CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Delineating the In Vitro Biological and Qualitative Analysis of Selected Herbal Teas

by

Anila Sajjad

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This study is wholeheartedly dedicated to: The sake of Allah, my Creator and my Master,

And

My great teacher and messenger, Muhammad (May Allah bless and grant him), who taught us the purpose of life,

And

My beloved parents, and my dearest husband, who stands by me when things look bleak and leads me through the valley of darkness with light of hope and support,

My beloved kids: Humna, Ali and Shaheer Whom I can't force myself to stop loving,

My friends who encourage and support me, and all the people in my life who touch my heart,

I dedicate this research.

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CERTIFICATE OF APPROVAL

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Abstract

The therapeutic utilization of naturally occurring compounds isolated from plants has been recorded over the centuries in mankind's history. As tea is most commonly used beverage in our daily life so this study has employed to investigate the medicinal potential of selected herbal teas by employing various biological and qualitative analysis. The selected herbal teas were: *Thymus serpyllum 1* (dried leaves), *Thymus serpyllum 2* (dried leaves and flowers), *Camellia sinensis* (green tea), *Camellia sinensis* (black tea), *Cymbopogon citratus* (lemon grass). These teas were screened for phytochemical properties, antioxidant, antibacterial, antifungal, cytotoxicity, protein kinase and alpha amylase inhibitory potential whereas qualitative analysis employed were FT-IR and GC/MS analysis. Ultrasonication assisted maceration was the extraction technique. The highest phenolic and flavonoid load were quantified in green tea with 70.88 ± 1.22 $\mu g\ GAE/mg$ extract and black tea 69.01 ± 0.49 $\mu g\ QE/mg$ extract respectively. The maximum free radical scavenging potential was observed in green tea with IC_{5_0} of 31.28 $\mu g/ml$. Highest reducing power was shown by green tea 266.8 ± 0.1$\mu g\ AAE/mg$ extract, whereas maximum antioxidant capacity was depicted by green tea extract with 99.65 ± 0.15$\mu g\ AAE/mg$ extract. On the contrary, lemon grass tea extract showed less phytochemical and antioxidant potential. All of the five extracts of teas were active against five bacterial strains tested, most active being the lemon grass with 20 ± 0.6mm (MIC >100) zone of inhibition against *K. pneumonia*, and 20 ± 0.5 mm (MIC >100) against *S. aureus*. Least antibacterial activity was observed by *T. serpyllum 2*. No antifungal activity was observed in all the tested samples. Lemon grass exhibited maximum activity in brine shrimp lethality assay with LC_{5_0} of 10.25$\mu g/ml$ while minimum activity was observed in black tea with LC_{5_0} of 123.2$\mu g/ml$. Lemon grass and green tea revealed the maximum protein kinase inhibition potential with a bald zone of inhibition 22.16 ± 1.75 mm, and 22.16 ± 0.76 mm respectively where as *T. serpyllum 1* presented a clear zone of inhibition i.e. 10.16 ± 0.76 mm. Black tea was found to be most proficient at inhibiting alpha amylase with 33.12 ± 0.15% inhibition, however least % inhibition was observed in green tea with 7.78 ± 0.30%. The present research study of tested

teas extracts confirmed the presence of functional groups that were identified by FT-IR spectroscopy analysis. In the current study, total 25 constituents from all the selected tea extracts were identified by GC/MS analysis. Crude extract of *T. serpyllum 1* and green tea showed the highest number of fatty acid compounds (6). Utilization of plants extracts as a substitute to chemically synthetic or artificial antimicrobials and antioxidants to fight against the food borne pathogenic microorganisms, inhibiting fat oxidation and extending the product shelf life, are increasing trends within the food trade. Our study exhibited promising perspective for the discovery of new bioactive molecules from teas. The results have shown that the extracts of these herbal teas could be safely used in pharmacy and other industries as well. Extended literature review confirmed the first time study of *T. serpyllum 2* (dried leaves + flowers).

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Abbreviations

AAE	Ascorbic acid equivalent
$AlCl_3$	Aluminium chloride
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BT	Black Tea
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
EC	Epicatechin
ECG	Epicatechin Gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin Gallate
FAO	Food and Organization
FC	FolinCiocalteu reagent
FCBP	Fungal Culture Bank of Pakistan
$FeCl_3$	Ferric chloride
FT-IR	Fourier Transform Infrared spectroscopy
GA	Gallic acid
GAE	Gallic acid equivalent
GC-MS	Gas chromatography-Mass spectrometry
GT	Green Tea
GTP	Guanosine Triphosphate
HCL	Hydrochloric acid
H_2SO_4	Sulfuric acid

IC_{5_0}	Median inhibitory concentration
K_2HPO_4	Dipotassium phosphate
KH_2PO_4	Monopotassium phosphate
LC_{5_0}	Median lethal concentration
LG	Lemon Grass
MIC	Minimum inhibitory concentration
MUFA	Monounsaturated Fatty acid
NA	Nutrient Agar
$N_{a_2}HPO_4$	Disodium hydrogen phosphate
$N_aH_2PO_4$	Monosodium dihydrogen phosphate
NB	Nutrient Broth
NIDDM	Non-Insulin-Dependent Diabetes Mellitus.
NIST	National Institute of Standards and Technology
PUFA	Polyunsaturated Fatty acid
QE	Quercetin equivalent
ROS	Reactive oxygen species
SDA	Sabouraud Dextrose Agar
SD	Standard deviation
SFAs	Saturated fatty acids
TAC	Total antioxidant capacity
TCA	Trichloroacetic acid
TFC	Total flavonoid contents
TFs	Theaflavins
TPC	Total phenolic contents
TRs	Thearubigins
TRP	Total reducing power capacity
TSB	Trypton soy broth
T2D	Type 2 Diabetes
T. s 1	*Thymus serpyllum 1*
T. s 2	*Thymus serpyllum 2*
USA	United States of America

Var	Variety
WHO	World health organization
ZOI	Zone of Inhibition
%FRSA	Percent free radical scavenging activity

Symbols

α	Alpha
β	Beta
γ	Gamma
μg	Microgram
mg	Milligram
ml	Milliliter
mm	Millimeter

Chapter 1

Introduction

With the progression in scientific disciplines, principles, skills, and innovative technologies, researchers are competent for discovering remedies for prevalent maladies and improve the quality of life and also expanding the life span. In any case, with every passing day unfamiliar or strange illnesses are being experienced and as a result we confront many challenges consistently to design novel medicines and also to secure ourselves. Because of changing the way of life, people are confronting numerous health related problems e.g. obesity, hypertension, diabetes, cancer and various other ailments because of mutations. Therefore, there is a need to change their life style and ensure to be much focused on plant based food ingredients contain natural antioxidants that are reported to be the therapeutic agents against free radicals [1], [2]. However, the tasks do not end at this stage. The prescribed medicines for the treatment of diseases also have some adverse effects. Some medicines have mild effects while some have severely permanent outcomes. To stay away from these deleterious outcomes, there is a need of time to explore some naturally occurring plant based products [3]. The therapeutic utilization of naturally occurring compounds isolated from plants, micro-organisms, and animals have been recorded over the centuries in mankind's history. Though, researchers were not succeeded in separating the biologically active compounds from different medicinal or herbal plants till the 19th century. Plants used for medicinal objective is an important element of folk tradition and heritage in the world mostly in

Asia and Africa. About more than three-fourth of population in the world depends directly on folklore medicine for health treatment [4]. WHO has listed more than 20,000 plant species with medicinal benefits providing remedies for various ailments such as pneumonia, colds, ulcers, bronchitis, diarrhea, and respiratory tract diseases [5]. Utilization of plants extracts as a substitute to chemically synthetic or artificial antimicrobials and antioxidants to fight against the food borne pathogenic microorganisms, inhibiting fat oxidation and extending the product shelf life are increasing trends within the food trade [6]. The most common among these are tea plants whose plant parts especially leaves have immense and everlasting reservoirs of bioactive molecules with medicinal and therapeutic potentials. Therefore now a day numerous research workers are trying to derive the active components from herbal tea plants for different particular disorders and manufacturing herbal based drug products, thus promoting the green herbal pharmacy [2]. Herbs teas are the most age-old herbs among all the different therapeutic plants, mostly utilized in China (Asia) [7].

Tea is most commonly used aromatic beverage around the world, produced from the *Camellia* sinensis leaves [8]. It belongs to Theaceae plant family. Tea is cultivated in more than 30 states. It is present in equilateral and semi tropic regions [9]. At present, tea has been planted in six continents [10], [11]. The two prime types cognated with *C. sinensis* are: *var. sinensis** and *var. assamica** [12]. Tea is categorized on the basis of its processing. Green, black, white and oolong are the most common [13]. But the most commonly utilized teas around the world are green and black teas. Young dried tea leaves are used for unfermented green tea (GT) production. It contains flavonoids that include catechins of epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC) [14]. It is grown in China and several countries of south East Asia. The link between green tea consumption and human health benefits has long been recognized as antimicrobial, anti inflammatory, oral health, cardiovascular health, anti carcinogenic and antioxidant [15]. Fully fermented leaves are utilized for producing Black tea (BT). Mostly, black tea consumption falls in higher proportions

in the USA. BT is fully fermented form of tea. Theaflavins (TFs) and Thearubigins (TRs) are the main BT polyphenols [16].These bioactive compounds of BT also have antioxidant activity and lower the possibility of certain cancers [17] and hardening of the arteries [18].

Thymus serpyllum, common name Tumoro, mostly consumed as tea beverage in Gilgit Baltistan areas of Pakistan [19]. It is also known as wild thyme, belongs to family lamiaceae [20]. They are non-woody perennials, small shrubs growing around the Mediterranea and are also found in North Africa, Asia, and Southern Europe at higher altitudes [21].It is regarded as a popular herb in Asia and Europe for its medicinal properties as antibacterial, antiviral, anti-inflammatory, antioxidant and also as stimulant, digestive, carminative and diuretics [22]. *Thymus serpyllum* tea is prescribed as expectorant and in the infections of pulmonary diseases especially in colds [22].

Cymbopogon citratus (lemon grass) is perennial aromatic plant, broadly utilized in making herb flavored teas due to its potential health benefits [23]. It belongs to the family poaceae, mostly used as herb throughout the world especially in South Asia and South East Asia [23]. It is most widely farming in the equatorial and subtropical with two common species, *C. flexuous* and *C. citratus* [24]. Lemongrass is also utilized in the preparation of baked goods, alcohol free drinks and confectionary products. Lemon grass herbal tea is most effectual treatment for various disorders and its effectiveness has been studied as antibacterial, antiamoebic, antifungal, antidiarrheal and anti-mutagenicity [25]. Cooling effect, slightly unsweetened and acrid flavor are the distinctive features of the tea [26]. Tea plays important role in cancer prevention, as anti-diabetic, antioxidant, antibacterial and anti-inflammatory effects. Teas have been substantiated to improve the insulin activity, helps in curing asthma, bone health, retard cataract, dental health, maintains fluid balance as well as improves the body mass index and maintain the body weight, prevents cellular damage and lowers the levels of stress hormone, etc. [7].

Extraction represents the separation of biologically active components of plant from the inert portion using appropriate solvent in standardized extraction techniques. Extraction is the foremost and critical step for obtaining bioactive constituents from natural sources [27].The extracts obtained after extraction are then thoroughly screened for their therapeutic potential.

Medicinal plants have been known for centuries for their numerous phytochemicals like tannins, glycosides, alkaloids, flavonoids, polyphenols and many others that play significant roles in maintaining human health [28]. The biochemical profiling of teas extracts can effectively be done with Qualitative analysis procedures i.e. FT-IR and GC/MS. As tea is considered as functional food, so it is important to study the fatty acids in tea from that perspective, by using GC/MS technique. A range of fatty acid compounds having therapeutic significance and significant functional groups demonstrated the presence of aromatic and organic compounds have been detected by using Qualitative screening approaches [29]. In ethno pharmacological research, the performance of bioassays is imperative to confirm the efficacy of potential drug candidates obtained from plant extracts. Bioassays also play a key role in determining the specific constitution of complex bioactive mixtures and it give valuable fingerprints in the quest of new drug discovery [30]. These procedures thus serve as efficient markers in the diagnosis of numerous health problems and in the large scale drug production, especially when chemical standardization tools are not easily accessible.

1.1 Problem Statement

Inflammatory disorders, oxidative stress induced disorders, pathogenic infections, drug resistance, cancer, cardiovascular disease, diabetes and etc. are the major risk factors for human health having increasing incidence all over the world [31]. Treatments of these diseases by synthetic drugs can put the patients under a lot of strain, cost burden and further damage their health due to the adverse side effects of these drugs. On the contrary, the use of herbal products or medicines is often

considered safe as it may be hardly diagnosed any severe case of hospital entry or deaths. At present, there is growing interest in herbal remedies due to the side effects associated with the synthetic drug treatment. Therefore, there is a focus on using alternative herbal treatments and therapies.

1.2 Significance of Solution

Tea as a drink after water is taken by almost many people in their routine daily life with lots of its hidden benefits. It is easily approachable, cost effective and has no or rare side effects. According to United Nations FAO unit report, Pakistan is listed among the top seven countries of the world where tea utilization has significantly increased [32]. Herbal teas have immense and everlasting reservoirs of bioactive molecules with medicinal and therapeutic potentials. The effective utilization of indigenous resources will not only be helpful in providing cost effective treatments to the socioeconomically deprived countries like Pakistan but it would also help in the global commercialization of the compounds of pharmaceutical importance. Biological research shows very little work on the flora of medicinal plants from Pakistan [33] so keeping this in view, this study was conducted to explore the therapeutic potentials of mostly consumed herbal teas. *Thymus serpyllum* (dried leaves + flowers) study was made first time in this work.

1.3 Objectives of Study

Herbal teas have a long history of safe use in humans. They not only add flavor to the food but are also helpful in the treatment of various disorders. The intention of the undertaken study is to explore additional benefits of the age-old herbal teas, *Thymus serpyllum* (Tumuro), *Camellia sinensis* (green tea, black tea), *Cymbopogon citratus* (lemon grass).

1.3.1 General Objective:

To explore the natural ethno medicinally significant properties of variety of locally/commercially available teas of Pakistan by employing various biological and qualitative analysis.

1.3.2 Specific Objectives:

- To collect the selected teas from different local areas of Pakistan.
- To apply the appropriate solvent for the extraction of teas.
- To screen all the tea extracts for the exploration of hidden bioactivities of medicinal significance by employing a set of *in vitro* bioassays i.e. phytochemical, antioxidant, antibacterial, antifungal, cytotoxicity, protein kinase inhibition potential and antidiabetic assays.
- To screen functional groups and fatty acid compounds in all the tea extracts by FT-IR and GC/MS techniques.

1.4 Scope of the Study

Current study has multi-dimensional scope as it covers the domains of Ethnomedicine, Pharmacology, and Bio-technology.

Chapter 2

Literature Review

2.1 What is Tea?

Tea was first originated in China about 2737 B.C where it was mainly used as a stimulating beverage and medicine [34]. Then after that it has been consumed in most of the countries of the world and now it has become the essential part of the world's culture. Tea has been consumed worldwide for many years and it is the habit of almost many people to take tea daily. This is most likely because of different types of tea and additionally the versatility of making different seasoned drinks by adjusting the infusion time. It is reported that tea is consumed daily by millions of people [35].Tea is cultivated in more than 30 states. It is present in equilateral and semi tropic regions [9]. At present, tea has been planted in six continents [10], [11]. In 2006, a record of more than 3 million tons of tea has been produced world widely, where of China, India, and Kenya were the biggest among the tea producing countries [34]. The tea production all over the world has been continually increasing; of that the annual production of black tea has been planned to grow at 1.9% to achieve about 3.14 million tons by 2017, while the annual green tea production has been planned to grow at the rate of 3.8% to attain approximately 1.57 million tons regarding the same time period [36]. Tea can be made in hot water by either infusing fresh or dried tea leaves. Infusing dried

leaves of tea is the most conventional way so far to make a cup of tea [10]. Dried tea is manufactured from fresh tea leaves that contain carbohydrates, protein, enzymes, caffeine, flavonoids, theanine and many other substances as well. The composition of tea leaf varied with the soil, variety, season, climate conditions, position of leaf, cultivation methodology, and also the age of leaf [8], [16]. Cooling effect, slightly unsweetened and acrid flavor are the distinctive features of the tea [26].Tea plays important role in cancer prevention, as anti-diabetic, antioxidant, antibacterial and anti-inflammatory effects [26].

2.1.1 Herbal Tea

Foundation to life itself is the capability to process or break down food into energy. For a healthy life style the choice of proper nutrients play a vital role. The backbone of life on earth is plants and greatly render to human life. Plants have been benefiting mankind since their origin. Human beings used plants not only because of their essential nutritional requirements but also for their curative purposes.

The utilization of herbal teas has been documented since Egyptian era [37]. Herbal tea exclusively comprises of flower and vegetative parts in dried form. Currently, there is colossal increase in marketing of herbal teas especially in Asian countries. The marketing of natural (herbal) teas has been tremendously increased throughout the world particularly in Asian side [38]. As indicated by economic evaluation, the utilization of herbal teas can assume a critical part in the support and prosperity of community by making reasonable earnings. The dietetic and therapeutic characteristics are credited to the herbal teas in perspective of its chemical components [39]. Various investigations are directed with respect to different parts of the plants utilized as herb teas. Herbal plant extracts are the valuable sources of many bioactive molecules which could serve as the antimicrobial agents against vast domains of microorganisms [40]. Tea polyphenolic compounds are considered to be contributed to antioxidant activities that neutralize the reactive oxygen species which are the major cause for development of many disorders in human body [41], [42].

2.1.2 Global Perspective of Herbal Teas

Herbal tea utilization has turned out to be worldwide tradition due to its numerous known and unknown health advantages. The utilization of medicinal or herbal tea is profoundly and broadly established among the South American populaces and currently conventional Chinese medicinal ways are being used for the preparation of herbs teas to improve the health conditions [43]. Medicinal or herbal plants are widely used for pharmacological purposes. The essential bioactive components of these plants are flavonoids, steroids, carotenoids, alkaloids, tannins, glycosides and terpenoids served as therapeutic agent and also the starting material for the development of drug [40]. The concentration of these bioactive components may differ in different plants therefore different plants exhibit distinctive medicinal properties. In Turkey, the uses of aromatic or herb teas have become deeply rooted because of their charming fragrance [24]. The utilization of herbal teas has long been accepted for various health issues. In addition to the flavoured teas and folklore herb teas, different herb based teas have turned out to be progressively well-known due to their aroma, antioxidant, and antimicrobial properties. Herb teas used for medicinal objective is an important element of folk tradition and heritage in the world mostly in Asia and Africa. In North America, herbal teas are recommended during cancer treatments to patients for quick and better health improvements [44].

2.2 *Camellia sinensis*: The Tea Plant

Tea is most commonly aromatic beverage around the world, produced from the *Camellia sinensis* leaves [8]. It belongs to Theaceae plant family. *Camellia sinensis* tea plant is an evergreen large shrub with white-yellow flowers and long indented leaves. Flowering period starts from October to February, and fruiting season starts in August till October. Its maximum growth is about 8 meters but for harvesting purpose plant is fixed to 1.5 meters [16]. Geographical areas and climatic conditions for cultivation around the world arises the variance among teas

FIGURE 2.1: *Camellia sinensis* tea plant [46].

[8], [16]. Taxonomic classification of *Camellia sinensis* is given in table (Table 2.1). The two prime varieties cognated with *Camellia sinensis* are: *var. sinensis** and *var. assamica** [12].

- ***Var. sinensis*:** The plant is bushy with small leaves. It is grown in China and several countries of south East Asia. It requires cold climate for its production. The leaves are mainly used to yield Chinese green and black tea [45].

- ***Var. assamica*:** This type has larger leaves, with hanging and flouresced occurrence. It is mainly cultivated to yield Indian and Assamica black tea [45].

Both varieties are grown throughout the world, though mostly in tropic and sub-tropic areas of the world [9]. Leaves of tea plants (Figure 2.1) are used for tea purposes. Best tea will be produced by young and light colored leaves which grow at the top [16].

TABLE 2.1: Taxanomic classification of *Camellia sinensis* [12].

Kingdom	**Plantae**
Class	Magnoliopsida
Subclass	Dilleniidae
Order	Theales
Family	Theaceae
Genus	Camellia L
Species	Camellia sinensis
Vernacular name	Kuntze- Tea

Tea is categorized on the basis of its processing (Table 2.2). Green, black, white and oolong teas are the most common [13]. In the world wide, the percentages contributed to the utilization of tea are: 78% to black, 20% to green and 2% to oolong tea [47], [48], [49], [50]. Very young or immature tea leaves or buds are used for the production of **White tea**. It is lighter in flavor than green and black tea. Its color is light yellow despite of its name. It contains flavonoids, fluoride and tannins. Fluorides are present in considerable amounts nearly 34% which could be helpful in maintaining dental health. Primarily it is produced in China and recently Nepal, Thailand, Sri lanka, India and Taiwan are the producers of white tea [51]. Young dried tea leaves are used for unfermented **Green tea** (GT) production. It contains flavonoids that include catechins of EGCG, EGC, ECG, and EC [14].It has antioxidant capacity because of flavonoids, the compounds that derived from tea plant [52]. Partially fermented leaves are used for the production of **Oolong tea** and fully fermented leaves are utilized for producing **Black tea** (BT) [53]. Mostly, the black tea consumption falls in higher proportions in the USA. BT is fully fermented form of tea. Theaflavins (TFs) and Thearubigins (TRs) are the main BT polyphenols [8], [16]. These bioactive compounds of BT also have antioxidant activity and lower the possibility of certain cancers [17] and hardening of the arteries [18]. Cooling effect, slightly unsweetened and acrid flavor are the distinctive features of the tea [26].

TABLE 2.2: Processing steps of *Camellia sinensis* tea leaves [16], [51]

	1st Step	2nd Step	3rd Step	4th Step	Product
Fresh Tea Leaves	Fixed	Rolled	–	Dried	Green Tea
	Withered	Bruised	Partially fermented	Dried	Oolong Tea
	Withered	Rolled/Cut	Fermented	Dried	Black Tea
	Leaves with more fine hairs/flosses		Withered	Dried	White Tea

2.2.1 Green Tea

Young tea leaves are used for the production of green tea (Figure 2.1). Manufacturing methods are different for both Black and Green tea. These methods preserve naturally occurring polyphenols that are contributing to the health-benefiting properties. It also contains flavonoids which consist of catechin components and their derivatives that are EGCG, ECG, EGC, EC, the major references of Green tea antioxidants [15].The link between green tea consumption and human health benefits has long been recognized as antimicrobial, anti-diabetic, anti-inflammatory, oral health, cardiovascular health, anti-carcinogenic, and antioxidant. Tea antioxidants in tea beverages play a significant role in scavenging ROS and free radicals as peroxide, superoxide and hydroxyl radicals [8], [54]. Additionally, Green tea is an organic fount of fluoride that may play significant role in the cariostatic activity together with the other compounds of green tea [55]. Green tea antimicrobial effects has been observed against extensive range of gram positive and gram negative micro-organisms, fungi and viruses [56].A variety of chemical components are found in green tea, including polyphenols i.e catechins and flavonoids,alkaloids, amino acids, polysaccharides, volatile oils, vitamins (vitamin C), an inorganic elements (manganese, fluorine, and aluminium), and lipids etc. These components play a vital role in green tea efficacy [8].

2.2.1.1 Processing of Green Tea

The nature of green tea is influenced by the various phases associated with the manufacturing process [45].The manufacturing of Green tea involves newly plucked leaves that are quickly subjected to steam (10-15 min) and dried, this process deactivate the enzymes (polyphenol oxidase, peroxidase, ascorbic acid oxidase, and catalase) in the leaves to prevent the catechin oxidation, allowing the tea leaves to sustain its green color as well as to inhibit fermentation, producing a dried and stable product(Table 2.2). As the enzymes become inactivated, they could not deliver TFs and TRs. Tea polyphenols thus conserve in monomeric structure [45].

2.2.1.2 Green Tea Composition

Green tea leaves are enriched by a variety of compounds and having complex composition (Table2.3) involving proteins accounted for 15-20% of total dry weight of tea leaf, carbohydrates for 5-7% (glucose, cellulose, sucrose, fructose and pectins), trace elements and minerals accounted for 5% (Calcium, chromium, iron, strontium, copper, molybedenum, sodium, magnesium, phosphorus, potassium, cobalt, zinc, manganese, nickel, aluminium , fluorine and selenium), amino acids for 1-4% contain theanine, aspartic acid, glutamic acid, lysine, tryptophan, leucine, serine, threonine, tyrosine, glycine and arginine and very small numbers of lipids almost 7%, sterols, vitamins including vitamin B, C, and E, pigments such as carotenoids and chlorophyll, volatile compounds and xanthic bases. In addition alkaloids are accounted for 3-4% also called methylxanthines (caffeine, theobromine, theophylline), phenolic acids and polyphenols 30% that containflavonoids (flavones, flavonols (catechins)), flavandiols, flavanones, isoflavones and anthocyanins) and phenolic acids (gallic acids) are also present [8], [15]. In table 2.3, the * Values refer to dry weight of green tea leaves.

TABLE 2.3: Green tea composition [8].

Compound	**Green Tea***
Proteins	15-20
Amino acids	1-4
Fiber	26
Other Carbohydrates	5-7
Lipids	7
Pigments	2
Minerals	5
Phenolic compounds	30
Oxidized phenolic compounds	0

2.2.1.3 Green Tea Therapeutic Significance

Tea is considered to be a nature's defensive mechanism that works silently in our body. In the course of recent decades, researchers have investigated the green tea constituents, its health advantages and disadvantages.

Tea is enriched by tea antioxidants that are contributed to overall health benefits. In green tea, (-) - epigallocatechin-3-gallate (EGCG) is the principle polyphenol and constitute upto 60-70% of total catechin component [8], [54]. Proper regulation of reactive oxygen species (ROS) has a marked effect on human health issues. An over production or reduced scavenging of ROS has been accounted in the pathogenesis of various diseases such as neurodegenerative disorders, cancer, diabetes, and atherosclerosis (hardening of arteries) [17]. Interaction of catechins and EGCG to peroxyl radicals, promote the anthocyanin like compound and a B-ring present in flavonols-3-ols and a C-ring compound [57] so the frequent tea intake reduces the risk of oxidative status of human body cells [49]. In EGCG and EGC, the main site for antioxidant reaction is trihydroxyphenyl B ring [57]. Oxidative stress is responsible for the onset of cancer. Tea possesses anti-cancer, antioxidant, and anti-mutagenic properties that could reduce the pervasiveness of cancer and secure the people [7], [50]. A few surveys have outlined some back

data reports which described that pancreatic, prostate, bladder, and gastrointestinal carcinomas could be prevented by green tea catechin compounds [7], [17], [49]. Obesity is regarded as an inflammatory disease that stimulates the occurrence of insulin resistivity and type 2 diabetes (T2D) [58]. Epidemiological data analysis revealed that diabetes and obesity risk factors were likely to be prevented by the consumption of tea. It is obvious that addition of sugar in tea increases the risk of diabetes and body weight gain, so the very low quantity of sugar in tea should be practiced [58]. Bioactive components of tea as tea catechins have been shown neuro-protective activities pass through the brain barrier and protect the brain from neuronal cell death (neuronal degeneration) in a large scale array of cellular and animal models of neurological dysfunction [59], [60]. So it is manifested that tea catechins have antioxidant, mental chelating and anti-inflammatory capacities.

2.2.1.4 Side Effects of Green Tea

No doubt, many health advantages are associated with green tea, although its efficacy may be valuable within specific dosage and tea over utilization (green or black) may induce deleterious effects. Consuming green tea in higher quantities may initiate cytotoxicity in liver [48]. It is necessary to avoid drinking very warm tea because it may damage the esophagus and may lead to esophageal cancer [60]. In addition, it is not necessary that tea may effect in the same way to all the individuals.

2.2.2 Black Tea

Among all the consumed tea beverages around the world, tea from *C. sinensis* plant leaves particularly black tea touches the highest consumption level. It is considered as a worldwide favourite drink of many people. As tea of *C. sinensis* is categorized into four distinct types as green, black, oolong and white tea according to different processing procedures from unfermented to semi fermented and completely fermented categories of tea and each type is chemically comprised

of different components with different sensory qualities of color, aroma, flavor and taste [13]. Amongst all these, the completely fermented form is black tea. The highest consumption of black tea contributed to 78% of all the teas and utilized abundantly in most of the East Asian countries (Japan , China), Western countries (North America, Europe) and South Asian countries (Sri lanka and India) also [60]. The tea production all over the world has been continually increasing; of that the annual production of black tea has been planned to grow at 1.9% to achieve about 3.14 million tons by 2017 [36]. Matured *C. sinensis* leaves are used for the black tea production. Oxidized black tea possesses slightly bitter/strong taste and aroma than green tea because of the presence of TFs and TRs, the main black tea polyphenols. Moreover, caffeine content is also higher in black tea than green tea [59]. Mainly western black tea is prepared without milk but in southern Asian countries, there is a little trend of the use of black tea without milk. People of Asian countries mostly like to take tea with milk and sugar.

2.2.2.1 Processing

Black tea, unlike green, oolong and white teas, undergoes complete oxidation process (commonly referred to as fermentation) during manufacturing process that the interaction of oxygen to the cell wall of tea plant causes to change the leaves color from darkish brown to black color hence the tea famous for its name. Once selecting the fresh and healthy leaves from tea plant, these are allowed to wither for a day until the moisture is lost (55% of original leaf weight) from the leaves so that leaves become soft and easily rolled.

The rolling process followed the leaves membranes to be broken down, permitting the essential oils and secretions that provide the characteristics aroma to tea. After the rolling step, tea leaves are dispersed over cool and humid places wherever fermentation of poly phenols is initiated [61]. The colors of leaves become darker throughout the oxidation process (Table 2.2).

Oxidation provides black tea a unique nutty and spicy flavor because some of the catechins during fermentation are converted to TFs and TRs and some phenolic

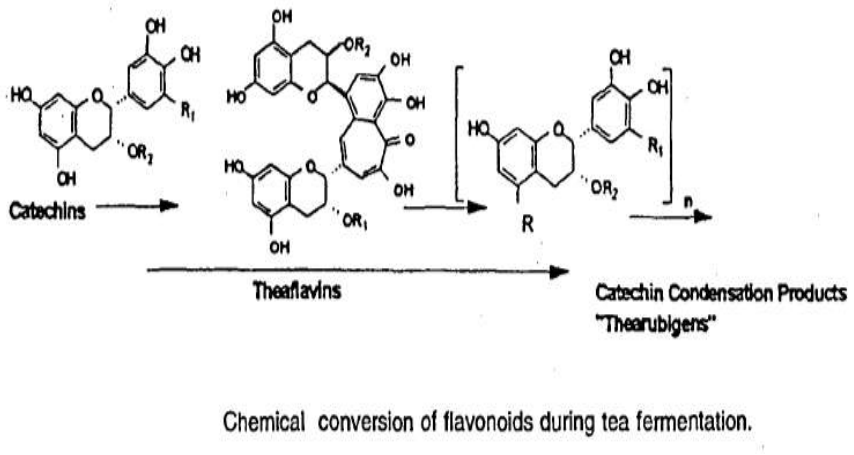


FIGURE 2.2: Conversion of catechin into black tea flavonoids [61]

acids (Figure 2.2) due to the peroxidase and polyphenol oxidase enzymes action result in lowering the catechins contents [62]. When the color, flavor and aroma of tea have completely developed then the process of oxidation must be ceased by drying the leaves at higher temperatures i.e. 900°C-1300°C .The flavorsome juicy secretions dried on leaf surface until making tea in hot water.

2.2.2.2 Black Tea Composition

Black and Green tea composition is comparatively similar but the main difference that exist between these teas being the synthetic changes that happen during the production process. The tea polyphenolic compounds include significant measures of flavonoids overwhelm the structural components of black tea [63]. Tea polyphenols are ordered into six categories of compounds that include: flavanols, flavones, flavonols, anthocyanins, flavanones, iso-flavones [63].

Polyphenols add the notable acrid, sharp, and sweet flavor to tea and represent green tea polyphenols for 16-30%, oolong tea for 8-20% and black tea for 3-10%

TABLE 2.4: Black tea chemical constituents [7]

Constituents	**Concentration (%)**
Catechins	3-10
Theaflavins	3-6
Carbohydrates	15
Thearubigins	12-18
Protein	1
Flavonols	6-8
Mineral matter	10
Phenolic acids	10-12
Volatiles	1
Amino acids	13-15
Methylxanthine	8-11

[61]. The fundamental bioactive components of tea associated with the polyphenols and these active components on the basis of dry weight are contributed as catechins 9%, TRs, and TFs 23% [8],[16]. Normally a Black tea cup contains around 200 mg of flavonoids. Clinical research have reported a noteworthy relation between the daily tea intake of more than 3cups per day and lowered the possibility of many diseases, suggested the significance of tea polyphenols [61].

Flavanols comprised of TFs, TRs, and unoxidizedcatechin components wherever quercetin, myricetin and kaempferol are the constituents of flavonols. Caffeic, gallic, and quinic acids are tea phenolic acids whereas theobromine, theophylline, caffeine and numerous flavoured compounds enhanced with linalool [63]. Additionally tea also contains widely studied bioactive compounds i.e aminoacids and theanine. Black tea chemical constituents are given in table 2.3.

Composition of tea is probably going to be related with the origin, geographical location, environmental conditions and fermentation processing [61].

2.2.2.3 Black Tea Therapeutic Significance

Tea has been related to several health edges since its discovery. Tea represents an important role as antioxidants help protecting the cells from oxidative stress, improving the beneficial micro-flora of intestines and inhibit the dental caries. Various health advantages of tea include cancer prevention, anti-diabetic, antioxidant, anti-viral, antibacterial and anti-inflammatory effects. Cardiovascular, anti-aging, neurological behavior and effect on metabolic syndrome wesre also reported. These health advantages are believed to be related specifically with the manifestation of tea polyphenols.

The significant antioxidant characteristics of black tea are ascribed to the flavonoids compounds including TFs, theaflavic acid and bisflavanols [52]. From epidemiological study analysis, it is obvious that complex diseases such as cardiovascular disease (CVD), artherosclerosis and certain type of cancers evoked by cu^{2+} induced lipoprotein oxidation, are prevented by flavonoid found in green and black tea [49]. So, proposed an inverse relationship between these diseases and dietary flavonoid intake attributed to the antioxidant capacity of flavonoids [49]. Many researchers have found a significant relationship between the consumption of black tea and cancer prevention as black tea possesses anti-initiating property. Baker *et al.* (2007) reported the cancer preventive efficacy of black tea in the case of prostate, ovarian, and rectal cancers development [64]. Many studies suggested that black tea polyphenols contributed to the strong anti-oxidant action that may lower the possibility of cancer by minimizing the DNA damage and also progression of cancer directing to malignant tumor [65]. Regular intake of black tea is related to the decreased possibility of breast, bladder and ovarian cancers in females [66].

Black tea components as catechins, polyphenols, theaflavins, Gallic acid, polysaccharides and anthocyanin render the carbohydrate metabolism disorder by inhibiting the alpha-glycosidase and alpha-amylase i.e. carbohydrate-hydrolyzing enzymes. Black tea extract and caffeine are worthwhile for prevention control of

obesity caused by feeding of high fat diet (HFD), resultant effect may be contributed to inhibit the formation of adipose tissues and adipose tissue mass development [67]. Tea also affects the physiological behavior by reducing the stress and accelerates the relaxation rate because of the existence of amino acid thiamine, a component of black tea, contributed to the bitter taste of tea infusion [68]. Flavonoids are the bioactive components of tea may impact worthwhile effects in the central nervous system (CNS), protect the neurons from the stress and also reduces the neuro inflammatory responses by improving the mental performance through alteration in synaptic plasticity [69]. Black tea extracts are the valuable sources of many bioactive molecules which could serve as the antimicrobial agents against vast domains of microorganisms.

Black tea possesses interesting health advantages however more analysis should have been directed on the both humans and animals.

2.2.2.4 Black Tea Side Effects

Caffeine in tea can cause arrhythmias so its consumption must be limited (1 cup/-day) for pregnant and/or breast feeding ladies [41]. As aluminium is present in tea so tea intake must be limited to individuals with renal disorders since aluminium levels can be increased in the body that elicited neurological disorders, therefore it is important to avoid the foods that contain aluminium [37]. Moreover, flavonoids components in black tea are considered to have an affinity for Fe so black tea extract can prompt a critical reduction of the Fe bioavailability from diet. Thus the aforementioned findings manifested that tea intake should also be limited to anaemic patients [37]. Intake of caffeinated tea (250 mg) increases the attentiveness, restiveness, and the blood pressure and high intake of tea might affect the sleep quality and duration as well [48].

2.2.3 *Thymus serpyllum linn* (Tumuro)

Thymus serpyllum L. (Figure 2.3) belongs to lamiaceae family which contains about 7534 species [70], including genus *Thymus L.* comprises 220 species [71]. *Thymus serpyllum linn*, also known as creeping thyme or wild thyme, and Breckland thyme [20]. In Pakistan, it is naturally growing herb, found in Gilgit, Swat, Chitral, Kashmir, and Kaghan. Its local names are 'Tumuro' and 'Ben ajvain' [19]. The herb is utilized as tea by local inhibitants for different functions as expectorant, anthelmintic, sedative, antiseptic, and carminative [22]. Taxonomic classification of *Thymus serpyllum* is given in table 2.5. They are non-woody perennials, small shrubs growing around the Mediterranea and are also found in North Africa, Asia, and Southern Europe at higher altitudes [20]. The plant grows to approximately 10-25cm height with small, simple, and oblong leaves arranged in whorl form. The color of leaves is light green having the length of approximately 1-2cm and leaf width is about 0.5-0.8cm. Dark and light purple colored flowers are growing on the stem peak and flowering period continues from start of May to mid-September [72].

TABLE 2.5: Taxonomical classification of *Thymus serpyllum* [20].

Kingdom	**Plantae**
Class	Magnoliopsida
Subclass	Asteridae
Order	Lamiales
Family	Lamiaceae
Genus	Thymus L.
Species	Thymus serpyllumL.
Vernacular name	wild thyme, Tumuro, pahariajwain

FIGURE 2.3: *Thymus serpyllum*[20]

2.2.3.1 Phytochemical Constituents of Wild Thyme

The phytochemical constituents of *T. serpyllum* and its essential oil production are thought to be influenced by geographical origin, developmental stage, harvesting season, and its habitat [73]. Carvacol [74] and thymol [75] are the key constituents of thyme essential oil whereas other constituents are p-cymene, borneol, linalool, caryophyllene, citral, geraniol, γ -terpinene, citronellal, α-pinene, terpinyl acetate, isobutyl acetate, 1,8-cineole, citronellol and -bisabolene [74]. The pharmacological action of *T. serpyllum* is considered to be associated with carvacol and thymol, both belong to monoterpene phenolic group with effective germicide properties [76]. As indicated by the European Pharmacopoeia, *T. serpyllum* herb contain around 1.2% of essential oil that include more than 40% of total thymol and carvacol content [76]. Thyme oil is considered as one of the world's best essential oil and used in food preservation [77]. Besides essential oil composition, wild thyme further comprises flavonoids, tannins, phenolic carboxylic acids, and triterpenes. The polyphenolic compounds in the wild thymus contained phenolic

acids, rosmarinic acid, luteolinglucuronide, apigeninglucuronide, salvianolic acid, and luteolinglucoside. Flavonoids compounds include flavonones and flavones [78]. In Pakistan, Thyme essential oil composition is reported by Ahmad *et al.* (2006) as thymol is present in 53.3%, carvacol is 10.4% followed by p-cymene with 8.8%, however amount of these compounds are higher in fresh flowers than dried flowers and leaves [79].

2.2.3.2 *Thymus serpyllum* Therapeutic Significance

Plant species of the lamiaceae family are well-recognized for their aromatic and medicinal purposes.The aerial parts of Thymus species and its volatile constituents are highly suggested throughout the past. These are used in various ailments to cure the diseases and also used as spice, condiments and herbal tea for their curative value [77].

The therapeutic properties of *T. serpyllum* have been broadly utilized as a part of modern and folk lore medicines for a long time and hundreds of years respectively [80]. From the upper ground part of the wild thyme, dried and freshly picked herbs gathered during the blooming period of plant. These ethereal parts of the herbs possess some specific curing properties because of presence of thyme essential oils [80]. Recently, there has been developed great enthusiasm for ethnobotanical, pharmacological, and phytochemical investigations of *T. serpyllum* and also concerning to its therapeutic properties. Mostly wild thyme is utilized in herbal therapies as syrups, tea, decoctions, oil, and tinctures [72]. The pharmacological roles of wild thyme have also been investigated against drug resistant micro-organisms [81]. It has many successful therapeutic applications, and additionally utilized in food, cosmetics, and pharmaceutical industries. Moreover, the increasing demand of herb based products as health boosted supplements from customers and also the clinical use of these herbal supplements as an alternative to synthetic drugs has further prompted the research circle into numerous aromatic and medicinal plants study of which wild thyme possesses a vital place [77].

Herbal infusion is oftenly utilized in the treatment of various ailments especially in the respiratory and gastrointestinal disorders [82]. The English herbal pharmacopeia characterizes *T. serpyllum* as a therapeutic plant and mentioned the symptoms for its utilization are bronchitis, sore, whooping cough, and catarrhal bronchitis. In case of sore throat, it is used for gargling along with blackberry leaves [83]. *T. serpyllum* has a critical role in improving circulation of blood and afterward stimulate the immune system and lowers the cholesterol, therefore its feedings help to cure a number of diseases [83]. In *T. serpyllum*, high ratios of phenolic acids especially rosmarinic acid and flavonoids are accountable for the manifestation of antioxidant activity [84]. The essential oil contain powerful compounds which enhances the immune system and to fight the infections. Moreover, it is also affirmed by the studies of Nikoli'c *et al.* [2014] that *T. serpyllum* essential oil possesses the highest anti-tumor potential [74]. The oil is also used in rheumatoid arthritis and hearing-impairment (deaf) [78]. *T. serpyllum* is broadly utilized in making herb flavoured teas and in cuisine mostly to flavor fish and meat [72].

2.2.4 *Cymbopogon citratus* (Lemon Grass)

The consumption of herbal tea has become global tradition because of its various renowned and undocumented health benefits. According to folklore medicines, a few plants have traditionally restorative advantages and *C. citratus* commonly called lemon grass stayed one of them. *Cymbopogon citratus* is also known as lemon grass (Figure 2.4). *Cymbopogon citratus* is perennial aromatic plant belong to the family poaceae, in which around 500 Genus and nearly 8000 herbal species are included [85]. Plant is mostly used as a herb throughout the world especially in South Asia and South East Asia. It is native to Indonesia, Southeast Asia, Philippines, Southern India and Srilanka [23]. Lemongrass also known as citronella, sereh, fever grass, takrai, serai and is most widely farming in the equatorial and subtropical with two common species, *C. flexuous* sand *C. citratus* [24]. The plant grows to approximately 90cm height with linear leaves having length of approximately 1m and leaf width is about 1-2 cm. The average life span of Plant is

FIGURE 2.4: *C. citratus* [86]

approximately 5-6 years for economic use [86]. Flowering period of plant is short, starts from September to November. It is a lemony flavoured herb commonly utilized in making flavoured teas [87]. Taxonomic classification of *Cymbopogon citratuc* is given in table 2.6.

TABLE 2.6: Taxonomical classification of *Cymbopogon citratus* [88].

Kingdom	**Plantae**
Class	Liliopsida
Subclass	Commellinidae
Order	Cyperales
Family	Poaceae
Genus	Cymbopogon
Species	Cymbopogon citratus
Vernacular name	Lemon grass

2.2.4.1 Phytochemical Constituents of Lemon Grass

C. citratus chemical composition varied with respect to geographical locations, genetic variations, plant part used, extraction methodology, maturity phase of plant, and harvesting period [89], [90]. Regardless of all these variances, various compounds have been discovered including alkaloid, phenolics, saponins, anthraquinones, flavonoids, and tannins. *Cymbopogon citratus* leaves extracts yields yellow or amber liquid of aromatic and essential oil contain aldehydes (75-85%), mainly citral that consists of neral and geranial [91]. Essential oil obtained from lemon grass extract contains citral (citral-α, citral-β), limonene, myrecene, linalool, geraniol, geranial, neral and citronellal, caffeicacid, geranyl acetate, elemicin, and α-terpineol [88]. The phytochemicals in *Cymbopogon citratus* are phenolic and flavonoid compounds which are quercetin, apiginin, luteolin and kaempferol, and isoorientin 2'-O-rhamnoside [92]. The most biologically active component in lemon grass is citral contribute to the bio-efficacy of the plant [93]. Regardless of its (lemon grass) occurrence in different areas of the world, *C. citratus* (lemon grass) contains citral in highest percentage of about 80% and this higher content of citral legitimizes the *C. citratus* cultivation on commercial scale in many countries [91]. Many factors could influence the quality of essential oil yield from the lemon grass i.e. water content of the soil and salinity [89]. Harvesting time period (early or late) is another important factor that influenced the *C. citratus* citral content and essential oils [94]. Maximum range of citral seemed to achieve by harvesting *C. citratus* after planting of 6 months to yield the best quality of essential oil and minimize the cost production. *C. citratus* also contains macro nutrients, vitamins, minerals and electrolytes [95].

2.2.4.2 Processing Steps of Lemon Grass

The detailed processing steps of lemon grass are given in the figure 2.5.

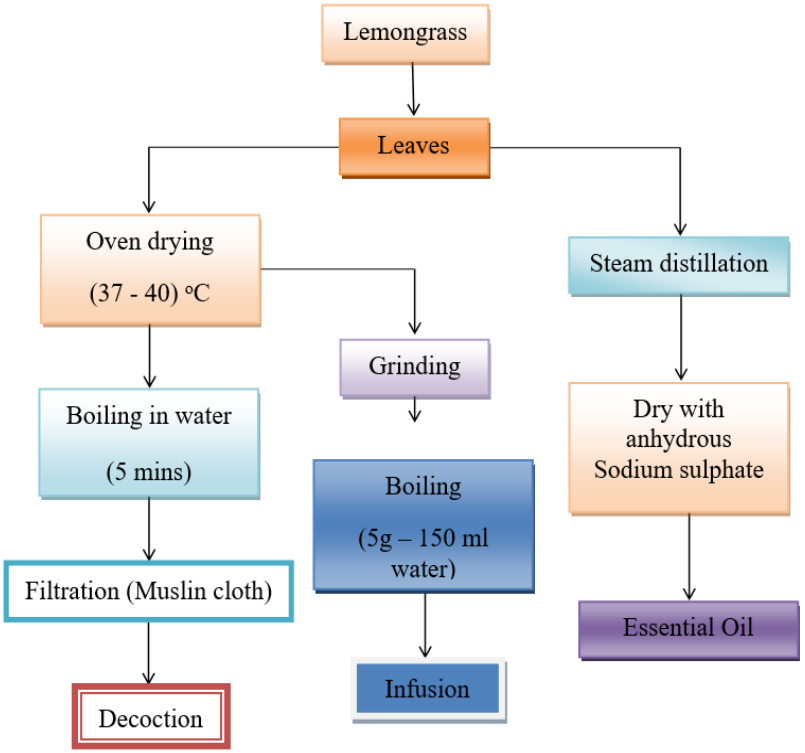


FIGURE 2.5: Different processing steps followed to isolate the bioactive components from lemon grass [96]

2.2.4.3 *Cymbopogon citratus* (lemon grass) Therapeutic Significance

C. citratus is of great importance because of its valued essential oils, its utilization in customary pharmaceutics and additionally in the food technology. Many herbal teas are now associated with preventing and treating many health conditions [97]. Lemon grass contains many biological active compounds found in the leaves that play significant role in various health problems. Lemon grass aqueous infusion is mostly used for making aromatic beverage like tea and the whole plant is used into traditionally food because of its lemony flavor as well as in folklore medicines [87]. Lemon grass aqueous extract is a traditional cure for headache, flu, pneumonia, leprosy, elephantiasis, malaria, and gingivitis [88]. Lemon grass herbal tea is most effectual treatment for various disorders and its effectiveness has been studied as antibacterial, antiamoebic, antifungal, antidiarrheal and anti-mutagenicity. Antioxidant, anti-malarial, anti-inflammatory and neuro-stimulant properties have also been investigated [88]. Scientists have recognized the antioxidant activities of lemon grass and also reported its capacities to minimize the effects of ROS [98].

Mirghani *et al.* (2012) also demonstrated the antioxidant activity of lemon grass extracts of both stalk and leaves and the desired outcomes are associated with dose dependency [99]. Lemon grass tea contains sufficient citral concentrations that are found to be effective to initiate apoptotic process in tumour cells without causing damage to healthy cells [100]. The protective effect of citral has been observed on healthy liver cells during the chemotherapy treatment of liver cancer [101]. *C. citratus* extract has been investigated in several studies for its possible potentials as hypoglycemic and hypolipidemic agent that may lessens the obesity and hypertension risks [91].

Fresh lemon grass is used typically in Sri Lanka and Southeast Asia. Lemon grass essential oil or plant extract is considered to be safe for human use [24]. Lemon grass possesses cleansing properties and is regarded as efficient detoxifier that detoxifies gastrointestinal tract, liver, bladder, pancrease, and kidney. It regulates the levels of uric acid and cholesterol, reduces extra fat, body mass, and different body toxins [99]. However, it stimulates the lactation, food digestion, and circulation but gastroenteritis and heartburn are also reported by its severe use [96]. Aqueous infusion of lemon grass is considered helpful in curing skin problems and reducing blood pressure. In addition, lemon grass has strong anti-tumor properties [100].

2.2.4.4 Side Effects

Lemon grass toxicity and gastric resistance were investigated in young rats. No toxic effects were observed at lower dose (5-1500mg/kg) but some abnormalities and toxicity were reported when comparatively administered high dose (2000-3000mg/kg). So lower dose of essential oil are considered harmless but high dose could cause liver cells necrosis and also affect the morphological structure of stomach in rats [102].

2.3 Phytochemical Analysis of Herbal Teas

Medicinal plants have been known for centuries for their numerous phytochemicals like tannins, glycosides, alkaloids, flavonoids, polyphenols and many others. Nature is considered as an abundant pharmaceutical stores existing on this planet owing to their ability to produce various secondary metabolites with a broad spectrum of bioactivities [103]. Employing various phytochemical analysis techniques many plant based chemicals have been characterized. Phytochemicals are non-nutritive plant constituents which play a prophylactic and defensive job against health complications both in animals and plants. Thousands of chemicals proven to be active against pests and diseases have been discovered [104]. Polyphenols are a part of plant secondary metabolites capable of playing effective role in neutralizing free radicals, quenching singlet oxygen. These compounds have antioxidant capability and are able to reduce the oxidative stress [105]. Polyphenols are the most abundant and ubiquitous molecules occurring in plants. Mode of action of phenolics involves the inactivation of free radicals by donating hydrogen specie which inhibits lipid peroxidation reaction and as a result prevention against oxidative degradation takes place [106]. Phenolics are also a weapon of plant defense systems as they have proven to be active against varied pathogenic microorganisms. Most frequently used protocol for total phenol content estimation is a Folin-Ciocalteu reagent based method [107].

Flavonoids exist as glycones, aglycones and methylated derivatives. Discovery of more than 4000 flavanoids make it the largest group of polyphenols. They act as antioxidants by quenching free radicals. They exert multiple pharmacological actions like antiinflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities [108]. Factors to classify these compound are; molecular framework and antioxidant potential. Different classes are anthocyanins, catechins, flavanones, flavones, flavonones and isoflavonoids. They are a part of plant defense mechanism against microbial infections [109]. Total flavonoids content is determined by $AlCl_3$ Colorimetric method [107].

2.4 Antioxidant Analysis of Herbal Teas

Free radicals are highly reactive species and are capable of damaging cellular proteins, membrane lipids and in the nucleus DNA [110] (Figure 2.6). Humans have protective mechanisms for combating these free radicals but the imbalance between antioxidant defense mechanisms and free radical generation causes defined as oxidative stress. Natural sources serve as a storehouse of different kinds of antioxidants which have been separated and used continually without any harmful effect [111]. According to an estimation, oxygen free radicals (OH) and other ROS attack each human cell 10,000 times per day. This oxidative stress modifies genetic makeup and serves as the basis of development of mutagenesis [112]. No single antioxidant assay is capable of evaluating complete antioxidant potential of a sample. A variety of bioassays are executed to determine antioxidant potential. So, different types of antioxidant assays were performed, each type signifies different mechanism e.g. disintegration of peroxide, scavenging of free radical and prevention of chain initiation.

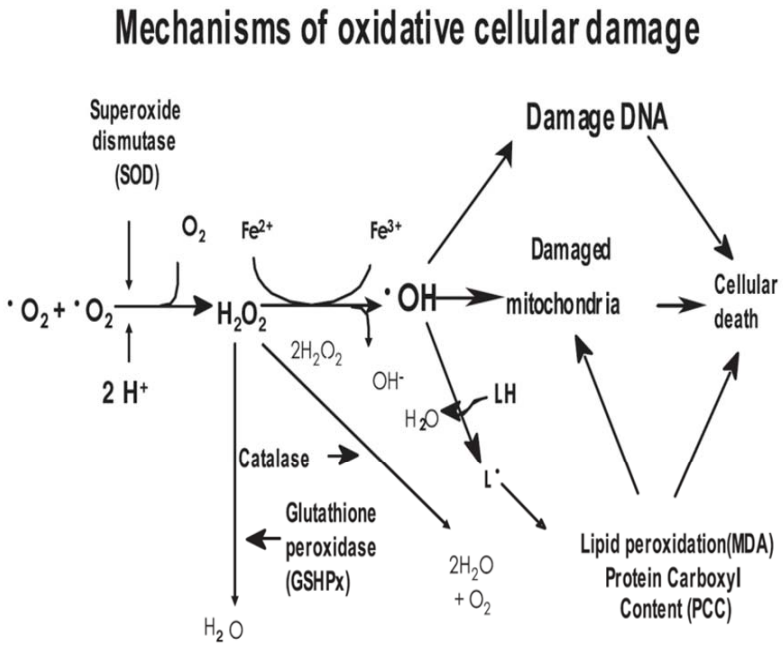


FIGURE 2.6: Mechanism of oxidative cellular damage [113]

2.5 Antimicrobial Analysis of Herbal Teas

Various microbes i.e. bacteria, fungi and algae have proven to be pathogenic in plants and animals. Folk medicines have always shown promising results against these transmittable diseases since antiquity. Haphazard usage of antimicrobials induces resistance in infectious organisms regardless of advancement in medical knowledge [114]. Spread of diseases throughout the world compels scientists to establish more advance and systemized novel to face such calamities. Folk knowledge about the remedial properties of natural products including plants authenticates anti-infectious properties could be helpful for treating various ailments [115].

In order to evaluate antimicrobial and antifungal screening of test extracts, disc diffusion method is followed. To determine MIC, both agar dilution and broth dilution method is adopted.

2.6 Cytotoxicity of Herbal Teas

Cancer is amongst the deadliest known diseases which have inflicted tremendous loss to mankind throughout its medical history. A number of plant secondary metabolites including alkaloids, flavonoids and terpenes etc have been used as effective anticancer agents [116]. Much attention has been shifted recently to natural; especially plant based anticancer agents due to notorious side effects associated with synthetic drugs. The general toxicity and anticancer assays have been performed since last thirty years to evaluate if a plant is generally toxic or not [117]. Brine shrimp lethality assay is employed to evaluate a broad spectrum of biological activities taking into account that pharmacology is toxicology at high doses. Positive correlation might be evident between cytotoxicity to human cells and fatality to nauplii of brine shrimps [118]. Harvesting the nauplii (shrimp larvae) is highly recommended before being used for the test [119].

2.7 Enzyme Inhibition of Herbal Teas

Eukaryotic protein kinases constitutes a large family of proteins that are responsible for catalyzing the transfer of gamma phosphate group of donor molecule (ATP) or (GTP), the energy packets of cell, to the tyrosine, serine and threonine in the recipient protein. Multiple cellular cascades rely on the phosphorylation of the proteins involved in the biological pathways [120]. Protein phosphorylation by protein kinases at serine/threonine and tyrosine residues is a significant governing mechanism in many biological processes i.e. cell proliferation, apoptosis, metabolism and cell differentiation (Figure 2.7). Around 518 protein kinases have been discovered in humans so far and are categorized in 20 families based on the amino acid sequence [121]. Research has proved that dysregulation of protein kinases hinders the cellular processes relevant during disorders like neoplasia, therefore they hold pro-oncogenic potential [122]. Several molecules which inhibit the protein kinases are under investigation for their potential role in cancer therapeutics.

Protein kinase inhibitory activity is determined by disc diffusion method using *streptomycesactinobacter*. Inhibition of hyphae formation of this strain can be correlated to the inhibition of protein kinase, a regulatory class of enzymes for cell's metabolic pathways as described by Yao *et al.* ., (2012) [123].

Alpha amylase is a vital enzyme involved in carbohydrate digestion. It is responsible for the breakdown of long chain carbohydrates (Figure 2.8). Inhibition of alpha amylase activity is an effective method to control diabetes as its inhibition helps in preventing the formation of simpler sugars (dextrin, glucose and maltose etc). Thus alpha amylase inhibitors delay glucose uptake rate and help hyperglycemic individuals in maintaining serum glucose level. They are also known as starch blockers as they prevent starch hydrolysis and ultimately prevent or slow its absorption into the body [125]. Many plants have been studied for their alpha amylase inhibition potential and are being the part of herbal therapies. So the research is inclined towards the plant sources capable of inhibiting this enzyme [126]. Alpha amylase inhibition assay is used to assess antidiabetic activity of test samples.

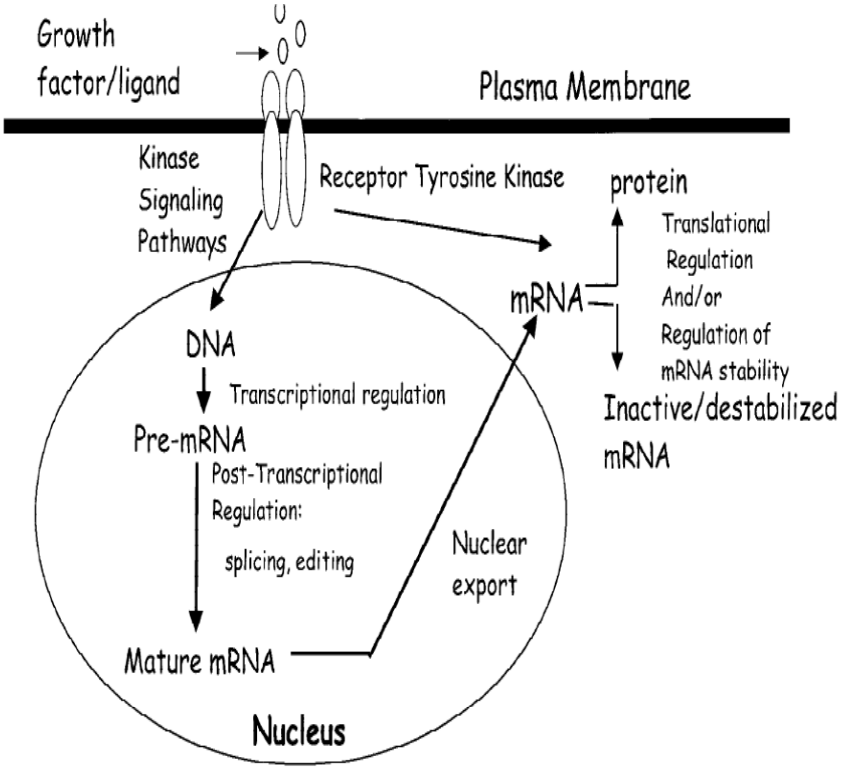


FIGURE 2.7: Regulatory control points in gene expression by growth factors [124]

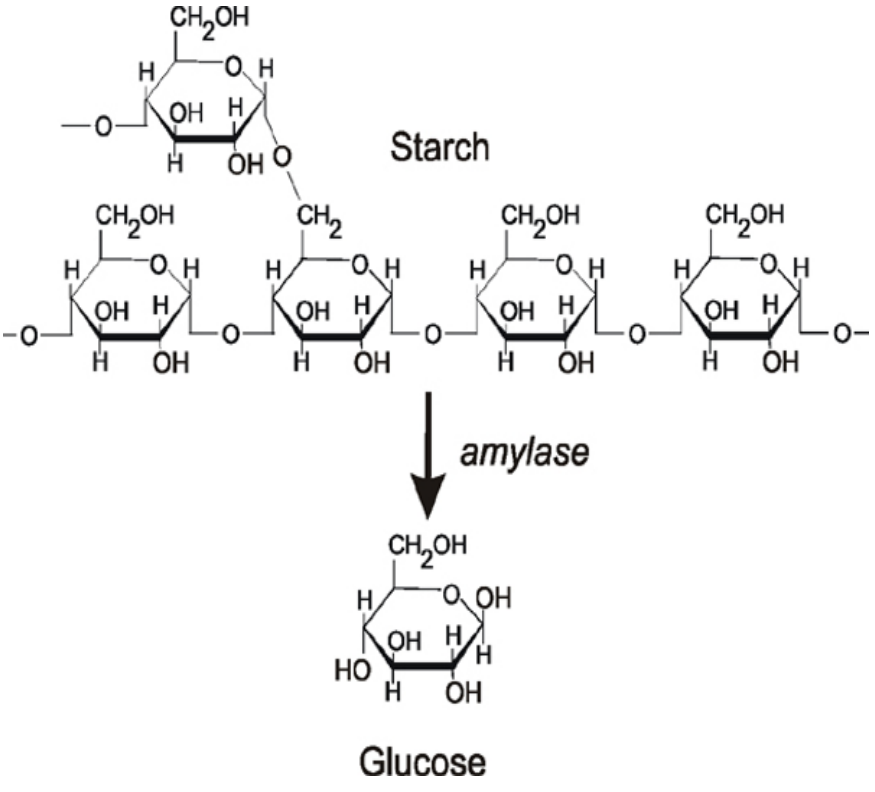


FIGURE 2.8: Break down of starch by Alpha Amylase enzyme [127]

2.8 Qualitative Analysis of Herbal Teas

FT-IR technique indicates the bonds existed in the compound and consequently be used to determine functional groups of the molecule present in teas to better characterize the field of examination of these natural products [128].

The principle of FT-IR is based mainly on the absorption of Infra red radiations by the examined material. By the identification of vibration frequencies of chemical bonds, it is possible to analyze the chemical functions exhibited by the tested materials [128].

The study of extracted plant materials assumes an essential part in the advancement and modernization of standards necessary for herbal preparations. GC-MS investigation is solely used to figure out the significant fatty acid constituents present in tea extract [29]. As tea is considered as functional food, so it is important to study the fatty acids in tea from that perspective. However, the total lipid content found in tea is lower, almost 1-3% on the basis of dry weight. Subsequently, lipids or fatty acids emanates as an important domain for research and quality control. The characteristic flavor and aroma of tea are mainly contributed by fatty acid compounds. Lipid contents of plants are significant sources of nutrition, pharmaceutical and industrial importance. The major biochemical functions of lipids can be categorized into three main areas i.e. energy storage, structural component of cell membrane, and signaling for biochemical activities [29]. Dietetic lipids and fatty acids (FAs) plays vital role in maintaining health and also in the prevention of diseases [129]. Variance in fatty acid compounds could assume an important part in the choice of cultivars with enhanced tea production potential [130].

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Chemicals and Reagents

Ethyl acetate, Methanol, and Chloroform (Sigma Aldrich, Germany); Distilled water prepared freshly was also used; DMSO (Sigma Aldrich, USA); Acarbose (Glucobay® 100; Bayer Pakistan Pvt. Ltd Karachi); Hydrochloric acid (HCl), Iodine (I), Potassium iodide (KI) and Surfactin (Sigma Aldrich, USA); Aluminium chloride ($AlCl_3$), Ammonium molybdate, Ascorbic acid, Doxorubucin, DPPH reagent, Ferric chloride ($FeCl_3$), Monosodium dihydrogen phosphate ($N_aH_2PO_4$), Nutrient agar, Potassium acetate, Potassium ferricyanide, Sea salt, Tween-20 (Merck-Schuchardt, USA), Sabouraud dextrose agar (Oxoid, England), Tryptone soy broth, Standard antibiotics (roxithromycin and cefixime), Standard antifungals (clotrimazole), Trichloroacetic acid, Sulphuric acid (Sigma Aldrich, Germany); Dried instant yeast (Fermipan BDH, England); Folin-Ciocalteu reagent (FC) and Phosphate buffer saline (PBS) (Riedel-de-Haen, Germany); -amylase enzyme (Unichem Laboratories: Pharmaceutical Company India); Brine shrimp *"Artemia salina"* eggs were acquired from (Ocean star Int, USA) and Medium ISP4 (Prepared in laboratory).

3.1.2 Apparatus and Equipments

Ultrasonicator (sweep zone technology, USA), incubator (Memmert, USA), pasteur pippetes, bicompartment perforated tray, microplate reader (Elx 800, Biotek, USA), neubaeur chamber (marine Germany), compound light microscope (Irmeco, Germany), centrifuge (B.Bran, Germany), 5% CO_2 incubator (Sanyo MCO-17AIC, Japan), FT-IR spectrophotometer (Bruker-Tensor 27, US), Shimadzu QP2010 Ultra (Japan), Vernier caliper (tailin, Japan), Freezer 9170 WB M (Dawlance Pakistan) and rotary evaporator (Bibby Sterlin, England). Erlenmeyer flasks, whattmann filter paper, tripod stand, 96-well plate (SPL life science, Korea), beakers, muslin cloth, petri dishes, magnifying glass, magnetic stirrer, funnel, micropippete (Sartorius, France).

3.1.3 Animals and Cultures

3.1.3.1 Bacterial Strains

Gram positive bacterial strains: *Staphylococcus aureus* (ATCC-6538), *Bacillus subtilis* (ATCC-6633) , and *streptomyces 85E*. Gram negative bacterial strains: *Escherichia coli* (ATCC-25922), *Pseudomonas aeruginosa* (ATCC-15442), *Klebsiella pneumonia* (ATCC-1705).

Bacterial culturing was done by using nutrient agar; incubation was done at 37°C, however stock cultures were preserved at 4°C. *Streptomyces strain 85E* used for protein kinase inhibition assay was cultured on ISP4 media and refreshed in Tryptone soya broth (TSB).

3.1.3.2 Fungal Strains

The fungal strains against which the samples were tested included *Aspergillus niger* (FCBP-0198), *Aspergillus flavus* (FCBP-0064), *Fusarium solani* (FCBP-0291), *Aspergillus fumigatus* (FCBP-66), and *Mucor species* (FCBP-0300).

Culturing of tested fungal strains was maintained on SDA (sabouraud dextrose agar); incubation was done at 28-30°C, however stock culture was maintained at 4°C. Brine shrimps eggs (Ocean star Int., USA).

3.2 Methods

3.2.1 Research Methodology Outlines

Figure 3.1 shows the outlines of our research methodology.

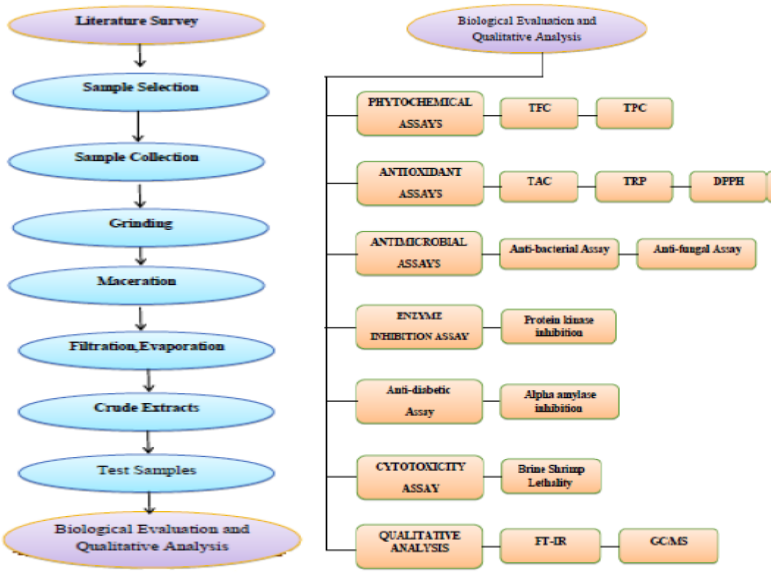


FIGURE 3.1: Research methodology outlines

3.2.2 Collection and Identification of Samples

In the recent study, five different tea samples were collected from different areas of Pakistan. *Thymus serpyllum linn*, local name Tumuro was obtained from Giligit (Northern areas of Pakistan). Two types of T. *serpyllum herbal* teas were used: *Thymus serpyllum 1* (dried leaves) and *Thymus serpyllum 2* (dried leaves + flowers) also named Tumuro 1 and Tumuro 2 respectively. *Camellia sinensis* (Green Tea) was collected from Shinkiari. *Camellia sinensis* (Black tea) (Brooke bond

supreme) and Lemon grass (*Cymbopogon citratus*) were purchased from local super store. All the samples were identified by Dr. Rizwana (Department of Plant sciences) from Quaid-e-Azam University, Islamabad. All the tea samples were in dried form, properly kept in a cool and dry place before the experiments. Shinkiari and Gilgit are the northern areas of Pakistan.

3.2.3 Extraction

The collected tea samples were pulverized separately by commercial miller to coarse powder. The grinded powder of different teas was employed for successive extraction by using ethyl acetate solvent of medium polarity. Extraction technique employed was sonication assisted maceration. Accurately weighed (20 g) powdered tea samples were subjected to sonication aided maceration with analytical grade ethyl acetate (1:4) for 72 hours with frequent sonication cycles of 30 minutes in ultra-sonic bath at room temperature. Third day, the solvent was strained using muslin cloth. Through Whatmann No.1 filter paper filtration was done and filterate was concentrated by rotary evaporator at 40°C. The recovered solvent along with fresh solvent was again used in extracting the marc for further 24 hours. Fourth day the solvent was filtered in the same manner and the marc was dried fully. The filtrate was concentrated by rotary evaporator and finally dried at 45°C in a vacuum oven to procure the crude extracts of all the tea samples [131].

3.3 Biological Evaluation

3.3.1 Phytochemical Analysis

3.3.1.1 *Total Phenolics Content (TPC) Quantification*

Stock solutions

Folin-Ciocalteu (FC) reagent was diluted with distilled water to get 10% solution. 6% Sodium carbonate stock solution in distilled water was prepared. Tested tea extracts were weighed and stock solution (4 mg/ml) of each tea extract was made in dimethyl sulfoxide. Gallic acid (positive standard) stock solution was prepared separately in DMSO (4 mg/ml).

Procedure

Most frequently used protocol for total phenolic content estimation is a *Folin-Ciocalteu* reagent based method previously described by Haq *et al.* . (2013) [107]. Microtiter 96-well plate was used for this assay and whole procedure was run in triplicate. From each stock solution, 20 μl of tested tea samples were taken and transferred to respective wells followed by the addition of FC reagent (90 μl). For 5 minutes, then incubate the resultant mixture at room temperature, an addition of 90 μl of $N_{a_2}CO_3$ solution (6%) was made. At 37°C, the incubation period was lasted for 30 minutes and then measured the absorbance at 630 nm with the help of microplate reader. The positive control employed in this procedure was Gallic acid in two fold serial dilutions (2.5, 5, 10, 20, 40 $\mu g/ml$) to attain calibration curve while negative control employed was dimethyl sulfoxide. Results were demonstrated as μg Gallic acid equivalent (GAE) per mg of extracts ($\mu gGAE/mg$ extract).

3.3.1.2 *Total Flavonoids Content (TFC) Quantification*

Stock solutions

Different stock solutions prepared for this assay include; 10 g aluminium chloride ($AlCl_3$) in 100 ml distilled water, 98.15 g potassium acetate per litre of distilled water to make 1M solution to be used in the given assay. Crude extracts were weighed and a stock solution of 4 mg/ml of each test extract and quercetin (positive standard) were prepared separately in DMSO.

Procedure

For the quantification of total flavonoid content, following procedure was followed as described by Haq *et al.* . (2013) [107]. The 96-well plate was used for this assay and whole procedure was run in triplicate. From each stock solution, 20 μl of tested tea samples were taken and transferred to respective wells. Then 10 μl (10%) $AlCl_3$ and (1M) Potassium acetate solutions was also pipette in above mentioned wells respectively. 160 μl of distilled water was also added in above wells. For 30 minutes, then incubate the resultant mixture at room temperature and measured the absorbance at 415 nm with the help of microplate reader. Quercetin was employed as positive control at concentrations 40, 20, 10, 5, 2.5$\mu g/ml$, in order to obtain the calibration curve while negative control employed was DMSO. The resultant flavonoids (TFC) were demonstrated as μg Quercetin equivalent (QE) per mg of extracts (μg QE/mg extract).

3.3.2 Antioxidant Assays

A variety of bioassays will be executed to determine antioxidant potential.

3.3.2.1 *DPPH assay (Free Radical Scavenging Assay; FRSA)*

Stock Solutions

9.2 mg DPPH was added in 100 ml methanol in order to freshly prepared DPPH solution. Preparation of ascorbic acid solution was done by adding 1 ml of DMSO to 1 mg of ascorbic acid. Crude test extracts were weighed and a stock solution of 4 mg/ml of each test extract was prepared in dimethyl sulfoxide.

Procedure

FRSA of each tea extract was assessed by standard protocol previously illustrated by Khan *et al.* . (2015) [132]. Free radical extinguishing capability of extracts or samples is assessed by DPPH reagent based assay. A change in absorbance values is detected because antioxidants in test samples cause production of hydrazine which renders the discoloration of purple color of DPPH reagent. The 96-well plate was used for this assay and whole procedure was run in triplicate. From each stock solution, tested tea sample (10 μl) was taken and transferred to respective wells in the microtiter plate followed by the addition of DPPH reagent (190 μl). For 60 minutes, then incubate the resultant mixture at 37°C in a pitch dark surrounding and measured absorbance at 517 nm with the help of microplate reader and % scavenging activity of each tea sample was find out by the given formula:

$$\%Scavening = (1 - Abs/Abc)100 \tag{3.1}$$

Where, Abs is Absorbance of sample containing DPPH reagent, Abc is Absorbance of negative control containing DMSO and DPPH reagent.

The samples showing more than 50% scavenging at tested concentration ($200\mu g/ml$) were analyzed at lower concentrations (three fold serial dilutions) to calculate IC_{5_0}. Graph pad prism5 soft ware was used to calculate the IC_{5_0}. Standard ascorbic acid was employed as positive and and Dimethyl sulfoxide (DMSO) as negative control.

3.3.2.2 *Reducing Power Evaluation*

Stock Solutions

Preparation of Phosphate buffer of 6.6 pH and 0.2 molarity was done by dissolving sodium dihydrogen phosphate ($N_aH_2PO_4$, 1 g) and disodium hydrogen phosphate dehydrate ($N_{a_2}HPO_4.2H_2O$, 1.42 g) in 50 ml deionized distilled water. Similarly solutions of 1% potassium ferricyanide ($1g/100ml$), 0.1% ferric chloride ($0.1g/100ml$), and 10% trichloroacetic acid ($10g/100ml$) were prepared in distilled water. Crude tea extracts were weighed and a stock solution of 4 mg/ml of each tea extract was prepared in dimethyl sulfoxide. Preparation of ascorbic acid solution was also done by adding 1 ml of DMSO to 1 mg of ascorbic acid.

Procedure

A well elaborated procedure revealed by Khan *et al.* . (2015) [132] was adopted for determining total reducing power of tested tea extracts. Briefly, an aliquot of 100 μl pipetted from each tea extract and was taken in respective eppendroff tubes. Then 200 μl of phosphate buffer (0.2 molar) with pH 6.6 and 1% potassium ferricyanide (250 μl) were also taken in respective eppendorf tubes. For 20 minutes, incubation of admixture was done at 50°C. In the above mixture, 10% trichloroacetic acid (200 μl) addition was made and then centrifugation of whole mixture was done at 3000 rpm for 10 minutes at room temperature. Aliquot (150 μl) from supernatant was taken and transferred into the wells of 96-well plate containing 0.1% $FeCl_3$ (50 μl), after that absorbance was measured at 630 nm. Standard ascorbic acid (1 mg/ml) at final concentrations of 25, 12.5, 6.25, and $3.125\mu g/ml$ was employed as positive control however negative control employed was dimethyl sulfoxide. A calibration curve was obtained using ascorbic acid at different concentrations ($y = 0.025x + 0.665$, $R^2 = 0.995$). The resultant reducing power of tea samples were demonstrated as g ascorbic acid equivalent per mg of extracts ($\mu g\ AAE/mg$ extract).

3.3.2.3 *Total Antioxidant Capacity (TAC) Evaluation*

Stock Solutions

Crude tea extracts were weighed and a stock solution of 4 mg/ml of each tea extract was prepared in dimethyl sulfoxide. Preparation of ascorbic acid solution was also done by adding 1 ml of DMSO to 1 mg of ascorbic acid. To prepare TAC reagent freshly, first of all 1.67 g of sodium monobasic phosphate, 0.247 g of ammonium molybdate and 1.63 ml of sulphuric acid was dissolved in small amount of distilled water respectively to have final volume 50 ml.

Procedure

Phosphomolybdenum based analysis technique as narrated by Fatima *et al.* . (2015) [133] was adopted for estimating total antioxidant capacity of test extracts. First of all, 100 μl of each test extract was taken in eppendorff tubes. TAC reagent of 900 μl was also added in eppendorff tubes. For 90 minutes, incubation of reaction mixture was done at 95°C in water bath. After cooling reaction mixture at 37°C, 200 μl from each reaction mixture was pipetted into each well of 96- well plate. With the help of microplate reader at 630 nm, measured the absorbance of assay plate. Ascorbic acid was employed as positive control at final assay concentrations (50, 25, 12.5, and 6.25 $\mu g/ml$) and DMSO employed as negative control (blank). A calibration curve was obtained using ascorbic acid at different concentrations ($y = 0.043x + 0.124$, $R^2 = 0.997$). The procedure was run in triplicate and the total antioxidant capacity of all tea samples was demonstrated as g ascorbic acid equivalent per mg of extracts ($\mu gAAE/mg$ extract).

3.3.3 Antimicrobial Assays

3.3.3.1 *Antibacterial Assay*

Stock Solutions

The 20 mg/ml stock solutions of all tea extracts were prepared in DMSO. Roxithromycin and cefixime (positive standards) stock solutions were prepared as 4 mg/ml in DMSO.

Inoculum Preparation

The culture was refreshed by taking 10 ml aliquot of sterile nutrient broth inoculated with sterile loopful of bacterial colonies maintained at 37°C for 24 hrs. Turbidity was checked according to McFarland 0.5 turbidity standard.

Procedure

Antibacterial potential/activity of each tea extract was determined by previously documented disc diffusion protocol by Khan *et al.* . (2015) [132]. By taking 50 μl aliquot from 24 hrs refreshed bacterial cultures was used to prepare lawn on NA petri plates. 5 l of each tea extract was infused on discs of filter paper (sterilized) and then placed on properly labeled seeded agar plates. Positive controls cefixime monohydrate and roxithromycin (5 μl from 4 mg/ml DMSO) were also infused on discs and placed on plates. At 37°C for 24 hrs incubation was done. Around each disc (tea samples + controls), zone of inhibition was examined, measured in milli meters (mm) by vernier caliper and then recorded. The assay was run as triplicate analysis.

MIC Determination

MIC was determined according to the procedure depicted by Fatima *et al.* . (2015) [133]. Samples which exhibited significant zone of inhibition i.e.≥12 mm were further subjected to MIC determination by microbroth dilution method. Density of bacterial inoculums was maintained at ($5x10^4$ CFU/ml). Three fold serial dilutions of test samples with final well concentrations of (100, 33.33, 11.11, and 3.70 $\mu g/ml$) were prepared with sterile NB. Subsequently, 195 μl of bacterial culture was added into each well of 96-well plate followed by incubation for 24-36 hrs at 37°C. The results were verified by visible antibacterial activity determination. The MIC is the minimum concentration which shows visibly clear wells.

3.3.3.2 *Antifungal Assay*

Stock Solutions

Accurately weighed 20 mg test extracts were dissolved in 1 ml of DMSO to make 20 mg/ml solutions. Stock solution of standard drug clotrimazole was prepared as 4 mg/ml DMSO (final concentration 20 $\mu g/disc$).

Inoculum Preparation

Spores of fungal strains were harvested from stock cultures on sterile SDA plates. At 28°C, incubation of plates was done for 7 days. After incubation spores were suspended in Tween-20 solution (0.02% v/v) in distilled water. Turbidity was finally checked according to McFarland 0.5 turbidity standard.

Procedure

Antifungal assay was performed as previously illustrated by Fatima *et al.* . (2015) [133]. Petri plates having sterile sabouraud dextrose agar (20-25 ml) were swabbed with 100 μl refreshed inoculum. 5 μl of each tea extract (20 mg per ml of DMSO)

was infused on discs of filter paper (sterilized) and then placed on properly labeled seeded agar plates. Sterile filter discs infused with 5 μl of DMSO and clotrimazole (4 mg per ml of DMSO) respectively and were placed on plates. At 37°C for 24-48 hrs incubation was done. Around each disc (tea samples + controls), zone of inhibition was examined, measured in milli meters (mm) by vernier caliper and then recorded. The assay was run as triplicate analysis.

3.3.4 Cytotoxicity Potential

3.3.4.1 *Brine Shrimp Lethality Assay*

Stock Solutions

The 20 mg/ml stock solutions of all tea extracts were prepared in DMSO. Standard drug doxorubicin stock solution was prepared as 4 mg/ml in DMSO. Simulated sea water was prepared by dissolving 6 mg yeast and sea salt (38 mg) in 1 liter distilled water.

Procedure

The preliminary cytotoxicity of crude extracts against brine shrimps (*Artemia salina*) larvae was determined by 24 hours lethality test as described previously by Haq *et al.* . (2013) [107]. *Artemia salina* eggs were hatched in specially designed bi-compartment perforated tank that was filled with simulated sea water. The compartment containing eggs was completely covered with aluminium foil while other was lightened with a light source. The tank was incubated at 30-32°C for 24-48 hours. After specified incubation period, the eggs were hatched and nauplii started moving towards the lightened compartment of the tank through small perforations. The hatched nauplii were then collected with Pasteur pipette and placed in beaker containing sea water. Two-fold serial dilution of test extracts was made up to the final concentrations (200, 100, 50, 25 $\mu g/ml$). 10 mature nauplii were transferred and 150 μl of sea water was added to each well. To made the

final volume of each well (up to 300 μl) sea water was used. Serial concentrations of positive control doxorubicin (10, 5, 2.5, 1.25 $\mu g/ml$) was made while negative control used was DMSO (1%). After incubating 96 well plate at 37°C for 24 hrs, dead nauplii were counted using inverted microscope. The whole experiment was performed thrice. The LC_{5_0} was calculated by graph pad prism5 software.

The percent lethality of each extract was determined using formula:

$$\%mortality = no.of deadshrimps/totalno.of shrimps \times 100 \tag{3.2}$$

3.3.5 Enzyme Inhibition Potential

3.3.5.1 *Protein Kinase Inhibition Assay*

Stock Solutions

A stock solution was prepared for each sample by dissolving 20 mg crude extract per ml DMSO. Surfactin was used as standard and its 4 mg/ml stock solution was prepared in DMSO. Tryptone soya broth (TSB) dissolved in distilled water was used to refresh culture.

Inoculum Preparation

Streptomyces 85E strain spores were inoculated in sterile TSB and for 24 hours incubation was done at 37°C. McFarland 0.5 turbidity standard was used to adjust the turbidity of refreshed culture.

Procedure

A well elaborated procedure revealed by Fatima *et al.* . (2015) [133] was employed to test the ability of herbal tea extracts to inhibit protein kinases. *Streptomyces* 85E strain was used in this assay. By swabbing of 100 μl culture, bacterial lawn

was formed on sterile petri plates having ISP4 medium. 5 μl of each tea extract (20 mg per ml of DMSO) was infused on discs of filter paper (sterilized) and then placed on properly labeled seeded agar plates. The final concentration of test sample on disc was $100\mu g/disc$. Positive control (surfactin) was also applied on discs and the final concentration of surfactin on disc was $20\mu g/disc$. Negative control (DMSO) infused discs were also placed. For 72 hrs, incubation of assay plates was done at 28-30°C to allow the hyphae formation. Around each disc (tea samples + controls), the results were recorded by measuring the clear and bald zones of inhibition to nearest mm. The bald zone around samples indicated protein kinase inhibition potential by inhibiting the hyphae formation while clear zone indicated cytotoxicity of test samples.

3.3.6 Antidiabetic Assay

3.3.6.1 *Alpha Amylase Inhibition Potential*

Stock Solutions

A stock solution was prepared for each sample by dissolving 4 mg crude extract per ml DMSO. Phosphate buffer was prepared by mixing 26.5 ml of KH_2PO_4 and 23.5 ml of K_2HPO_4 and making the final volume up to 1 liter and confirming the desired pH. By dissolving of enzyme (alpha amylase, 14.37 units) per ml of phosphate buffer pH 6.8, a stock solution of enzyme was prepared. The 1 M hydrochloric acid solution was prepared having 82 μl HCL in 1000 μl of distilled water. By the addition of 20 mg starch per 10 millilitre (ml) of potassium phosphate buffer (pH 6.8), a starch solution was prepared and was slightly heated to get the clear solution. Stock solution of iodine reagent contained iodine ($5mM$, 6.3 mg) and potassium iodide ($5mM$, 8.3 mg) in $10ml$ of phosphate buffer pH 6.8. Acarbose was used as a standard and its 250 μM stock solution was prepared in DMSO.

Procedure

A slightly modified alpha amylase inhibition assay was used to assess antidiabetic activity of tested tea samples [134]. To each well of 96 well plate was pipetted phosphate buffer (15μl), enzyme (25 μl), tea samples (10 μl), and starch (40 μl). For 30 minutes, incubation of reaction mixture was made at 50°C with subsequent addition of HCL (20 μl, 1M) for the cessation of reaction. The assay was further preceded by the addition of iodine reagent (90 μl). Same procedure was followed for the preparation of negative control just by replacing the test extract with equal quantity of DMSO where as acarbose was added instead of test extract in case of positive control. Preparation of blank was carried out by adding equal quantity of buffer in place of test extract and α-amylase enzyme solution. With the help of microplate reader at 540 nm, measured the absorbance of assay plate and calculated the results after triplicate analysis. By using the following equation, enzyme inhibition was calculated.

$$\%Inhibition = [(Abs - Abn)/(Abb - Abn) \times 100] \tag{3.3}$$

Where, Abs = absorbance of tested tea extracts, Abb = absorbance of blank and Abn = absorbance of negative control.

3.3.7 Qualitative analysis

Mainly, two tools/techniques were used in order to determine the functional groups and structures of organic molecules present in our test extracts. These two tools are GCMS and FT-IR.

3.3.7.1 *Fourier Transform Infrared (FT-IR) Spectroscopy Analysis*

FT-IR technique indicates the bonds existed in the compound and consequently be used to determine functional groups of the molecule.

Procedure

All the herbal tea extracts were analysed by ***FT-IR Qualitative Analysis*** (KBr pellet method) by using Fourier Transform Infrared Spectrometer (Bruker-Tensor 27) instrument under the following appropriate conditions:

- **Instrument**: Bruker-Tensor 27; FT-IR.
- **Spectral range**: 515 cm^{-1} – 4000 cm^{-1}.
- **Resolution**: 4 cm^{-1}.

The acquired spectra for the products were examined and construed for particular infrared absorption frequencies with a table to characterize the functional groups for organic and carbonyl compounds. Each functional group has different absorption frequencies and Omnic software 8.2 was used for the interpretation of FT-IR spectra [128].

3.3.7.2 *Gas Chromatography/Mass spectroscopy (GC/MS) Analysis*

Stock Solutions

Crude herbal teas extracts were weighed (75 mg) and dissolved in 75 ml chloroform and kept in vials. The vials were kept in sonicator for a while and then for 10 minutes centrifuged the reaction mixture at 5000 rpm to develop the phase separation. 800 μl of organic phase/supernatant was withdrawn carefully into other properly labelled vials for each herbal tea for GC/MS fatty acid content analysis. It was assured that samples were prepared just before analysis and if not to be tested for more than 1 hour they were stored at 4°C.

Chromatographic Conditions

A well elaborated procedure revealed by Kumar *et al.* . (2012) [135] was employed for the fatty acid content determination in selected herbal teas extracts

using GC/MS analysis. By using Shimadzu QP2010 Ultra (a GC/MS system with GC 2010), prepared herbal teas samples were examined. A DB-5m column was used with 30.0 m length, 0.25 mm diameter, and 0.25 μm thickness. Helium was used as carrier gas with the coloumn flow rate of 1.39 mL/min. Injection mode was split with the injection temperature of 270.0°C. 1μl of each sample was injected automatically into machine. The temperatures of oven were programmed as: initial temperature was 100°C and it remained constant for two (2) minutes, then temperature was increased (200.0°C at 15°C per minute), and again remained constant for the next two minutes. Finally, again temperature was increased up to 240.0°C at the rate of 20C per minute and remained constant for eighteen minutes. 230°C temperature was the temperature of ionization source and 280°C was the interface temperature. Identification of compounds was made with the help of NIST library.

Chapter 4

Results and Discussion

4.1 Biological Evaluation

4.1.1 Phytochemical Analysis

4.1.1.1 Total Phenolics Content (TPC) Quantification

Plants have an endless ability to synthesize aromatic secondary metabolites. Flavones, flavonoids, flavonols, phenolic acid and tannins are the important subclasses of this group. The polyphenols in the plant extracts react with the redox reagents and form a blue colored complex consisting of Phosphomolybdenum or phosphotungstic complex [136]. *Total phenolic content* (TPC) was determined for all the herbal teas extracts prepared in Ethyl acetate solvent (Figure 4.1) and expressed as Gallic acid equivalent ($\mu gGAE/mg$ extract).

Maximum phenolic content was determined in green tea i.e. $70.88 \pm 1.22\ \mu gGAE/mg$ extract followed by *T. serpyllum 1* and black tea with 63.78 ± 1.56 and $63.70 \pm 4.78\ \mu gGAE/mg$ extract respectively. *T. serpyllum 2* has phenolic load with $36.99 \pm 2.40\ \mu gGAE/mg$ extract where as lowest phenolic content was observed in lemon grass with $13.67 \pm 0.96\ \mu gGAE/mg$ extract (Table 4.1). In all the teas extract, TPC decreased in the following manner:

$$Greentea > T.\ serpyllum\ 1 > Blacktea > T.\ serpyllum\ 2 > Lemongrass \quad (4.1)$$

Selected herbal teas extracts were investigated to find out their phenolics load mathematically. The highest phenolics content was computed in the green tea (Table 4.1) which represents its usefulness as the potential source of antioxidants. Tea poly phenols contribute to protective and effective roles against reactive oxygen species damage or oxidative stress and also increase the antioxidant capacity by feeding of tea infusions [137]. Comparatively lower levels of black tea polyphenols are due to the reason that during fermentation of black tea, some of the catechin components converted into theaflavins and thearubigins. However, Theaflavins play significant inhibitory role in certain diseases such as inflammation, certain type of cancers [18].The highest phenolic contents in green tea, *T. serpyllum*, and black tea might be accredited to different functional groups and fatty acid compounds present in these teas as verified by FT-IR and GC/MS analysis. Lemon grass tea extract showed lower phenolic load, the reason may be different compounds present in different plants, contribute to bioefficacy of these plants or there might be some other factors such as geographical regions, climate conditions in which plant grow [89], [90].

Previous studies [138] showed that seperate research was made on dried flowers and leveas of *T.serpyllum*. However, no combine study was conducted before this work therefore this may be the first study regarding this work.

Natural antioxidants in food plants have always proven to be beneficial in management of oxidative stress. Therefore, a variety of different assays were performed to find out antioxidant capacity based on different mechanisms i.e free radical scavenging, inhibition of chain initiation and peroxide decomposition.

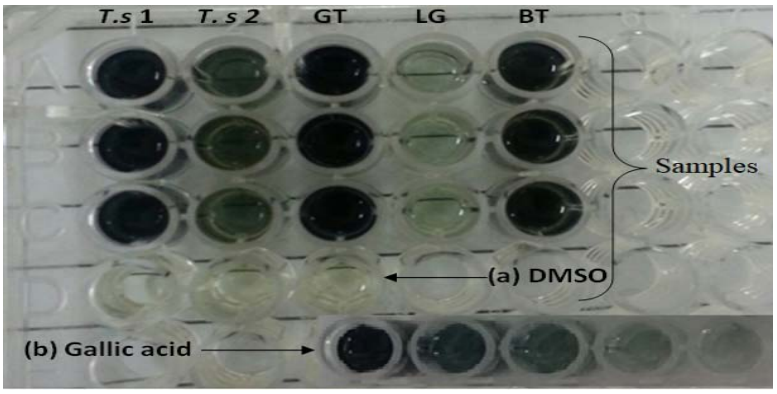


FIGURE 4.1: Total phenolics content assay executed on selected herbal teas from T. s 1 to BT in 96 well plate. (a). represents DMSO used as a ve control. (b). represents Gallic acid used as +ve control.

4.1.1.2 Total Flavonoids Content (TFC) Quantification

Nature is considered as an abundant pharmaceutical stores existing on this planet owing to their ability to produce various secondary metabolites with a broad spectrum of bioactivities [103]. For all the herbal teas extracts, flavonoid content was finding out by TFC assay (Figure 4.2) and expressed as μg Quercetin equivalent per mg extract of plants leaves ($\mu g\ QE/mg$ extract). The results revealed that black tea has maximum flavonoid content i.e. 69.01 $\pm$ 0.49 $\mu g\ QE/mg$ extract, followed by *T. serpyllum 1* and *T. serpyllum 2* with 40.71 $\pm$ 0.17 and 40.28 $\pm$ 1.45 $\mu g\ QE/mg$ extract respectively. Green tea showed higher phenolic content but in the determination of flavonoid content, green tea contained 25.65 $\pm$ 0.71 $\mu g\ QE/mg$ extract. Lemon grass has lowest flavonoid content as 11.48 $\pm$ 0.22 $\mu g\ QE/mg$ extract (Table 4.1). TFC of all the teas extracts decreased in the following manner:

$$Blacktea > T.\ serpyllum\ 1 > T.\ serpyllum\ 2 > Greentea > Lemongrass \quad (4.2)$$

Figure 4.3 shows the graphical representation of Total phenolic content (TPC) and total flavonoid content (TFC) of herbal teas extracts. The results of our study are in close conformity with the previous findings [139] that showed higher flavonoids content in black tea extract than green tea extract. The significant antioxidant characteristics of black tea are ascribed to the flavonoids compounds including

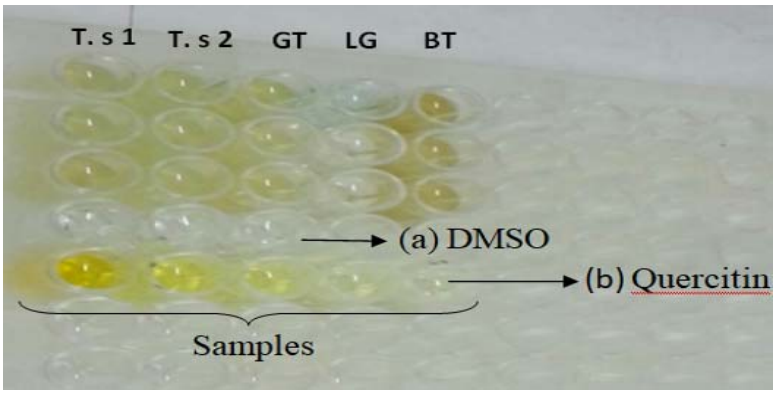


FIGURE 4.2: Total flavonoids content assay executed on selected herbal teas from T. s 1 to BT in 96 well plate. (a). represents DMSO used as a ve control. (b). represents Quercitin used as +ve control.

TFs, theaflavic acid and bisflavanols [52]. Dietitic flavonoids percentages are higher in black tea and those who consume black tea have more intake of flavonoids [140]. Complex diseases such as cardiovascular disease (CVD), artherosclerosis and certain type of cancers evoked by cu2+ induced lipoprotein oxidation, are prevented by flavonoid found in green and black tea [141]. So, proposed an inverse relationship between these diseases and dietary flavonoid intake [49] attributed to the antioxidant capacity of flavonoids. Black tea showed greater flavonoids content that may be due to the presence of carboxylic acid, aromatics, alcohols, esters, ketones, ethers, and aldehyde functional groups as determined by FT-IR analysis. In *T. serpyllum*, high ratios of phenolic acids especially rosmarinic acid and flavonoids are accountable for the manifestation of antioxidant activity [84]. The capableness of polyphenols and flavonoids to eradicate harmful free radicals and quench singlet oxygen species is owing to their antioxidant prospect in living systems.

4.1.2 Antioxidant Potential

4.1.2.1 DPPH assay (Free Radical Scavenging Assay; FRSA)

Stability and accessibility inside the cells make DPPH free radical a perfect criterion to check scavenging potentiality and consequently, and also antioxidant ability in test extracts. DPPH reagent is of dark purple color and it has the capability

TABLE 4.1: Values of Total phenolic content (TPC) and total flavonoid content (TFC) of teas extracts.

Samples Name (Scientific & Local)	**TPC** ($\mu g\ GAE/mg$ **extract)**	**TFC** ($\mu g\ QE/mg$ **extract)**
T. serpyllum 1 (Tumuro 1)	63.78 ± 1.56	40.71 ± 0.17
T. serpyllum 2 (Tumuro 2)	36.99 ± 2.40	40.28 ± 1.45
C. sinensis (Green Tea)	70.88 ± 1.22	25.65 ± 0.71
C. sinensis (Black Tea)	63.70 ± 4.78	69.01 ± 0.49
C. citratus (Lemon Grass)	13.67 ± 0.96	11.48 ± 0.22

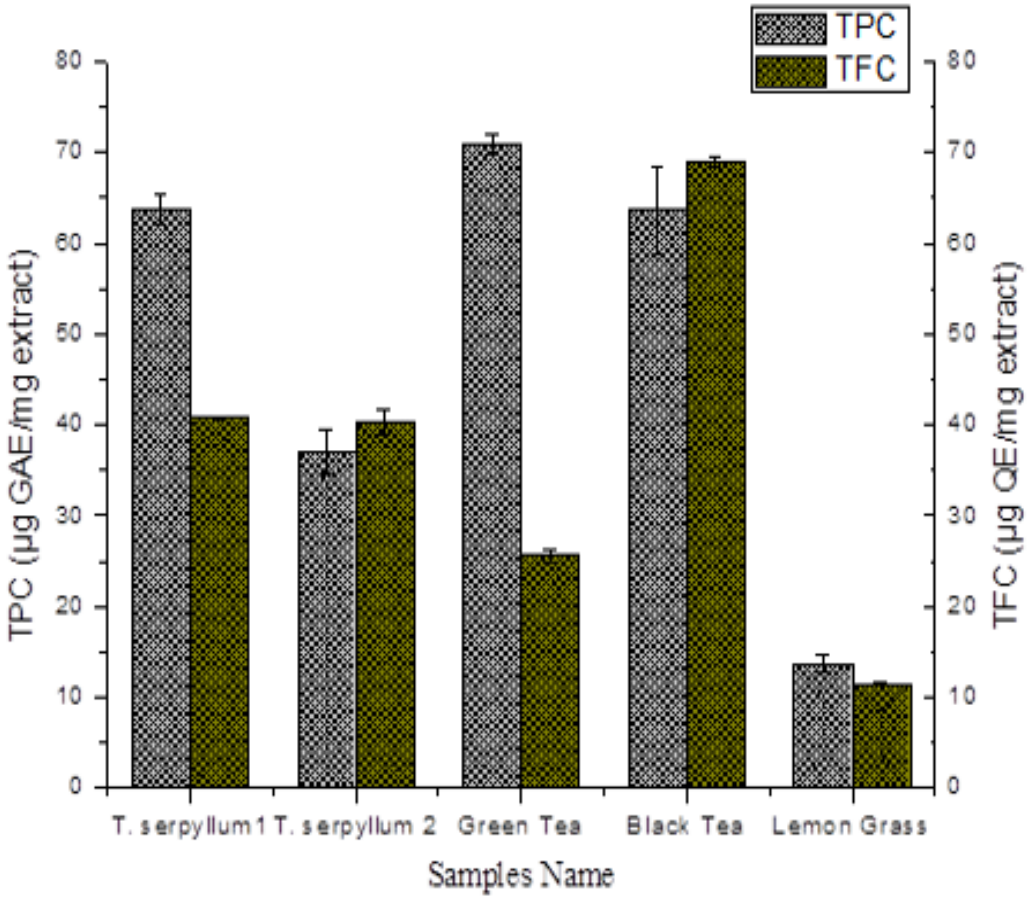


FIGURE 4.3: Graphical representation of Total phenolic content (TPC) and total flavonoid content (TFC) of herbal teas extracts.Values for extracts presented are expressed as mean of triplicate ± Standard Deviation (SD).

TABLE 4.2: % FRSA and IC_{5_0} values of selected teas extracts.

Samples Name (Scientific & Local)	**%FRSA**	**IC50(g/ml)**
T. serpyllum 1 (Tumuro 1)	75.13 ± 0.072	169.6
T. serpyllum 2 (Tumuro 2)	15.88 ± 0.125	–
C. sinensis (Green Tea)	85.60 ± 0.125	31.28
C. sinensis (Black Tea)	72.84 ± 0.216	64.71
C. citratus (Lemon Grass)	8.635 ± 1.25	–

to gain an electron from donor antioxidants resulting in change of color from dark purplish to light purple up to light yellow. This decolorization is owing to the presence of antioxidants in test extracts which can be quantified by computing changes in absorbance values at 517 *nm* by spectrophotometer [142]. The potential free radical scavenging activity of all the teas extracts was determined by DPPH assay (Figure 4.4). The assay was performed by using 96 well plate method and FRSA was noted by the discoloration of 2, 2-diphenyl-1-picrylhydrazyl free radical to a yellow colored 2, 2-diphenyl-1-picrylhydrazyl molecule. The results of DPPH assay revealed that noteworthy percentage of free radical scavenging was observed in green tea with the value of 85.60 ± 0.125. The % scavenging of all the tea samples were as follows (Table 4.2).

The IC_{5_0} values of all the active samples were calculated using Graph pad prism5 software. The IC_{5_0} value of *T. serpyllum 1*, green tea and black tea were 169.6, 31.28 and 64.71 respectively (Table 4.2). The free radical scavenging activity of all the active samples in terms of IC_{5_0} followed in the order:

$$Greentea > Blacktea > Thymus\ serpyllum\ 1 \tag{4.3}$$

In vitro characterization of tea extracts have been found out on the basis of scavenging of stable free radicals by using DPPH assay. DPPH technique is a method that permits the reactivity of radical DPPH with all the antioxidants present in the plant sample, these antioxidant compounds are not only the polyphenolic

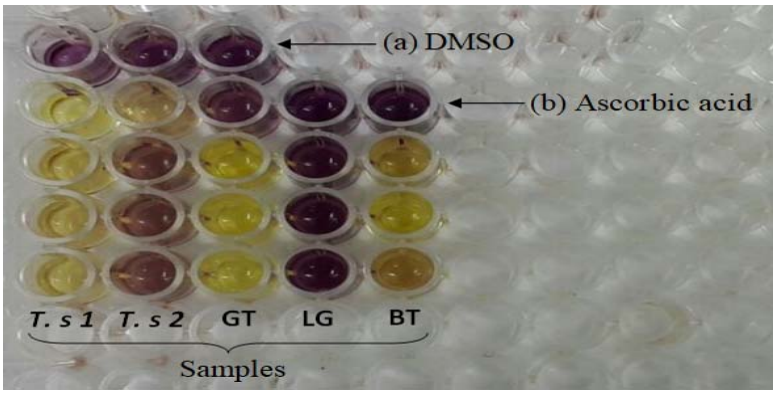


FIGURE 4.4: DPPH free radical scavenging activity of selected teas extracts from T.s 1 to BT executed in 96-well plate. (a). represents DMSO used as a ve control. (b). represents Ascorbic acid used as +ve control.

compounds but also the flavonoids too, may function as antioxidant [143]. In the DPPH assay, % scavenging of green tea in terms of IC_{5_0} was higher than black tea and *T. serpyllum 1* (Figure 4.5), which might be ascribed to the different functional groups (carboxylic acid, aromatics, alcohols) present in green tea extract as confirmed by FT-IR analysis. According to Williams *et al.* . (1995), lower the values of IC_{5_0} contribute to higher the antioxidant activity [144]. Our results showed that green tea possessed powerful antioxidant activity as it had IC_{5_0} values below 50 $\mu g/ml$ and it might be because of the polyphenolic compounds especially catechin components as epigallocatechin gallate is the principle polyphenol (EGCG) and contributed to higher free radical scavenging activity where as black tea have strong antioxidant activity as it had IC_{5_0} values between 50-100 $\mu g/ml$. As for *T .serpyllum* it showed weak antioxidant activity with the IC_{5_0} values between 150-200 $\mu g/ml$, also confirmed from previous findings [75], and the reason might be some factor as geographical regions, climate conditions in which plant grow, cultivating and harvesting time periods, moisture, storage or product shelf life [145]. Our results are in close agreement with the previously reported work where maximum free radical scavenging activity was observed in Bangladesh green tea [146]. Tea poly phenols contribute to protective and effective roles against reactive oxygen species damage or oxidative stress and also increase the antioxidant capacity by feeding of tea infusions [136].

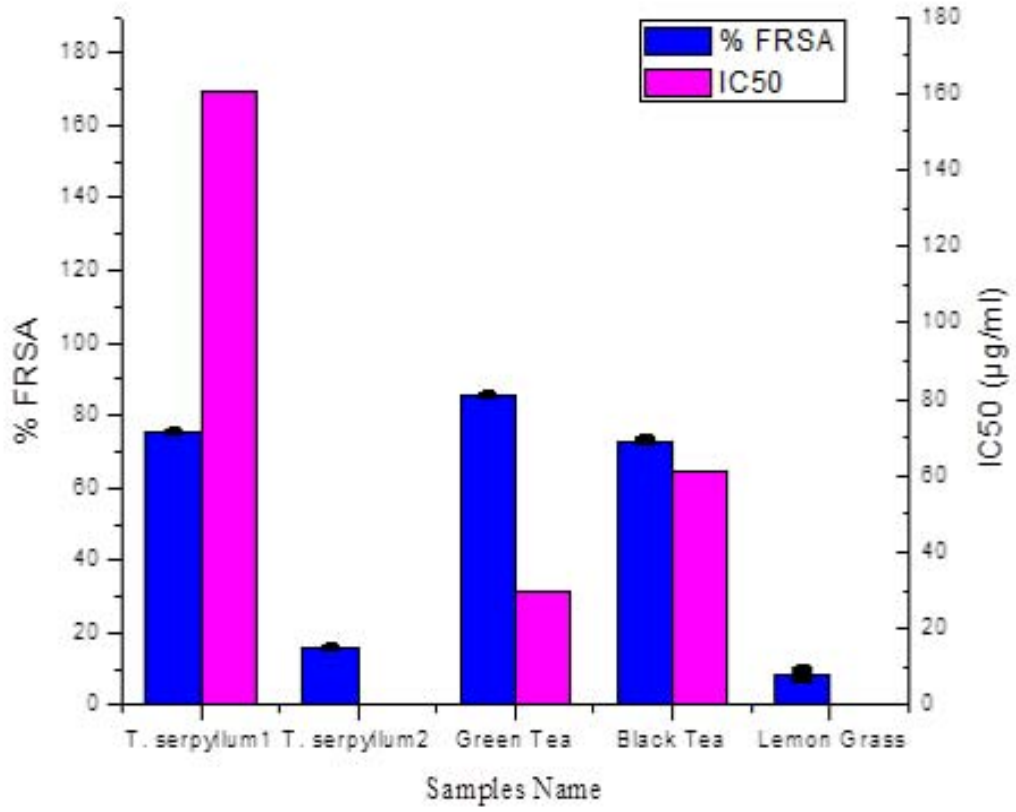


FIGURE 4.5: Graphical representation of % FRSA and IC_{5_0} values of selected teas extracts. Values for extracts presented are mean of triplicate ± SD.

4.1.2.2 Total Reducing Power (TRP) Evaluation

Total reducing efficacy in test extracts was explored in undertaken research so that supporting assumptions can be drawn regarding antioxidant powers of our selected herbal teas. The reducing activity of assay can be find out on the basis of reduction of potassium ferricyanide complex to the potassium ferrous cyanide due to the presence of reductants i.e. antioxidants in the test samples. The analysis of tea samples was made by ferric reducing power assay. The assay was carried out by using 96 well plate (Figure 4.6) and the total reducing potential was expressed in terms of ascorbic acid equivalent per mg extract ($\mu g\ AAE/mg$ extract). The results revealed the highest reducing potential in green tea with $266.8 \pm 0.1\ \mu g\ AAE/mg$ extract. Black tea has also high reducing potential with

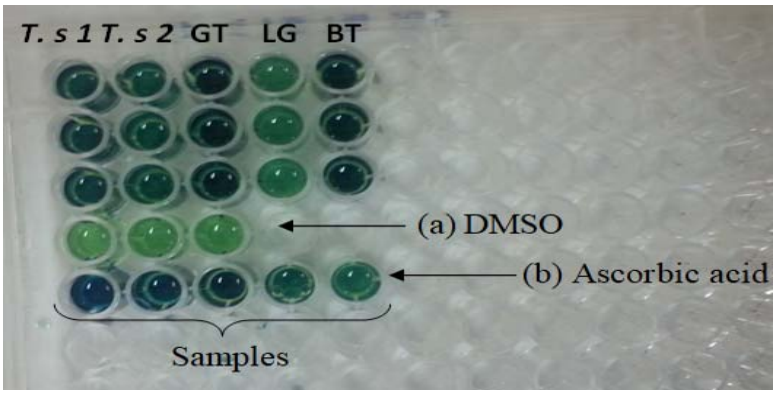


FIGURE 4.6: Total reducing potential estimation of herbal teas extracts from T.s 1 to BT executed in 96-well plate. (a). represents DMSO used as a ve control. (b). represents Ascorbic acid used as +ve control.

233.73 ± 0.152 $\mu g\ AAE/mg$ extract followed by *Thymus serpyllum 1* with 187.4 ± 0.2 $\mu g\ AAE/mg$ extract. On the other side, *Thymus serpyllum 2* has lower reducing potential i.e. 76.33 ± 0.321 $\mu g\ AAE/mg$ extract followed by Lemon grass with lowest reducing potential i.e. 17.7 ± 0.251 $\mu g\ AAE/mg$ extract (Table 4.3). So, results of total reducing power of selected herbal teas samples followed in the order:

$$Greentea > Blacktea > T.\ serpyllum\ 1 > T.\ serpyllum\ 2 > Lemongrass \quad (4.4)$$

Total reducing efficacy in teas extracts was evaluated in further research work that supported the consideration of antioxidant potential in all the teas extracts especially in the green tea with the remarkable value of 266.8 ± 0.1 $\mu g\ AAE/mg$ extract. TRP levels of black tea were also observed remarkably greater than the other herbal teas tested (Figure 4.8) because of the presence of certain compounds like theaflavins and thearubigins. Studies revealed that a black tea component Theaflavins (TFs) especially TF-4 and TF-3 have strong antioxidant potential equals to the antioxidant potential attributed to EGCG in green tea [18]. Thymol is the biological active component of *Thymus serpyllum* contributed to the antioxidant potential [75]. The antioxidant potential was also quantified in *C. citratus* in lower values. The reason might be each plant had a variety of different

compounds that exhibit unique capacities to show the maximal potential in these mechanisms; however biological active compounds are present in leaves of lemon grass as phenols, tannins, flavonoids and alkaloids [147]. The findings of our study are in conformity with the many previous findings that reported the higher levels of green tea as compared to other teas [146].

4.1.2.3 Total Antioxidant Activity (TAC) Evaluation

Over-expression of the natural phenomenon of oxidation in biological system leads to production of highly reactive free radicals i.e. hydrogen peroxide, hydroxyl radical, hypochlorous acid, superoxide radical etc. Any irregularities in the supply of antioxidants are hypothetically associated with the development of numerous health hazards including cardiovascular diseases, cancer and liver injuries due to DNA mutations, lipid peroxidation, oxidative inactivation of numerous enzymes and protein damage [148]. The total antioxidant capacity of crude extracts of all tea samples was analyzed by Phosphomolybdenum based assay (Figure 4.7). Higher the absorbance by tea extracts indicates higher the antioxidant potential/activity. The antioxidant capacity of all the tea samples was analyzed by applying phosphomolybdenum based technique by which samples were evaluated by measuring the formation of green coloured complex. Antioxidant entities present in test extracts make phosphomolybdate ion to get reduced itself. This reaction leads to a change in color which can be measured by measuring the absorbance at 630 nm using spectrophotometer. The maximal antioxidant capacity was observed in green tea and calculated as μg ascorbic acid equivalent per mg of extract ($\mu g\ AAE/mg$ extract) i.e. $99.65 \pm 0.15\ \mu g\ AAE/mg$ extract. *T. serpyllum 2* also showed remarkable antioxidant potential with $93.66 \pm 0.11\ \mu g\ AAE/mg$ extract followed by Black tea, *T. serpyllum 1* and Lemon grass with antioxidant potential 77.51 ± 0.088, 67.90 ± 0.11, $52.92 \pm 0.177\ \mu g\ AAE/mg$ extract respectively (Table 4.3). So the results of total antioxidant capacity of the tea samples followed the particular order:

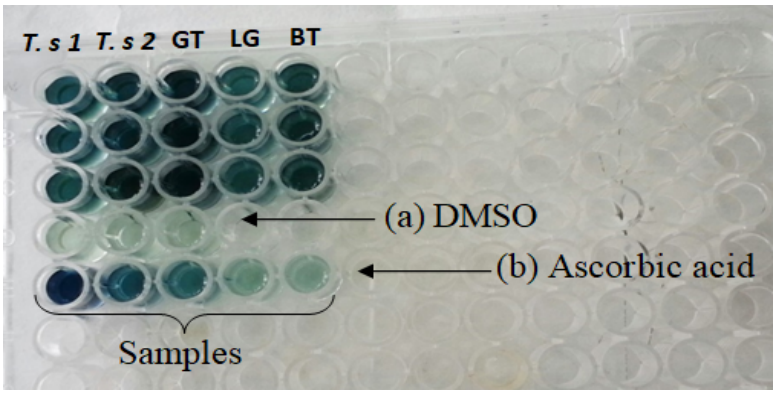


FIGURE 4.7: Total antioxidant capacity determination of selected herbal teas extracts from T.s 1 to BT executed in 96-well plate. (a). represents DMSO used as a ve control. (b). represents Ascorbic acid used as +ve control.

$$Greentea > T.\ serpyllum\ 2 > Blacktea > T.\ serpyllum\ 1 > Lemongrass \quad (4.5)$$

Evaluation of TAC of different teas showed notable values especially for green tea and *T. serpyllum 2* i.e. 99.65 ± 0.15 and 93.66 ± 0.11 $\mu g\ AAE/mg$ extract respectively. For the reason that green tea catechins such as EGCG, EGC, and ECG attributed to the antioxidant capacity of green tea. In EGCG and EGC, the main site for antioxidant reaction is trihydroxylphenyl B ring attached to the flavan-3-ols structure contributed to the antioxidant capacities and free radical scavenging activities of green tea [57]. It is reported by Rice-Evans (1999) that antioxidant potential in green and black tea is 3.5 times higher than antioxidants found in vitamins [52]. It is also reported that antioxidant potential of *T. serpyllum* not only because of certain active components but it is also due to the synergistic of many compounds present in lower levels [149]. GC/MS analysis of green tea, *T. serpyllum 2*, and black tea revealed that these teas extract may contain certain bioactive compounds which have antioxidant activity such as palmitic acid, oleic acid, and petroselinic acid. From literature study it is revealed that phenolic and alcoholic compounds as terpinem-4-ol, p-cymene-8-ol and thymol are assembled in reproductive part of the plant than vegetative part.

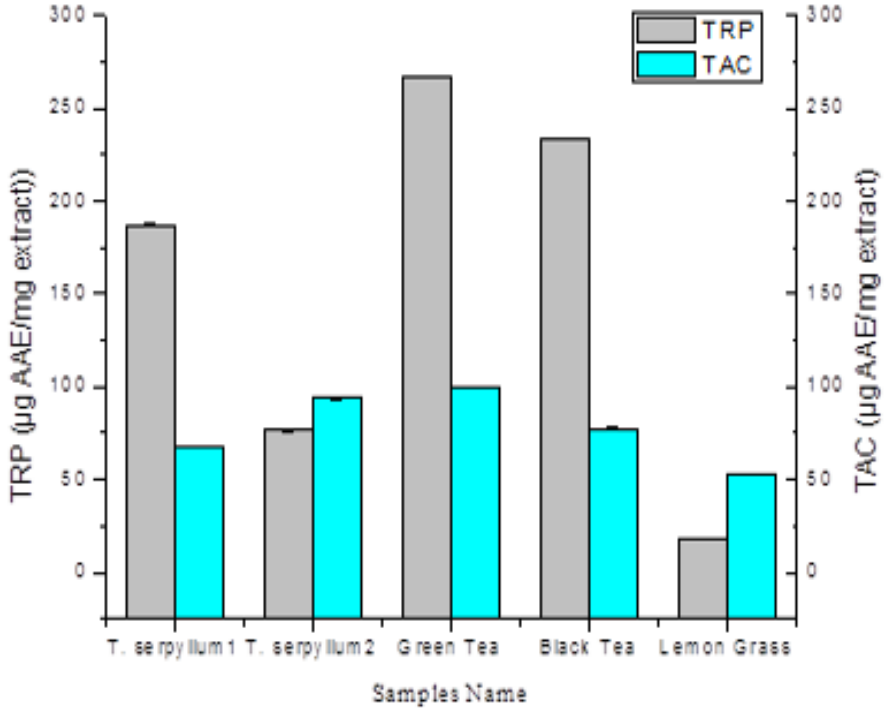


FIGURE 4.8: Graphical representation of TRP and TAC of selected herbal teas extracts. Values for extracts presented are mean of triplicate ± SD.

Where p-cymene and thymol methyl ether are amassed, thus confirmed the medicinal significance of flowers than leaves[149]. In lemon grass citral is most active component contribute to the bio effectiveness of plant. Infusion time of tea is also important to get the maximal antioxidant capacity of tea [150].

The quantitatively estimation of antioxidant potential of these five teas have shown that these possessed greater antioxidant properties except lemon grass tea which has less antioxidant potential than others according to the data shown in table 4.3.

Comparatively green tea extract possesses the highest antioxidant potential in all the assays than other teas. On the contrary, lemon grass tea extract contribute to

TABLE 4.3: % FRSA and IC_{5_0} values of selected teas extracts.

Samples Name (Scientific & Local)	**%FRSA**	**IC_{5_0} ($\mu g/ml$)**	**TRP (μg AAE/mg extract)**	**TAC(μg AAE/mg extract)**
T.serpyllum 1 (Tumuro 1)	75.13 ± 0.072	169.6	187.4 ± 0.2	67.90 ± 0.11
T.serpyllum 2 (Tumuro 2)	15.88 ± 0.125	–	76.33 ± 0.32	93.66 ± 0.11
C.sinensis (Green Tea)	85.60 ± 0.125	31.28	266.8 ± 0.1	99.65 ± 0.15
C.sinensis (Black Tea)	72.84 ± 0.216	64.71	233.73 ± 0.1	77.51 ± 0.08
C.citratus (Lemon Grass)	8.635 ± 1.25	–	17.73 ± 0.25	52.92 ± 0.17

less antioxidant potential (Figure 4.8, 4.9). The results elicited that the antioxidant potential of the plant not only depend upon its biological active compounds i.e. polyphenols and flavonoids but also influenced by the geographical region and climate conditions in which plant grow and also during the preparation of samples, extracting and analysis technique. Moreover, each plant had a variety of different compounds that exhibit unique capacities to show the maximal potential in these mechanisms.

4.2 Antimicrobial Potential

4.2.1 Antibacterial Activity

The importance of plant derived antimicrobial agents have long been accepted and proved. Medicinal Plants provide an inexhaustible source of antimicrobial agents used by mankind since the dawn of medicine. Increasing irrational use of antimicrobials has resulted in severe antimicrobial resistance even to the drugs of last resort like carbapenems [151]. Hypersensitivity reactions, myelosuppression

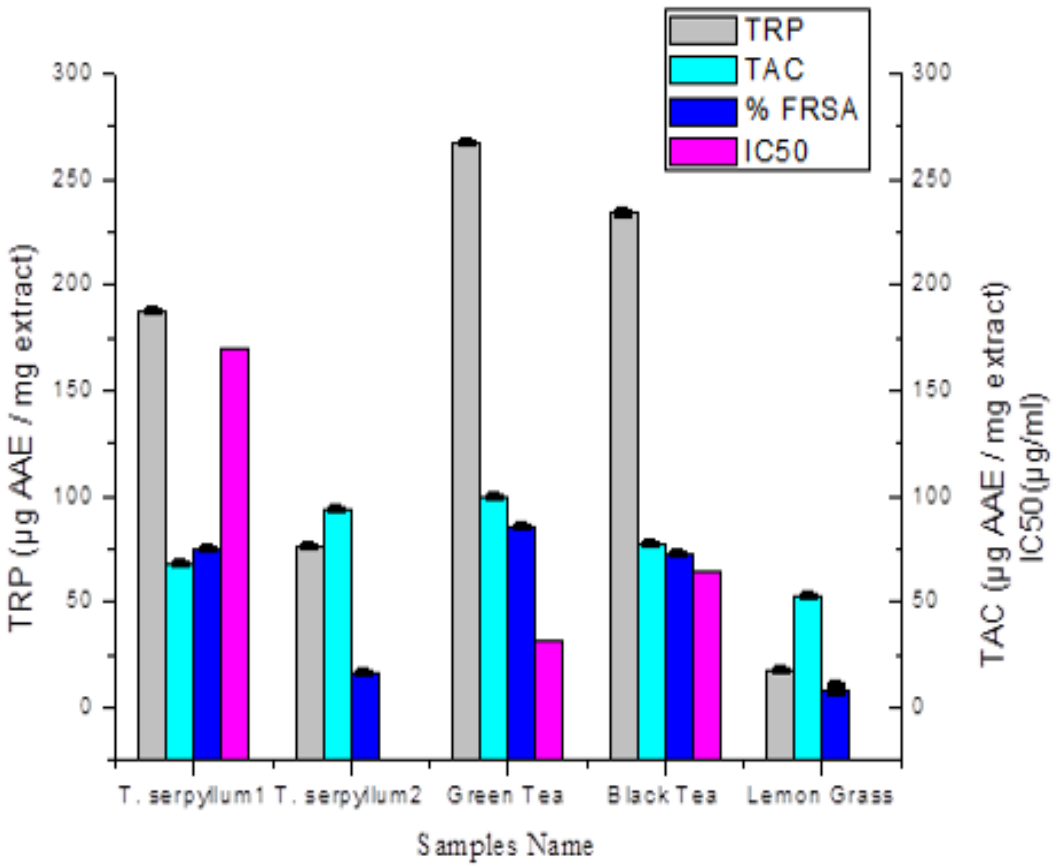


FIGURE 4.9: Graphical representation of TRP, TAC, % FRSA and IC_{50} of selected herbal teas extracts. Values for extracts presented are mean of triplicate ± SD.

and allergic reactions are additional complications beside resistance. Plants are huge reservoirs of valuable molecules possessing anti-infectious property. The antibacterial properties of medicinally significant herbal plants mostly depend upon the type of solvent used, the tested products or organisms and part of plant used. However, the discrepancy in findings depends upon the different bacterial strains, different concentrations and type of extract used by varied research centers [145].

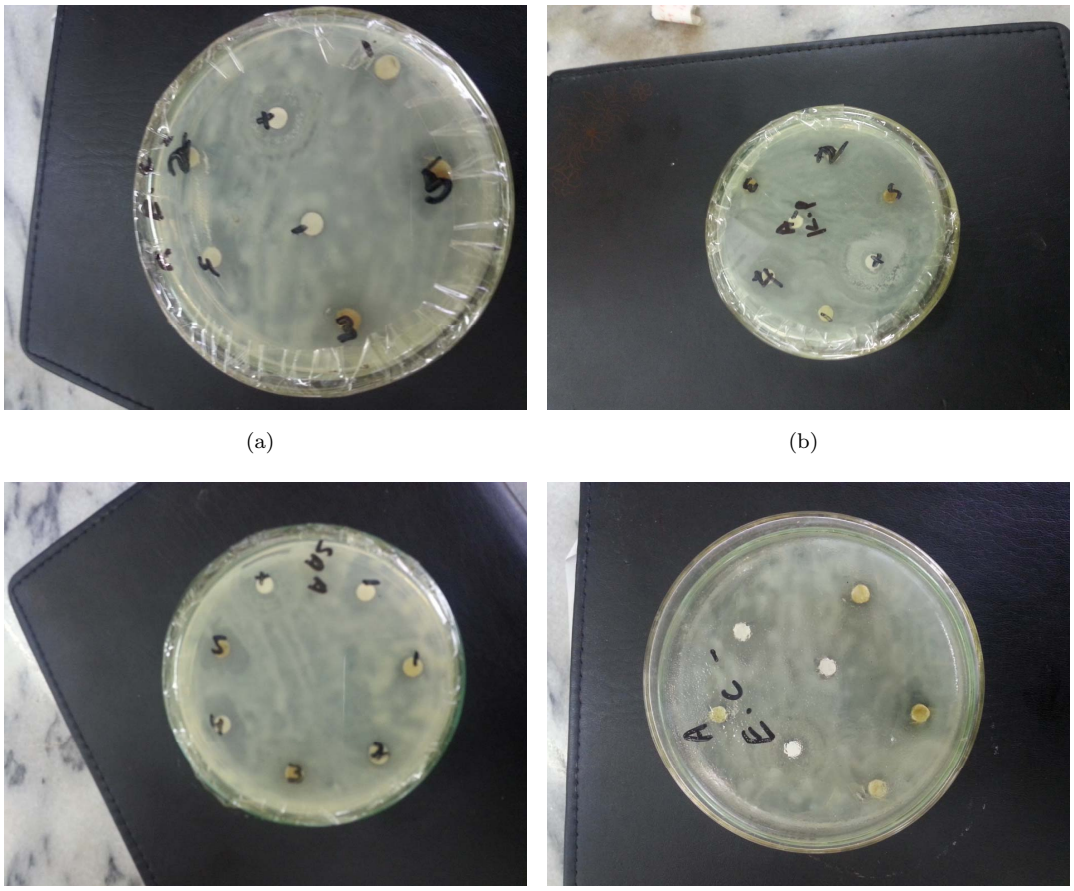
Anti-bacterial potential tested by disc-diffusion method showed significant activity against the bacterial strains employed in terms of zone of inhibition (mm ± SD) as shown in table 4.4. The test samples which showed a zone of inhibition greater than 12 *mm* were further tested for determining MIC at lower concentrations by micro broth dilution method.

Thymus serpyllum 1 showed maximum activity against *K. pneumoniae* i.e. 14 ± 0.5mm and *P. aeruginosa* (12 ± 0.5mm) and *E. coli* (12 ± 0.5mm). The weakest activity of *T. serpyllum 1* was observed against *B. subtilis* and *S. aureus* i.e. 9 ± 0.5mm and 10 ± 1mm respectively (Table 4.4).

Thymus serpyllum 2 was found active only against *S. aureus* i.e. 13 ± 1mm while weakest activity was observed against *P. aeruginosa* ($7 \pm 1mm$) followed by *E. coli* (10 ± 1), *S. aureus* (10 ± 1mm) and *K. pneumoniae* ($11 \pm 0.5mm$) (Table 4.4). The results of our study are in harmony with Wani *et al.* . (2013) manifested the inhibitory effect of *T. serpyllum* against *K. pneumonia* and *E.coli* [152]. Sokolic-Mihalak *et al.* . (2013) revealed that phenolic compounds of *T. serpyllum* can be contributed to the antibacterial activity, causing the release of intracellular membrane components as amino acids, proteins, pentose and phosphates leading to the membrane perturbation and permeability and also inhibited lipid peroxidation [153]. These findings confirmed the results of our study that *T. serpyllum* has antibacterial potential against vast domains of gram positive and gram negative bacteria (Figure 4.10).

Green tea exhibited highest activity against *E. coli* with the ZOI $15 \pm 1mm$ followed by *S. aureus* $12 \pm 0.5mm$, *P. aeruginosa* and *B. subtilis* each with the ZOI of $10 \pm 1mm$ and the lowest ZOI was found against *K. pneumoniae* i.e. $9 \pm 0.5mm$ (Table 4.4). Black tea manifested strongest antibacterial potential against *S. aureus* i.e. $18 \pm 0.5mm$ followed by *E.coli* i.e. $14.5 \pm 0.5mm$ (Figure 4.10) where as black tea exhibited weakest antibacterial activity against *P. aeruginosa* i.e $10 \pm 1mm$. Anti bacterial activity of black tea against *K. pneumoniae* and *B. subtilis* was the same $12 \pm 0.5mm$ (Table 4.4).

The results of our study demonstrated that green tea and black tea extracts exhibited the potential antibacterial activities that might confirm their use and efficacy against various infections. As stated by antibacterial assay, green tea possessed strongest antibacterial activity against *E. coli* and *S. aureus* (Table 4.4; Figure 4.10) with the same MIC values of 100 $\mu g/ml$. our study results are in compliance with the results of *Ponmurugan* et al. . (2016), demonstrated the antibacterial



(a) (b)

(c) (d)

FIGURE 4.10: Antibacterial activity of selected teas extracts against (a) *B. subtilis*; (b) *K. pneumonia*; (c) *S. aureus*, (d) *E. coli*

activity in ethyl acetate extract of green tea against *E. coli*, *B. subtilis* and *K. pneumonia* [154]. Saleh *et al.* (2014) also reported antibacterial activities of green tea are in line with our results [155].

Factors that may affect the antibacterial potential of green tea include origin and geographic locations of plant, vegetation type and preparation of extracts [145]. It is reported that outer membrane of gram negative bacteria serves as a barrier for many substances such as antibiotics [156]. The current findings may be ascribed to catechin and polyphenolic compounds present in green tea extract. Lee *et al.* . (2009) reported that green tea inhibit the attachment of bacterial pathogens to the host cell membrane [157]. The possible mechanistic approach has been reported towards the action of green tea extract of leaves manifested the bacterial cell membranes disruption inhibit the bacterial attachment to the host cells, inhibited

the formation of biofilm on host cells [158]. The destruction of bacterial cell membranes result the bacterial inability to produce toxins by inhibiting the fatty acid synthesis and critical enzyme activity, followed by other inhibitory effects on bacterial functions as release of membrane components i.e. k^+, inhibition of respiration and cell lysis caused by tea polyphenolic compounds [159].

Black tea extract screened in the current study exhibited maximum antibacterial activity against *E. coli*, *S. aureus*, *B. subtilis* and *K. pneumoniae* proved the efficacy of black tea (Figure 4.10). Our test results are in conformity with Michalcyzk and Zawislak (2008) and Pulkit *et al.* (2015) evaluated that black tea extract had the strongest antibacterial activity than green tea proved the confirmation of our results [160], [161]. Many scientific studies have evaluated that Theaflavins (TFs) and Thearubigins (TRs) the main polyphenols of black tea contributed to its pharmacological potential[8], [16]. The bioactive components of black tea lower the risk of certain cancers such as liver, pancrease, colon and prostate [162]. Apart from this, now researchers have been put more focus on the therapeutic and nutritional value of these bio active compounds of green and black tea because of their broad spectrum actions.

Lemon grass extract showed strongest antibacterial activity i.e. 20 ± 0.6 mm and $20 \pm 0.5mm$ against *K. pneumoniae* and *S. aureus* (Figure 4.10) with the MIC value of 100 $\mu g/ml$ respectively, and 19.5 ± 0.5 mm against E.coli. The intermediate susceptibility was seen against *P.aeruginosa* and *B. subtilis* i.e 12 ± 0.5 and 12.5 ± 0.5 mm respectively (Table 4.4). The result of our study was in conformity with Hamad *et al.* (2017) that lemon grass showed strongest inhibitory effect against *S.aureus* [163]. Our result was also in agreement with Ewansiha *et al.* (2012) that lemon grass leaf extract showed maximium antibacterial activity against *E.coli* with the activity index of 16.33 ± 0.58 mm [90]. Lemon grass has been utilized against gastrointestinal disorders. Aldehyde compounds of lemon grass essential oil such as geranial, neral and β-citronellal were contributed to the antibacterial activity of plant against both Gram +ive and Gram ive bacterial strains [92]. In addition, phenolic compounds and tannins were also found to be involved in the bacterial growth inhibition.

TABLE 4.4: Antibacterial activity and MIC values of selected tea extracts.

Sample Name	Antibacterial Assay									
	Diameter of zone of inhibition in mm (Mean ± SD) * (MIC: μg /ml)									
	E. coli	***MIC***	***P. aeruginosa***	***MIC***	***K. pneumoniae***	***MIC***	***S. aureus***	***MIC***	***B. subtilis***	***MIC***
T. serpyllum 1	12 ± 0.5	100	12 ± 0.5	100	14 ± 0.5	100	10 ± 1	–	9 ± 0.5	–
T. serpyllum 2	10 ± 1	–	7 ± 1	–	11 ± 0.5	–	13 ± 1	–	10 ± 1	–
Green tea	15 ± 1	100	10 ± 1	–	9 ± 0.5	–	12 ± 0.5	100	10 ± 1	–
Black tea	14.5 ± 0.5	100	10 ± 1	–	12 ± 0.5	> 100	18 ± 0.5	100	12 ± 0.5	100
L. grass	19.5 ± 0.5	100	12 ± 0.5	> 100	20 ± 0.6	> 100	20 ± 0.5	100	12.5 ± 0.5	> 100
Controls										
Roxithromycin	–	–	–	–	–	–	19 ± 1	33.3	17 ± 0.5	33.3
Cefixime	20 ± 1	33.3	15 ± 0.5	11.11	25 ± 1	33.3	–	–	–	–
DMSO	–	–	–	–	–	–	–	–	–	–

In table 4.4, $--$ = No activity, Final concentration of extracts and positive controls was $100\mu g/disc$ and $20\mu g/disc$ respectively. DMSO served as negative control. Values for extracts are presented as ZOI $\pm$ SD, the assay was run in triplicate.

The standard antibiotics Roxithromycin and Cefixime were used as positive control against gram positive and gram negative bacteria respectively revealed maximum antibacterial potential against bacterial strains as shown in table 4.4 and figure4.10. DMSO was used as negative control showed no antibacterial activity (Table 4.4) that proved its harmless effects on the tested bacterial strains (Figure 4.10). Figure 4.11 shows the graphical representation of antibacterial activity of tea extracts against *E. coli*, *P. aeruginosa*, *K. Pneumoniae*, *S. aureus*, *B. subtilis*. The results of our research work confirmed the presence of different bioactive compounds in our tested extracts that might contributed to antibacterial activity, as verified by GC/MS and FT-IR techniques. As a result of this study it's clear that tea as a drink utilized by millions throughout the world has been manifested to be a versatile antimicrobial agent against micro-organisms in vitro and may validate to be a naturally functional antimicrobial in an era wherever antibiotic resistance is becoming ever more pervasive.

4.2.2 Antifungal Activity

By employing disc diffusion method, crude extracts of all the herbal teas were investigated for their antifungal potential against fungal strains. None of the extract was found to have significant antifungal activity. Clotrimazole, the standard antifungal drug and its final concentration used was $10\mu g/disc$. The growth ZOI of standard was 34.33 ± 1.52. The assay was run as triplicate analysis.

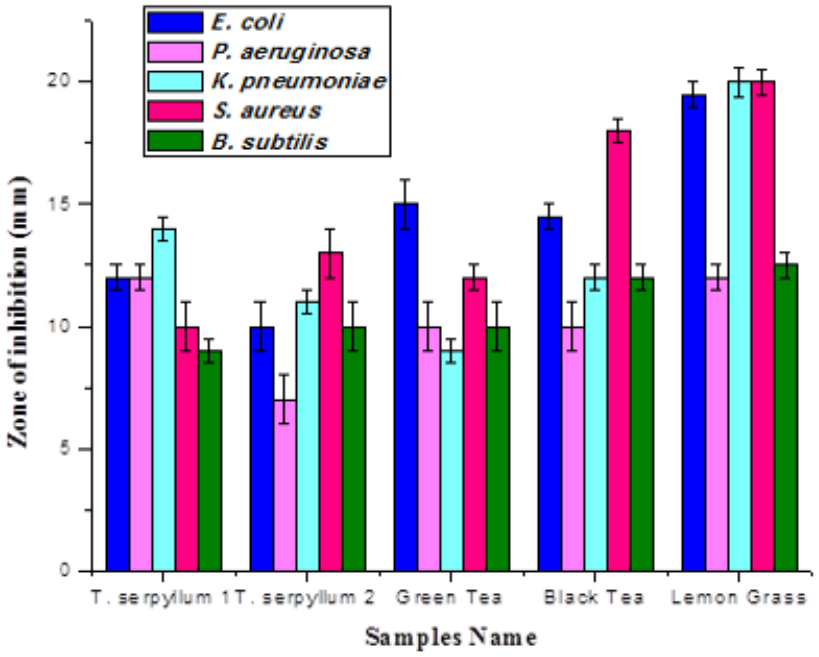


FIGURE 4.11: Graphical representation of antibacterial activity of tea extracts against *E. coli*, *P. aeruginosa*, *K. Pneumoniae*, *S. aureus*, *B. subtilis*.

4.3 Cytotoxicity Potential

4.3.1 Brine Shrimp Lethality Assay

Preliminary cytotoxicity of the herbal teas was assessed against *Artemia salina nauplii* (brine shrimp larvae) and the obtained results were analyzed to determine the lethality profile of the selected herbal teas by employing the brine shrimp lethality test (Figure 4.12). This assay is based on the ability of samples to kill the brine shrimp larvae. This assay has been considered as an efficacious probe for the bioactivities of different plants extracts [164]. Overall crude extracts exhibited significant mortality and results were depicted in table 4.5. Lemon grass showed maximum cytotoxicity with LC_{50} value of $10.25\mu g/ml$, followed by *T. serpyllum 1* with LC_{50} value of $11.60\mu g/ml$, and *T. serpyllum 2* with LC_{50} value of $17.19\mu g/ml$ respectively. Green tea exhibited cytotoxic potential with LC_{50} value of $50.88\mu g/ml$ whereas the minimum cytotoxicity was observed in black tea extract with LC_{50} value of $123.2\mu g/ml$ (Table 4.5). The cytotoxic potential of all

(a) (b)

FIGURE 4.12: Brine Shrimp lethality assay.
(a) Artemia salina eggs in bicompartment perforated tank; (b) Hatched nauplii in beaker containing sea water

the herbal teas extracts arranged in the following manner:

$$Lemongrass > T.serpyllum1 > T.serpyllum2 > Greentea > Blacktea \quad (4.6)$$

It is commonly inferred that brine shrimps or *Artemia salina* larvae and carcinoma cells of mammals behave in the same manner in many aspects that is why cytotoxic effects of undertaken test extracts might become potential candidates for antitumor and anticancer activities; possible biological activities of test extracts against malarial parasites, pests, tumors and harmful microbes [165]. The activity of samples were based on concentration dependent manner and as there was decrease in concentration of samples, the percent (%) mortality rate also decreased confirmed the prior studies by using the brine shrimps larvae as a test model (Figure 4.12) [166].

Among all the extracts lemon grass and *T. serpyllum 1* were the most active which suggests the presence of some cytotoxic phytoconstituents which makes them an active candidate for screening against cancer cell lines. This strengthens the idea of cytotoxic activity of these extracts against various cancer types. These herbal teas are worth seeing for the potential anticancer compounds which could add therapeutic arsenal to treat various types of cancers. According to a recent report by Shah (2011), in lemon grass many novel phytochemical compounds are found

TABLE 4.5: Brine shrimps lethality potential of selected teas extracts.

Samples Name	**%Mortality (concentration: $\mu g/ml$)**				**LC_{5_0} μg/ml**
	200	**100**	**50**	**25**	
T. serpyllum 1 (Tumuro 1)	100 ± 5.77	100 ± 5.77	80 ± 5.77	70 5.77	11.60
T. serpyllum 2 (Tumuro 2)	90 ± 5.77	80 ± 5.77	70 ± 5.77	60 ± 5.77	17.19
C. sinensis (Green Tea)	60 ± 5.77	50 ± 5.77	40 ± 5.77	30 ± 5.77	50.88
C. sinensis (Black Tea)	90 ± 5.77	70 ± 5.77	40 ± 5.77	30 ± 5.77	123.2
C. citratus (Lemon Grass)	100 ± 5.77	100 ± 5.77	80 ± 5.77	70 ± 5.77	10.25

including phenolic acids, flavonoids, terpenes, esters, and ketones that possess anticancer, anti-mutagenic and anti-inflammatory potentialities [88]. Thymol and carvacol are the main components of different species of genus Thymus considered as having cytotoxicity effects [153]. Green tea was found less cytotoxic with comparatively higher LC_{5_0} value. Our results are in close agreement to another study by Yang *et al.* (2000) who also endorsed the non-cytotoxic features of tea and proposed its utilization as anti-cancer and anti-mutagenic agent [167]. The LC_{5_0} of green tea and *T. serpyllum 2* also showed cytotoxic nature of teas extracts. This may be due to the presence of different bioactive constituents i.e. palmitic acid, petroselinic acid, oleic acid, linoleic acid, and stearic acid (confirmed by GC/MS), carboxylic acid, alcohols, aromatics, ketones, and aldehyde groups (confirmed by FT-IR). The standard employed also showed significant cytotoxity which accounts for its use in the treatment of various cancers as it is being investigated for treating cancers of different etiologies and it has shown positive results. LC_{5_0} of positive conrol (Doxorubucin) was $5.93 \mu g/ml$. DMSO was used as negative control.

4.4 Enzyme Inhibition Assays

4.4.1 Protein Kinase Inhibition Assay

Protein kinases are regarded as oncogenic in human beings. The important role of protein kinases is the formation of aerial hyphae. This led to the basic principle of the assay in order to measure the kinase inhibition potential of our tested samples. All the herbal teas were assessed for their possible protein kinase inhibition potential by observing the formation of bald zone through disc diffusion method, the test strain used was *streptomyces* 85E (Figure 4.13). A notable hyphae formation inhibition was observed for lemon grass, green tea and black tea i.e. 22.16 $\pm$ 1.75 mm, 22.16 $\pm$ 0.763 mm, and 21.13 $\pm$ 1.02 mm bald zones at $100\mu g/disc$ respectively. The significant bald zone was also observed for *T. serpyllum 2* i.e 12 $\pm$ 1.04 mm whereas *T. serpyllum 1* presented a clear zone of 10.16 $\pm$ 0.76 mm. No growth inhibition was observed around DMSO affirmed its non-toxic effect whereas Surfactin was used as positive control and it showed 17mm bald zone (Table 4.6).

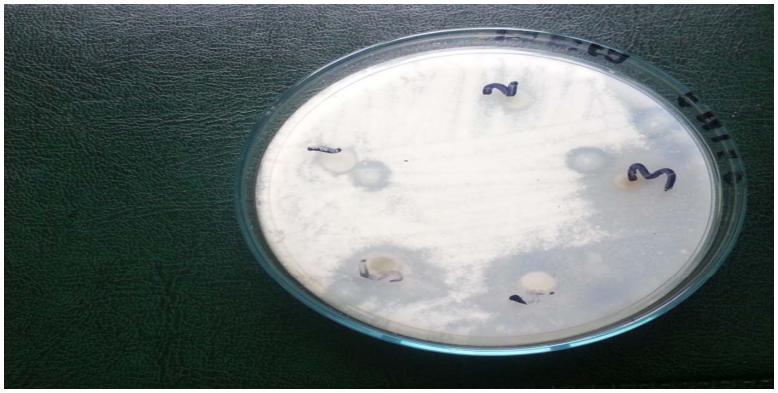


FIGURE 4.13: Protein kinase inhibition potential of selected teas extracts.

Inhibitors of protein kinases represent distinguished entities which might help in discovering new cancer therapeutic agents. The targeted therapy are being searched for nowadays, directed against the cancer cells and signaling pathways, have reduced nonspecific side effects [168]. The findings of undertaken research of selected herbal teas depicted that green tea, lemon grass, and black tea possessed highest potential of protein kinase inhibition. This indicated that herbal teas have

appreciable activity against the test strain and these succeeded in inhibiting the hyphae formation (Figure 4.13). This also suggests the presence of anti cancer compounds in these teas which still needs to be explored further. A few surveys have outlined some back data reports which described that pancreatic, prostate, bladder, and gastrointestinal carcinomas could be prevented by green tea catechin compounds [7], [166]. Many studies suggested that black tea polyphenols contributed to the strong anti-oxidant action that may lower the possibility of cancer by minimizing the DNA damage and also progression of cancer directing to malignant tumor [169]. Lemon grass tea contains sufficient citral concentrations that are found to be effective to initiate apoptotic process in tumour cells without causing damage to healthy cells [100]. The inhibitory activity exhibited by extracts of green tea, lemongrass, and black tea might be due to various numerous functional groups and fatty acid compounds as examined by GC-MS and FT-IR analysis of the tested tea samples.

Number of kinases coded in human genome reached to about 500. Test extracts which possess the ability of allosterically adhering with either active or inactive site of any of these kinases can be regarded as potential and major sources to establish new treatment regimens against cancer [167]. This assay is advantageous in many regards as it quickly identifies compound being cytotoxic and also pinpoint potential inhibitors which hinders signal transduction in infections and cancers. The results of protein kinase inhibition assay recorded for the tested samples are presented in the table.

TABLE 4.6: Protein kinase inhibition potential of selected teas extracts.

Samples Name (Scientific & Local)	**Diameter of growth zone inhibition [($mm \pm$ SD at 100 $\mu g/disc$ (MIC: $\mu g/disc$)]**		**MIC**
	Clear Zone	**Bald Zone**	
T. serpyllum 1 (Tumuro 1)	10.16 ± 0.76	–	–
T. serpyllum 2 (Tumuro 2)	–	12 ± 1.04	>100
C. sinensis (Green Tea)	–	22.16 ± 0.76	11.11
C. sinensis (Black Tea)	–	21.13 ± 1.02	>100
C. citratus (Lemon Grass)	–	22.16 ± 1.75	>100

−− = No activity, Values (Mean SD) is average of triplicate assays. DMSO: negative control, Surfactin: positive control (20 $\mu g/disc$; 17 mm zone)

4.5 Antidiabetic Assay

4.5.1 Alpha Amylase Inhibition Assay

Alpha amylase and alpha glucosidase are the carbohydrate hydrolyzing enzymes and inhibition of these enzymes is considered as an efficacious strategy in order

to maintain the glucose levels in blood within the acceptable range. Alpha amylase inhibition assay was used to access the anti diabetic activity of herbal teas (Figure 4.14) [170]. Researchers round the globe are searching for plant derived antidiabetic drugs in order to overcome the side effects linked with insulin and oral hypoglycemic drugs [171].The antidiabetic potential of herbal teas is well established and these herbal teas have been the part of herbal remedies to reduce hyperglycemia. Results showed that black tea exhibited maximum amylase inhibition activity with 33.12 ± 0.15%, followed by *T. serpyllum 1*, *T. serpyllum 2,* and lemon grass with the values of 31.65 ± 0.85 % inhibition, 29.28 ± 0.30 % inhibition, and 21.25 ± 1.2 % inhibition respectively (Table 4.7). Least % inhibition was observed in green tea with 7.78 ± 0.30 % inhibition. The obtained results were arranged in the following manner:

$$Blacktea > T.\ serpyllum\ 1 > T.\ serpyllum\ 2 > Lemongrass > Greentea \quad (4.7)$$

Figure 4.15 shows the graphical representation of alpha amylase inhibition potential of selected herbal teas extracts.The enzyme inhibition caused by our test samples might be due to the presence of certain fatty acid compounds such as caffeine, palmitic, oleic acid, linoleic, and stearic acid identified by GC/MS, and aldehydes, aliphatics, carboxylic acid, esters, aromatics, ethers, alcohols, and ketones functional groups identified by FT-IR which could well be responsible for the positive results. In the present study, black tea showed highest inhibitory potential suggesting their useful role as an adjunct therapy in the treatment of NIDDM (Type II diabetes). According to the laboratory study report of 2009 [172], scientists found that black tea compounds were more potent than compounds extracted from green tea and from oolong tea in regard to slowing the blood sugar absorption, proved the efficacy of our results. The characteristic feature of black tea also supported our results of GC/MS and FT-IR analysis that also verified the presence of certain bioactive compounds and functional groups in the black tea extract. It was also suggested that black tea intake for longer time period associated with the reduced risk of diabetes [173]. *T. serpyllum* exhibits antidiabetic properties and

TABLE 4.7: Alpha amylase inhibition potential of selected herbal teas extracts.

Samples Name (Scientific & Local)	**Alpha amylase inhibition (%)**
T. serpyllum 1 (Tumuro 1)	31.65 ± 0.85
T. serpyllum 2 (Tumuro 2)	29.28 ± 0.30
C. sinensis (Green Tea)	7.78 ± 0.30
C. sinensis (Black Tea)	33.12 ± 0.15
C. citratus (Lemon Grass)	21.25 ± 1.2

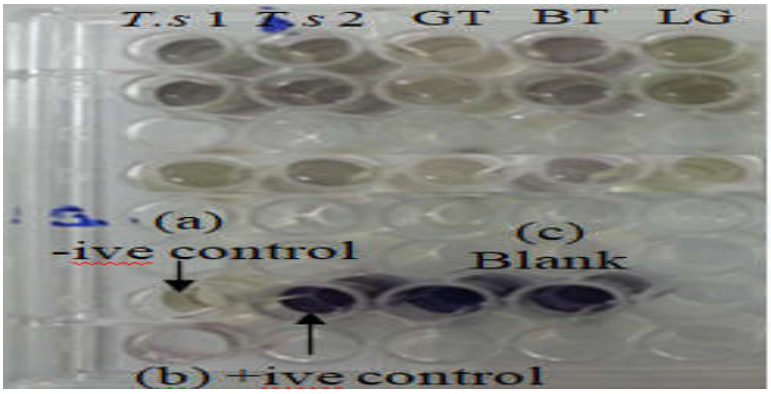


FIGURE 4.14: Alpha amylase inhibition assay of selected teas extracts from T. s 1 to LG. (a) represents ive control. (b) represents +ive control. (c) represents Blank.

antioxidant properties [80].

Numerous current examinations have demonstrated that plants which possess both antioxidant and anti-diabetic properties are supposed to be best for therapeutic treatments when contrasted with others treatments for diabetes [174]. It is also reported from another study that there is no association between green tea consumption with the reduction in type 2 diabetes risk factor; however, daily black tea consumption reduces 14% the risk of diabetes [175]. Further investigations may offer functional compounds (natural) to be utilized in managing the diabetes.

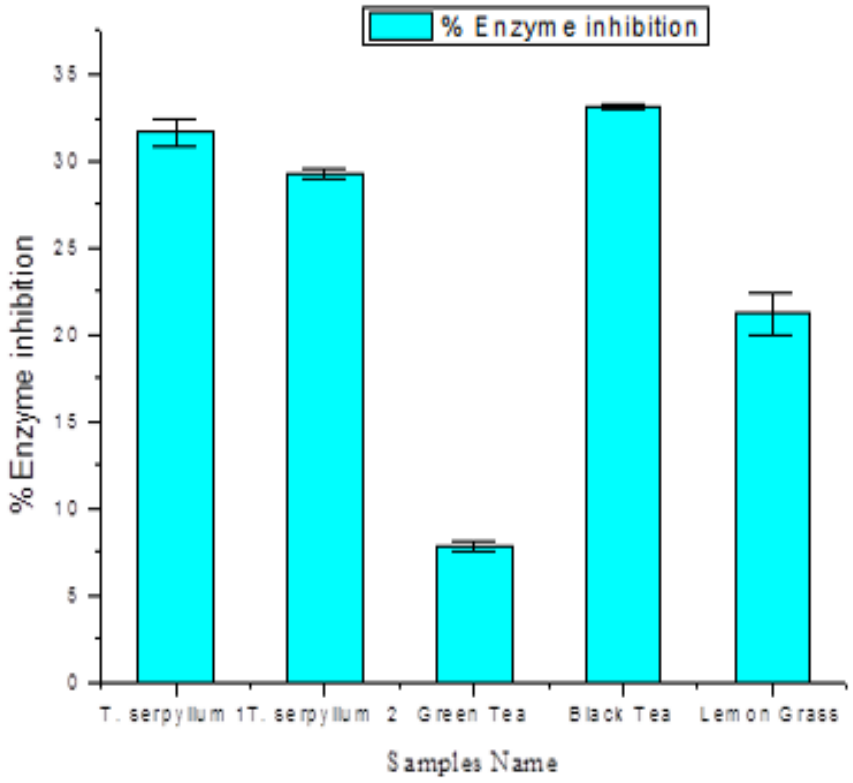


FIGURE 4.15: Graphical representation of alpha amylase inhibition potential of selected herbal teas extracts.

4.6 Qualitative analysis

4.6.1 Determination of functional groups using FT-IR spectroscopy

For the identification of functional groups, the most common widely used technique is FT-IR spectroscopy. FTIR spectroscopy is speedy, versatile and responsive technique that has been utilized for illustrating the structure and physiochemical properties of investigated material [176]. In this technique, functional groups can be detected depending on the extract composition and also on the solvent polarity.

For the characterization of crude extracts of herbal teas, FTIR spectroscopy was conducted (Figure 4.16). The present research study confirmed the presence of

functional groups that were identified by FT-IR spectroscopy analysis. Figures and table presented the infra red spectrum of each herbal tea and characteristic bands were observed ranging from 4000 cm^{-1} $515cm^{-1}$ in all herbal teas spectrum.

TABLE 4.8: FTIR analysis of herbal teas extracts; T. s1 and T.s 2 (*Thymus serpyllum 1* and *2*), GT (green tea), BT (black tea), LG (lemon grass).

Sr.No.	**Frequency of band (cm^{-1})**	**Experimental Frequencies (cm^{-1}) of Teas**	**Bond**	**Functional groups**
1	3500-3200	3359.04 T.s 1 3355.04 T.s 2 3260.17 GT 3341.65 BT 3310.30 LG	O – H Stretch, H-bonded	Alcohols, Phenols
2	3000-2850	2917.82 T.s 1 2917.10 T.s 2 2917.54 GT 2917.17 BT 2917.07 LG	C – H Stretch	Alkanes
3	3300-2500	2849.44 T.s 1 2849.15 T.s 2 2849.48 GT 2849.27 BT 2849.14 LG	O – H Stretch	Carboxylic acid
4	1740-1720	1731.42 BT	C = O Stretch	Aldehydes, Saturated aliphatics
5	1760-1690	1694.92 GT 1695.63 LG	C = O Stretch	Carboxylic acid
6	1760-1665	1687.72 T.s 1	C = O Stretch	Carbonyls (general)

Table 4.8 – continued from previous page

Sr.No.	**Frequency of band (cm^{-1})**	**Experiment Frequencies (cm^{-1}) of Teas**	**Bond**	**Functional groups**
7	1710-1665	1708.81 T.s 2 1702.97 BT	C = O Stretch	, unsaturated aldehydes, Ketones
8	1680-1640	1654.41 BT 1645.53 LG	-C = C- Stretch	Alkenes
9	1650-1580	1627.86 GT	N – H Bend	1 amines
10	1550-1475	1514.77 T.s 2 1550.07 BT 1555.84 LG	N – O Asymmetric stretch	Nitro compounds
11	1500-1400	1455.72 T.s 1 1462.02 T.s 2 1453.77 GT 1461.91 BT 1454.25 LG	C – C Stretch (in ring)	Aromatics
12	1370-1350	1376.18 T.s 1 1376.08 T.s 2 1361.89 BT 1365.33 LG	C – H Rock	Alkanes
13	1335-1250	1254.30 T.s 1 336.80 GT	C – N Stretch	Aromatic amines
14	1300-1150	1160.72 T.s 1 1164.59 T.s 2 1188.37 GT	C – H Wag	Alkyl halides

Table 4.8 – continued from previous page

Sr.No.	**Frequency of band (cm^{-1})**	**Experiment Frequencies (cm^{-1}) of Teas**	**Bond**	**Functional groups**
15	1250-1020	1239.41 T.s 2 1144.06, 1032.62 GT 1163.43 BT 1237.09, 1187.75, 1145.76 LG	C – N Stretch	Aliphatic amines
16	1320-1000	1030.43 T.s 1 1030.72 T.s 2 1032.62 GT 1030.48 BT 1031.20 LG	C – O Stretch	Alcohols, Carboxylic acid, Esters, Ethers
17	1000-650	970.83 BT	= C – H Bend	Alkenes
18	850-550	T.s 1, T.s 2, GT,BT,LG	C – Cl Stretch	Alkyl halides
19	690-515	T.s 1, T.s 2 , GT, LG	C – Br Stretch	Alkyl halides

The results summarized in the table 4.8 show the presence of highest absorption band in the region of 3500-3200 cm^{-1} in all the herbal teas. This band is caused by the presence of alcohol and phenolic groups and/or the H-bonded O-H stretch in hydration water. It means teas fibers possessed hygroscopic characteristic and exhibit hydrophilic nature [177]. Below 3000 cm^{-1}, the saturated hydrocarbons C-H stretch occurs. The strong bands appear at 2917 cm^{-1} in all the tea spectra indicated the asymmetric and symmetric stretching of C-H in aliphatic CH3 groups

[178]. Another strong absorption band at 2849 cm^{-1} was also observed due to O-H stretching, indicated the presence of carboxylic acid group in all the herbal teas. Carbonyl group is the significant functional group consist of C=O. In the spectra, carbonyl compounds are the strongest bands lie in the region of 1760 cm^{-1} 1665 cm-1indicated the presence of aldehydes, saturated aliphatics, Carboxylic acid, α , β unsaturated aldehydes, Ketones and Carbonyls (general). For the functionality of double bond, conjugation plays significant role in the observing carbonyl frequency. The band between 1500 cm^{-1} 1400 cm^{-1} in *Thymus serpyllum 1, 2,* green tea, lemon grass, and black tea indicated the presence of aromatic compounds that contributed to antioxidant and other biological activities of herbal teas, supports the confirmation of our results (Table 4.8). The another strongest band was also observed at 1030 cm-1confirmed the presence of esters, carboxylic acid, ether and alcoholic compounds in our all test extracts that also proved their strong aroma, taste and these compounds play significant roles in bio activities of herbal teas. Many small peaks were observed between 1370 cm^{-1} 1020 cm^{-1} and 970 cm^{-1} 522 cm^{-1}, confirmed the presence of many functional groups. Similar results were obtained in previous research work that also showed O-H (alcohols, phenols), C-H (aliphatic), C=O (carbonyl), C-O-C (esters), C-N (aliphatic amines) [179], [180]. These previous findings precisely coordinate with the present results justifying our perspective.

Present research work regarding FT-IR evaluation of herbal teas is in favor of all elements as the particular bands demonstrate the presence of aromatic and organic compounds, reconfirmed the antioxidant and other biological activities of selected herbal teas extracts. So it was clear from table and spectra of these herbal teas that there were many similarities related to functional groups of these teas, support the result of our study for different biological activities. These results of herbal teas have shown that the extracts of these herbal teas could be safely used in pharmacy and other industries as well.

4.6.2 Biochemical Analysis of Samples via FT-IR

The significant spectral range present between 3500-1000 cm^{-1} gives the way to distinguish different teas and the all the organic compounds found in these teas extracts that contribute to significant biological roles with different compositions [181]. In the present study, a novel effort has been made to correlate the functional groups present in teas extracts and phytochemical and different biological activities manifested by these extracts.

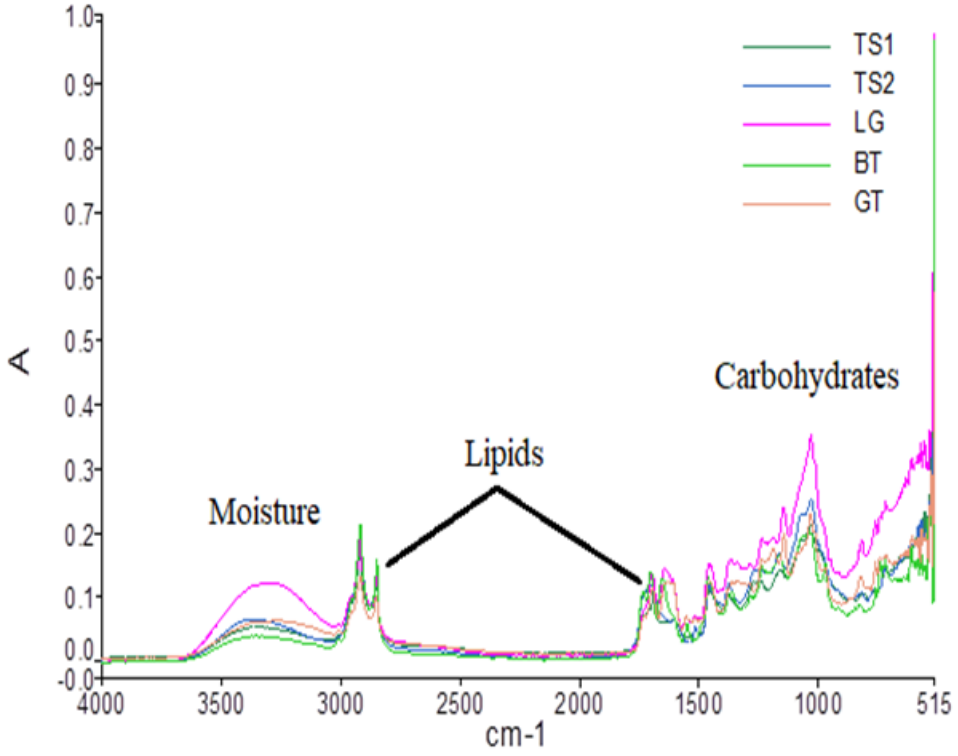
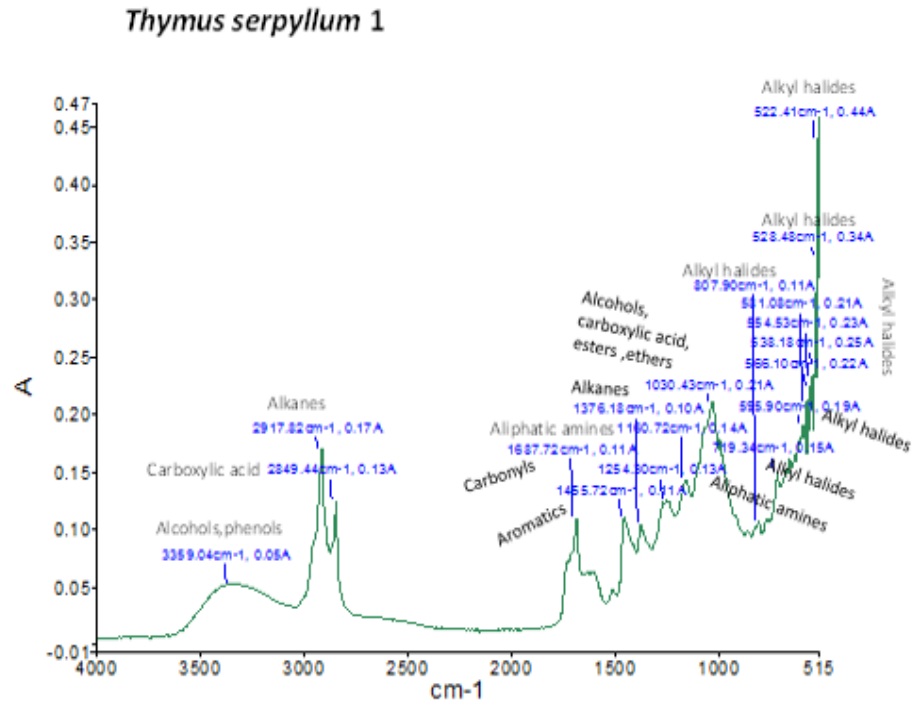
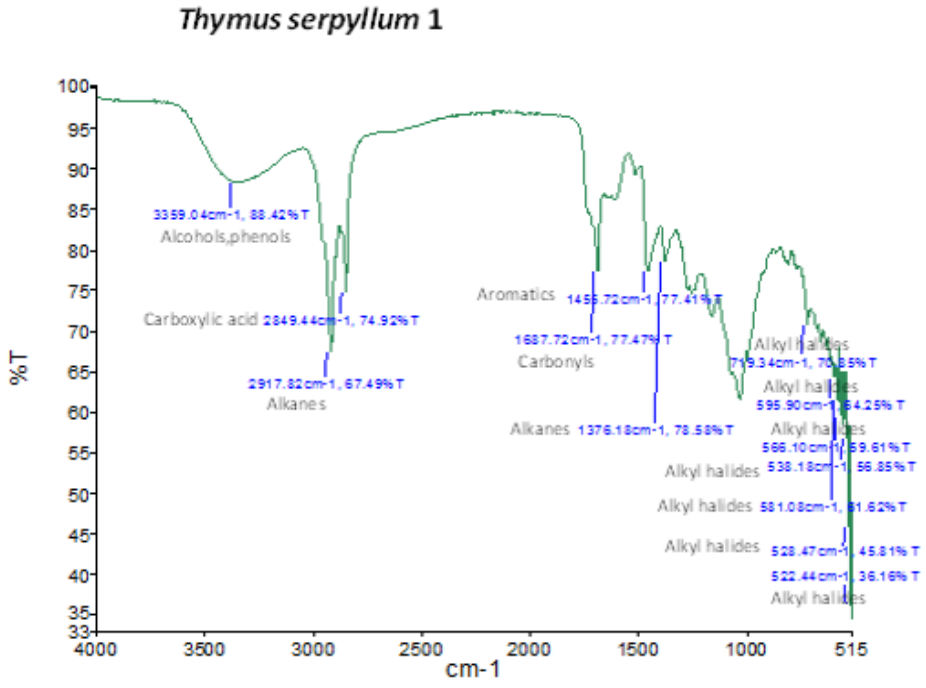


FIGURE 4.16: FT-IR spectra showing the moisture, lipid and carbohydrate contents in tested teas extracts.

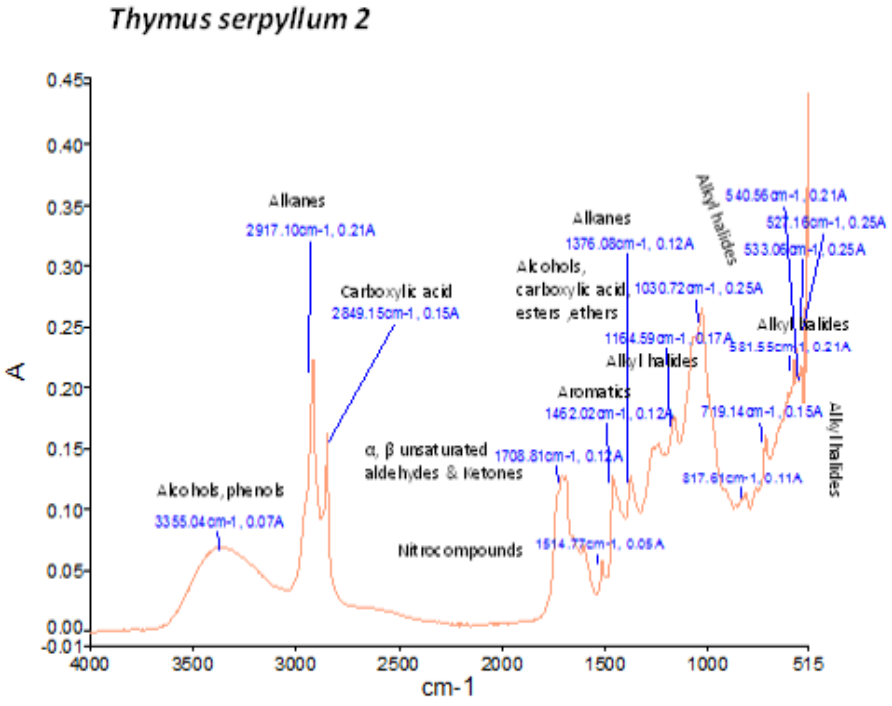


(a)



(b)

FIGURE 4.17: (a) Absorption and (b) Transmission spectra of *Thymus serpyllum 1*. FT-IR spectrum of *Thymus serpyllum 1* showing significant functional groups for phytochemical, antioxidant, antimicrobial, cytotoxicity, and antidiabetic activities.



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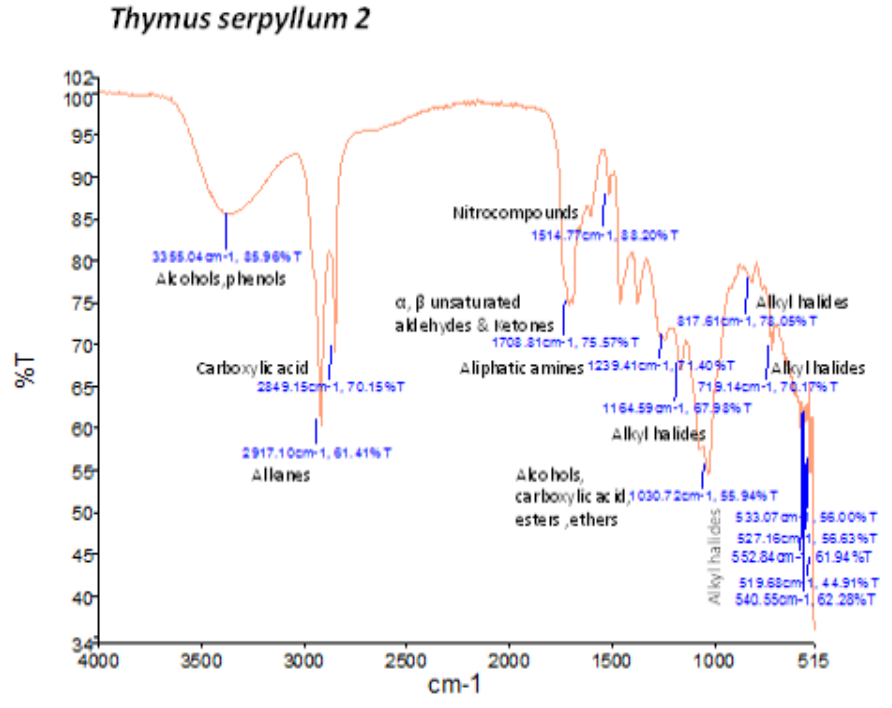
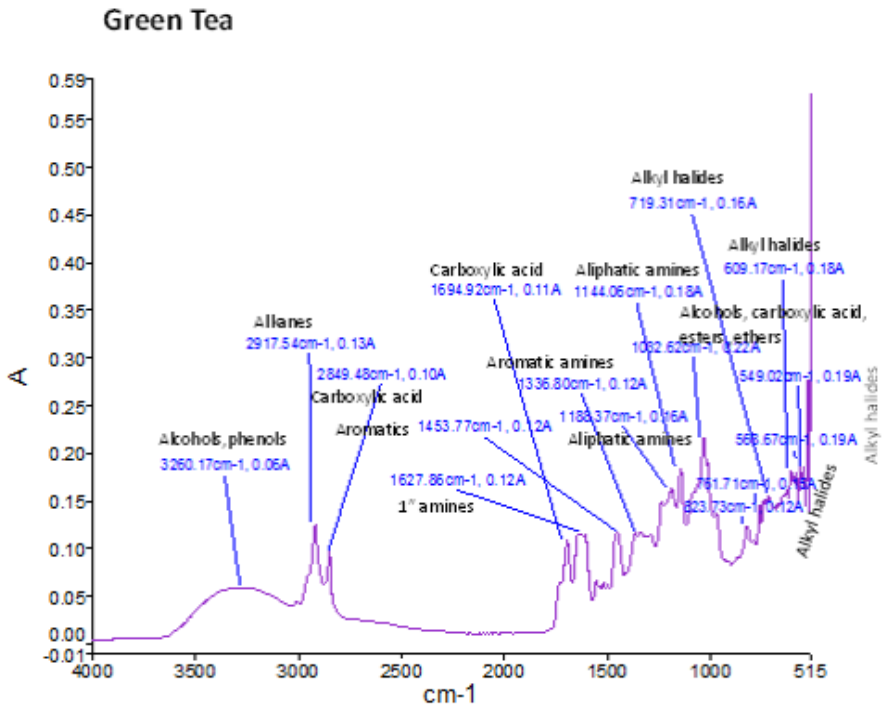
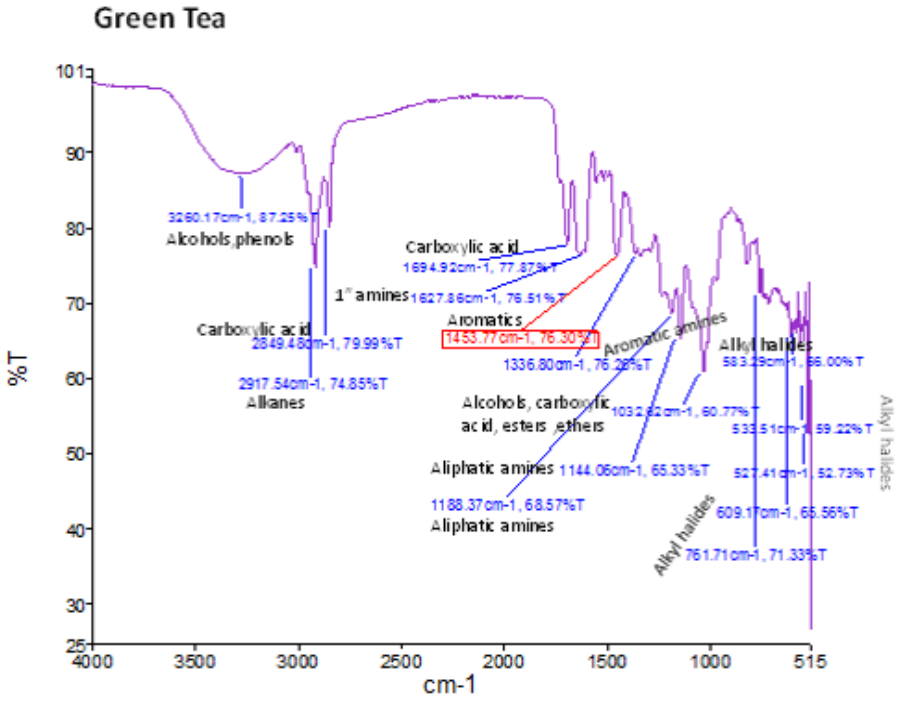


(b)

FIGURE 4.18: (a) Absorption and (b) Transmission spectra of *Thymus serpyllum 2*. FT-IR spectrum of *Thymus serpyllum 2* showing significant functional groups for phytochemical, antioxidant, antibacterial, cytotoxicity, protein kinase inhibition, and anti-diabetic activities.

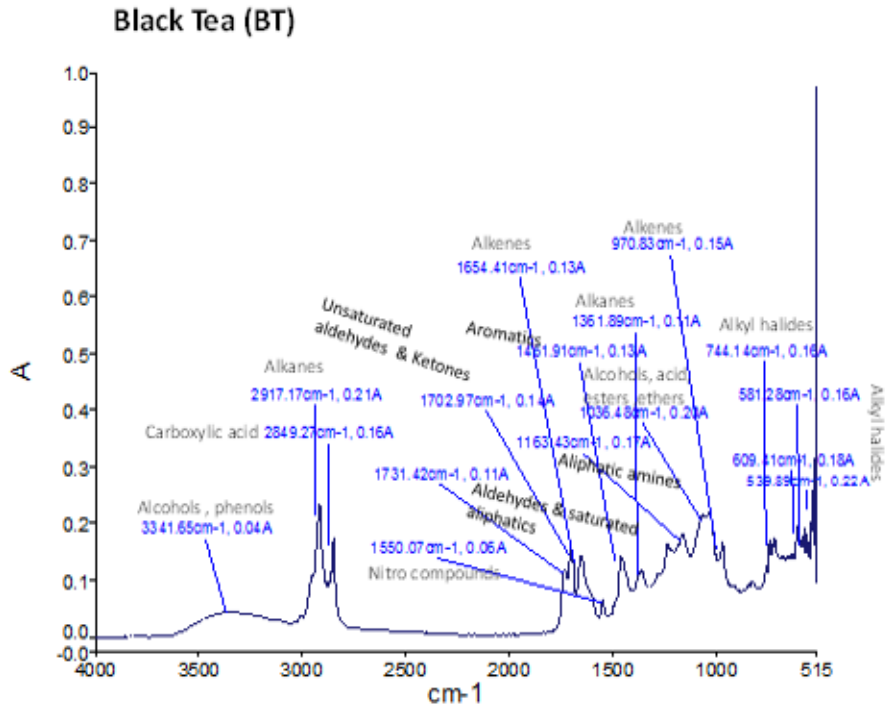


(a)



(b)

FIGURE 4.19: (a) Absorption and (b) Transmission spectra of Green Tea. FT-IR spectrum of Green Tea showing significant functional groups for phytochemical, antioxidant, antibacterial, cytotoxicity, and protein kinase inhibition activities.



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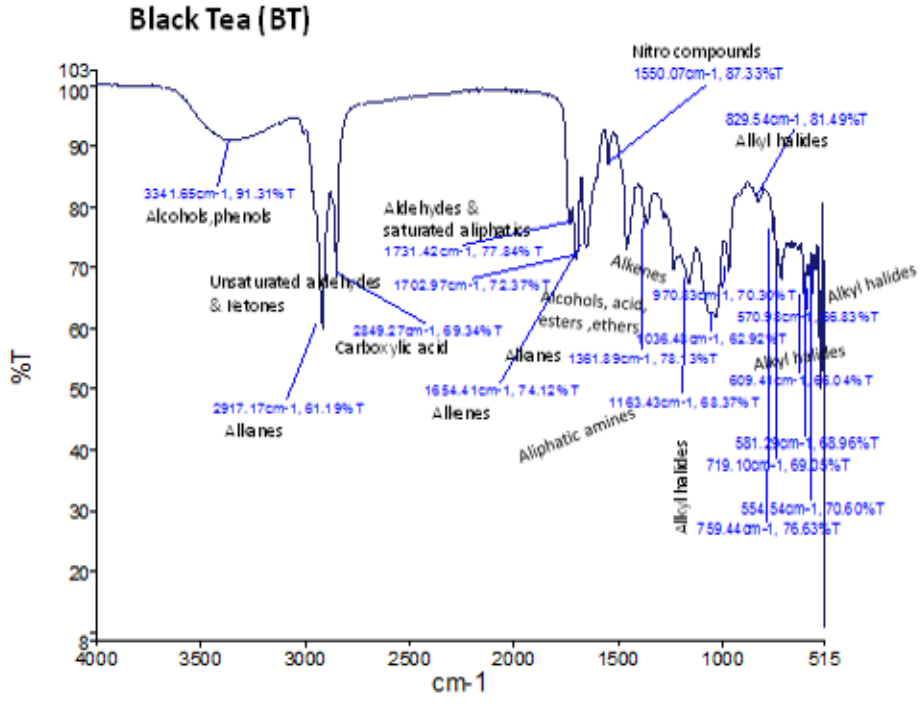
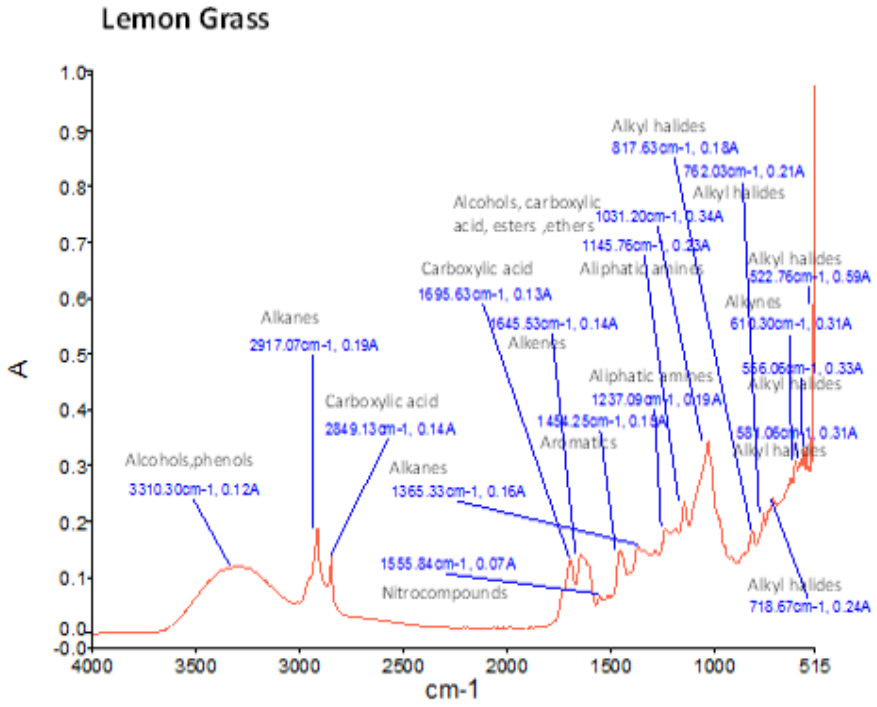


(b)

FIGURE 4.20: (a) Absorption and (b) Transmission spectra of Black Tea. FT-IR spectrum of Black Tea showing significant functional groups for phytochemical, antioxidant, antibacterial, cytotoxicity, protein kinase inhibition, and anti-diabetic activities.



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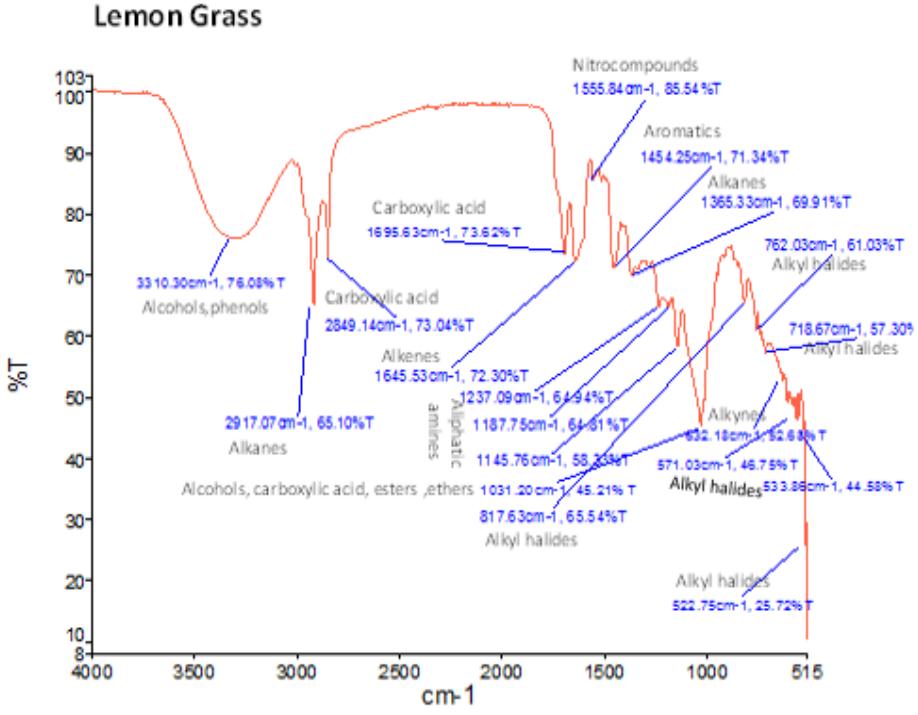

(b)

FIGURE 4.21: (a) Absorption and (b) Transmission spectra of Lemon Grass. FT-IR spectrum of Lemon Grass showing significant functional groups for antioxidant, antibacterial, cytotoxicity, protein kinase inhibition, and anti-diabetic activities.

4.7 Fatty acids Profile in Selected Teas by Using GC/MS

The study of extracted plant materials assumes an essential part in the advancement and modernization of standards necessary for herbal preparations. As tea is considered as functional food, so it is important to study the fatty acids in tea from that perspective. Therefore by the use of GC/MS, the present investigation was attempted to figure out the significant fatty acid compounds present in all the tea extracts. In the present research work, total 25 compounds were identified from all the selected tea extracts. Crude extract of *T. serpyllum 1* (Tumuro 1) and green tea showed the highest number of compounds (6), following the lemon grass with 5 compounds whereas *T. serpyllum 2* (Tumuro 2) has 4 compounds, and in black tea total 3 compounds was observed respectively. Among them more than 5 compounds were present commonly in all theses tea extracts. The active volatile compounds along with their retention time, peak %, and common names are given individually in table 4.9-4.13. GC/MS chromatograph of each tea extract with compound name is presented in figure 4.22-4.27. Mass spectrum of each identified compound is also presented in figure 4.28-4.37.

4.7.1 *Thymus serpyllum 1* (Tumuro 1)

The results of *T.serpyllum 1* showed that the most abundantly present compound is cis 9-hexadecenal (Palmitolealdehyde) followed by n-hexadecanoic acid (Palmitic acid), 9, 12-octadecadienoic acid (Linoleic acid), n-octadecanoic acid (stearic acid), 9-octadecanoic acid, 12-hyd (Ricinoleic acid), and 12 oxo-octadecanoic acid (Methyl ester) (Figure 4.22, 4.23). Cis-9-hexadecenal belongs to fatty aldehydes, a class of organic compounds. These are aliphatic, long chain aldehyde compounds play a role in fatty acid degradation. Strong aroma or fragrance is the characteristic of this volatile compound [182]. Therefore, leaves of *T.serpyllum* are used for making tea, flavoring food. The compound palmitic acid is saturated fatty acid (SFA) and linoleic acid is poly unsaturated fatty acid (PUFA) compound. Methyl

TABLE 4.9: Profile of volatile flavor compounds in *Thymus serpyllum 1* along with their retention time and peak%

Sr. No.	Peak No.	R. time (min)	Area%	Compound Name	Common Name
1	3	36.934	11.04	n-Hexadecanoic acid	Palmitic acid
2	4	38.285	23.06	Cis 9-Hexadecenal	Palmitolealdehyde
3	5	38.410	4.14	n-Octadecanoic acid	Stearic acid
4	6	38.573	6.52	9,12-Octadecadienoic acid	Linoleic acid
5	7	38.722	1.49	9,12-Octadecadienoic acid	Linoleic acid
6	8	38.851	3.76	9, 12-Octadecadienoic acid	Linoleic acid
7	9	39.647	0.80	9-Octadecenoic acid , 12-hyd	Ricinoleic acid
8	10	39.735	0.41	12 oxo-Octadecenoic acid	Methyl ester

esters are also present in the extract, characterized by strong aroma. Particularly, sweet and fresh fruity flavor of acid compounds indicate the presence of leading volatile flavor compounds in the extracts of teas [182], [183]. The tea leaves showed diverse fatty acid composition from SFAs and PUFA to MUFA, fatty aldehydes and methyl esters (Table 4.9) proved its potential as antioxidant [135], anti androgenic [184], anti cancer [185], anti inflammatory [184], hypocholesterolemic [186], and flavor, hemolytic [184], make the *T. serpyllum* an exceptional material for pharmaceutical, nutritional, and other applications as well [187].

4.7.2 *Thymus serpyllum 2* (Tumuro 2)

Many works have been conducted on the essential oil composition of *T. serpyllum* from the standpoint of their composition, aroma and flavor (food flavoring). There is no previous research found for determining the fatty acid profile of *T. serpyllum*

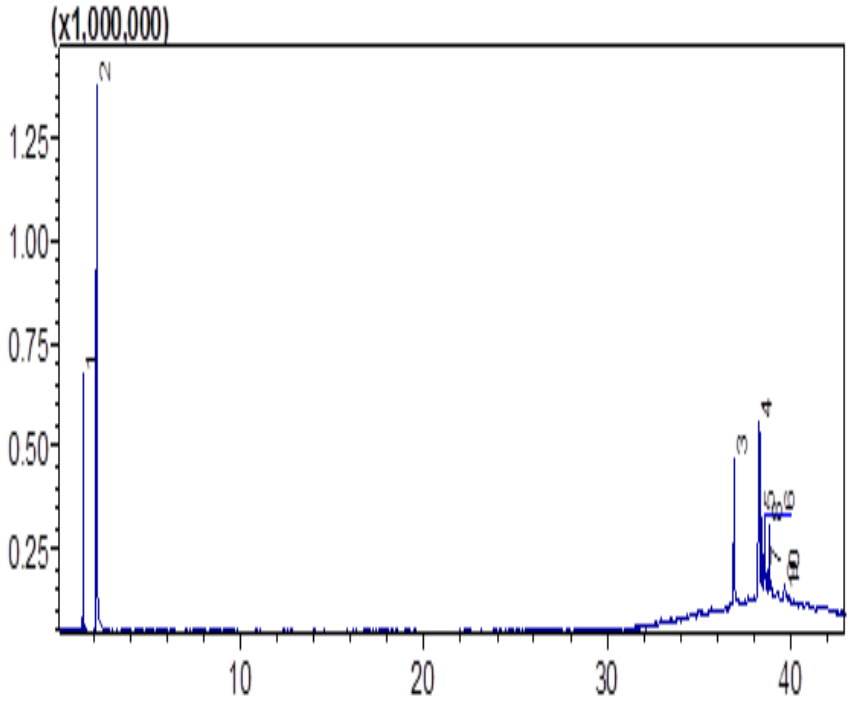

FIGURE 4.22: GC/MS chromatograph of *T. serpyllum 1.*

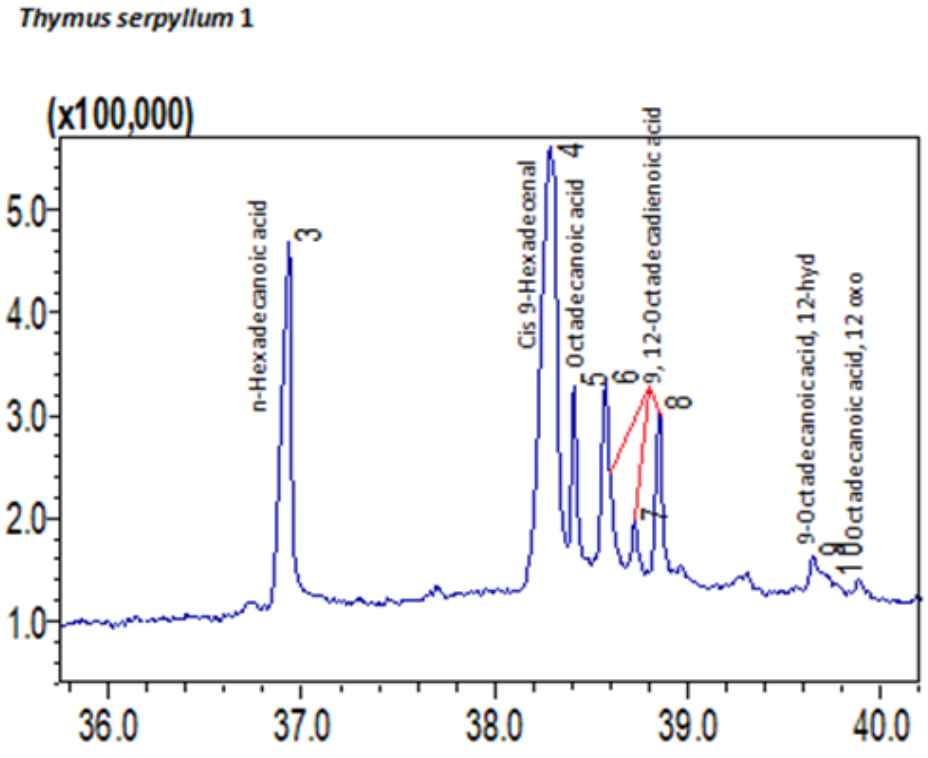

FIGURE 4.23: Identified GC/MS chromatograph of *T. serpyllum 1.*

leaves and flowers collectively. This may be the first study regarding the fatty acids compounds present in *T. serpyllum 2* leaves and flowers collectively.

The results of *T. serpyllum 2* showed that palmitic acid, petroselinic acid, stearic acid, and linoleic acid were present in test extract (Figure 4.24). The most prevalent fatty acid was petroselinic acid (MUFA). The percentages of saturated fatty acids (SFAs) are greater than MUFAs in *T. serpyllum 2* (Table 4.10). Petroselinic acid is atypical monounsaturated fatty acid, and a positional isomer of oleic acid. Its occurrence is higher in some oils obtained from plants seeds. The most important feature of this compound is that it is involved in the lipolysis when combined with the triglycerides; hence the oils rich in petroselinic acid may provide a low fat choice instead of traditional vegetable oils [188]. Petroselinic acid could restrain the vasoconstriction in case of over production of arachidonic acid and also inhibits its synthesis [189]. Petroselinic acid from omega-12 fatty acid group, used in food supplements with the significant role of anti-inflammatory, and anti-aging effects and also used in confectionaries. The presence of this compound in the plant is also associated with its remedial nature as an anti aging and a skin irritant lowering agent, therefore commonly used in cosmetics products as a moisturizer [190].The obtained results could indicate the higher levels of SFAs in thymus extract (Table 4.10). The presence of these fatty acids justifies the utilization of whole plant suggested for medicinal significance. It is regarded as a popular herb in Asia and Europe for its medicinal properties as antibacterial, antiviral, anti-inflammatory, antioxidant and also as stimulant, digestive, carminative and diuretics [22]. It is also used as tea in colds and prescribed as expectorant and in the infections of pulmonary diseases [75]. Therefore, presence of these compounds in *T. serpyllum 2* may be beneficial for various health purposes.

However further research is needed to explore the bioactivities of *T. serpyllum 2*. Many factors influenced the fatty acid composition i.e. climate, latitude, geographical location for production and cultivars [191].

TABLE 4.10: Profile of volatile flavor compounds in *Thymus serpyllum 2* along with their retention time and peak%

Sr. No.	Peak No.	R. time (min)	Area%	Compound Name	Common Name
1	3	36.893	5.55	n-Hexadecanoic acid	Palmitic acid
2	4	38.269	11.83	6-Octadecanoic acid	Petroselinic acid
3	5	38.384	1.49	n-Octadecanoic acid	Stearic acid
4	6	38.543	2.05	9, 12-Octadecadienoic acid	Linoleic acid
5	7	38.827	1.12	9, 12-Octadecadienoic acid	Linoleic acid
6	8	38.851	3.76	9, 12-Octadecadienoic acid	Linoleic acid
7	9	39.647	0.80	9-Octadecenoic acid , 12-hyd	Ricinoleic acid

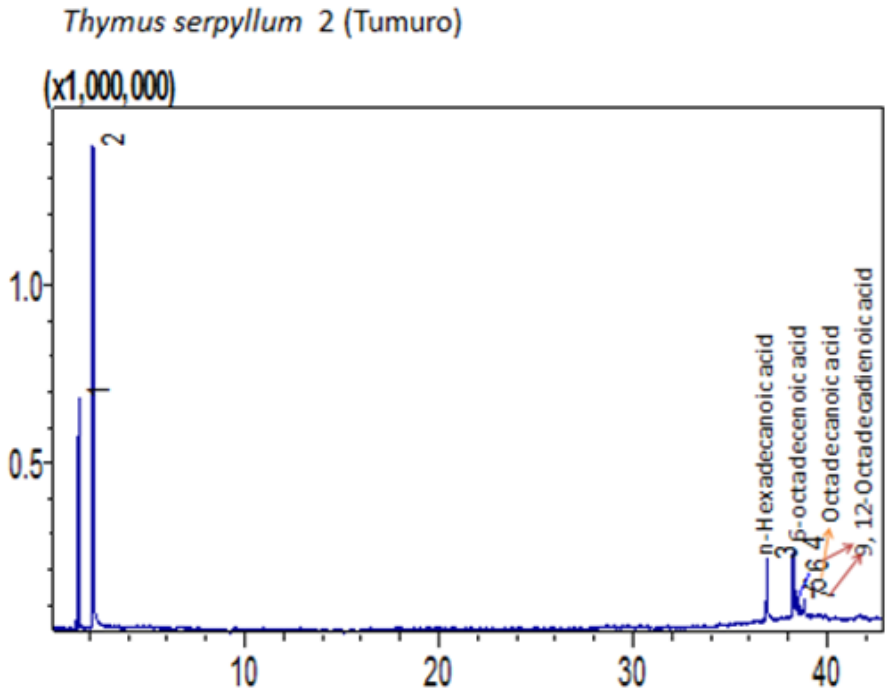

FIGURE 4.24: Identified GC/MS chromatograph of *T. serpyllum 2*.

4.7.3 *Camellia sinensis* (Green Tea)

From green tea extract following fatty acids were examined by GC/MS; Palmitic acid, 13-hexyloxacyclotridec-10-en-2-one, Oleic acid, stearic, linoleic, and Ricinoleic acids (Figure 4.25). Among them, the most predominant fatty acid was oleic acid, followed by palmitic and ricinoleic acids (Table 4.11). Our results are in consistent with past reports on the fatty acid composition in green tea [192]. Oleic acid belongs to omega-9-fatty acid group has many health benefitting properties including lowering bad cholesterol levels, raising good cholesterol levels, lower the blood pressure, and also reduces the risk of cardiovascular disease and stroke [193]. The results of this study demonstrated that green tea extract contains high unsaturated FA content, so it can also be concluded that green tea oil could be used as edible oil because of predominant unsaturated FA (oleic acid, ricinoleic acid, and linoleic acid). Ricinoleic acid is an unsaturated hydroxy acid belongs to the omega-9-fatty acid group [194]. It has anti-inflammatory anti fungal properties and also acts as analgesic [195]. It also acts as stimulant laxative [196]. 13-hexyloxacyclotridec-10-en-2-one (macrolactone) is synthesized by the cycling of an unsaturated hydroxyl acid compound, ricinoleic acid [197]. It was studied that this compound possessed chemotherapeutic potential, act as an antitumor agent [198]. It is reported that linoleic acid along with palmitic acid and oleic acids are the most prevalent fatty acids in triaglycerols of fat and plasma lipoproteins [199]. It was also reported that during the manufacturing proceeses and long time storage of green tea, a change in its fatty acid contents (% of dry weight) was observed that might also affect the quality of tea i.e. flavor of tea [200]. Another study also revealed that in tea leaves, fatty acids composition were notably influenced by growth condition, cultivar, cultivation, plucking time period of tea leaves, methods of processing, and lastly the storage of tea [191], [201].

TABLE 4.11: Profile of fatty acid compounds in green tea along with their retention time and peak%

Sr. No.	Peak No.	R. time (min)	Area%	Compound Name	Common Name
1	3	12.383	10.89	n-Hexadecanoic acid	Palmitic acid
2	4	13.288	1.68	13-Hexylocyclotridec -10-en-2-one	Macrolactone
3	5	13.987	22.42	9-Octadecenoic acid	Oleic acid
4	6	14.177	2.37	n-Octadecanoic acid	Stearic acid
5	7	14.419	1.63	9, 12-Octadecadienoic acid	Linoleic acid
6	8	14.878	0.96	9, 12-Octadecadienoic acid	Linoleic acid
7	9	16.924	5.40	9-Octadecenoic acid, 12-hyd	Ricinoleic acid

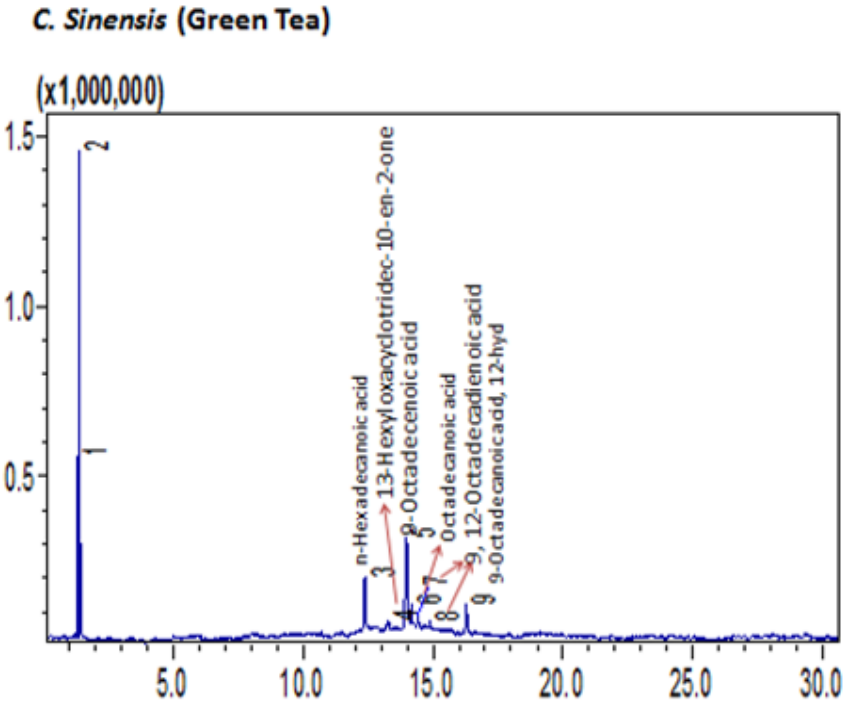

FIGURE 4.25: GC/MS chromatograph of *C.Sinensis* (Green Tea) with compounds name.

4.7.4 *Camellia sinensis* (Black Tea)

The results revealed that black tea contain 3 main compounds i.e. Caffein, palmitic acid and oleic acid (Figure 4.26). However, the most predominant fatty acid was oleic acid, followed by palmitic acid and caffeine (Table 4.12). It was reported that caffeine contents were found greater in black tea than green tea and coffee beans [15]. A cup (8oz) of green tea contains 8-30 mg caffeine where as a black tea cup (8oz) contains 25-110mg caffeine level [15]. Briskness is the quality of black tea and this quality is attributed by caffeine [16]. Caffeine increase the mental alertness, elevates the mood [202], decreases the risk of type 2 diabetes, stroke, certain cancers, and a muscle relaxant [203]. Palmitic acid exerts various significant bioactivities such as antioxidant activity, anti androgenic, and hypocholesterolemic [135]. So, it may be concluded that antioxidant property of our black tea extract might be due to the presence of palmitic acid, proved the health beneficial effects of black tea. Our results are in alliance with the previous results, found the similar fatty acids in their study [204]. From the previous study, it was also confirmed that higher fatty acid contents was observed in dry time periods/season and lower contents was found in rainy time periods [191]. Significant loses of fatty acids were seen in the wilting and firing processes. A minimal change was observed in fatty acid contents during the other phases of processing i.e. rolling, fermentation [29]. However, the formation of volatile flavor compounds during black tea manufacturing and also the change in lipid content of tea during the long time storage have been accounted so far.

4.7.5 *Cymbopogon citratus* (Lemon Grass)

Table 4.13 illustrated the fatty acids profile in lemon grass extract. Among them, palmitic and stearic acids are saturated fatty acids whereas oleic acid is MUFA, and linoleic acid is PUFA (Figure 4.27). Ricinoleic acid is an unsaturated hydroxy acid belongs to the omega-9-fatty acid group [194]. Oleic acid is most abundantly found fatty acid in lemon grass extract than palmitic, linoleic, and stearic acids.

TABLE 4.12: Profile of fatty acid compounds in black tea along with their retention time and peak%

Sr. No.	Peak No.	R. time (min)	Area%	Compound Name	Common Name
1	3	11.480	2.74	1H-Purine-2, 6-dione	Caffein
2	4	12.380	5.48	n-Hexadecanoic acid	Palmitic acid
3	5	13.961	12.07	9-Octadecenoic acid	Oleic acid

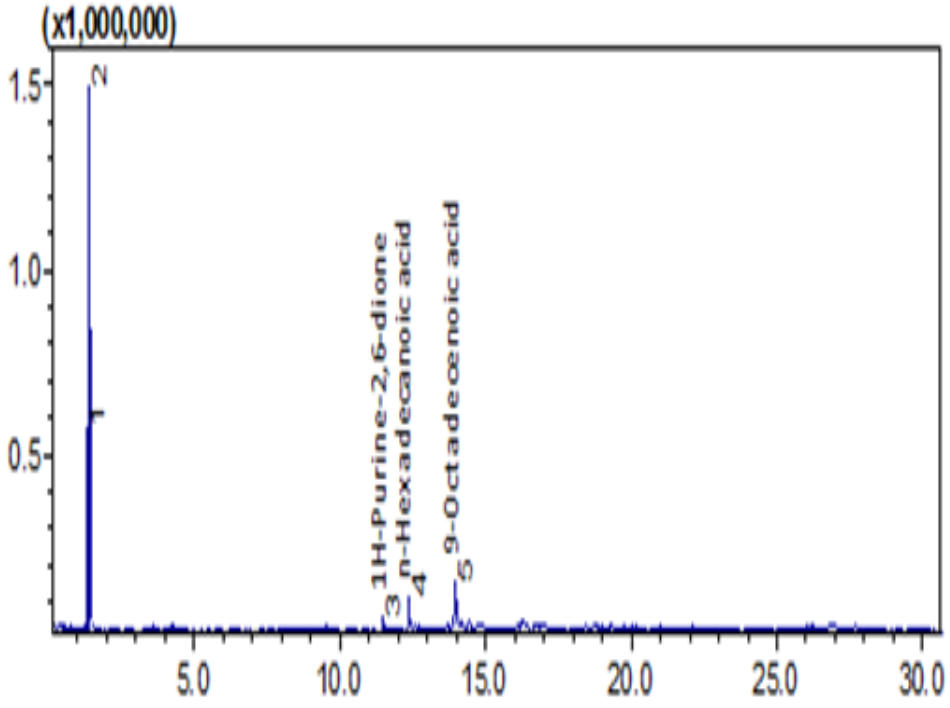

FIGURE 4.26: GC/MS chromatograph of *C.Sinensis*(Black Tea) with compounds name

From this study, it could be deduced that in lemon grass extract, unsaturated and saturated fatty acids were present (Table 4.13). However, the lemon grass extract was mostly composed of highly unsaturated fatty acids. Linoleic acid from the omega-6-fatty acid group is an essential fatty acid. It is important for the better development and strengthens of nervous system [205]. Linoleic and linolenic acids are the two unsaturated FAs indispensable for human health. These fatty acids are not synthesized by humans, so these must be acquired from food sources known as essential fatty acids. Ricinoleic acid has anti-inflammatory anti fungal properties and also acts as analgesic [195]. Lipids and fatty acids from food sources play significant roles for disease prevention and improve the health status [206]. Our results are in accordance with the previous findings that also illustrate the presence of fatty acids in lemon grass [207]. The perennial nature and easy cultivation are the key features of lemon grass. Thus, it will give economically feasible yields with better future for nutritional and medicinal values [207]. Generally in folk medicines, lemon grass is mostly used for the cure of gastrointestinal and nervous disorders [208]. From the recent study, it can be concluded that presence of fatty acids compounds in lemon grass extract might proved its potential as antimicrobial, antioxidant, anti-diabetic, and cancer preventive. Bioactivities of different fatty acid compounds are given in the table 4.14.

The fatty acid contents of these teas were evaluated by dissolving extracts into chloroform. The chloroform fraction contained the neutral lipids as identified in our study. Here the FTIR results were also found to be supported the GC/MS findings, as major part of the FTIR spectrum of all the tea extracts represent the lipid content. The results of this analytical technique manifested the potential uses of these herbal teas for future nutritional and medicinal values.

Conclusively, the extraction and identification of biologically active elements from medicinally important plants is exceptionally fundamental for novel drug discovery with effective therapeutics. Recently, there is a great demand of natural plant based medicines due to the cognizance of severe side effects of synthetic drugs or additives.

TABLE 4.13: Profile of fatty acid compounds in lemon grass along with their retention time and peak%

Sr. No.	Peak No.	R. time (min)	Area%	Compound Name	Common Name
1	4	23.600	6.03	n-Hexadecanoic acid	Palmitic acid
2	5	25.397	17.07	9-Octadecenoic acid	Oleic acid
3	6	25.585	1.66	n-Octadecanoic acid	Stearic acid
4	7	25.800	3.20	9, 12-Octadecadienoic acid	Linoleic acid
5	8	26.180	1.67	9,12-Octadecadienoic acid	Linoleic acid
6	9	27.267	1.27	9-Octadecenoic acid,12hyd	Ricinoleic acid

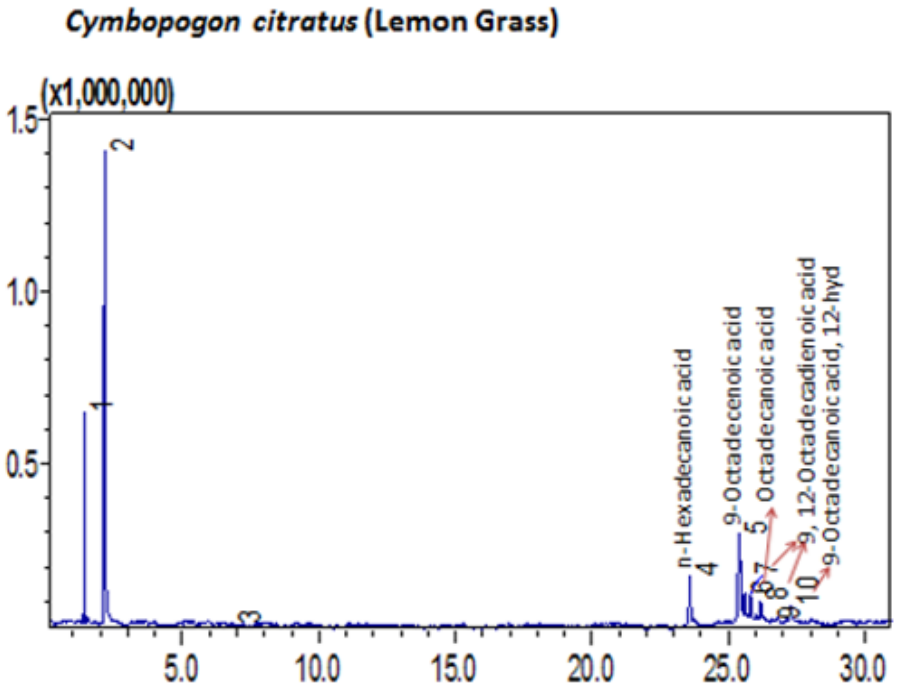

FIGURE 4.27: GC/MS chromatograph of *Cymbopogon citratus* (Lemon Grass) with compounds name

TABLE 4.14: Bioactivity of some Fatty acid compounds identified in selected herbal tea extracts.

Compound Name	**Biological activity**	**References**
n-Hexadecanoic acid Palmitic acid	Antioxidant,5-Alpha reductase inhibitor, anti-androgenic, hypocholesterolemic, nematicide	[135], [199]
9-Octadecenoic acid Oleic acid	Cancer preventive, anti-inflammatory, 5-Alpha reductase inhibitor, anti-androgenic, dermatitigenic, flavor, hypocholesterolemic	[193], [199]
n-Octadecanoic acid Stearic acid	Hypocholesterolemic, cosmetic, 5-Alpha reductase inhibitor, flavor	[135]
Cis-9, cis-12- Octadecadienoic acid Linoleic acid	Nervous system , anti eczemic, hepatoprotective, anti-acne, hypocholesterolemic, anticoronary	[186]
9-Octadecanoic acid, 12hyd Ricinoleic acid	stimulant laxative, antifungal, analgesic , anti-inflammatory	[195], [196]
6-Octadecanoic acid Petroselinic acid	anti-inflammatory, lipolysis, anti aging , cometics, control vasoconstriction, confectionaries, skin irritating lowering agent	[188], [189], [190]
1H-Purine-2,6-dione Caffein	Mental alertness, elevates the mood, prevents T2D, cancer, bronchopulmonary, stroke, muscle relaxant	[201] , [202], [208]
Cis 9- Hexadecenal Palmitolealdehyde	Fatty acid degradation, strong aroma	[182]
13-Hexylocyclotridec- 10-en-2-one Macrolactone	Anticancer properties	[198]
Methyl ester	Flavor	[182], [183]

4.8 Mass Spectra of Identified Compounds

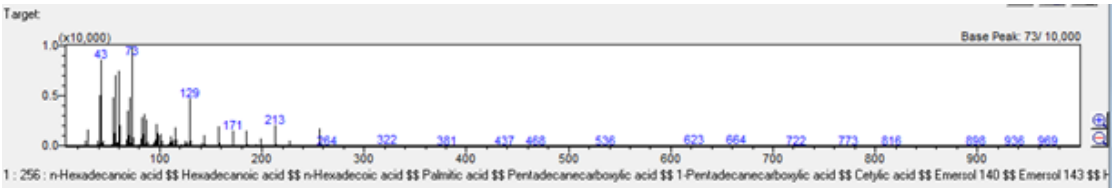

FIGURE 4.28: Mass spectrum of n-Hexadecanoic acid.

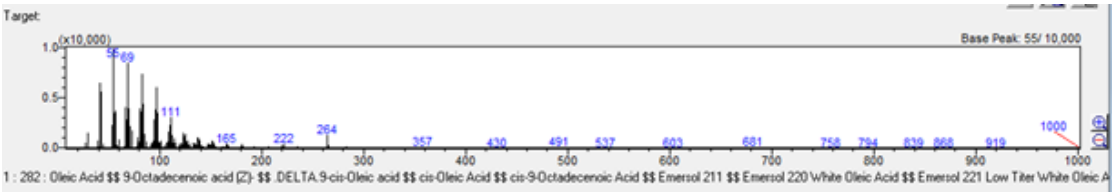

FIGURE 4.29: Mass spectrum of 9-Octadecenoic acid.

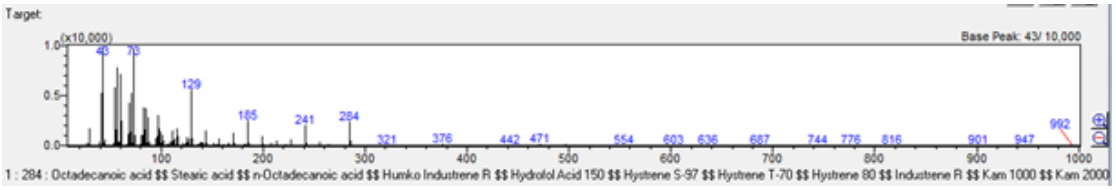

FIGURE 4.30: Mass spectrum of n-Octadecanoic acid.

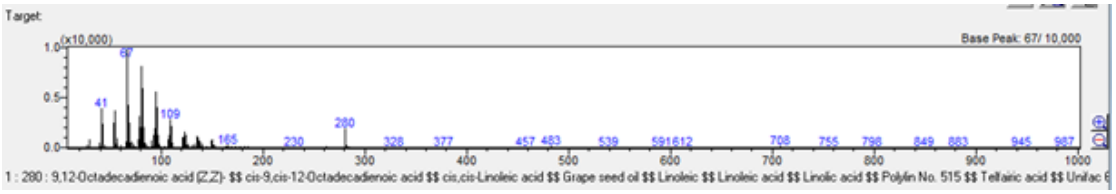

FIGURE 4.31: Mass spectrum of cis-9, cis-12-Octadecadienoic acid.

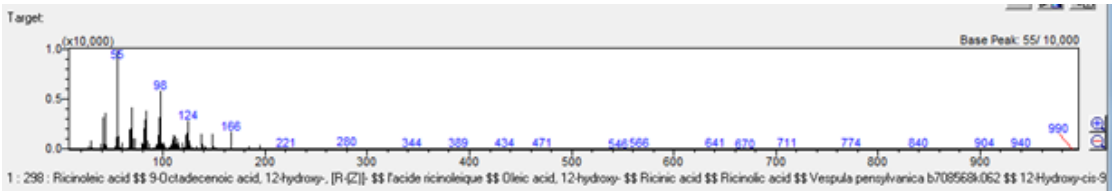

FIGURE 4.32: Mass spectrum of 9-Octadecenoic acid, 12hyd.

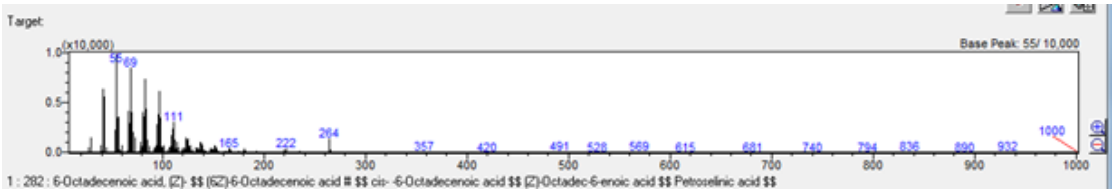

FIGURE 4.33: Mass spectrum of 6-Octadecanoic acid.

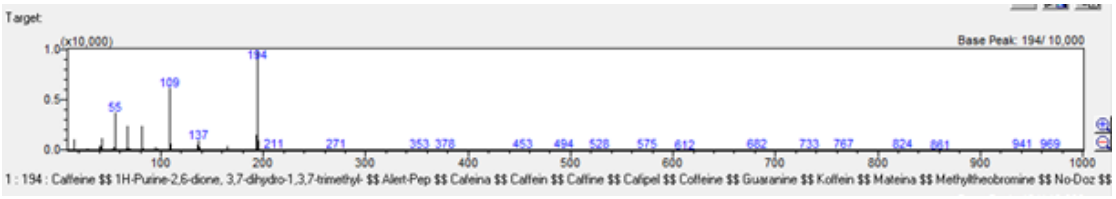

FIGURE 4.34: Mass spectrum of 1H-Purine-2,6-dione.

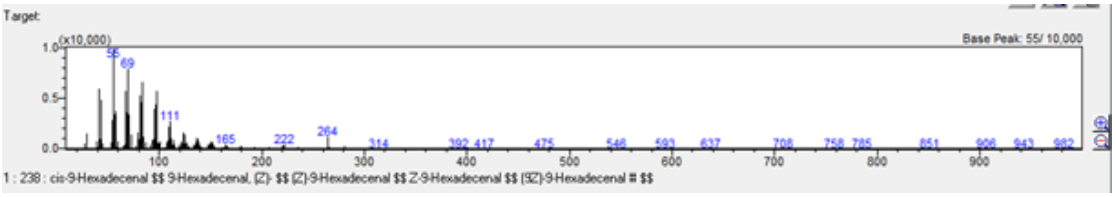

FIGURE 4.35: Mass spectrum of cis-9 Hexadecenal.

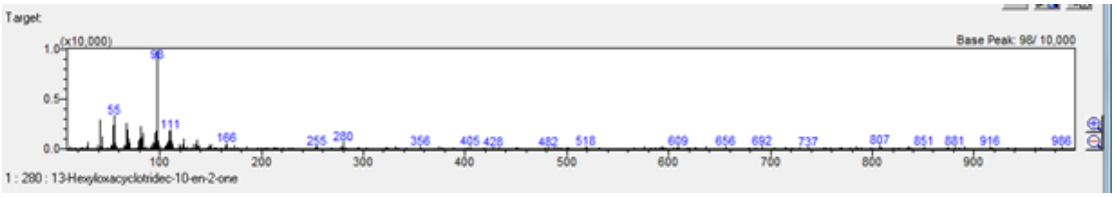

FIGURE 4.36: Mass spectrum of 13-Hexylocyclotridec-10-en-2-one.

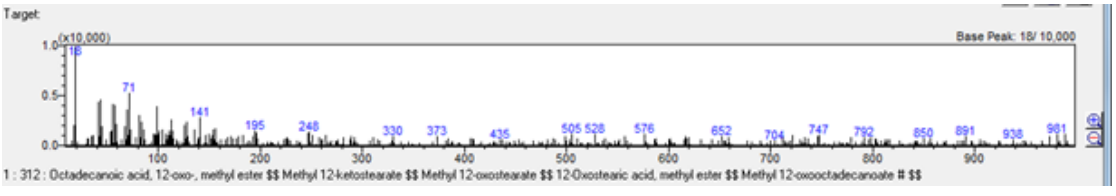

FIGURE 4.37: Mass spectrum of 12 oxo -Octadecanoic acid.

Chapter 5

Conclusion and Future Prospects

5.1 Conclusion

Tea plants used for medicinal objective are an important element of folk tradition and heritage in the world. Herbal tea manifested varied composition of bioactive compounds which are responsible for its putative biologic activities. The results of our study alleviate the conventional use of aromatic herbal tea plants in reducing the oxidative stress levels that is the major cause of various diseases. In the present study, the biological activities of extracts assessed through various bioassays depicted better results in phytochemical, antioxidant, antibacterial, enzyme inhibition and antidiabetic assays whereas qualitative analysis of these extracts confirmed the presence of significant functional groups and fatty acid compounds in these teas extracts.

In conclusion, assaying of phytochemicals displayed highest phenolic load in the green tea extract and flavonoid load in black tea. Maximum antioxidant aptitude narrated as ascorbic acid equivalent was also computed highest in green tea extract whereas lemon grass extract showed less phytochemical and antioxidant potential. In antibacterial assay, all of the five extracts of teas were active against five bacterial strains tested that might confirm their use and efficacy against various infections. Among them, remarkable activity was shown against *S. aureus* by

lemon grass and black tea extracts however; modest activity was observed against *P.aeruginosa* and *B. subtilis* by all tested samples. Least antibacterial activity was observed by *T. serpyllum 2*. Subjected tea samples showed no antifungal activity against the strains tested in our study.

Cytotoxicity profile established using brine shrimp lethality assay confirmed the highest proficiency of lemon grass and *T. serpyllum 1* extracts that may proposed their utilization as anti-cancer and anti-mutagenic agents while minimum activity was observed in black tea. Remarkable protein kinase inhibitory action was quantified in lemon grass and green tea extracts with bald zone of inhibition, pressing a need for bioactivity guided isolation of potentially active compounds. *T. serpyllum 1* presented a clear zone of inhibition indicated its less significant inhibition potential. Black tea extract showed the best alpha amylase inhibitory action that might suggesting its useful role as an adjunct therapy in the treatment of T2D however least % inhibition was observed in green tea.

All the tested teas extracts confirmed the presence of significant functional groups that were identified by FT-IR spectroscopy analysis. By the use of GC/MS, the present investigation was attempted to figure out the significant fatty acid compounds present in all the tea extracts. Results of our detailed screening led us to the conclusion that the probing of herbal teas has unveiled the additional benefits of these teas and also exhibited promising perspective for the discovery of new bioactive molecules from teas. The results have shown that the extracts of these herbal teas could be safely used in pharmacy and other industries as well. So, more investment and research is needed for the screening of bioactive compounds of traditional herbal teas which could serve as an effective means for therapies.

5.2 Future Prospects

- By employing polarity based solvent system, extensive biological screening of traditional teas will provide better results.

- *Thymus serpyllum 2* (dried leaves + flowers) which was studied first time, might give better results by optimized lab protocols.

- The high protein kinase inhibition potential of lemon grass and green tea could be used as an adjunct therapy for treatment of cancer hence emphasizing further evaluation to support our claim.

- Bioactivity guided isolation should be the most logical extension of our study in order to isolate, identify and characterize potentially active components responsible for observed biological actions.

- Future in *vivo* investigations might certify and strengthen the reported *in vitro* findings.

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