to tea especially green tea.

Major catechins are epicatechin gallate (EGC), epigallocation gallate (EGCG). The most active and abundant catechin in green tea is EGCG. Black tea contains much lower concentrations of these catechins than green tea. Oolong tea contains a mixture of simple polyphenols, such as catechins and complex polyphenols. Black, Green and Oolong tea are all extremely good source of vitamin C (Namita *et al.*, 2012).

Fresh leaves from Assam contain 22.2% polyphenols, 17.2% protein, 4.3% caffeine, 27.0% crude fiber, 0.5% starch, 3.5% reducing sugars, 6.5% pectin's, 2.0% ether extract and 5.6% ash. Per 100 g, the leaf is reported to contain 293 calories, 8.0 g water, 24.5 g protein, 2.8 g fat, 58.8 g total carbohydrate, 8.7 g fiber, 5.9 g ash, 327 mg Ca, 313 mg P, 24.3 mg Fe, 50 mg Na, 2700 µg beta-carotene equivalent, 0.07 mg thiamine, 0.8 mg riboflavin, 7.6 mg niacin, and 9 mg ascorbic acid (Duke and Atchley, 1983).

Leaves also contain carotene, riboflavin, nicotinic acid, pantothenic acid and ascorbic acid. Caffeine and tannin are among the more active constituents. Ascorbic acid, present in the fresh leaf, is destroyed in making black tea. Malic and oxalic acids occur, along with kaempferol, quercitrin, theophylline, theobromine, xanthine, hypoxanthine, adenine, gums, dextrin's, and inositol. Chief components of the volatile oil (0.007-0.014% fresh weight of leaves) is hexenal, hexenol, and lower aldehydes, butyraldehyde, isobuteraldehyde, isovaleraldehyde, as well as n-hexyl, benzyl and phenyl ethyl alcohols, phenols, cresol, hexoic acid, n-octyl alcohol, geraniol, linalool, acetophenone, benzyl alcohol, and citral. Certain constituents, especially catechin, epigallocatechin, and epigallocatechin gallate are said to have antitoxidative properties (Leung, 1980).

2.1.4 Health benefits of Tea

Tea is one of the most popular drinks due to its pleasant taste and perceived health effects. Although health benefits have been attributed to tea consumption since the beginning of its history, scientific investigation of this beverage and its constituents has been under way for about 30 years (McKay and Blumberg, 2002).

Consumption of tea, in particular green tea, has been correlated with low incidence of chronic pathologies in which oxidative stress has been reported to be involved, such as cancer (Chung *et al.,* 2003)and cardiovascular diseases (Babu and Liu, 2008). The health benefits ascribed to the consumption of teas may be related to the high content of bioactive ingredients such as polyphenols. Polyphenols have been reported to possess antioxidant, antiviral, and anti-inflammatory activities; modulate detoxification enzymes; stimulate immune function and decrease platelet aggregation (Lampe, 2003).

Among all tea polyphenols, epigallocatechin gallate (EGCG) has been found to be responsible for much of the health-promoting ability of GT (N. Khan *et al.*, 2006).In general, GT has been found to be superior to black tea (BT) in terms of health effects, owing to the higher content of EGCG, although the role of thearubigins and theaflavins contained in black tea have not been properly investigated (Cabrera *et al.*, 2006). Over the last few years, clinical studies have revealed several physiological responses to tea that may be relevant to the promotion of health and the prevention or treatment of some chronic diseases (Crespy and Williamson, 2004).

2.1.5 Processing of Tea Leaves

There are different types of tea depending on botanical varieties, geographical origin and processing (Mejia *et al.*, 2009). Concerning to the level of "fermentation", it can be categorized into three major types: not fermented (green and white tea), partially fermented (oolong tea) and completely fermented (black tea).

To produce green tea, the leaves are rolled and steamed to minimize oxidation and inactivate polyphenol oxidase prior to drying (McKay and Blumberg, 2002). In black tea production, after the leaves are rolled, which disrupts cellular compartmentation and brings phenolic compounds into contact with polyphenol oxidases, they undergo oxidation for 90–120 min. Hence the color of tea is changed to reddish copper from green. Oolong tea is produced with a shorter "fermentation" period than black tea and has a taste and color somewhere between green and black teas. In White tea production, plant materials are steamed and dried immediately after picking to prevent oxidation,

giving it a light and delicate taste. Each kind of tea has its characteristic flavor and appearance (Kosińska and Andlauer, 2014).

The processing of tea leaves determines the contents of bioactive ingredients, hence it should be expected that each variety of tea, black, red or green, will represent a different package of compounds of physiological importance (Czernicka *et al.*, 2017). Fig. 2.1 shows the schematic representation of tea processing.

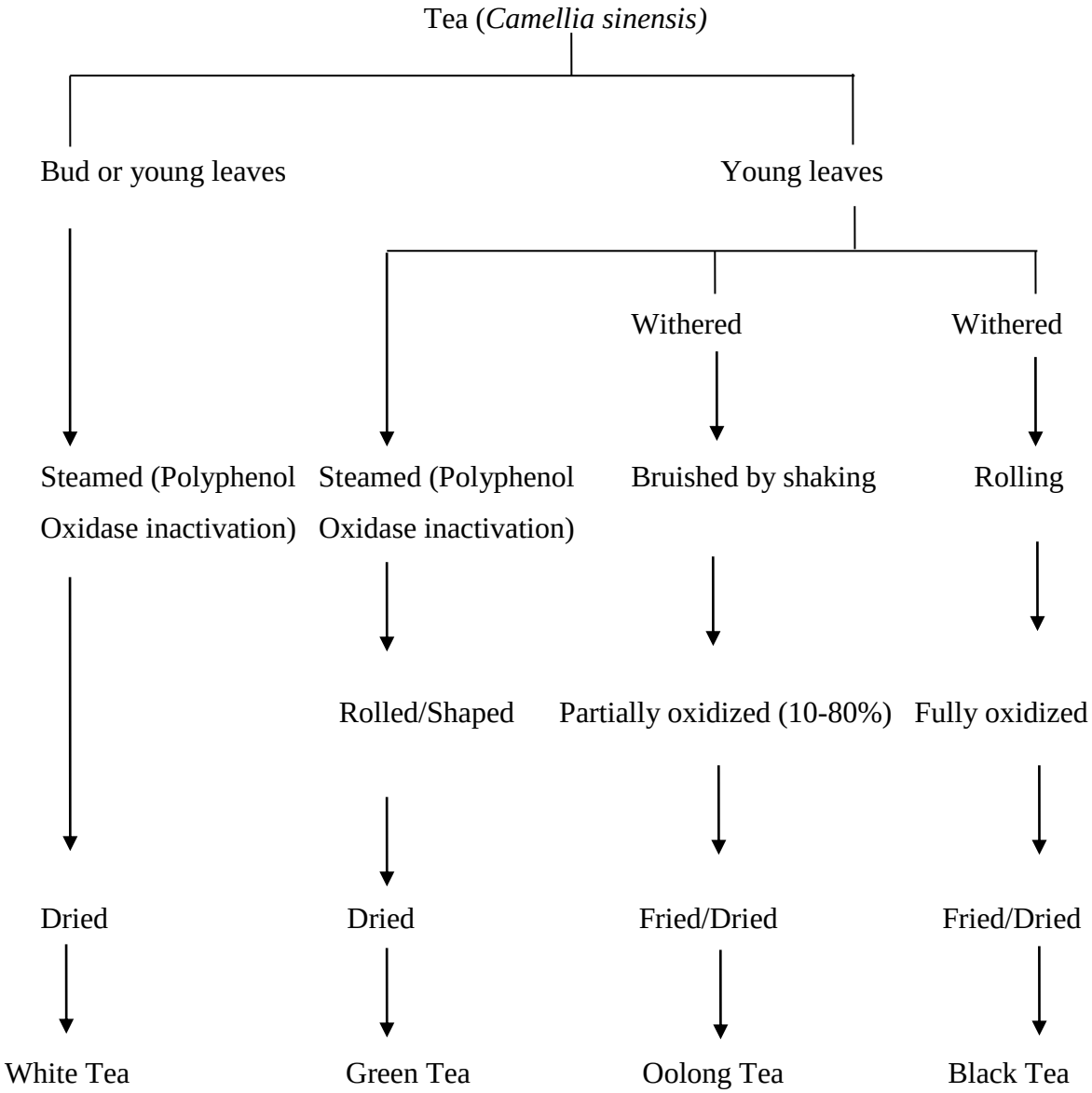


Fig 2.1. Schematic representation of tea processing (McKay and Blumberg, 2002)

The composition of tea can be influenced by several parameters associated with growth conditions, such as genetic strain, season, climatic conditions, soil profile, growth altitude, horticultural practices, plucking season, shade growth, and with the region in which tea has been cultivated. The other factors that can influence the profile of bioactive compounds are manufacturing process (withering, steaming/pan-firing, rolling, oxidation/fermentation and drying) and storage (Y.-S. Lin *et al.*, 2003).

2.1.5.1 Picking/ Plucking

The method of harvesting tea shoots is termed plucking. Tea leaves and flushes, which include a terminal bud and two young leaves, are plucked from *Camellia sinensis* bushes typically twice a year during early spring and early summer or late spring-autumn; winter pickings of tea flushes are much less common, though they occur when climate permits. Tea leaves are plucked by hand or are mechanically harvested by a plucking machine. Many high-quality green teas are hand plucked (Ahmed and Stepp, 2012).

2.1.5.2 Withering

The tea leaves will begin to wilt soon after picking, with a gradual onset of enzymatic oxidation. Withering aims to remove moisture and soften the leaves to prepare them for further rolling. The leaves can be either put under the sun or left in a cool breezy room to pull moisture out from the leaves. The leaves sometimes lose more than a quarter of their weight in water during withering. The process is also important in promoting the breakdown of leaf proteins into free amino acid and increases the availability of freed caffeine both of which changes the taste of tea. It leads also to the development of aroma and partial oxidation due to a breakdown of cell walls caused by moisture loss. Duration of withering depends on type of tea: white tea leaves are withered for 4–5 h, whereas green, oolong and black tea are withered for at least twice as long (R. S. S. Kumar *et al.*, 2012).

Depression in polyphenol oxidase (PPO) activity during the withering of tea leaf (*Camellia sinensis*) in black tea manufacture affected the oxidative-condensation of tea flavones in forming theaflavins (TF) and thearubigins (TR), which are associated with

brightness, briskness and ‘body’ of tea liquors. In contrast to conventionally manufactured CTC teas, unwither fresh leaf CTC produces a higher proportion of TF and lower TR, resulting in bright, brisk and thin liquors. Hardness of wither was accompanied by a further depression in PPO activity, and formation of TF also declined with a concomitant loss in brightness and briskness. The TR content increased up to a certain degree of withering and thereby improved the ‘body’ of the liquor. Very hard wither, accompanied by a large reduction in moisture content, and restricts PPO activity. It appears that in the fresh leaf, high moisture levels together with dissolved oxygen accelerate the enzyme oxidation and produce large amounts of TF. Senescence of the leaf seems to facilitate the production of TR (R. Ullah *et al.*, 1984).

2.1.5.3. Rolling

The rolling/twisting of tea leaves was originally done manually, whereas nowadays it is performed by machines (Kosińska and Andlauer, 2014). The objective of the rolling is to break the leaf cells and release the oxidases, including polyphenol oxidase and peroxidase, and initiate the process of catechin oxidation with oxygen in the air. The rolling method provides a unique taste and flavors. Some specific compounds such as 2,6-nonadienal, 3-hexen-2-one, dodecanal, 2,5-octanedione and methylpyrazine were identified to differentiate the rolling system (Wan et al., 2009).

Black tea is usually processed in two different ways, orthodox and cut-tear-curl (CTC)processing, and the main difference between them is the rolling phase of the fermentation process. Orthodox rolling refers to hand processing or rolling with machines that imitate hand rolling. CTC machines enable more rapid and extensive leaf disruption, producing smaller particles and therefore greater surface areas for enzymatic oxidation. Tea obtained by this method is used mainly for commercial tea in the form of teabags (Kosińska and Andlauer, 2014).

Experiments were conducted on black tea processing to study the effects of rolling, fermentation and drying on the quality carried out at National Tea Research Institute (NTRI), Shinkiari, Mansehra, during 2002. Tea leaves were rolled from 20-30 minutes depending upon the intensity of withering and raw material. Fine plucking (2 leaves

and a bud) had bright red color both in plain and milky tea. The taste and aroma was pleasant as compared to coarse plucking. Quality of coarse leaves was very poor in terms of taste, aroma, color, strength and infusion. Rolling time (25 minutes) gave better results as compared to 20or30 minutes time. Fermentation (4 hours and 5 minutes) gave best results in terms of taste, aroma and strength. Drying of black tea at 110^{o}C temperature produce good quality tea (Naheed *et al.,* 2019).

2.1.5.4 Fermentation/Oxidation

Rolled leaves are placed on trays and left in a room with controlled temperature, humidity, and aeration for 2-4 h. The process is traditionally called fermentation; however, it is mainly enzymatic oxidation of polyphenols by polyphenol oxidases. The initial green color of leaves turns into light brown, and deep brown in the course of their oxidation, which indicates formation of oligomeric theaflavins and polymeric thearubigins (Kosińska and Andlauer, 2014). Practically, the completion of fermentation is judged by the change in color (from green to coppery) and the pleasant aroma that develops (R. S. S. Kumar *et al.,* 2012).

2.1.5.5 Drying

Drying is a process in which the moisture is removed from the fermented, its color changes from coppery red to black, and fermentation is arrested. Moisture in the fermented leaf is reduced from 55% to 3% to suppress microorganism growth. The two objectives of drying are to terminate the chemical changes and to remove moisture to impart better storage quality (R. S. S. Kumar *et al.,* 2012). It is very important to dry the fermented leaves for the correct amount of time in order to form a good quality tea. Late drying or early drying will certainly cause the deterioration of black tea quality. Furthermore, many quality oriented flavors are formed during drying. Oxidation of leaf components continues through the drying stage while some chemical components such as amino acids and simple carbohydrates increase. Drying can be done in a myriad of ways including sunning, air drying, or baking. Great care must be taken to not overcook the leaves. The drying of the produced tea is responsible for many new flavor compounds particularly important in green teas (Xu and Chen, 2002).

2.1.5.6 Storage/Packing

Tea is a hygroscopic material and it absorbs moisture during cooling and sorting. The amount of moisture uptake depends on the ambient temperature and humidity to which it is exposed. Tea leaves have a considerably long shelf-life due to their low moisture content. In general, drying tea to 3% moisture level is advisable and when packed, the moisture level should not be higher than 5% to 6%. Above this, the keeping quality will be impaired and in extreme cases it will go moldy before reaching the market. Hence, tea should not be stored in open conditions in the sorting and packaging rooms. Bins are essential to store the finished product (R. S. S. Kumar *et al.*, 2012).

2.1.6 Effect of fermentation on quality of tea

Fermentation of black tea is the series of chemical changes that happen under the assistance of enzyme during making process, mainly refers to the oxidization of polyphenols. Fermentation is the key process determining black tea's quality. It promotes the oxidization of polyphenol in the tea leaf with the help of enzyme. Meanwhile other chemical substance will change too making the green tea leaves into red color. The unique aroma and flavor of black tea will then be formed (Kosińska and Andlauer, 2014).

The same plant, *Camellia sinensis*, is used to produce all types of tea, and the differences among the various types arise from the different processing steps that are used. Based on the degree of fermentation, tea can be classified as black, green, white, or oolong tea. The oxidized polyphenol compounds such as theaflavins (Altemimi *et al.*)and thearubigins (TR) formed during fermentation are responsible for the color, taste, flavor, and aroma of black tea. The concentrations of TF and TR as well as desirable quality characteristics increase as fermentation time increases, reaching optimum levels and then degrading if the fermentation time is prolonged. It is also necessary to control the environment for oxidation. There are no established environment conditions that must be maintained during the fermentation of the ruptured tea leaves. However, in most cases, the process is performed at a temperature of 24-29 ^{o}C for 2-4 h or 55-110 min for orthodox tea or crush, tear, and curl (CTC) black tea,

respectively, under a high relative humidity of 95-98% with an adequate amount of oxygen (Pou and Jolvis, 2016).

Studies showed that the caffeine content in tea leaves increased reasonably after treating leaves with microorganisms for a period of time (i.e. orthodox pile-fermentation), and the amount of caffeine content increase varied significantly between black and green teas (27.57% and 86.41%). These results suggested that the change of caffeine content in tea leaves during the pile-fermentation depended not only on the growth and reproduction of microorganisms, but also on the tea composition (X. Wang *et al.*, 2005).

2.2 Mint (*Mentha piperita)*

2.2.1 Introduction

The genus *Mentha* belongs to the Lamiaceae family and comprises a large number of species. This specie is herbaceous; the composition of the essential oil varies a lot among the varieties, during the year and at different stages of its development, but is mainly composed of monoterpenes as menthol (70% -90%), which is the major substance (Souza1 *et al.*, 2014).

Mint is a rapid growing perennial herb with many varieties that grow up to 3 feet tall and are quite invasive. Mint grows best in full sun to partial shade, should be planted early in the growing season and is generally hardy to -20° F. Mint prefers moist soil conditions, but excess water will promote root and leaf diseases. Mint are of different varieties. Culinary varieties include those listed above and those with mint-like flavors like red raripila mint, ginger mint (red mint), horsemint, and pineapple mint. Medicinal mint types widely used in teas and medicinal preparations include water mint, corn or field mint, and pennyroyal (Buckland and Drost, 2009).

Fig 2.2 Figure of mint plant

2.2.2 Chemical constituents

The volatile constituents from the aerial parts of *Mentha piperita* L. (peppermint) is extracted by hydro distillation and headspace/solid-phase micro-extraction (HS/SPME) methods and analyzed by gas chromatography/mass spectrometry (GC/MS). The main components in the hydro distillation method were menthol (45.34%), menthone (16.04%), menthofuran (8.91%), cis-carane (8.70%), 1, 8-cineole (4.46%), neo-menthol (4.24%), and limonene (2.22%). The main components in the HS/SPME method were menthol (29.38%), menthone (16.88%), cis-carane (14.39%), menthofuran (11.38%), 1,8-cineole (9.45%), trans-caryophyllene (2.76%), neo-menthol (2.37%), β-Pinene (2.26%), α-Pinene (1.55%), germacrene-D (1.41%), trans-sabinene hydrate (1.28%), and neoisomenthyl acetate (1.02%) (Taherpour *et al.*, 2017).

Most of the aqueous extracts of local mint species contained alkaloids, flavonoids, phenolic and saponins. The results were consistent with the findings of (Al-Okbi *et al.*, 2015). *Mentha* extract (ME) has been reported to have antioxidant and antiperoxidant properties. It has been found that essential oil exhibited antimicrobial activity and it could be a better natural antioxidant. Hence, the essential oil of mint may be exploited as a natural source of bioactive phytochemicals bearing antimicrobial and antioxidant potentials (Sharafi *et al.*, 2010).

2.2.3 Health benefits and other uses

Mentha species, one of the world's oldest and most popular herbs, are widely used in cooking, in cosmetics, and as alternative or complementary therapy, mainly for the treatment of gastrointestinal disorders like flatulence, indigestion, nausea, vomiting, anorexia, and ulcerative colitis. *Mentha* leaves have traditionally been used as tea in the treatment of headache, fever, digestive disorders and various minor ailments (Salehi *et al.*, 2018). Furthermore, it is well documented that the essential oil and extracts of *Mentha* species possess antimicrobial, fungicidal, antiviral, insecticidal, and antioxidant properties (Brahmi *et al.*, 2017).

2.3 Asuro *(Justicia adhatoda)*

2.3.1 Introduction

Justicia adhatoda is an erect, evergreen, shrub with few branches to many branches. It usually grows up to 2.5 meters tall, but exceptional specimens to 6 meters can be found (Huxley, 1992). It grows best in areas where annual daytime temperatures are within the range 20–27°c. Found on a variety of sites from moist river banks to dry slopes and disturbed areas, at elevations from sea level to 1,300 meters (Dhankhar *et al.*, 2011).

Fig 2.3 Figure of asuro plant

2.3.2 Chemical constituents

The leaves portion of the plant has higher contents of total carbohydrate (16.1 mg/g fresh wt.). The leaf also has high soluble protein (7.82 mg/g fresh wt.), total phenolic (32.1 mg/g dry wt.) and flavonoids (37.9 mg/g dry wt.) than the flower parts which has soluble protein (2.5 mg/g fresh wt.), total phenolic(22.8 mg/g dry wt.) and flavonoids (29.2 mg/g dry wt.). But in alkaloid content it is having more or less same content (1.09 and 1.08 mg/g dry wt.) (Gupta *et al.*, 2014).

A study for screening of phytochemicals and to compare antioxidant and anti-inflammatory activity of methanol and chloroform extract of *J. adhatoda* shows that methanol extract showed highest percentage inhibition of DPPH free redical. Anti-inflammatory activity was in the same pattern as antioxidant activity with highest percentage inhibition of inflammation for methanol extract (Dhakal, 2016).

2.3.3 Health benefits and other uses

The whole plant is anti-inflammatory, antispasmodic, febrifuge, and pectoral. The roots and leaves are widely used in the Ayurvedic and Unani systems of medicine in India and Thailand for treating bronchitis, asthma, fever and jaundice. The leaves are antiseptic. About 50 g of the freshly collected root is boiled and mixed with cow milk (125 ml), then given orally once a day as a treatment for diabetes. The plant is potential anti-diabetic due to the presence of the compounds vasicine and vasicinol. The plant is said to have definite expectorant activity and is used in the treatment of bronchitis (I. Khan *et al.*, 2018).Methanol extract of *Justicia adhatoda* also showed considerable inhibition of cancer cells (Batool *et al.*, 2017).

2.4 Lemongrass *(Cymbopogon citratus)*

2.4.1 Introduction

Cymbopogon citratus (Lemongrass) is a widely used herb in tropical countries, especially in Southeast Asia. The essential oil of the plant is used in aromatherapy. *Cymbopogon* is a genus of about 55 species, which are indigenous in tropical and semi-tropical areas of Asia. Lemongrass is equally versatile in the garden. This tropical grass

grows in dense clumps that can grow to 6ft (1.8 m) in height and about 4ft (1.2 m) in width, with a short rhizome (Shah *et al.*, 2011).

Fig 3.3 Figure of lemongrass plant

2.4.2 Chemical constituents

The chemical composition of the essential oil of *Cymbopogon citratus* varies according to the geographical origin, the compounds as hydrocarbon terpenes, alcohols, ketones, esters and mainly aldehydes have constantly been registered. The essential oil (0.2–0.5%, West Indian lemon grass oil) consists of, mainly, citral (Rauber Cda *et al.*, 2005). Lemongrass consists of luteolin and its 6-C and 7-O–glycosides, isoorientin 2-O-rhamnoside and isolation of the flavonoid quercetin, kaempferol and apiginin from the aerial parts. The phenolic compounds elimicin, catecol, chlorogenic acid, caffeic acid and hydroquinone are also isolated from the plant (Miean and Mohamed, 2001). DPPH scavenging activity was highly elicited by the extract of *C. citratus*. Chloroform, methanol and water extracts of *C. citrates* leaves effectively decreased the extent of DNA damage (Balakrishnan *et al.*, 2014).The antioxidant effect is correlated with the compounds present in the polyphenol fraction and it corroborates the use of lemongrass in folk medicine (Costa *et al.*, 2013).

2.4.3 Health benefits and other uses

Lemongrass essential oil which has citral as its main component, has exhibited anti-inflammatory effect in both animal and human cells (X. Han and Parker, 2017). *Cymbopogon citratus* a tropical perennial herb plant that is widely cultivated to be eaten either fresh with food or dried in tea or soft drink has been reported to possess a number of medicinal and aromatic properties. This study aimed at evaluating the protective effects of *C. citratus* aqueous extract against liver injury (Rahim *et al.*, 2014).

The commercial and medicinal uses of the various species of *Cymbopogon* are well documented. Ethno pharmacology evidence shows that they possess a wide array of properties that justifies their use for pest control, in cosmetics and as anti-inflammation agents. These plants may also hold promise as potent anti-tumor and chemo preventive drugs (Avoseh *et al.*, 2015).

2.5 Curry leaf *(Murraya koenigii)*

2.5.1 Introduction

Murraya koenigii of family Rutaceae, commonly known as curry leaf tree, closely associated with south India where the word "curry" originates from the Tamil "Kari" for spiced sauces (Math and Balasubramaniam, 2004).A plant of the moist tropics, where it is found at elevations up to 1600 meters. It grows best in areas where annual daytime temperatures are within the range 27-41°c (Barwick, 2004).

Fig 3.4 Figure of curry leaf plant

2.5.2 Chemical constituents

Gas Chromatography and Gas Chromatography-Mass Spectrometry (GC-MS) analysis indicates the 33 different compounds representing 97.56 % of the total oil. Major compounds detected in the oil were Linalool (32.83%), Elemol (7.44%), Geranyl acetate (6.18%), Myrcene (6.12%), Allo-Ocimene (5.02), α-Terpinene (4.9%), and (E)-β-Ocimene (3.68%) and Neryl acetate (3.45%). From the identified compounds, they were classified into four groups that are oxygenated monoterpenes (72.15%), monoterpene hydrocarbons (11.81%), oxygenated sesquiterpenes (10.48%) and sesquiterpenes hydrocarbons (03.12%) (Rajendran *et al.*, 2014).

The antioxidant properties of different extracts (water, alcohol, hexane or chloroform extract) of curry leaves (*Murraya koenigii L.*) were evaluated using various assays. The alcohol water (1:1) extract of curry leaves showed the highest antioxidant and free redical scavenging activity. It inhibited membrane lipid peroxidation by 76%, at 50 µg/ml, scavenged 93% of superoxide's at 200 µg/3 ml and scavenged approximately 90% of hydroxyl and DPPH redicals at 4-5-fold lower concentrations (Ningappa *et al.*, 2008).

2.5.3 Health benefits and other uses

Murraya koenigii, a plant widely distributed in Eastern-Asia. Through systematic research and pharmacological evaluation of different parts of the plant extracts has been shown to possess antiviral, anti-inflammatory, antioxidant, antidiabetic, antidiarrheal, antileishmanial, and antitumor activity (Samanta *et al.*, 2018).

Curry leaf contains several medically active constituents including a glycoside called koenigin, an essential oil and tannins. It is a warming, strongly aromatic herb that improves appetite and digestion. The leaves, roots and bark can all be used internally in the treatment of digestive problems. It has been shown that the leaves increase digestive secretions and relieve nausea, indigestion and vomiting. The leaves can be used internally in treating constipation, colic and diarrhea. The antibacterial activity of essential oil has pronounced by Disc Diffusion Method against various pathogenic microbes (Jain *et al.*, 2017).

2.6 Synergic effects of herbs

Generally, synergy is defined as the interaction of two or more agents to produce a combined effect greater than the sum of their individual effects (Vuuren and Viljoen, 2011). The concept of synergy classified into two main categories based on the mode of actions pharmacodynamics and pharmacokinetic synergy. The first type of synergy describes two or more agents that work on the same receptors or biological targets that result in enhanced therapeutic outcomes through their positive interactions. The second type of synergy results from interactions between two or more agents during their pharmacokinetic processes (absorption, distribution, metabolism and elimination) leading to changes of the agents quantitatively in the body and hence their therapeutic effects (Spinella, 2002).

In a study was done to determine the immediate effect of green tea, cinnamon, ginger and combination of them on postprandial glucose levels. Herbs combination exerted GI of 60, which was the lowest. Combination of these herbs showed the best lowering effect on postprandial glucose levels as compared with each herb alone. A potential synergism from the active ingredients of blended herbs was determined (Azzeh, 2013). The use of herbal therapy in inflammatory bowel disease is increasing worldwide. Herbal therapies exert their therapeutic benefit by different mechanisms including immune regulation, antioxidant activity, inhibition of leukotriene B4 and nuclear factor-kappa B, and antiplatelet activity (Triantafyllidi *et al.*, 2015).

Many of the herbs and herbal preparations are used by cancer patients for their ability to stimulate immunity and improve the quality of life. Mistletoe *(Viscum album)*, *Curcumalonga*, garlic (*Allium sativum*) are the prominent ones. Besides possessing antiproliferative activity such herb are reported to potentiate the anticancer effect of other herbs or drugs. Curcumin in combination with catechins, the polyphenolic compounds of green tea (*Camellia sinensis*) can synergistically inhibit the proliferation of HCT 15, HCT 116 of human colon adenocarcinoma and human larynx carcinoma HepG-2 cells efficiently through induction of apoptosis. Ginger is another traditional herb known to possess hypolipedemic, antioxidant and hepatoprotective

properties. The combined effect of ginger extract and atorvastatin were investigated on lipid profile and atorvastatin-induced hepatic toxicity (Kapoor and Singla, 2015).

The recent literature discusses thoroughly the mechanisms underlying synergistic actions of herbal ingredients. Results have revealed that the multi-component nature of medicinal herbs makes them particularly suitable for treating complex diseases and offers great potential for exhibiting synergistic actions. The exploration of synergistic mechanisms of herbal ingredients will not only help researchers to discover new phytomedicines or drug combinations but also help to avoid the possible negative synergy (Y. Yang *et al.*, 2014).

2.7 Antagonist effects of herbs

The adverse effects and drug interactions associated with herbal remedies are largely unknown. Ginkgo biloba extract, advertised as improving cognitive functioning, has been reported to cause spontaneous bleeding, and it may interact with anticoagulants and antiplatelet agents. St. John's wort, promoted as a treatment for depression, may have monoamine oxidase–inhibiting effects or may cause increased levels of serotonin, dopamine and norepinephrine. Although St. John's wort probably does not interact with foods that contain tyramine, it should not be used with prescription antidepressants. Ephedrine-containing herbal products have been associated with adverse cardiovascular events, seizures and even death. Ginseng, widely used for its purported physical and mental effects, is generally well tolerated, but it has been implicated as a cause of decreased response to warfarin (Cupp, 1999).

The Quinine tree (*Rauvolfia caffra)* is used as a medicinal plant among traditional communities in many countries to manage tumors and other diseases associated with oxidative stress. *R. caffra* showed promise as a cure, with antioxidant activity comparable to the commercially used drug. However, we found two phytochemicals with possible antagonistic effect: co-occurrence of alkaloids and saponins significantly reduced antioxidant activity (alkaloids only63%; alkaloids plus saponins 15%; steroids, terpenoids and cardiac glycosides = 82%), thus alkaloids and saponins should be exclusive to each other in drug formulations (Milugo *et al.*, 2013).

2.8 Herbal tea

Herbs are valued for its specific aroma, taste, putative physiological effect and medicinal properties which appeal to sense of taste, smell, and sight and therefore promote continuous development of functional foods and drinks (Yokozawa *et al.*, 1998). Such herbal remedies often consumed in the form of tea, where boiling water are added to steep infusion of dried plant parts. In Asian countries, tea drinking is a ritual and life style, however in European countries tea consumption is occasional and usually European choose a wide variety of fruit teas or traditional herbal infusions. Till date, tea consumption depends primarily on the type and mode of preparations (Horžić *et al.*, 2013).

Herbal tea, according to many, look like tea and is brewed as the same way as tea, but in reality it is not considered a tea at all. This is due to the fact that they do not originate from the *Camellia sinensis* bush, the plant from which all teas are made. There are several kinds of herbal teas that have been used for their medicinal properties. Some of them being consumed for its energizing properties to help induce relaxation, to curb stomach or digestive problems and also strengthen the immune system. Some of the popular herbal teas are Black tea, Green tea, Chamomile tea, Ginger tea, Ginseng tea, Peppermint tea, Cinnamon tea etc. (Ravikumar, 2014).

Herbal tea is defined as an infusion of leaves, fruits, stems, roots, etc. made from plant parts other than *Camellia sp*. Herbal tea is a polyherbal formulation of different medicinal plants that is a rich source of antioxidant. According to (Quispe *et al.*, 2012) increasing consumption of herbal teas is a worldwide trend because supplementation of human diet with herbal provides high antioxidant compounds that may have beneficial effects. Additionally, due to the advance development of technology and time constraint, people have started to seek for convenient herbal products. Herbal tea has been used for health care and diseases prevention for thousands of years in many countries because according to (Tschiggerl and Bucar, 2012) herbal teas are convenience to take, easy to prepare, mild in action and in most cases with negligible side effects besides, cheap in price and rich in resource. It is well reported that the total amount of phenolic compounds may have a direct contribution in the defense against

oxidative stress and could be considered to be active metabolites involved in the antioxidant activity of herbs (Zhao *et al.*, 2013).

2.9 Antimicrobial activity

Tea polyphenols are well-known for their antioxidant properties. Green tea has greater antioxidant potential than oolong and black teas. Black tea is also known to have potent antioxidant properties which are manifested by its ability to scavenge free redicals, inhibit lipid peroxidation, and chelate metal ions. Tea polyphenols are also known for their antibacterial activity. In general, antibacterial activity decreases when the extent of tea fermentation is increased. Tea catechins, particularly EGCG and ECG, have antibacterial activity against both Gram-positive and Gram-negative bacteria. Green tea can prevent tooth decay by inhibiting oral bacteria. The antibacterial activity of black tea has also been reported (Chan *et al.*, 2011).

In Eastern and Western traditional medicine peppermint and its oil have been used as an antispasmodic, aromatic, antiseptic and also in the treatment of cancers, colds, cramps, indigestion, nausea, sore throat and toothaches. Peppermint oil and menthol have moderate antibacterial effects against both Gram-positive and Gram-negative bacteria. Peppermint is also found to possess antiviral and fungicidal activities (Singh *et al.*, 2015).

The key active constituents of lemongrass oil (LGO) giving distinct aroma are citral (65-86%), neral and geraniol. It was identified that lemongrass oil bears antidepressant, antioxidant, antiseptic, sedative, nervine, bactericidal, and fungicidal properties. As a bactericidal agent, the LGO was found to be effective against many bacterial species including *Acinetobacterbaumanii*, *Aeromonasveronii*, *Enterococcusfaecalis*, *Escherichi a coli*, *Staphylococcus aureus* and so on (Naik *et al.*, 2010).

Curry leaves are natural flavoring agents with numerous health benefits. They contain several medicinal properties that include it being anti-diabetic, antioxidant, antimicrobial, anti-inflammatory, anti-carcinogenic and with hepatoprotective properties. In a study curry leaf extract showed a broad spectrum of very significant

antibacterial activity by producing a clear zone of inhibition against *Staphylococcus, E.coli, Streptococcus* and *Proteus* (Irfan and Ali, 2016).

The compound isolated from ethyl acetate fraction of *Justicia adhatoda* flowers extract has a significant antibacterial activity against bacteria and fungi. Four bacterial strains like *S. typhi, E. coli, E. faecalis* and *B. cereus* and two fungal strains such as *C. lunata* and *C. albicans* were tested by using disc diffusion method (Duraipandiyan *et al.*, 2015).

2.10 Drying

Drying is possibly the oldest method of food preservation (Shrestha, 2001). It has been known that the various fruits and vegetables can be enjoyed out of season if the freshly harvested material is preserved by drying.

2.10.1 Mechanism of drying

When hot air is blown over a wet food, heat is transferred to the surface, and latent heat of vaporization causes water to evaporate. Water vapor diffuses through a boundary film of air and is carried away by the moving air. This creates a region of lower water vapor pressure at the surface of the food, and a water vapor surface gradient is established from the moist interior of the food to the dry air. This gradient provides the driving force for water removal from the food. Water moves to the surface by the following mechanisms:

- Liquid movement by capillary forces.
- Diffusion of the liquid which is adsorbed in layers at the surface of solid components of the food.
- Diffusion of the liquids, caused by difference in the concentration of solutes in different region of the food.
- Water vapor diffusion in air spaces within the food caused by vapor pressure gradients.

Movement of moisture during drying is illustrated in Figure 2.3

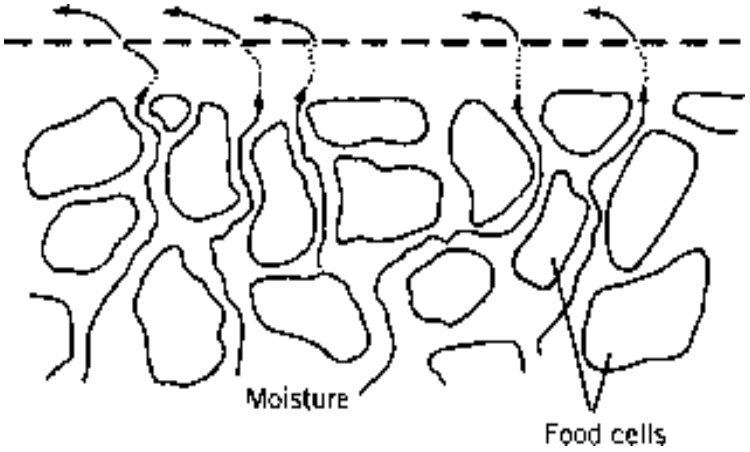


Fig 2.2 Movement of moisture during drying (Kharel, 2004)

In foods, water is present as free water and bound water. Free or unbound water is present within the pores and spaces between plant cells. It has the same characteristics as pure water. It exerts the same vapor pressure and same latent heat of vaporization as pure water. It is easily removed during drying. Bound water is held on the surfaces of solid compounds, such as the cellulose, hemicelluloses, in the cell wall by molecular interactions between water and the solid. It exerts a vapor pressure less than that of pure water, it does not evaporate easily, and the latent heat of vaporization is greater than that of the pure water at a given temperature. It requires more heat to release and therefore bound water is not usually removed during drying (Heldman and Hartel, 1997).

During drying of a wet solid in a heated air, the air supplies the necessary sensible and latent heat of evaporation of the moisture and also act as a carrier gas for the removal of the water vapor formed from the vicinity of evaporating surface (Kharel, 2004).

2.10.2 Different methods of drying

2.10.2.1 Sun drying

Sun drying is a natural method of drying making use of exposure to the sun and requires time and some effort by the man to spread and collect the produce (Hall, 1970).

2.10.2.2 Solar Drying

Solar drying refers to methods of using sun's energy for drying but excludes open air sun drying. Solar drying is more effective than sun drying and has lower operative cost than mechanized dryers.

2.10.2.3 Cabinet drying

Cabinet drier consist of an insulated cabinet fitted with shallow mesh or perforated trays, each of which carries a thin layer of food. Hot air is circulated through the cabinet tray. A system of duct and baffles is used to direct air, over and/ or through each tray, to promote uniform air distribution. Cabinet drying are usually used for small scale operations comparatively inexpensive, low maintenance cost, commonly used to dry fruit and vegetable pieces (Fellows, 2000).

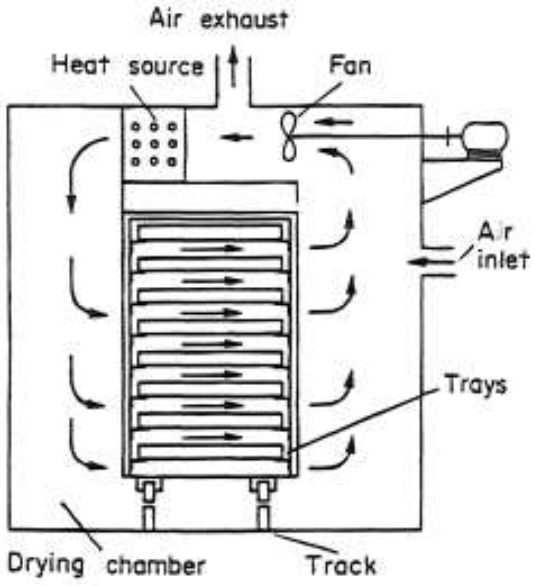


Fig 2.3 Cabinet dryer

2.10.3 Effect of drying on phytochemicals and antioxidant activity

Production of phytochemicals in plant is affected by many pre and postharvest factors including farming practices, environmental factors, storage and processing conditions, but temperature is the primary factor among all. The metabolism of phytochemical begins right after the harvest and can involve complex biochemical reactions. This

reaction can lead to significant changes in plant attributes and health promoting phytochemicals, such as those with strong antioxidant activities (Li *et al.*, 2012).

Different phytochemicals are affected by temperature differently. Carotenoids are very sensitive to heat and can incur significant losses during different vegetable processing steps. Ascorbic acid is also a natural antioxidant which gets destroyed even at mild temperature (Palermo *et al.*, 2014). Flavonoids and other phenolic compounds are relatively stable over long storage (Vallejo *et al.*, 2003).

At low temperature, deterioration of phytochemicals slowed down. Opposed to lower temperature, high temperature also brings a significant change in total phenolic, flavonoid, tannin content and antioxidant activity compared with its fresh form. However their concentration may vary according to the drying methods used and the duration of exposure to hot air (McSweeney and Seetharaman, 2015). Exposure to high temperature leads to discharge of phenolic compounds through disintegration of cellular constituents which then results in the migration of components, leading to losses by leakage or breakdown by various chemical reaction involving enzymes, light and oxygen as well as (Nayak *et al.*, 2013).

Tea contains many valuable compounds such as phenolic, flavonoids (catechins), amino acids, minerals, vitamins (B, C, E), xanthic bases (caffeine, theophylline), pigments (chlorophyll, carotenoids) and volatile compounds (aldehydes, alcohols, esters, lactones, hydrocarbons) (Vuong *et al.*, 2012). Most of the previous researches on tea were carried out on limited drying methods (Hirun *et al.*, 2014). Furthermore, drying treatments can affect nutritional and qualitative characteristics of tea such as vitamins, color, chlorophyll, total flavonoid, total phenolic content and antioxidant activity of final product. Drying methods caused a significant decrease in total phenolic and antioxidant capacity of tea leaves.

To select the best drying method, different criteria such as plant species, energy consumption, cost, and final color of dried plant, nutritional value and the time of drying should be considered. Since the consumption of tea has been increasing during the past decades, selecting the best treatments in respect to qualitative and nutritional

characteristics of this product is very important. The higher temperatures and longer drying time led to more color damage. Totally, freeze drying increased or maintained color characteristics and vitamin C, while oven drying was more efficient to elevate TPC, TFC and antioxidant activity of tea (Roshanak *et al.*, 2016).

Herbal preparation developed using oven drying was found to have inferior phytochemicals content. Nevertheless, the herbal preparation developed using all treatments still retain appreciable amount of phytochemicals (Mahanom *et al.*, 1999).

2.11 Phytochemicals

Phytochemicals are non-nutritive bioactive chemical compounds found naturally in plants that protect against diseases. A number of phytochemicals are known, some of which include: alkaloids, saponins, flavonoids, tannins, glycosides, anthraquinones, steroids and terpenoids. They do not only protect the plants but have enormous physiological activities in humans and animals. These include cancer prevention, antibacterial, antifungal, antioxidative, hormonal action, enzyme stimulation and many more (Doss and Anand, 2012).

2.11.1 Classes of phytochemicals

Phytochemicals are not essential nutrients and are not required by the human body for sustaining life, but have important properties to prevent or to fight some common diseases. Many of these benefits suggest a possible role for phytochemicals in the prevention and treatment of disease, Because of this property; many researchers have been performed to reveal the beneficial health effects of phytochemicals. The purpose of the present review is to provide an overview of the extremely diverse phytochemicals presents in medicinal plants. While phytochemicals are classified by function, an individual compound may have more than one biological function serving as both an antioxidant and antibacterial agent (Saxena *et al.*, 2014). Bioactive and disease preventing phytochemicals present in plant are shown in Table 2.3

Table 2.1: Bioactive phytochemicals in medicinal plants

Phytochemical classes	**Phytochemicals**	**Sources**	**Nutritional benefits**
Carotenoids	β-carotene& carotene, lutein, lycopene	Tomato, pumpkin, Carrot, watermelon. Guava, dark yellow pink and red colored vegetative fruits	- Act as antioxidant - Reduce level of cancer - Producing enzymes - Inhibit spread of cancer
Polyphenols	Tannins	Fruits, Legumes, Green vegetable, black Tea	-Exhibit anti-microbial and antioxidant activities help speed excretion of carcinogen from the body
Flavonoid	Anthocyanin, Anthoxanthin	Beans, citrus fruits	Block access of carcinogen, prevents Malignant change in cells. Prevent cancer

Saponins	Panaxadiol, Panaxatriol	Potato, tomato, soybean, beans	Reduce glucose and glycerol uptake in the gut.
Phytosterols	β-sitosterol, campesterol, stigma sterol	Potatoes, tomatoes, vegetable oils, alfalfa, sprout	Block excess uptake of dietary cholesterol and facilitate cholesterol excretion
Terpenes	Mono-terpenes	Garlic, maize, ginger	Help detoxify carcinogens, inhibit spread of cancer
Detoxifying agents	Reductive acids Tocopherols Aromatic isc coumarins Flavones Carotenoids Retinoid Cyanates Phytosterols	Cruciferous vegetables including cabbage, cauliflower, broccoli Onion, Garlic, Turnips, Broccoli, Kale, Berries, Pomegranate	proliferation of cancer-fighting enzymes. Inhibitors of procarcinogen activation Inducers of drug binding of carcinogens Inhibitors of tumourogenesis

2.11.2 Phenolic / Polyphenols

Phenolic phytochemicals are the largest category of phytochemicals and the most widely distributed in the plant kingdom. Structurally, they contain aromatic ring containing one or more hydroxyl groups (O' Connell and Fox, 2001). Based on the number of carbon atoms present in its structure, phenolic are categorized into five major groups.

i. C_6 group: This group of phenol includes simple phenols and benzoquinones with six carbon atoms.
ii. C_6Cn group: Phenolic acid and hydroxycinnamic acid derivatives are included in this group.
iii. C_6CnC_6 group: The largest group of phenolic compound includes flavonoids which have low molecular weight and are further of five types (flavones, flavonols, flavanols, flavones and anthocyanin's) on the basis of substitution pattern of carbon ring.
iv. $(C_6Cn)_3$groups: This group consists of lignin's and lignans.
v. Tannins: Tannins are high molecular weight phenols and classified into two main categories (hydrolysable and condensed tannins) (O' Connell and Fox, 2001)

2.11.2.1 Significance of phenolic compounds

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

- As Antioxidant Compounds: The main and most important role of phenol is their antioxidant property. They act as free redical scavengers which are formed due to high UV radiation.
- As Structural Polymers: Lignin is the most important and widely distributed phenolic compounds which act as structural unit of plants.
- As Defensive Compounds: Due to presence of tannins, plant develops an astringent taste. Tannins interact and precipitate with proteins which results in bitter taste of plants. Consequently, they act as feed deterrents in most of the cases.

- As Signal Compounds: Many biochemical metabolic pathways have phenolic compounds as their signal molecules. For instance, in salicylic acid pathway, methyl salicylate (phenolic compound) acts as a signaling compound. Another phenolic signaling compound reported is de-hydrodiconiferyl alcohol glucosidase (DCG).
- As Pollinator Attractors: Simple phenolic acids with low molecular weight are responsible for aroma and attractive coloration of flowers which attract pollinating agents. Phenols with these functions are anthocyanin's, flavonoids etc.
- As UV screen: The phenolic present in plant cuticle play an important role in screening the amount of UV radiations reaching earth through ozone layer.

2.11.3 Flavonoids

Flavonoids are the largest group of phenolic compounds and have a basic skeleton composed of three rings (C6-C3-C6). They are classified into six major classes according to their substitution pattern in the B- and C-rings, which are flavan-3-ols, anthocyanin's, flavones, isoflavones, flavones and flavonols (Harbone and Baxter, 2000). The flavonoid polymers are also known as pro anthocyanidins. Flavonoids occur as plant secondary metabolites that are involved in pigmentation, antioxidants, antimicrobials, antistressors, and UV irradiation protection (Vaya and Aviram, 2001). More than 4000 flavonoids have been described so far within the parts of plants normally consumed by humans and approximately 650 flavones and 1030 flavanols are known (Ghamsemzadeh *et al.*, 2010).

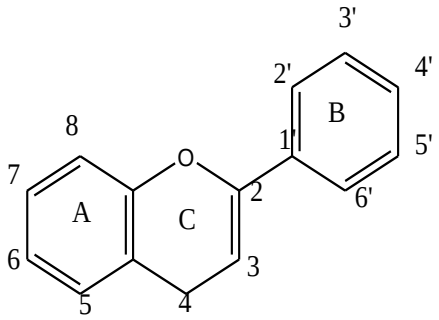


Fig.2.4 Structure of Flavonoid (Nishiumi *et al.*, 2011)

2.11.3.1 Biological activity of flavonoid

Flavonoids have been reported to exert multiple biological property including antimicrobial, cytotoxicity, anti-inflammatory as well as antitumor activities but the best-described property of almost every group of flavonoids is their capacity to act as powerful antioxidants which can protect the human body from free redicals and reactive oxygen species. The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free redical scavenging activities (Kelley *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 peroxynitrite redicals, stabilizing them and giving rise to a relatively stable flavonoids redical (S. Kumar and Pandey, 2013).

Mechanisms of antioxidant action may include

- Suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free redical generation.
- Scavenging ROS and
- Regulation or protection of antioxidant defenses

2.11.4 Tannins

Tannins are polyphenols sometimes called plant polyphenols although originally the name tannin was given to the plant extracts exhibiting astringency, without knowing their chemical structures (Haslam, 1989). The features distinguishing tannins from plant polyphenols of other types are basically the properties of the former: binding to proteins, basic compounds, pigments, large-molecular compounds and metallic ions, and also antioxidant activities, etc. (Okadu and Ito, 2013). These are widely distributed in plant flora. They are phenolic compounds of high molecular weight. Tannins are soluble in water and alcohol and are found in the root, bark, stem and outer layers of

plant tissue. They form complexes with proteins, carbohydrates, gelatin and alkaloids. On the basis of their structural characteristics it is therefore possible to divide the tannins into four major groups: Gallotannins, ellagitannins, complex tannins, and condensed tannins (Saxena *et al.*, 2014).

Gallo tannins are all those tannins in which galloyl units or their meta-depsidic derivatives are bound to diverse polyol-, catechin-, or triterpenoid units.

Ellagitannins are those tannins in which at least two galloyl units (C–C) are coupled to each other, and do not contain a glycosidically linked catechin unit.

Condensed tannins are all oligomeric and polymeric pro anthocyanidins formed by linkage of C-4 of one catechin with C-8 or C-6 of the next monomeric catechin.

Complex tannins are tannins in which a catechin unit is bound glycosidically to a gallo tannin or an ellagitannin unit.

2.11.4.1 Activity of tannins

Tannins have diverse effect on biological system since they are potential metal ion chelators, protein precipitating agents and biological antioxidants. Because of the varied biological roles that tannins can play and because of the enormous structural variation, it has become difficult to develop models that would allow an accurate prediction of their effects in any system (Skowyra, 2014).

The tannin-containing plant extracts are used as astringents, against diarrhea, as diuretics, against stomach and duodenal tumors and as anti-inflammatory, antiseptic, antioxidant and hemostatic pharmaceuticals (Dolara *et al.*, 2005). In the food industry tannins are used to clarify wine, beer, and fruit juices. Other industrial uses of tannins include textile dyes, as antioxidants in the fruit juice, beer, and wine industries, and as coagulants in rubber production (Gyamfi and Aniya, 2002). Recently the tannins have attracted scientific interest, especially due to the increased incidence of deadly illnesses such as AIDS and various cancers. The search for new compounds for the development of novel pharmaceuticals has become increasingly important, especially as the

biological action of tannin-containing plant extracts has been well documented (Palavy and Priscilla, 2006).

2.11.5 Tea polyphenols

Tea leaves are, apart from wine, fruit and vegetables, a very good source of polyphenolic compound. Tea is reported to contain nearly 4000 bioactive compounds of which one third is contributed by polyphenols. Other compounds are alkaloids (caffeine, theophylline and theobromine), amino acids, carbohydrates, proteins, chlorophyll, volatile organic compounds (chemicals that readily produce vapors and contribute to the odor of tea), fluoride, aluminum, minerals and trace elements (Ho and Wang, 2009).

In tea leaves three basic polyphenol groups can be distinguished: catechin, theaflavins and thearubigenes. Green tealeaves consist of flavonoids as well as phenolic acids which can make up to 30% of fresh leaves dry weight and only 10% of dry weight of black tea. Both green and black tea contains a similar quantity of flavonoids, differing in respect of their chemical structures. Other products of polyphenol oxidation are tannins. Tannins were divided into two groups: hydrolysable, water-soluble, including simple phenolic acids, gallic acid esterified into the polyols, and non-hydrolysable tannins(condensed), the polymers of elementary flavonoids particles (Z.-m. Chen and Lin, 2015).

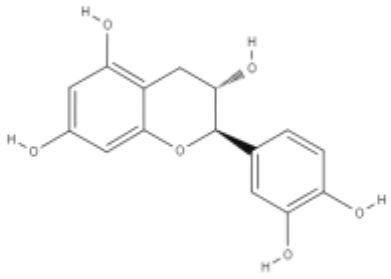
2.11.5.1 Flavan-3-ols

Green tea is one of the most abundant food sources of catechins, members of flavan-3-ols, a subclass of flavonoids. Flavan-3-ols have two chiral centers C2 and C3, which results in four isomers for each level of B ring hydroxylation. (+)-Catechins and (–)-epicatechin are widespread in nature whereas (–)-catechins and (+)-epicatechin are rare. The most abundant catechins in tea are (−)-epigallocatechin gallate (EGCG), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), (−)-epicatechin (EC), (+)-catechin (C), and (−)-gallocatechin gallate (GCG) (Xu *et al.*, 2004).

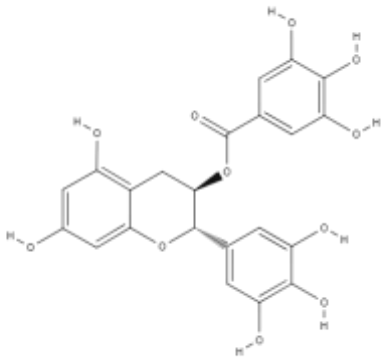
The chemical structures of major polyphenols present in green tea are shown in Fig. 2.2. The following order of antioxidant activity of tea catechins emerged from a number of studies: epigallocatechin ~epigallocatechin gallate >> epicatechin gallate = epicatechin >catechins (Łuczaj and Skrzydlewska,2005). Catechin is a compound which does not evaporate and it contained about8-15% from the dry weight of plant (Farhoosh *et al.*, 2007). Table 2.2 shows a clear picture of the predominance of catechins in tea across all varieties.

Table 2.2 Predominance of catechins in tea across all varieties

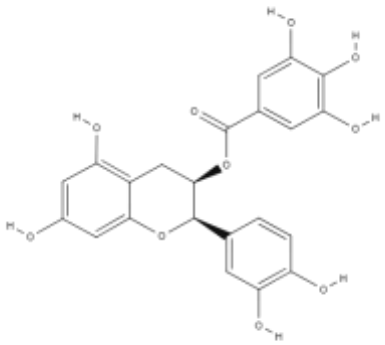
Tea	**Content of polyphenols**
Green tea	Catechins
White tea	Catechins
Black tea	Catechins, Theaflavins, Thearubigins
Oolong tea	Catechins, Theaflavins



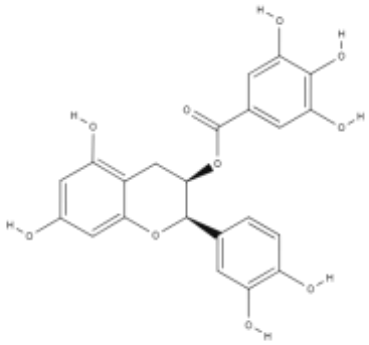
Catechin



Epicatechin gallate



Epigallocatechin gallate



Epicatechin gallate

Fig 2.5 The chemical structures of major polyphenols present in green tea (Malaguti *et al.*, 2013).

2.11.5.2 Phenolic acids

Gallic acid and its quinic acid ester, theogallin, are the most abundant simple polyphenols present in tea. During processing of black tea, the amount of gallic acid significantly increases due to oxidative degallation of phenolic esters during the fermentation. Recently, 15 different phenolic acid derivatives have been identified in black tea by(L. Z. Lin *et al.*, 2008).

2.11.5.3 Flavonols and Flavones

Quercetin, myricetin, kaempferol and their mono-, di-, and tri-glycosides are the most abundant flavonols in tea. Recently, three flavonols, 19 *O*-glycosylated flavonols, 28acylated glycosylated flavonols, and 7 C-glycosylated flavonols have been identified in white, green, oolong, and black tea samples (L. Z. Lin *et al.*, 2008).

2.11.5.4 Theasinensins

Theasinensins are dimeric gallocatechins linked by C–C bonds between two B rings forming a biphenyl. They are present mainly in oolong tea. Theasinensin A is a product of oxidation of two EGCG molecules, B of EGCG and EGC, C of two EGC molecules,

and F of EGCG and ECG. Additionally, theasinensins might be formed from gallocatechins inthe gut during digestion of tea (L. Z. Lin *et al.*, 2008).

2.11.5.5 Theabrownins

Theabrownins are pigment compounds formed during oxidation of tea leaves. They dissolve easily in water and not in organic solvents like ethyl acetate. They are the main bioactive components in pu-erh tea, and are characterized by high molecular weight and complex structure. It is known that theabrownins are formed by oxidation of polyphenols. However, the mechanism of their formation is not clear. It was reported that the cellulases and pectinases produced by microorganisms, together with polyphenol oxidase, play an important role in theabrownin formation (Y. Wang and Ho, 2009).

2.11.5.6 Theaflavins and Thearubigins

Thearubigins are the most abundant group of phenolic pigments found in black tea, accounting for an estimated 60-70% of the solids in a typical black tea infusion. Thearubigins isolated from 15 commercial black teas have been analyzed using a strategy combining standard chemical characterization along with a series of modern complementary mass spectrometry techniques, including MALDI-TOF-MS, FTICR-MS, LC/TOF-MS and LC/MS/MS. Fifteen molecular formulas have been matched to constituents of fresh tea leaf that have survived processing and 21 to diametric transformation products such as theasinensins, theaflavins, theaflavates, theanaphthoquinones, theacitrins and oolongtheanins, which were further confirmed by ESI MS/MS. MALDI-TOF-MS data revealed an average of 5000 additional thearubigin components in the mass range between m/z 1000 to 2100 clearly defining the molecular weight range of the thearubigin fraction (Kuhnert *et al.*, 2010).

Estimation of TF and TR in black tea is generally done using a spectrophotometer. The chemistry underlying the changes which occur during tea leaf fermentation is reviewed and used as a basis for proposals for the structure of thearubigins, the major pigments of black teas (Haslam, 2003). Black tea contains two

major groups of pigments, theaflavins (TFs) and thearubigins (TRs). TFs contain a bis-flavan substituted 1, 2-dihydroxy-3, 4-benzotropolone moiety (Menet *et al.*, 2004).

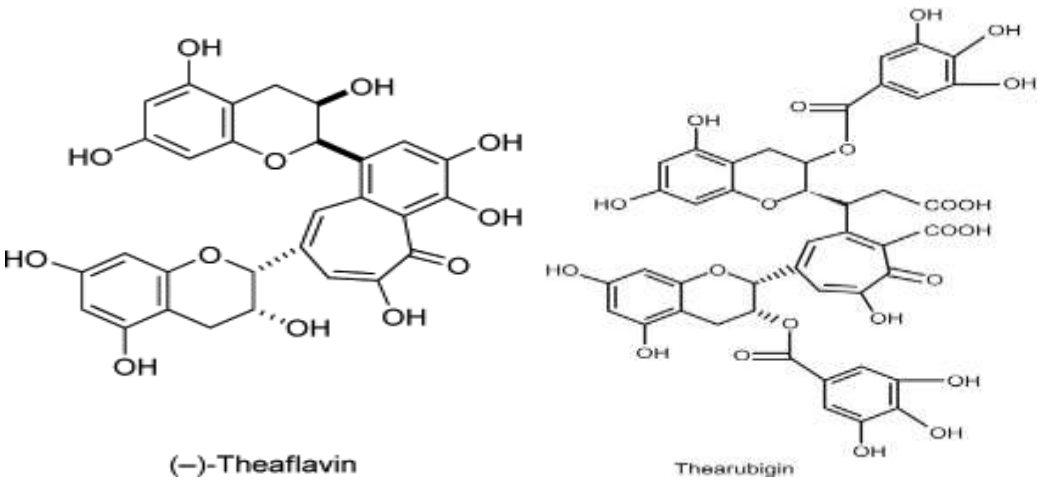


Fig 2.6: Chemical structure of Theaflavin and Thearubugin present in black tea (Haslam, 2003)

It is generally believed that polyphenols such as theaflavins and thearubigins as well as catechins as major constituents of black tea are mainly responsible for antioxidant actions. Antioxidative properties of black tea are manifested by its ability to inhibit free redical generation, scavenge free redicals, and chelate transition metal ions. Black tea, as well as individual theaflavins, can influence activation of transcription factors such as NFkappaB or AP-1. Theaflavins have been also proved to inhibit the activity of prooxidative enzymes such as xanthine oxidase or nitric oxide synthase (Luczaj and Skrzydlewska, 2005).

2.11.6 Phytochemical metabolism in human

Most phytochemicals found in foods exist in a variety of forms which influence their digestion and absorption. The absorption of most phytochemicals is thought to involve a carrier. Also, many glycosides are neither digested nor absorbed in the small intestines. Such phytochemicals not absorbed in the small intestine have been shown to undergo microbial degradation by colonic micro flora (Ross and Kasum, 2002). The

bacteria hydrolyze the glycosides, generating aglycones which may undergo further metabolism to form various aromatic compounds (Bradlaw *et al.*, 1999).

Once absorbed, most phytochemical metabolites get conjugated in the small intestine or in the Liver (Rhodes, 1996). These conjugated metabolites are then bound to plasma proteins such as albumin and are transported through the blood to various parts of the body the amount of these conjugated metabolites in the plasma varies considerably with the type of polyphenol consumed, the food source, and the amount ingested. However, after consumption of specific polyphenols, little is known about the metabolism of the different polyphenols in the body, and also about what metabolites are present in the plasma (Briskin, 2000).

2.11.7 Factors affecting phytochemical yield

Production of phytochemicals in plants is affected by many pre and post-harvest factors including farming practices, environmental factors (microclimate, location, growing season, soil type and nutrients), plant maturity, post-harvest storage and processing, but genetics is the primary factor among all.

2.11.7.1 Genetics

Genetics has perhaps the greatest effect on the production of plant secondary metabolites. Studies have also shown that the phytochemical content of a particular cultivar can vary significantly due to genetic and other factors. Mineral composition, soil type, temperature, light and water content are among the frequently reported factors that affect the total phytochemical contents in plants (Francisco *et al.*, 2012; Stephens *et al.*, 2009).

2.11.7.2 Post-harvest Storage and Processing Conditions

Changes in both the quality and phytochemical composition of plants can occur rapidly depending on postharvest handling such as storage and processing conditions (Tanko *et al.*, 2005). Carotenoids are very sensitive to heat, and can incur significant losses during different vegetable processing steps. The main cause of carotenoid degradation in foods is oxidation. Flavonoids and other phenolic compounds are relatively stable at

high temperature and over long storage. Phenolics in plants exist in both free and conjugated forms. Post-harvest loss of phenolics is mainly due to enzymatic oxidation by polyphenol oxidase and peroxidases (Tanko *et al.*, 2005).

2.11.7.3 Temperature and Humidity

Lower temperature and proper packaging can slow the deterioration of phytochemicals. More importantly, in most cases, higher antioxidant activities were maintained by fruits stored at 48 ºC, as opposed to 258 ºC. However, lowering temperature may have a different impact on the different groups of phytochemicals, and may not always result in increased phytochemical content or antioxidant activities. Low (chilling) temperature (18ºC) negatively affected the content of major carotenoids, except β-carotene (S. G. Lee *et al.*, 2013).

2.11.7.4 Controlled Atmospheres and Modified Atmosphere Packaging

Many studies have also shown that CA or MAP technologies offer the possibility to retard the respiration rate, maintain bioactive compounds and extend the shelf-life of fruits and vegetables as compared with conventionally stored or packaged samples (De Jesus et al. 2009). MAP could change the storage behavior of several constituents, such as flavonols and anthocyanins (Bengtsson 2010). CA inhibited the overall decline in polyphenol concentration (Kim et al. 2007).

2.12 Formation and degradation of radicals

Oxygen, an element indispensable for life, can under certain circumstances, adversely affect the human body. Oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called ‘free radical’. Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function. Most of the potentially harmful effects of oxygen are due to the formation of reactive oxygen species (ROS), which have a tendency to donate oxygen to other substances (Chima *et al.*, 2014). Reactive oxygen species (ROS) is a term which encompasses all highly reactive, oxygen-containing molecules, including free radicals. Free radicals are capable of attacking the healthy cells of the body, causing

them to lose their structure and function (Percival, 1998). ROS are free radicals produced as by-products of oxidation–reduction (redox) reactions (Dowling and Simmons, 2009).

Reactive oxygen species (ROS) are produced by living organisms as a result of normal cellular metabolism. At low to moderate concentrations, they function in physiological cell processes, but at high concentrations, they produce adverse modifications to cell components, such as lipids, proteins, and DNA1–6 (Free radicals, Free Radicals in Biology, Lipid peroxidation, 4-Hydroxynonenal formation, Role of oxidant, Lipid peroxidation). Types of ROS include the hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides. All are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage (Birben *et al.*, 2012).

The ROS are generated as a result of energy production from mitochondria (from the electron transport chain), as part of an antimicrobial or antiviral response, as well as detoxification reactions carried out by the cytochrome P 450 system (Cederbaum *et al.*, 2001; Weiss and LoBuglio, 1982). Environmental agents such as ultraviolet light, ionizing radiation, redox chemicals and cigarette smoke also readily generate ROS (Chima *et al.*, 2014). Humans have evolved a highly sophisticated and complex antioxidant protection system. Antioxidants are our first line of defense against free radical damage and are critical for maintaining optimum health and wellbeing. It involves a variety of components, that function interactively and synergistically to neutralize free radicals (Jacob, 1995).

An antioxidant is a substance that when present at low concentrations compared to that of an oxidizable substrate significantly delays or prevents oxidation of that substrate. Antioxidants can act by scavenging biologically important reactive oxygen species (O_2, H_2O_2, OH, HOCl, ferryl, peroxyl, and alkoxyl), by preventing their formation, or by repairing the damage that they do (Halliwell, 1991). The antioxidant defense system in most cells is composed of two components, the antioxidant enzymes component which includes enzymes such as superoxide dismutase, catalase and

glutathione peroxidase, and the low molecular weight antioxidant component that includes vitamins A and E, ascorbate, glutathione and thioredoxin. Oxidative stress occurs when the production of ROS exceeds the body's natural antioxidant defense mechanisms, causing damage to biomolecules such as lipids, proteins and DNA (Sahnoun *et al.*, 1997).

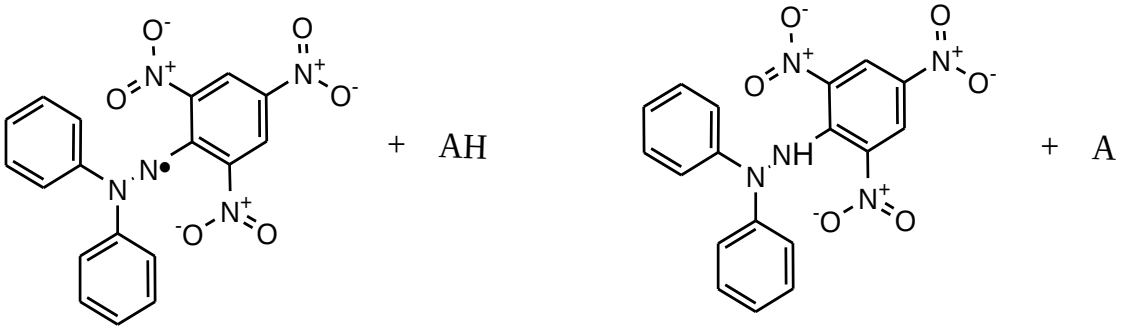
The shift in balance between oxidant/antioxidant in favor of oxidants is termed "oxidative stress." Oxidative stress contributes to many pathological conditions including cancer, neurological disorders, atherosclerosis, hypertension, ischemia/perfusion(Jenner, 2003), diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease and asthma (Andreadis *et al.*, 2003).

Medicinal plants and herbs are known to be an important source of various natural antioxidants. Many other non-nutrient food substances, generally phenolic or polyphenol compounds, display antioxidant properties and thus may be important for health. One of the ways to deal with such oxidative damage and disease is the adequate oral intake of antioxidants from external sources. The best known external antioxidants are vitamin E, vitamin C and the carotenoids (W. Chen *et al.*, 2016; Lobo *et al.*, 2010).

2.12.1 DPPH radical scavenging assay

DPPH assay measures the ability of a substance to scavenge the DPPH radical, reducing it to hydrazine. A number of methods are used to determine the radical scavenging effects of antioxidants. The DPPH method is a preferred method because it is fast, easy and reliable and does not require a special reaction and device (Fukumoto and Mazza, 2000). The DPPH antioxidant assay is based on the ability of 2, 2-diphenyl-1-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. DPPH free radical, which is at its maximum wavelength at 517 nm, can easily receive an electron or hydrogen from antioxidant molecules to become a stable diamagnetic molecule (Marxen *et al.*, 2007). The DPPH radical is one of the few stable organic nitrogen radicals, which produces solutions colored deep purple. A solution of DPPH radicals prepared in methanol is converted into DPPH-H (diphenylhydrazine)

molecules in the presence of an antioxidant agent, as shown in the following equation. Discoloration occurs due to the decreasing quantity of DPPH radicals in the environment. The discoloration of the DPPH therefore reflects the radical scavenging activity of the analyzed extract (Molyneux, 2003).



2,2-diphenyl-1-picrylhydrazyl 2,2-diphenyl-1-picrylhydrazine

Fig. 2.4 Reaction of DPPH-free radical with an antioxidant

Antioxidant efficiency is measured at ambient temperature and thus eliminates the risk of thermal degradation of the molecules tested. An antioxidant can be defined as "any compared to that of an oxidizable oxidation of that substrate" substance that, when present in low concentrations substrate, significantly delays or inhibits the oxidation of that substrate". Although the DPPH method is widely used, it does have some limitations. The radical portion of the molecule is a nitrogen atom located at the center of the structure. While this centralized location is freely accessible to small molecules, larger molecules may have limited access to the radical portion due to steric hindrances (Molyneux, 2003).

2.13 Antioxidants

Any substance which is capable of delaying, retarding or preventing the development of the rancidity or other flavors deterioration due to oxidation is called antioxidant (Coppen, 1983). Oxidation reactions are chemical reactions that involve the transfer of electrons from one substance to an oxidizing agent. Antioxidants can slow these reactions either by reacting with intermediates and halting the oxidation reaction directly, or by reacting with the oxidizing agent and preventing the oxidation reaction

from occurring (Pokorny, 2007). Much is known about antioxidants because of their functional importance, interest in antioxidants is high to protect edible oils, their derived products and also when used in food products to provide baking and culinary characteristics and nutritional benefits. Antioxidants are substances that generally prevent, delay or retard the onset of rancidity in food products due to oxidation of the unsaturated fatty acids incorporated in food products. The use of antioxidants helps to extend the shelf life of a food, minimizes waste and nutritional losses, and extends the scope of use of various fats/oils (D.K., 2003).

Antioxidants are generally effective, easily applied and inexpensive. Other justification for need of an antioxidant use are- an antioxidant can extend the shelf life of a food, reduce wastage and nutritional losses (oil soluble vitamins) and more important it can widen the range of fats which can be used (Allen and Hamilton, 1983).

The phenolic composition and antioxidant activities [TEAC, ORAC, FRAP] of consumer brews black tea from the were investigated. The main phenolic compounds identified were epigallocatechin gallate, four theaflavins, as well as epicatechin gallate, theogallin, quercetin-3-rutinoside and 4-caffeoyl quinic acid. Thearubigins represented an estimated 75-82% of the total phenolics. Further, polyphenol fractions were in decreasing order theaflavins, flavan-3-ols, flavonols, gallic acids and hydroxycinnamates. On average, a cup of a consumer brew of black tea is providing polyphenols at the level of 262mg GAE/serving, of which 65 mg were assigned to individual polyphenols. Treatment of the black tea brew with simulated gastric juice resulted in a significant increase of the identified theaflavins implying a partial cleavage of thearubigins in the environment of the gastric lumen. Therefore, black tea can be considered to be a rich source of polyphenols and/or antioxidants (Rechner *et al.*, 2002).

2.13.1 Mechanism of action

The antioxidants are active in lengthening the induction period in the process of oxidation of fats, probably due to the absorption of the activating energy of the chain reaction that result in the oxidation of antioxidants. An antioxidant act by reacting with

free redicals fatty acid (free radiator peroxy free redical) as they are formed, converting them back to the original substrate and then by terminating the chain propagation (or initiation). Free redicals of the antioxidant molecule are formed in the process, but the structure of an antioxidant so such that these are relatively stable and do not have enough energy to react with the fat to form further free redicals (Coppen, 1983). The process scheme (Dugan, 1986; Lundberg, 1961) is in Fig. 2.6

An antioxidant (AH) apparently reacts with free redicals in following manner:

R• + AH ----------RH + A•

RO• +AH ---------- ROH +A•

ROO• +A• ----------ROOH +A•

R• + A• ----------RA

RO• +A• ----------ROA

A• + ROO• ---------- ROOA (Non redical product)

A• + A• ---------- AA (Non redical product)

Fig. 2.6 Mechanism of action of antioxidant

Two principle mechanisms of action have been proposed for antioxidants (Rice-Evans and Diplock, 1993). The first is a chain- breaking mechanism by which the primary antioxidant donates an electron to the free redical present in the systems. The second mechanism involves removal of ROS/reactive nitrogen species initiators (secondary antioxidants) by quenching chain-initiating catalyst. Antioxidants may exert their effect on biological systems by different mechanisms including electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation (Krinsky, 1992).

According to Pan *et al.* (2003), tea polyphenols have been found to possess stronger antioxidant activity than the artificial antioxidants such as butylated hydroxyl toluene, butylated hydroxyl anisole, DL tocopherol. Besides tea polyphenols are also less toxic as compared to the artificial antioxidant. It has been found from the research that the

antioxidant activity decreased in the order of semi-fermentive tea > non-fermentive tea > fermented tea (C. S. Yang *et al.*, 2009).

2.13.2 Types of antioxidant

2.13.2.1 Synthetic (artificial) antioxidant

Most of the synthetic antioxidants used are phenolic compounds among which Butylated Hydroxytoluene (BHT) and Butylated Hydroxyanisole (BHA), Tert. Butylated Hydroquinone (TBHQ), Propyl Gallate (PG) is common in use. A quantitative tolerance limits for this synthetic antioxidant in Federal Regulations are limited not exceeds total content of 0.02% of fat or oils either alone or in combination (Bauernfeind and Cort, 1974).

2.13.2.2Natural Antioxidant

Antioxidants are widely distributed in nature. Natural antioxidants impart no adverse effect in its long run of use; and do not have a quantitative tolerance limit in federal regulations. It imparts no adverse effect in its long run of use; natural antioxidants do not have a quantitative tolerance limit in federal regulations (Bauernfeind and Cort, 1974). Natural antioxidants seems to be more adequate for protection against oxidation and have many inherent qualities unsuppressed by the synthetic antioxidants (Loliger, 1983).

The tocopherols are slightly viscous paleyellow liquids freely soluble in most organic solvents; insoluble in water. To retard the development of oxidative rancidity and tocopherols are used in foods as antioxidants. Tocopherols have a molecular weight of 30-69 and boiling point 200-220 ^{o}c (0.1mm). In addition to food uses vitamin is used in food supplement and pharmaceutical dosage formulation. Tocopherols are readily oxidized and consequent protects the fat from oxidation (Meyer, 1987).

2.13.3 Synergistic Antioxidants

The preventive antioxidants which act by reducing the rate of chain initiation is called synergistic antioxidants although they have no effect as protectants when used along

with fats (F. A. Lee, 1975).These compounds helps to increase (improve) the ability of the phenolic antioxidants to retard rancidity (Furia, 1968)has presented an excellent review of the use of sequestrates (metal inactivators) in foods. Many components exhibit metal deactivating properties in edible triglyceride oils as evidenced by improvement in oxidative and/or flavor stability. Among these most important is citric acid (Dutton *et al.*, 1949).

All metal inactivating compounds have free hydroxyl for carboxyl groups that co-ordinate readily with metal forms salts readily proposed the metal inactivators, in effect complexes with peroxidant metal and hold them in a chelate or ring structure held winter co-ordination complexes, where the metal can no longer function as peroxidant (Schwab et. al. 1953)

Part III

Materials and Methods

3.1 Materials

3.1.1 Plant used

a. **Tea *(Camellia sinensis)*:** Tea was collected from Hile, Dhankutaand it is locally known as 'chiya patta'. The variety used was 'TAKHDAH'
b. **Lemongrass *(Cymbopogon citratus)*:** Lemongrass was collected from Jadibuti Prasodhan Kendra, Taraharaand it is locally known as 'kagate jhar'.
c. **Mint *(Mentha piperita)*:** Mint was collected from local market of Dharanand islocally known as 'pudina'.
d. **Curry leaf *(Murraya koenigii)*:** Curry leaves was collected from the locality around CCT, Dharan and it is locally known as 'kadi patta'.
e. **Asuro *(Justica adhotota)*:** Asuro was collected locally from Dharan locality and it is locally known as 'asuro'.

The plant under study was veified in Botanical lab at Central Campus of Technology, Dharan.

3.1.2 Chemicals

The following chemicals used were available in Central Campus of Technology. The list of chemicals used for the analysis is shown in Table 3.1

Table 3.1 List of chemicals used

Chemicals	**Supplier/Manufacturer**	**Other specifications**
Sodium hydroxide	Thermo fisher Scientific	Pellets, AR grade,

(NaOH)	India Pvt. Ltd.	98%
Hydrochloric acid (HCl)	Thermo Electron LLS India Pvt. Ltd.	36%, LR grade
Sulphuric acid (H_2SO_4)	Thermo fisher Scientific India Pvt. Ltd.	97%, LR grade
Oxalic acid	Merck (India) Limited	crystal
Sodium Carbonate (Na_2CO_3)	Qualigens fine chemicals	99.5%, LR grade
Sodium bicarbonate ($NaHCO_3$)	-	-
Methanol	Merck life science Pvt. Ltd	99% Liquid
Sodium nitrate ($NaNO_2$)	Thermo Fischer scientific India, Pvt. Ltd	98%
Aluminum chloride ($AlCl_3$)	S.D fine-chem Ltd	98% hygroscopic
Ferric chloride ($FeCl_3$)	Thermo Fischer scientific India, Pvt. Ltd	96% anhydrous
Anhydrous	-	-

hydrogen phosphate		
Folin-ciocalteu's reagent	Thermo Fischer scientific India, Pvt. Ltd	liquid
Acetic acid	Thermo Fischer scientific India, Pvt. Ltd	99% Liquid
Gallic acid	-	-
Ninhydrin solution	Central drug house Pvt. Ltd	powder
DPPH (2,2 diphenyl-1-picrylhydrazyl	Himedia laboratories (India) Pvt. Ltd	Amorphous

3.1.3 Equipment

The following equipment used were available in Central Campus of Technology**.**

1. Electric balance (Phoenix instrument, 620g)
2. Spectrophotometer (Labtronics, India)
3. Hot air oven (Victolab, India)
4. Incubator (Victolab, India)
5. Knives

6. Micropipette, pipette

7. Refrigerator

8. Grinder

9. Roller

10. Glassware (Beaker, Measuring cylinder, Volumetric flask, conical flask, Burette, Petridish, Porcelain basin, Crucible etc.).

3.2 Methods

3.2.1 Preparation of sample

Different combinations of tea and selected herbs (mint, lemongrass, asuro and curry leaf was made using random surface methodology(KC and Poudel, 2019). Optimization was done for the combinations as per DOE from APPENDICES 1which is presented in table 3.2

Table 3.2 Optimized new sample proportions

Sample	Tea(g)	Mint(g)	Lemongrass(g)	Asuro(g)	Curry leaf(g)	Fermentation Time (min)
A	0.313	0.1	0.1	0.1	0.387	180
B	0.383	0.1	0.315	0.1	0.1	180
C	0.348	0.1	0.309	0.143	0.1	180
D	0.361	0.1	0.339	0.1	0.1	180
E	0.291	0.1	0.409	0.1	0.1	180
F	0.316	0.248	0.1	0.236	0.1	180

3.2.2 Rolling, fermentation and drying

Each combination of sample was made then rolled in a mechanical roller and fermented at same time (180 min). After that those samples are subjected to cabinet dryer (50°c) till 6-7% moisture. Dried sample was carried out from the dryer.

3.2.3 Preparation of plant extracts

Plant materials were extracted as per(Ahmad *et al.*, 2014)with slight modification. Briefly, 10g of dried plant samples were stepped in 80% methanol (100ml) for 12 hours at room temperature. They were then filtered through (whatman no. 41) filter paper. Finally, extracts were transferred to brown colored glass bottles, sealed by using caps and stored at 4±2^{o} C until analysis. The extract concentration was determining by evaporating 5ml of extract (at 80^{o} C) to dryness and measuring the weight. The basic flow diagram of experimental methodology is shown in Fig 3.1

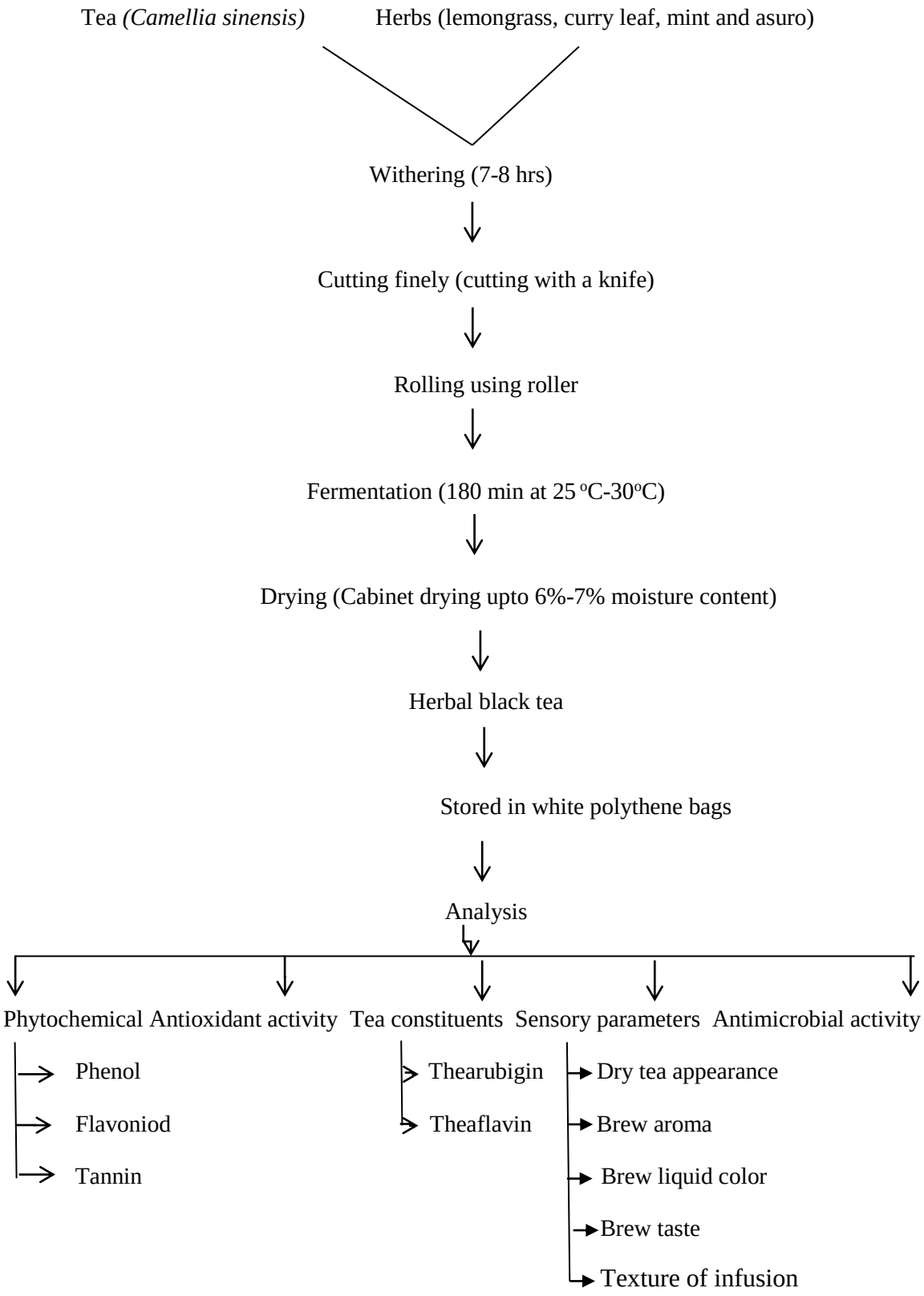


Fig 3.1 Flow diagram of experimental Methodology

3.3 Phytochemical qualitative analysis

The plant methanolic extracts were screened for the presence of the phytochemical classes by using the standard following methods (Jaradat *et al.*, 2015).

a. **Test for phenols and tannins**:

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

b. **Tests for flavonoids**

- Shinoda test: pieces of magnesium ribbon and HCl concentrated were mixed with crude plant extract after few minutes pink colored scarlet appeared that indicated the presence of flavonoids (Jaradat *et al.*, 2015).
- Alkaline reagent test: 2 ml 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).

3.4 Quantitative analysis

3.4.1 Determination of total phenol

Total phenolic content (TPC) in the plant methanolic extracts was determined using spectrophotometric method (Jaradat *et al.*, 2015). The reaction mixture was prepared by mixing 0.5ml of plant extract, 2.5 ml of 10% Folin Ciocalteu‘s reagent dissolved in water and 2.5 ml of 7.5% of Na_2CO_3 aqueous solution. The samples were thereafter incubated in a thermostat at 45ºC for 45 min. The absorbance was determined using spectrophotometer at wave length 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of Gallic acid (20, 40, 60, 80, 100µg/ml) and the calibration line was constructed. Based on the measured absorbance, the concentration of Gallic acid equivalent expressed in terms of (mg of GAE/g of extract).

3.4.2 Determination of flavonoids

Total flavonoid content was determined using a modified aluminum chloride assay method as described by (Barek and Hasmadi, 2015).Two ml of solution was pipette out in a test tube in which 0.2 ml of 5% Sodium Nitrate ($NaNO_3$) was mixed and stand for 5 minutes. 0.2 ml Aluminum Chloride ($AlCl_3$) was pipetted out mixed in the tube and allowed to stand for 5 min. This followed addition of 2ml of 1N Sodium Hydroxide (NaOH) in the tube and finally volume was made up to 5ml. The absorbance was measured after 15 minutes at 510nm against a reagent blank. The test result was correlated with standard curve of Quercetin (20, 40, 60, 80, 100µg/ml) and the total flavonoid content is expressed as mg quercetin equivalents (mgQE/g) (Barek and Hasmadi, 2015).

3.4.3 Determination of tannins

About 0.1 ml of the sample extracts added to volumetric flask(10ml) containing 7.5 ml distilled water and 0.5 ml folin-ciocalteu reagent,1 ml 35%Na_2Co_3 solution and dilute to 10 ml distilled water. The mixture is shaken well and kept at room temperature for 30min. A set of reference standard solution of Gallic acid (20, 40, 60, 80, 100µg/ml) are prepared in same manner as described earlier. Absorbance for test and standard solution are measured against blank at 725nm with an UV/visible spectrophotometer. The tannin content is expressed in terms of mg of GAE/g of extract (Mythili *et al.*, 2014).

3.4.4 Determination of DPPH redical scavenging activity

DPPH free redical scavenging activity (antioxidant activities) of extracts were determined by method described by (Vignoli *et al.*, 2011). Different dilutions of the extracts were made using 80%methanol. Then 1 ml of the extract was mixed with 2 ml of 0.1 mM DPPH solution. The absorbance was read at 517nm after 30 min incubation in the dark. Finally, percentage scavenging activity was determined using following equation

% scavenging activity = (Ac-As) × 100 /Ac

Where Ac is the absorbance of control and As is the absorbance of test sample.

Finally, the IC_{50} value, defined as the concentration of the sample leading to 50% reduction of the initial DPPH concentration, calculated from the separate linear regression plots of the mean percentage of the antioxidant activity of ascorbic acid against concentration of the test extract (µg/ml).

3.4.5 Determination of theaflavin and thearubigin

Biochemical assessment of black tea quality was done from estimation of TFs and TRs of tea brew. The absorbance were measured on a spectrophotometer. The tea sample 9g added to 375 ml of boiling water in a conical flask and the boiling continued for 10 min on a water bath. The tea infusion was filtered through cotton cloth and cooled to room temperature. The infusion 6 ml was mixed with 6 ml of 1% (w/v) aqueous solution of anhydrous disodium hydrogen phosphate (Na_2HPO_4) and the mixture extracted with 10 ml of ethyl acetate by quick repeated inversion for 1 min. The separated bottom layer drained, remaining was the ethyl acetate layer (the TF fraction) and diluted with 5 ml ethyl acetate (Borse and Rao, 2012).

Optical densities, E1, E2, and E3; were obtained on extracts prepared as follows:

E1 - TF extract (10 ml) were diluted to 25 ml with methanol.

E2 - Infusion (1 ml) diluted to 10 ml with water and made up to 25 ml with methanol

E3 - Infusion (1 ml) was mixed with aqueous oxalic acid (10% w/v, 1 ml), and water (8 ml) and made up to 25 ml with methanol

Optical densities of E1, E2, and E3 were measured at 380 nm.

% TF = 2.25 X E1
% TR = 7.06 (4 E3 -E1)

3.5 Antimicrobial activity test

Antibacterial activities of extracts were tested against two gram-positive *Staphylococcus aureus* and *Streptococcus pyogenes* and against two gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*. Antibacterial activity was measured using the disc-diffusion method. Inoculums (100 µl) were spread evenly onto 20 ml Mueller-Hinton agar set in 90-mm Petri dishes using a sterile cotton swab. 25 grams of sample were added in 250 ml of hot water. Stirred continuously for 3 times at 120 rpm for 1hr. Filtrate the mixture for extract collection. Lawn was prepared on MHA plates. Make three wells with a help of borer on each plate for three extract solutions. Pour 100 µl of sample extract in each well. Use Streptomycin as positive control. After incubation overnight at 37°C, the minimum inhibitory dose or lowest concentration of extract required to show a zone of inhibition was recorded (Fatima *et al.*, 2016).

3.6 Sensory Analysis

Three grams of tea sample was infused with 150 ml freshly boiled water for 5 min and covered with lid. It was left for 5 min. The grading system was based on 9point hedonic rating test which was indicated as,

9 = Like extremely; 8 = Like very much; 7 = Like; 6 = Like slightly; 5 = Neither like nor dislike;4 = Dislike slightly;3 = Dislike moderately;2 = Dislike;1 = Dislike extremely(Adnan *et al.*, 2013).

The total number of panelist were minimum of 12 in number. Judges were provided with prescribed A4 questionnaire sheet to record their cognitive (conscious intellectual activity such as thinking, reasoning or remembering) expert opinion on sensory observations. The information contained on the sensory sheet for ranking of dry tea appearance, brew aroma, brew liquid color, brew taste and texture of infusion.

3.7 Statistical Analysis

Analyses were carried out in triplicate. Statistical calculations were performed in Microsoft office Excel 2010. All the data obtained in the experiment were analyzed for

significance by Analysis of Variance (ANOVA) using statistical software Genstat Release v12 (Payne *et al.*, 2009). From this, means were compared using Fischer's protected LSD (Least Significance Difference) at 5% level of significance.

Part IV

Result and Discussion

All the samples were collected from the specific places as stated above. The overall results of phytochemical analysis, antioxidant property, antimicrobial activity and sensory parameters are from this work are expressed and discussed below.

4.1 Qualitative Phytochemical Screening of herbal black tea

During the experimental work, the methanol extract of herbal tea shown to have the total phenol, total flavonoid, tannin and antioxidant activity which is shown in Table 4.1

Table.4.1 Qualitative analysis for Phytochemicals

Test	Result
Phenols and Tannins	+ve
Flavonoid	+ve

+ve =positive test

The phytochemicals screening of methanol extract of sample showed that the leaves were rich in phenol, tannins and flavonoids.

4.2 Quantitative analysis of phytochemicals in herbal tea

Average phytochemical score of herbal tea sample extract are as shown in Table 4.2.

Table.4.2 Quantitative analysis of Phytochemicals*

Sample	TPC (mgGAE/g)	TFC (mgQE/g)	Tannins (mgGAE/g)
A	69.0 ± 6.601^{a}	24.02 ± 3.2^{bc}	39.37 ± 7.9^{a}
B	349.5 ± 11.8^{b}	43.84 ± 3.2^{d}	79.54 ± 66.6^{a}
C	63.4 ± 8.01^{a}	13.13 ± 0.4^{a}	58.58 ± 12.7^{a}
D	82.9 ± 4.3^{a}	18.52 ± 1.9^{ab}	42.17 ± 10.4^{a}
E	316.4 ± 7.1^{b}	27.67 ± 4.8^{c}	58.52 ± 6.3^{a}
F	71.4 ± 8.01^{a}	23.61 ± 1.3^{bc}	64.98 ± 10.3^{a}
Control	238.8 ± 3.4	22.2 ± 1.3	10.5 ± 8.3

*Values means are triplicate results, figures in the parenthesis are the standard deviations. Figures with same superscript within a column are not significantly different.

4.2.1 Total phenol content

The total phenol content in dried herbal tea leaf extract were determined as per the standard provided. The mean values for total phenol content in different sample A, B, C, D, E & F was found to be 69.0mgGAE/g, 349.5mgGAE/g, 63.4mgGAE/g, 82.9mgGAE/g, 316.5mgGAE/g, and 71.4mgGAE/g of liquid extract respectively which is presented in table 4.2.At the same time the value of control sample which possess only tea was 238.8 ± 3.4. In this study two samples had high total phenol value and this might be due to the incorporation of herbs. Other samples had low value of total phenol this might be sue to some technical fault during the experiment. The statistical analysis showed that there is significant difference in TPC within samples ($p\leq0.05$). Statistical analysis shows that there is no significance difference in sample B&E and A&C, A&D, and A&F but there were significance difference in sample B&A, B&C, B&D, B&F,

E&A, E&C, E&D and E&F. The highest value of total phenol was obtained for sample B.

According to (Mrad *et al*., 2012) the value of TPC will decrease during drying. (Ancos *et al.,* 2000) reported that the polyphenols compound may also deteriorate depending upon heat treatment.

According to the results of (Astill *et al.*, 2001)the variety, growing environment, manufacturing conditions, and grade (particle size) of the tea leaves each influence the tea leaf and final infusion compositions.

4.2.2 Total flavonoid content

The total flavonoid content in dried herbal tealeaves were determined as per the standard procedure. The obtained mean value for total flavonoid content in different sample A, B, C, D, E and F was found to be 24.02mgQE/g, 43.84mgQE/g, 13.13mgQE/g, 18.52mgQE/g,27.67mgQE/g and 23.61mgQE/g respectively. The statistical analysis showed that there is significant difference in TFC among samples ($p \leq 0.05$).Sample A&F were not significantly different while sample A&B, A&C, A&D, A&E, B&C, B&D, B&E, C&D, C&E and D&E were significantly different. The highest value for total flavonoid was obtained for sample B. In this study the total flavonoid value lies within a slight below and above the control sample and this might be due to the incorporation of herbs.

Black tea infusions are rich in total flavonoids content compared to green tea infusion. The difference in total flavonoid levels of various teas may be attributed to agronomic conditions, leafage, processing methods, and storage during and after transport, as well as the degree of fermentation (Bansode, 2015).

4.2.3 Tannin content

The total tannin content in dried herbal tea leaves were determined as per the standard provided. The obtained value for tannins in different sample A, B, C, D, E and F was found to be 39.37mgGAE/g, 79.54mgGAE/g, 58.58mgGAE/g, 42.17mgGAE/g, 58.52mgGAE/g and 64.98mgGAE/g respectively. The statistical analysis showed that

there is no significant difference in tannins among samples ($p \leq 0.05$).The highest value obtained for tannins was for sample B. Drying affects negatively phytochemicals was reported by (Nobosse *et al.*, 2017).

In epidemiologic studies, tannin compounds that inhibit iron bioavailability. Evidence from animal and single-meal studies suggests that tannic acid and tea consumption more consistently impair iron bioavailability than does the consumption of condensed tannins, although the connection between these studies' findings and individual iron status is not established (Delimont *et al.*, 2017).

4.3 Tea constituent

The thearubigin and theaflavin content in dried herbal tea leaves were determined as per the standard stated in the methodology. The obtained value for TR and TF percentage are as shown in the table 4.3. The statistical analysis showed that there is significant difference in TR and TF content among samples ($p \leq 0.05$). The highest value obtained in TF was for sample E and in TR is for sample B.

Table 4.3 Average Thearubigin and Thealavin content*

Sample	TF (%)	TR (%)
A	0.1339 ± 0.007^{c}	6.343 ± 0.16^{b}
B	0.1440 ± 0.006^{c}	7.258 ± 0.25^{c}
C	0.0292 ± 0.003^{a}	4.878 ± 0.36^{a}
D	0.0686 ± 0.004^{b}	4.741 ± 0.43^{a}
E	0.1643 ± 0.01^{d}	7.618 ± 0.17^{c}
F	0.1440 ± 0.006^{c}	6.975 ± 0.13^{bc}
Control	0.42 ± 0.008	9.67 ± 0.08

*Values means are triplicate results, figures in the parenthesis are the standard deviations. Figures with same superscript within a column are not significantly different.

Theaflavin and Thearubigin are two very important chemical constituents for the formation of tea liquor color and brightness and the estimation of these chemical constituents gives a fair idea about the quality of orthodox black tea. The ratio TF/TR is considered to be a good quality indicator of tea. In this study the control sample which has only tea sample have been found the highest value of TF/TR. The other herbal sample has lower value of TF/TR than control sample. This might be due to some herbal compound that binds with TF/TR and changes them into another form.

Thus, medium fermentation temperature and short temperature favor production of thicker and darker colored black tea. Fermentation at 30 ºC showed a marked increase in percent TR with a decline in TF (Owuor and Obanda, 2001). Thus, production of high quality black teas requires optimal fermentation temperature and/or duration. Cold fermentation (≤25 ºC) and/or optimum duration (60 min ≤time<90 min) are recommended for the achievement of high quality black teas (Owuor and Obanda, 2001; Tüfekci and Güner, 1997).

4.4 DPPH redical scavenging activity

Table 4.4 DPPH redical scavenging activity and IC_{50} value of samples

Sample	**DPPH Redical Scavenging Activity (% inhibition)**	**IC_{50}Value (µg/ml)**
A	47.50±24.89[a]	408.006
B	42.45±22.33[a]	506.40
C	50.32±19.44[a]	370.29
D	45.11±29.97[a]	438.97
E	55.41±29.97[a]	277.21
F	44.17±16.79[a]	510.23

*IC_{50} value from Ascorbic Acid standard curve is 820.4328µg/ml.

Antioxidant activity of dried herbal tea leaves were determined as per the standard procedure provided. The mean value for DPPH redical scavenging activity (% inhibition) in sample A, B, C, D, E and F were found to be 47.75%, 42.45%, 50.32%, 45.11%, 55.41%, 44.17%. Statistical analysis showed that there is no significant difference in antioxidant activity among samples ($p<0.05$).

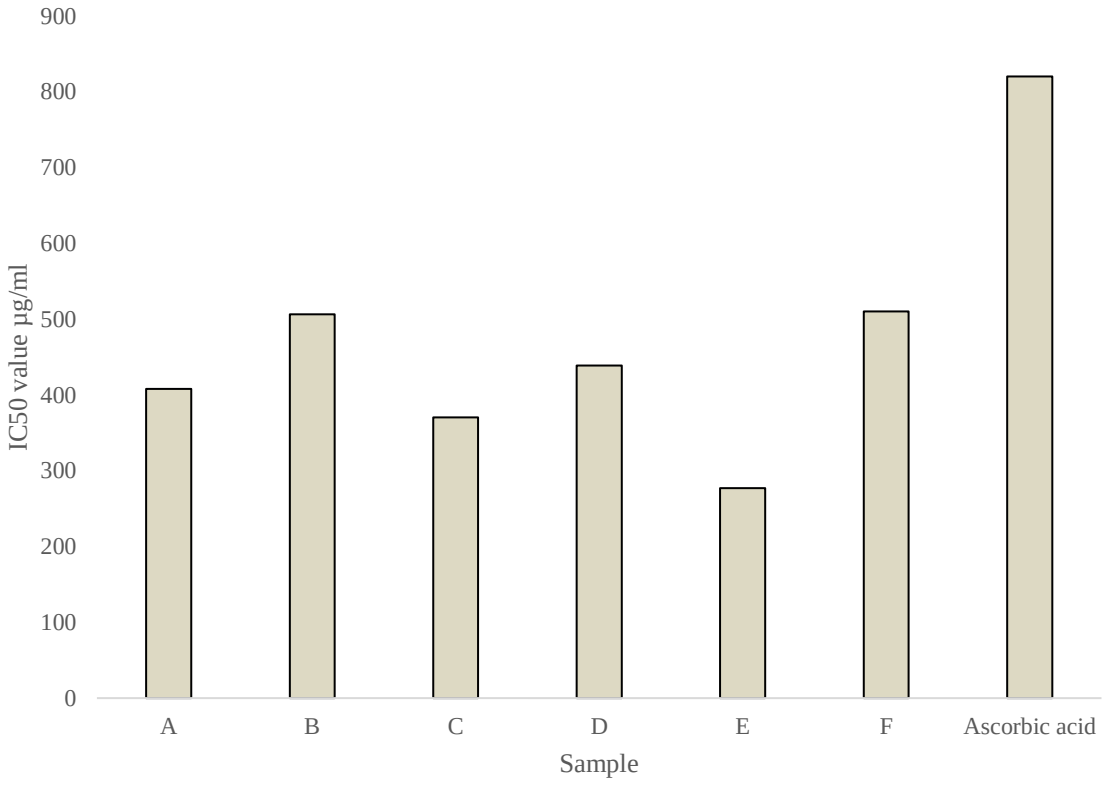


Fig 4.1 IC_{50} value of samples with respect to Ascorbic acid.

IC_{50}indicates that the amount of sample extract needed for 50% inhibition of DPPH radicals. The lowest IC_{50} value for DPPH redical scavenging activity was obtained 277.21µg/ml for sample E.

The antioxidant activity of tea increased significantly during various stages of fermentation. Study confirmed that antioxidant potential of tea samples, in general and in particular, varied scavenging activity with respect to green and black teas. It should be noted that in black tea processing, considerable amount of polyphenols converted

into theaflavins and thearubigins. These made tea pigments have their own antioxidant potential when compared to that of green tea (Nikniaz *et al.*, 2016).

Generally, variation in antioxidant activity may be influenced by phenomena such as environmental factors (sunlight, temperature, raining, etc.), collection period, variety, and chemical composition, maternity at harvest, growing condition, and soil state (Li *et al.*, 2012).

4.5 Antimicrobial activity

The results of the study showed that the sample extract of herbal tea indicates the presence of potent antibacterial activity, which confirms its use against microbial pathogens.

Table 4.5 Antibacterial pattern of herbal black tea

Sample	***Staphylococcus aureus*** **(mm)**	***Streptococcus pyogenes*** **(mm)**	***Escherichia coli*** **(mm)**	***Pseudomonas aeruginosa*** **(mm)**
A	4	0	0	0
B	5	5	6	5
C	6	4	4	0
D	0	3	0	0
E	4	7	4	4
F	0	4	0	0

The assessment of antimicrobial activity was evaluated by agar well diffusion technique in which herbal tea gives effective zones of inhibition. At the same dose of standard antibiotics used in this assay, streptomycin had better zones of inhibition (25 mm on *Staphylococcus aureus*, 20 mm on *Streptococcus pyogenes*, 20 mm on *Escherichia coli*, and 23 mm on *Pseudomonas aeruginosa* respectively(Fatima *et al.*, 2016).These observations may be attributed to herbal tea catechin compound and

polyphenols. Also, the solvent used in the extraction (Methanol) also has its own antimicrobial activity. The zone of inhibition obtained might also be influenced by the solvent.

Polyphenols act directly against microorganisms by inhibiting virulence factors. These compounds have been found to possess antibacterial action which protects the body from damage caused by free redical induced oxidative reactions. There are many health benefits that have been reported to consumption of the tea beverage, including, reduction of cholesterol, antibacterial, anti-diabetic, anti-inflammatory and antiviral. It is hoped that by use of herbal tea it may help to avoid the side effects of antibiotics. In future, the combined use of tea and antibiotics could be also useful in fighting emerging drug-resistant problem especially among enteropathogens (Fatima *et al.*, 2016).

4.6 Sensory Evaluation

Sensory evaluation was carried out for: dry tea appearance, aroma, liquid color, taste and texture of infusion by semi trained panelists using 9point hedonic scale. The statistical analysis (two-way ANOVA no blocking) was done. ANOVA was carried out using LSD at 5% level of significance. There was significant difference for most of the sensory attributes viz., dry tea appearance, aroma, liquid color, taste and texture of infusion at ($p<0.05$). The result of the sensory evaluation and statistical analysis is given in Appendix D.

4.6.1 Dry tea appearance

The mean sensory score for the six samples A, B, C, D, E, and F were found to be 6.25, 7.58, 6.83, 6.50, 6.58 and 6.50 respectively. The highest score for the dry tea appearance was obtained for sample B and least was obtained for sample A. LSD showed that A, C, D, E, F are not significantly different but sample B was significantly different from all other samples in dry tea appearance at 5% level of significance. So, on the basis of statistical analysis sample B was found superior.

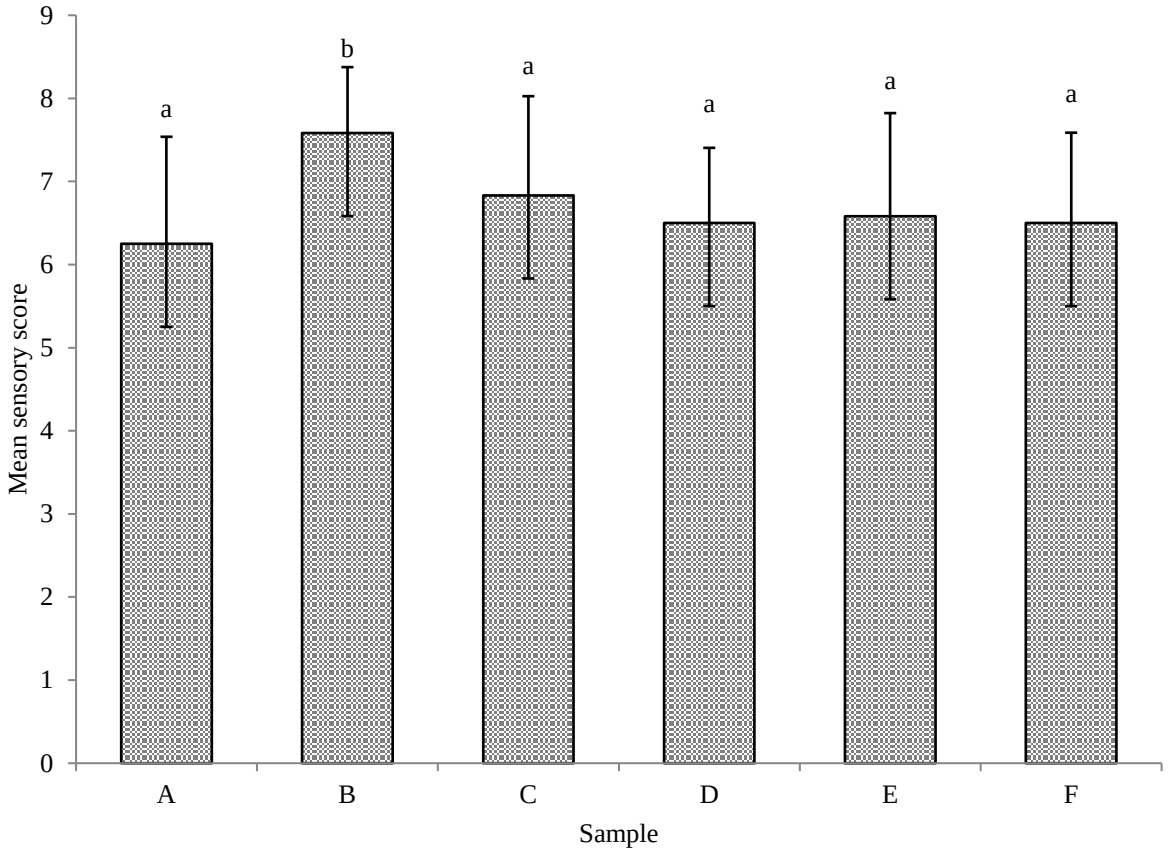


Fig. 4.2 Mean sensory score for dry tea appearance

The values in the figure are the mean sensory score for dry tea appearance. Values on the top of bar bearing similar alphabet are not significantly different at 5% level of significance. Vertical error bar represents standard deviation of scores.

The color of dry tea leaves itself offers clues about the processing the tea leaves went through. Dry tea appearance is one of the major quality parameters which affects in consumer acceptability of tea. Drying and fermentation changes the color of herbal tea from green to black. In this experiment the sample B was found more acceptable in dry appearance.

4.6.2 Brew aroma

Mean sensory score for aroma of samples A, B, C, D, E and F were found to be 5.91, 7.5, 6.66, 6.5, 7.16 and 7.08 respectively. The highest score for aroma was obtained for sample B and least was obtained for sample A. So sample B were superior on the basis of flavor from statistical analysis. The color of sample was significantly different ($p<0.05$). LSD showed that sample C&D and E&F are not sgnificantly different while other A and B are significantly different from each other at 5% level of significance.

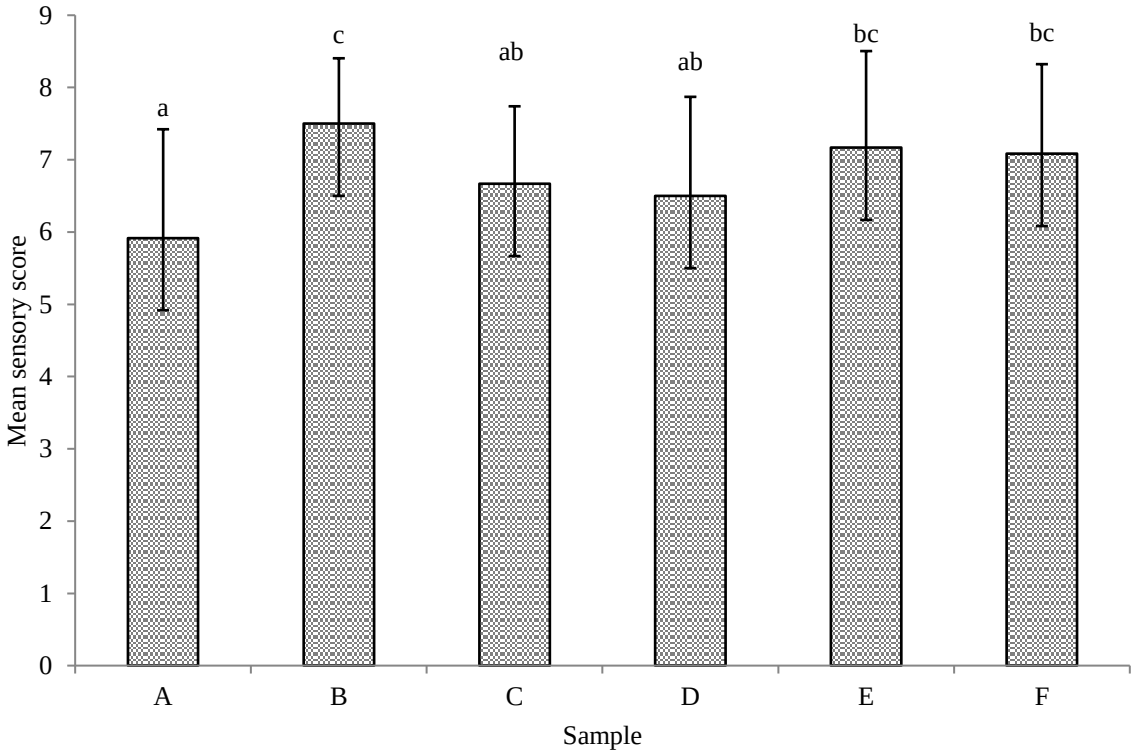


Fig. 4.3 Mean sensory score for aroma

The values in the figure are the mean sensory score for aroma. Values on the top of bar bearing similar alphabet are not significantly different at 5% level of significance. Vertical error bar represents standard deviation of scores.

Aroma is one of the critical aspects of tea quality which can determine acceptance or rejection of a tea before it is tasted (Mulder, 1838). The herbal tea sample contains no

additives and the aroma was coming only from sample itself. Serving temperature was held constant to avoid bias as temperature is known to influence aroma perception in foods. Tea sample B which contained 30gm mint, 94.5gm lemongrass, 30gm asuro and 30.6gm curry leaves had a significantly higher acceptability for aroma than other samples.

4.6.3 Brew liquid color

The mean sensory score for color of samples A, B, C, D, E and F were found to be 8, 7.9, 6.8, 6.3, 7 and 6.8 respectively. The highest score obtained for sample A and least obtained for sample D. The liquid color of the sample was significantly different ($p<0.05$). LSD showed that sample A&B and C&F are not significantly different while other D and E are significantly different from each other at 5% level of significance. Sample A and B was found superior on the basis of liquid color from statistical analysis.

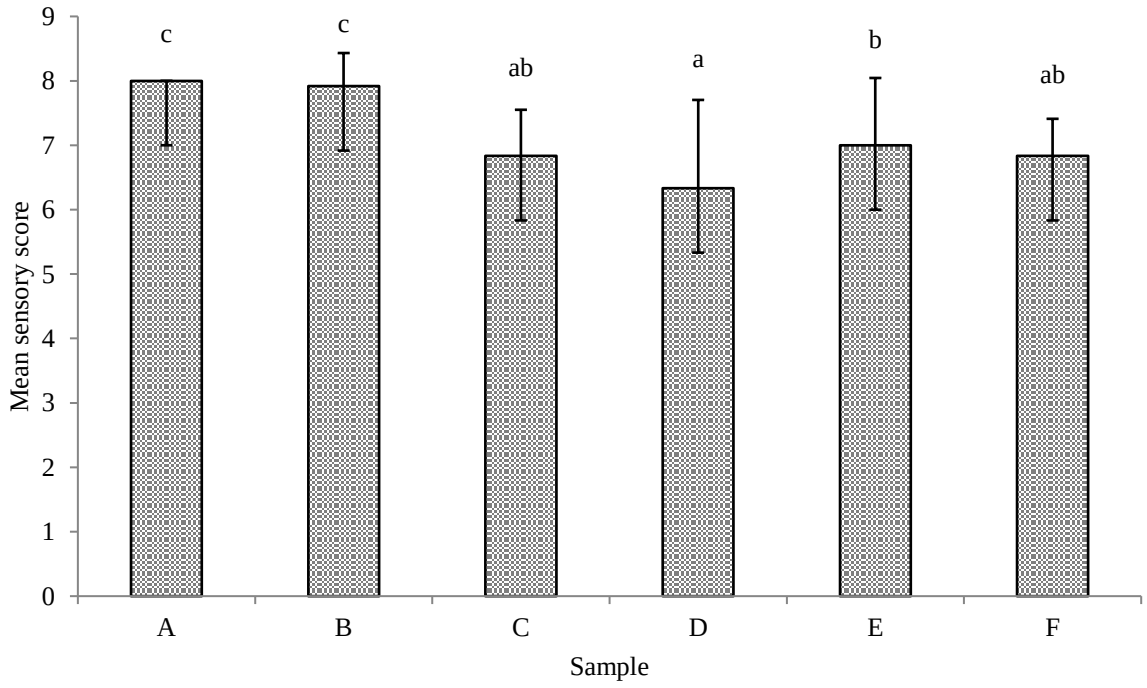


Fig 4.4 Mean sensory score for liquid color

The values in the figure are the mean sensory score for color. Values on the top of bar bearing similar alphabet are not significantly different at 5% level of significance. Vertical error bar represents standard deviation of scores.

Color is a sensation that forms part of the sense of vision and judges the appearance of a food product (Jellinek, 1985). Both leaf and liquor color are indicators of the amount of oxidation and roasting that tea leaves have gone through. A tea that is, by standard, processed in a certain way, should show color that is consistent with this processing. Colors can range from olive green to pale yellow, with ruddy inclusions from inconsistent oxidation. Black tea leaves become reddish brown and should appear consistently colored with no signs of differences in oxidation between leaves. All the samples were copper red in color due to fermentation and as mentioned in the literature the color might be due to the presence of thearubigin and theaflavin.

4.6.4 Brew taste

The mean sensory score for flavor of six samples A, B, C, D, E and F were found to be 5.8, 8.08, 5.8, 6.08, 6.5 and 6.6respectively. The taste of sample was significantly different ($P<0.05$). The least score for taste was obtained for sample A&C while the highest mean score was obtained for sample B. The samples A, C, D, E and F were not found to be significantly different while sample B were found to be significantly different from other samples at 5% level of significance. Sample B were the superior on the basis of taste from statistical analysis.

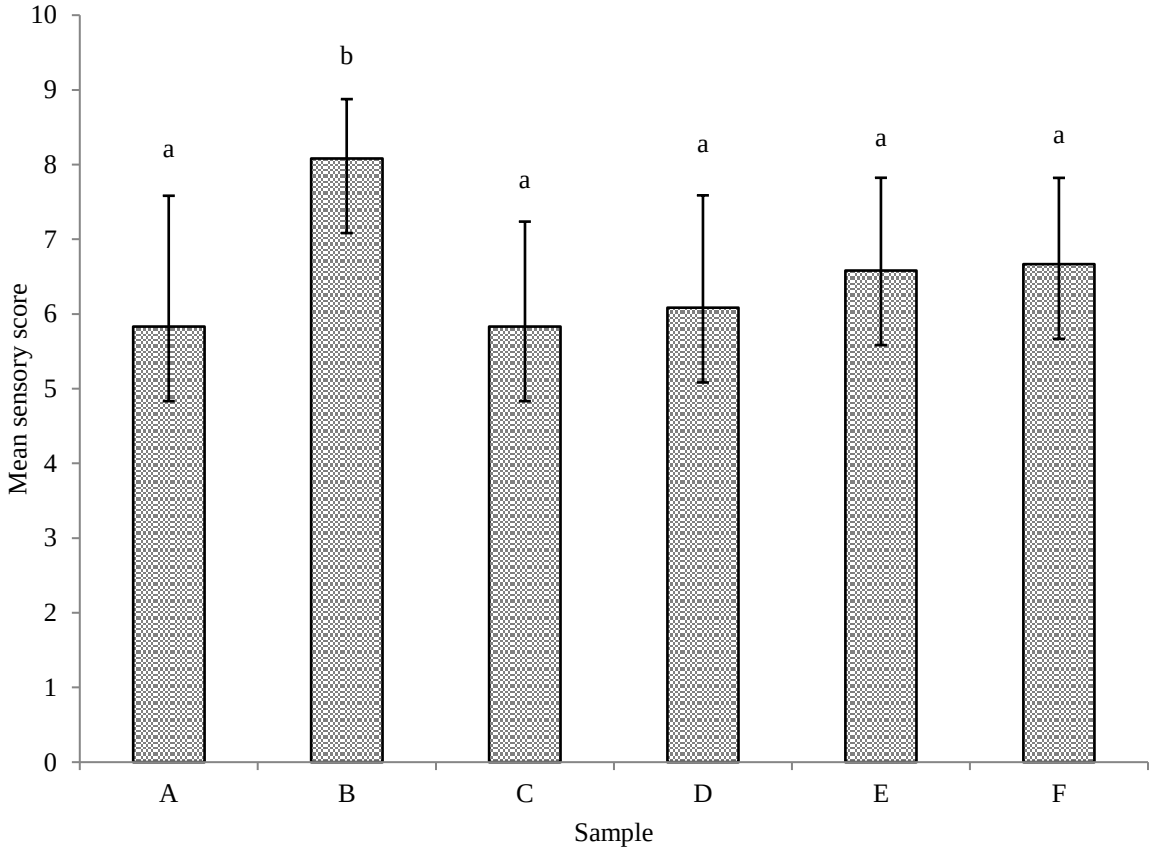


Fig 4.4 Mean sensory score for taste

The values in the figure are the mean sensory score for taste. Values on the top of bar bearing similar alphabet are not significantly different at 5% level of significance. Vertical error bar represents standard deviation of scores.

The sensation of taste is a result of the effect of water molecules interacting with receptors on the tongue and in the oral cavity (Carpenter *et al.*, 2000).Black tea has savory quality as its taste defining element, especially because of its strong and bruised taste. Black teas give a strong, full-bodied, reddish, and a bright brew. In tasting black tea special attention should be given to any sensations created on the tongue for example sweetness or savory and remember that bitterness is present in the majority of teas because of varying degrees of tannins and also the taste of black tea is influenced by thearubigin and theaflavin.

4.6.5 Body

The mean sensory score for infuse leaf of six samples A, B, C, D, E, and F were found to be 5.5, 6.7, 6, 6.08, 6.08 and 6.33 respectively. The infusion of leaves of the sample was significantly different ($p<0.05$). LSD showed that A&B, A&C, A&F, B&C, B&F and C&F and are significantly different while D and E are not significantly different from each other at 5% level of significance. Sample B were superior on the basis of overall infusion of leaves from statistical analysis.

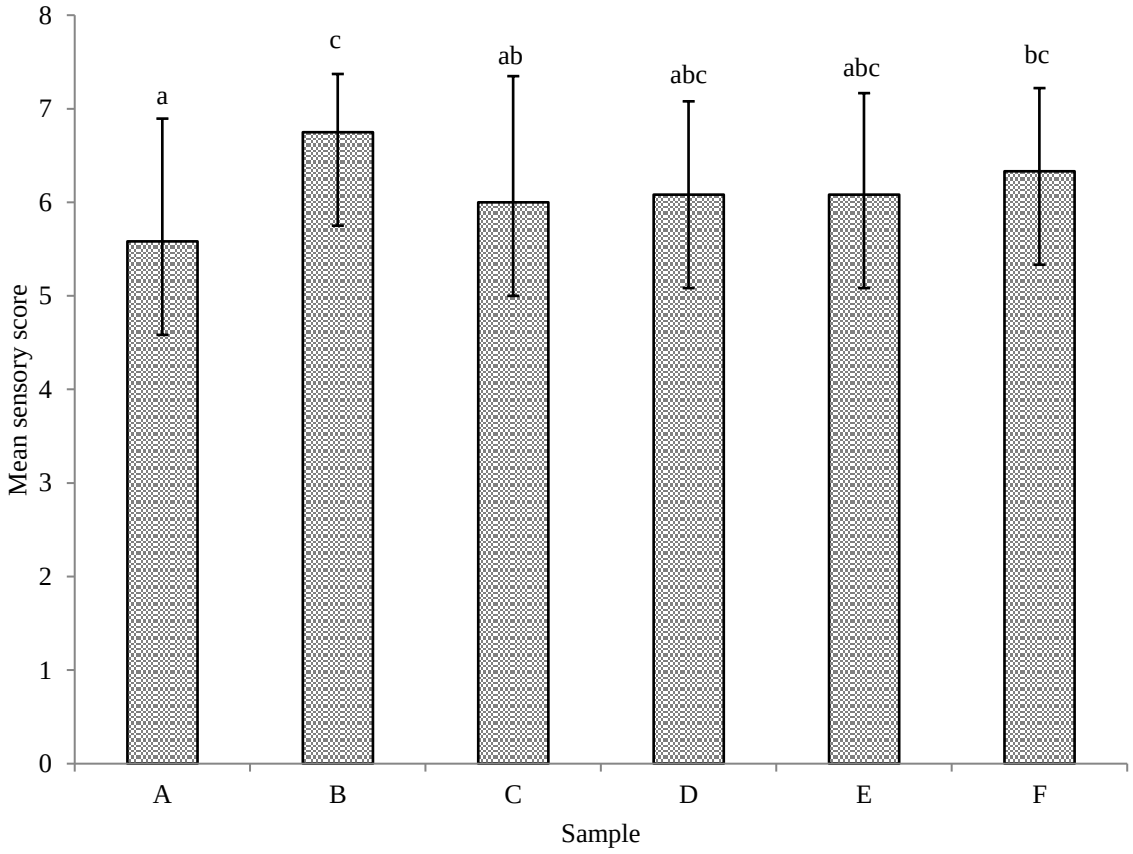


Fig 4.5 Mean sensory score for infuse leaf

The values in the figure are the mean sensory score for infuse leaf. Values on the top of bar bearing similar alphabet are not significantly different at 5% level of significance. Vertical error bar represents standard deviation of scores.

4.7 Discussion

The overall sensory evaluation explains that the all herbal tea samples are better in color and taste. Among six different sample the sample B was highly accepted and which also has high scoring on all sensory parameters. The bitterness and color of all samples are influenced by degree of fermentation and thearubigin and theaflavin which is the most essential part of black tea. According to the panelist they also had sensation on taste and aroma of two herbs namely lemongrass and mint used in on the tea. Generally, the lemongrass oil is in use in teas for increasing aroma and taste. In some places other types of herbs are also used to get a better mouthfeel of teas. Therefore, the use of herbs has been found increasing the sensory parameters of tea.

Part V

Conclusion and recommendations

5.1 Conclusion

Present work was carried out to estimation of phytochemical, antioxidant, antimicrobial and sensorial properties of herbal tea. The following were the conclusion drawn from the work.

1. The herbal infusion has been found to increase the phytochemical and antioxidant potential of tea.
2. All the herbal tea sample has moderate antimicrobial activity.
3. The herbal tea was found acceptable to consume.

5.2 Recommendations

From the present study following recommendations were made for further work.

1. Effect on quality parameters due to different processing operations on tea can be estimated.
2. Other varieties of tea leaves and herbs can be used.
3. Different solvents can be used for extraction.

Summary

Tea is widely cultivated in Nepal especially in eastern region. It serves worldwide as a nonalcoholic and second most consumable beverage which has many health benefits. Tea contains various types of natural antioxidants. Tea is categorized in different variations according to their processing procedure. Similarly Mint, Lemongrass, Asuro and Curry leaf has their own beneficial health potential and biochemical activity. All these herbs are available locally in Nepal. They are used in our cuisine especially for aroma (taste and flavor).

From the study all the samples of herbal tea has high in of total phenol, total flavonoid, tannin and DPPH % scavenging activity with mean value of 349.5mgGAE/g, 43.84mgQE/g, 79.54mgGAE/g and 55.41% respectively. Similarly, sample E had highest TF&TR content with mean value of 0.1642% and 7.618% respectively. Finally, all the samples were taken for sensory analysis where each sample was judged for dry tea appearance, brew aroma, brew liquid color, brew taste and body via Hedonic rating method. The best sample was determined by using two-way ANOVA (no blocking) at 5% level of significance. Mean sensory score for all samples showed that sample B which has the combinations of tea 0.383gm, mint 0.1gm, lemongrass 0.315gm, asuro 0.1gm and curry leaf 0.1gm was mostly preferred by the panelists. Thus, it is better to consume herbal black tea with the health benefits of tea and other herbs at a time.

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Appendices

Appendix A

Combination of the experimental runs as per DOE

Standard run	Tea(g)	Mint(g)	Lemongrass (g)	Asuro(g)	Curry leaf(g)	Fermentation Time(min)
1	0.2	0.4	0	0.4	0	40
2	0.2	0	0.8	0	0	40
3	0.6	0	0	0.4	0	180
4	0.2	0	0.4	0.4	0	110
5	0.6	0.4	0	0	0	40
6	0.6	0	0	0.4	0	40
7	0.6	0	0.4	0	0	180
8	0.2	0	0	0.8	0	110
9	0.6	0	0	0	0.4	110
10	0.2	0.4	0.4	0	0	40
11	0.2	0	0	0.4	0.4	110
12	0.2	0.4	0	0	0.4	180
13	0.2	0	0	0.4	0.4	180
14	0.2	0.4	0	0	0.4	400
15	0.2	0	0	0	0.8	180
16	0.2	0	0.8	0	0	180
17	0.2	0	0	0	0.8	400
18	0.6	0	0	0	0.4	180
19	0.6	0	0	0	0.4	40
20	0.2	0	0	0.8	0	40
21	0.2	0.4	0.4	0	0	180
22	0.2	0.8	0	0	0	110
23	0.6	0	0.4	0	0	40
24	0.2	0	0.4	0.4	0	180
25	0.2	0	0	0.4	0.4	40
26	0.2	0	0.4	0	0.4	180
27	0.2	0	0	0.8	0	180
28	0.2	0	0.8	0	0	110
29	0.2	0	0	0	0.8	110
30	0.2	0.4	0.4	0	0	110
31	0.2	0.4	0	0.4	0	110
32	0.2	0	0.4	0	0.4	40
33	0.6	0.4	0	0	0	180
34	0.2	0	0.4	0	0.4	110
35	0.2	0	0.4	0.4	0	40

36	0.2	0.4	0	0.4	0	180
37	0.6	0.4	0	0	0	110
38	0.6	0	0.4	0	0	110
39	0.6	0	0	0.4	0	110
40	0.2	0.4	0	0	0.4	110
41	1	0	0	0	0	40
42	1	0	0	0	0	180
43	1	0	0	0	0	110
44	0.2	0.8	0	0	0	40
45	0.2	0.8	0	0	0	180
46	0.36	0.16	0.16	0.16	0.16	75
47	0.36	0.16	0.16	0.16	0.16	145
48	0.28	0.18	0.18	0.18	0.18	40
49	0.28	0.18	0.18	0.18	0.18	180
50	0.28	0.18	0.18	0.18	0.18	110
51	1	0	0	0	0	180
52	0.6	0	0	0.4	0	180
53	0.6	0.4	0	0	0	40
54	0.6	0	0	0.4	0	40

Appendix B

SPECIMEN CARD FOR SENSORY EVALUTION

Hedonic rating test

Name of the panelist …….

Date ……

Name of the product: Herbal tea

Please taste the beverage samples provided to you and give points for your evaluation as given below, for each sensory quality parameters.

9 = Like extremely; 8 = Like very much; 7 = Like; 6 = Like slightly; 5 = neither like nor dislike; 4 = Dislike slightly; 3 = Dislike moderately; 2 = Dislike; 1 = Dislike extremely

	Sensory parameters				
Sample	Dry tea appearance	Aroma	Liquid color	Taste	Infuse leaf
A					
B					
C					
D					
E					
F					

Comment (if any)……

Signature……

Appendix C

Table B.1 Average sensory score*

Sample	Dry tea appearance	Brew aroma	Brew liquid color	Brew taste	Texture of infusion
A	6.25 ± 1.28^{a}	5.91 ± 1.50^{a}	8.00 ± 1.7^{c}	5.83 ± 1.74^{a}	5.58 ± 1.31^{a}
B	7.58 ± 0.7^{b}	7.50 ± 0.90^{c}	7.91 ± 0.51^{c}	8.08 ± 0.79^{b}	6.75 ± 0.62^{c}
C	6.83 ± 1.19^{a}	6.66 ± 1.07^{ab}	6.83 ± 0.71^{ab}	5.83 ± 1.40^{a}	6.00 ± 1.34^{ab}
D	6.50 ± 0.90^{a}	6.50 ± 1.37^{ab}	6.33 ± 1.37^{a}	6.08 ± 1.50^{a}	6.08 ± 0.9^{abc}
E	6.58 ± 1.24^{a}	7.16 ± 1.33^{bc}	7.00 ± 1.04^{b}	6.58 ± 1.24^{a}	6.08 ± 1.0^{abc}
F	6.50 ± 1.08^{a}	7.08 ± 1.24^{bc}	6.83 ± 0.57^{ab}	6.66 ± 1.15^{a}	6.33 ± 0.88^{bc}

*Figures in the parenthesis are the standard deviations. Figures with same superscript within a column are not significantly different.

Appendix D

Standard Curve of Gallic acid for phenol

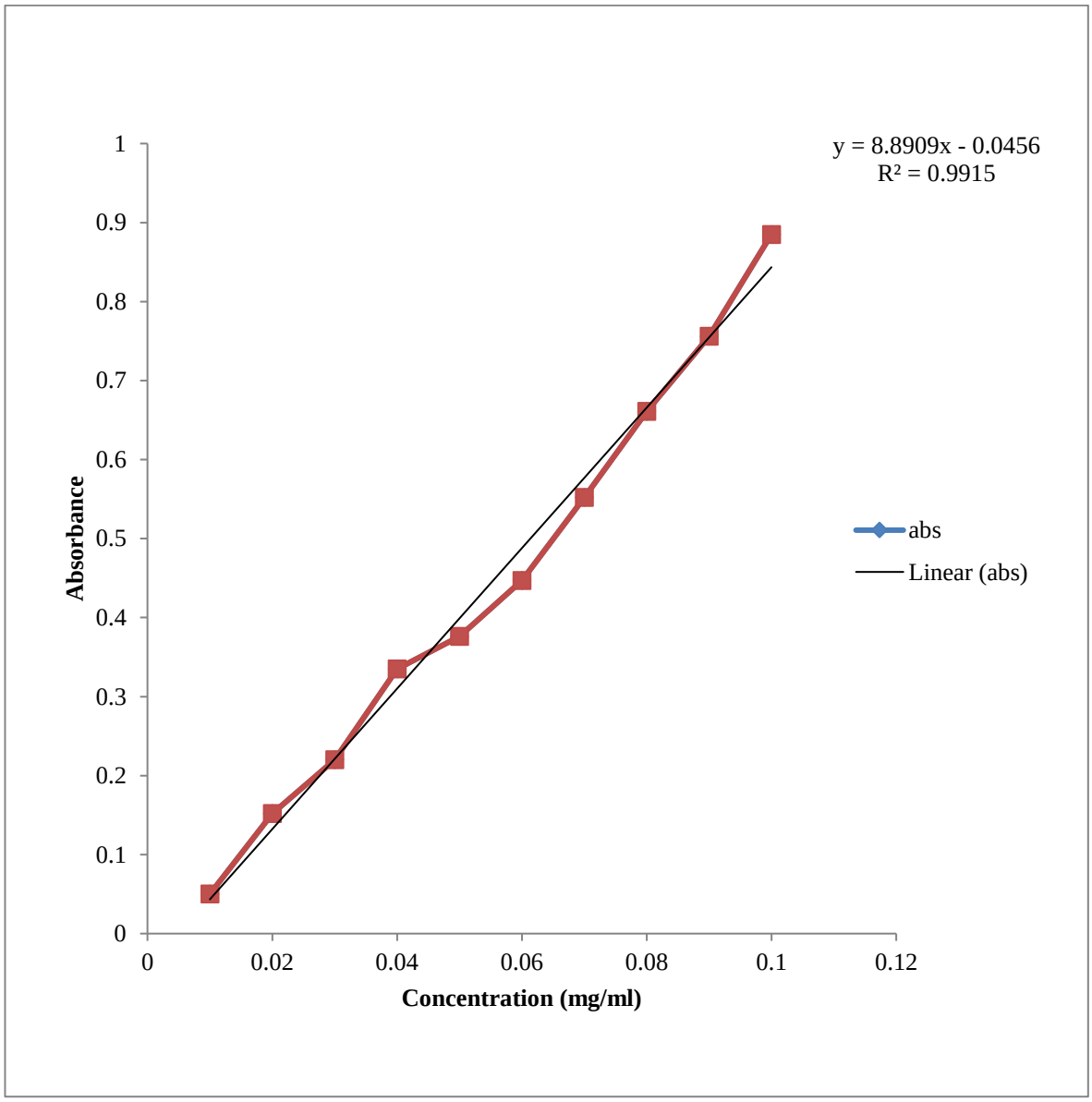


Fig C.1 Gallic acid standard curve

Standard Curve of Quercetin for flavonoid

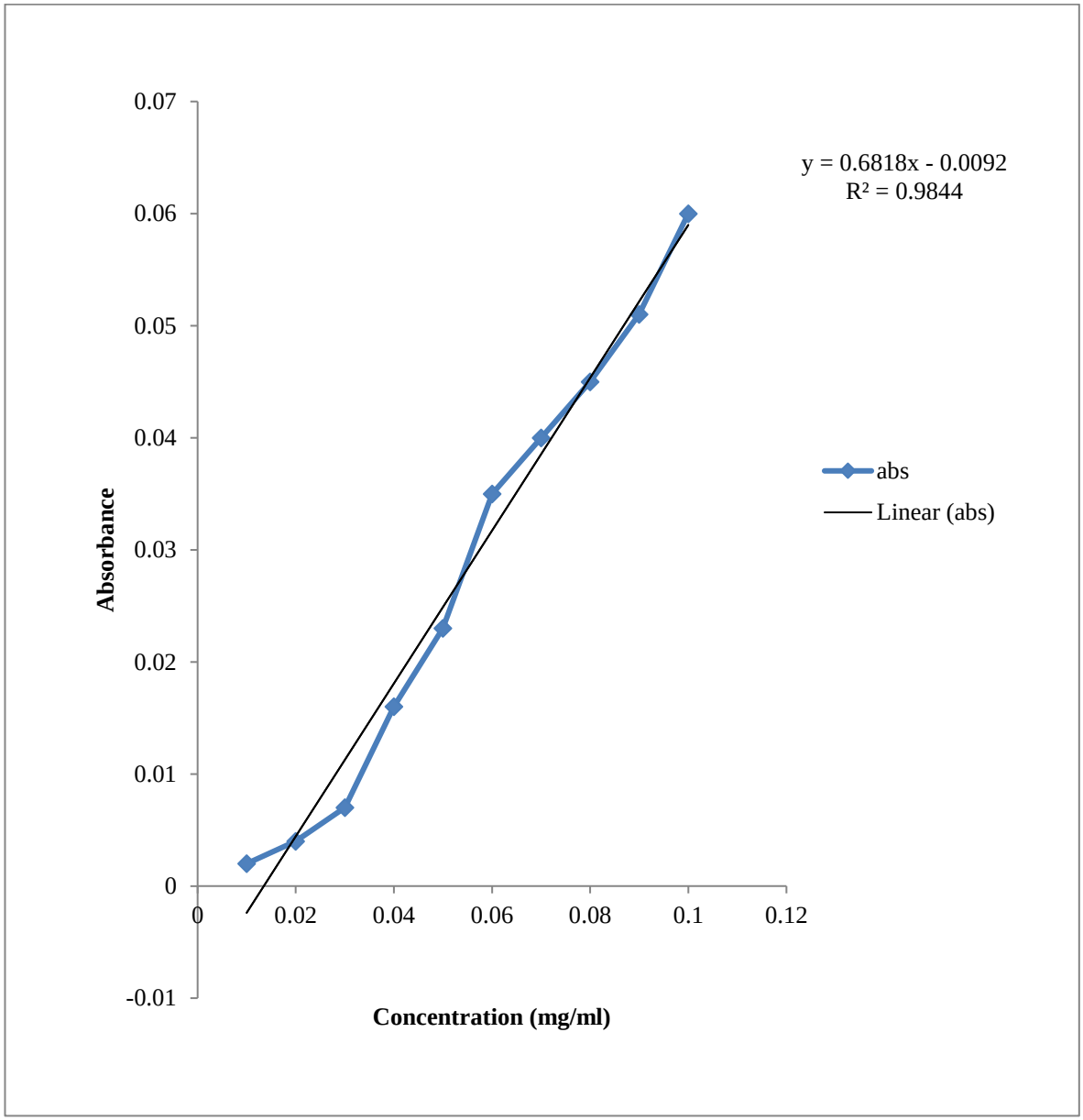


Fig C.2 Quercetin standard curve

Standard Curve of Gallic acid for tannin

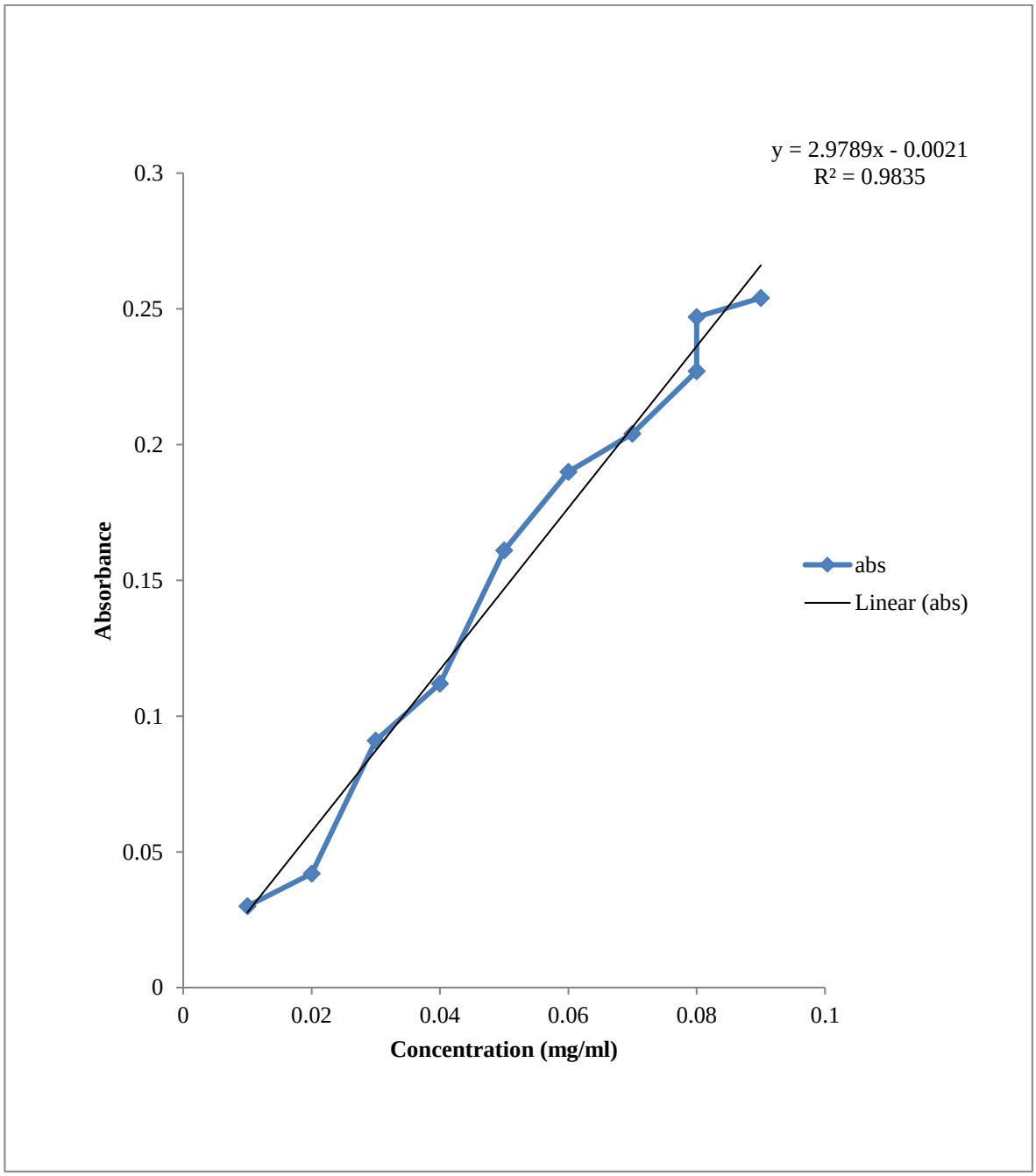


Fig C.3 Tannin standard curve

Ascorbic acid standard curve for IC_{50} value

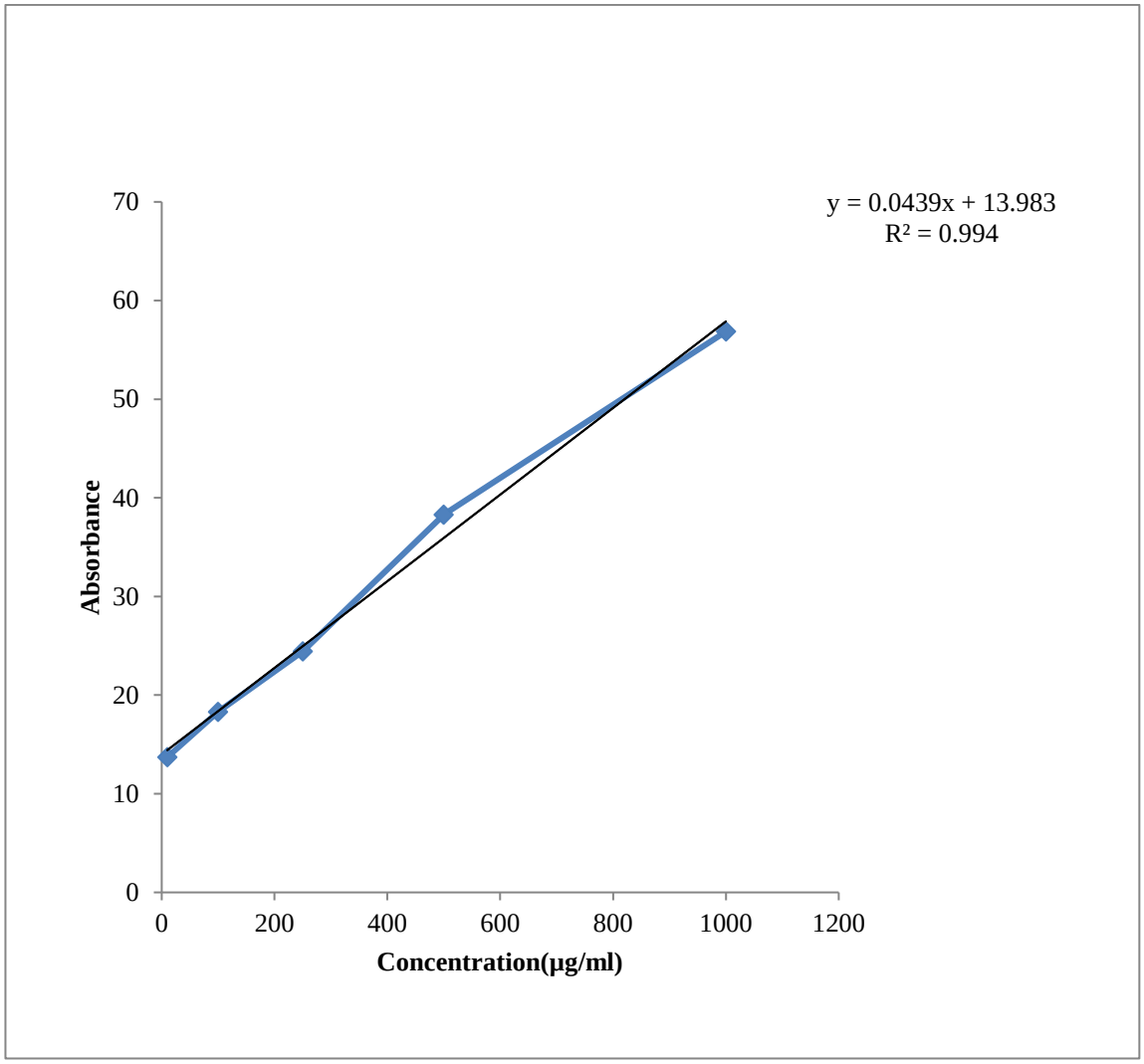


Fig C.4 Ascorbic acid standard curve

Appendix E

Table D.1 One-way ANOVA (no blocking) of Phenol content

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Sample	5	275281.4	55056.3	72.36	<.001
Residual	12	9129.9	760.8		
Total	17	284411.3			

Table D.2 One-way ANOVA (no blocking) for Flavonoid content

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Sample	5	1666.78	333.36	26.54	<.001
Residual	12	150.70	12.56		
Total	17	1817.48			

Table D.3 One-way ANOVA (no blocking) for Tannin content

Source of variation	**d.f**	**s.s**	**m.s.**	**v.r.**	**F pr.**
Sample	5	3958	792	0.60	0.704
Residual	12	15924	1327		
Total	17	19883			

Table D.4 One-way ANOVA (no blocking) for Antioxidant activity

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Sample	5	564.1	112.8	0.21	0.954
Residual	24	12804.6	533.5		
Total	29	13368.7			

Table D.5 One-way ANOVA (no blocking) for Theaflavin

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Sample	5	0.02792306	0.00558461	98.79	<.001
Residual	6	0.00033919	0.00005653		
Total	11	0.02826225			

Table D.6 One-way ANOVA (no blocking) for Thearubigin

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Sample	5	15.12672	3.02524	41.33	<.001
Residual	6	0.43917	0.07320		
Total	11	15.56589			

Table D.7 Two-way ANOVA (no blocking) for Dry tea appearance

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Sample	5	13.1250	2.6250	3.61	0.007
Panelist	11	39.7083	3.6098	4.96	<.001
Residual	55	40.0417	0.7280		
Total	71	92.8750			

Table D.8 Two-way ANOVA (no blocking) for Brew aroma

Source of variation	**d.f.**	**s.s.**	**m.s**	**v.r**	**F pr.**
Sample	5	19.1111	3.8222	4.03	0.003
Panelist	11	53.9444	4.9040	5.16	<.001
Residual	55	52.2222	0.9495		
Total	71	125.2778			

Table D.9 Two-way ANOVA (no blocking) for Brew liquid color

Source of variation	**d.f**	**s.s**	**m.s**	**v.r**	**F pr.**
Sample	5	26.4028	5.2806	10.99	<.001
Panelist	11	18.4861	1.6806	3.50	<.001
Residual	55	26.4306	0.4806		
Total	71	71.3194			

Table D.10 Two-way ANOVA (no blocking) for Brew taste

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Sample	5	43.236	8.647	7.76	<.001
Panelist	11	57.486	5.226	4.69	<.001
Residual	55	61.264	1.114		
Total	71	161.986			

Table D.11 Two-way ANOVA (no blocking) for Body

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Sample	5	8.9444	1.7889	2.59	0.036
Panelist	11	37.6111	3.4192	4.94	<.001
Residual	55	38.0556	0.6919		
Total	71	84.6111			

List of plates

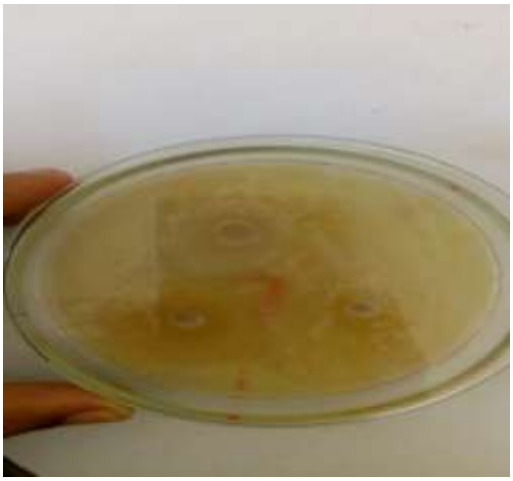
P 1: Fermentation

P 2: Dried sample

P 3: Absorption reading

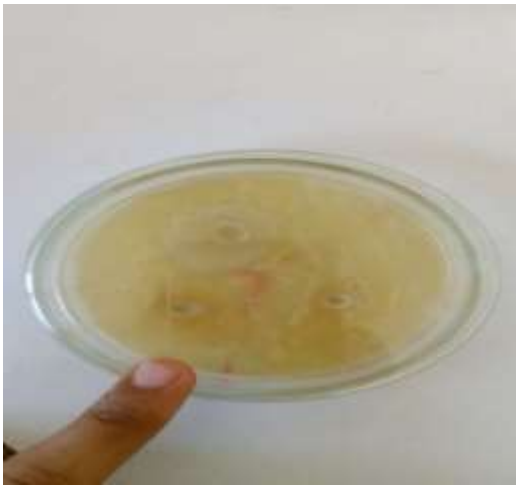
P 4: Estimation of TF&TR

P 5: Preparation of broth and MHA



P 6: Zone of inhibition

P 7: Zone of inhibition



P8: Zone of inhibition