Comparative Analysis of Biological Activity of *Zingiber officinale* and *Camellia sinensis* Polyphenols Using Wet Lab Approaches

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Dedicated to Maa and Paa ☺

for always being there for me, supporting me in every condition, believing in me

and

making me what I am today.

Marriam Khurshid

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Marriam Khurshid

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List of Abbreviations

WHO	World health organization
Z. Officinale	*Zingiber officinale*
C. sinensis	*Camellia sinensis*
UV	Ultraviolet
EGCG	(-)-epigallocatechin-3-gallate
GTP	Green Tea Polyphenols
MDR	Multi-Drug Resistant
MRSA	methicillin-resistant *Staphylococcus aureus*
DPPH	2, 2-di-phenyl-2-picryl hydrazyl hydrate
HBV	Hepatitis B virus
FDA	Food and drug administration
ACS	American Cancer Society
COX-1	cyclo-oxygenase
RTK	Receptor Tyrosine Kinase
Huh-7	Human Hepatocellular carcinoma cell line
DMEM	Dulbecco's Modified Eagle Media
FBS	Fetal Bovine Serum

PBS	Phosphate Buffered Saline
DMSO	Dimethyl sulfoxide
MTT	Methyl thiazole tetrazolium
BHA	Butylated Hydroxyanisole
AFIP	Armed Forces Institute of Pathology

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Abstract

Medicinal plants have been of great importance in therapeutics since ages. They contain some compounds called polyphenols in a significant amount and these polyphenols possess certain therapeutic activities. The focus of this research was to evaluate the polyphenolic activities of most commonly used medicinal plant in Pakistan. Tea is one of most commonly used beverage in Pakistan. It can be Ginger, Green or Black tea. Potential polyphenols and their activity was evaluated first using *in silico* analysis. The results found were then cross checked using wet lab approaches. Biological activities including anticancer, antimicrobial and antioxidant activities were evaluated. Extracts were made using two methods i.e. Maceration and soxhlet extraction, to compare the activity of extracts. Cell line used for anticancer evaluation was *HuH-7*. Bacterial strains used were clinical isolates including methicillin-resistant *Staphylococcus aureus* (MRSA) and multi-drug resistant *Pseudomonas aeruginosa, Escherichia coli, Salmonella Typhi* and *Shigella sonnei***.** Antioxidant activity was assessed using Hydrogen peroxide assay. The results obtained showed that extracts from *Zingiber officinale* and *Camellia sinensis* exhibit strong anticancer, antimicrobial and antioxidant activities. Comparatively, extracts taken from Maceration were more efficient than the ones taken from Soxhlet Extraction. Moreover, aqueous extracts of green tea were the most active of them all. These results suggested that polyphenols from *Z. officinale* and *C. sinensis* are potential drug candidates and they can be efficiently used in therapeutics.

COMPARATIVE ANALYSIS OF BIOLOGICAL ACTIVITY OF *ZINGIBER OFFICINALE* AND *CAMELLIA SINENSIS* POLYPHENOLS USING WET LAB APPROACHES

CHAPTER 1

INTRODUCTION

Herbal treatment has always been the greatest source to cure various diseases since ages. Extracts from different plants have been the key ingredient of different drugs. Almost 50% of the modern medicines are derived from plants or the chemical synthetic compounds of their phytochemical analogues. (Kunle *et al.*, 2012). Plants e.g. Ginseng, Ginkgo, Saw palmetto and Psyllium seed husk are popular for the treatment of several disorders (Alzaher, 2014). Antimicrobial, anti-cancer, anti-oxidant, anti-diabetic and various other biological activities of plants have been reported. Many medicinal plants have anticancer, antiviral and antimicrobial potential. (Liu & Du, 2012). Some plant extracts have shown biological activities against the strains which are resistant to conventional therapies. Huge number of phytochemicals and crude extracts having various potential have been identified (Rajasekaran *et al.*, 2014).

World health organization (WHO) is aiming to include medicinal plants into healthcare in the countries where use of plants for medical purposes is practiced (Nikam *et al.*, 2012). A beneficial use of plants can be in the production of protein based therapies, anti-cancer drugs, antibiotics and vaccines because several suggest that pharmaceutical grade proteins are developed from plant sources. Several reports suggest the use of crude plant extracts as remedy as most of the plants have low cytotoxicity is increasing every day.

Polyphenols are secondary metabolites produced by a lot of plants and they are well-known for their potential therapeutic effects as anticancer, anti-mutagenic, anti-microbial and anti-oxidant agents. The extent to which they are effective depends upon their food intake and bioavailability. Also their activity depend on their absorption by body because all polyphenols are not absorbed by equal efficacy (El Gharras, 2009). Polyphenols found in medicinal plants regulate cell activity by modulating the actions of enzymes and various other receptors, mechanism of which is still completely not known (Middleton & Kandaswami, 1993). Studies have shown that polyphenols are quite effective in in vitro analysis against carcinogen induced skin, lung, colon and duodenum cancers. (Adhami *et al.,* 2004). Multidrug resistant microbes have been an increasing problem now a days. Polyphenols with the combination of conventional antimicrobial agents have proved to be potent drug candidates (Daglia, 2012). Polyphenols have also been reported to help in prevention of cancers, diabetes, cardiovascular diseases, osteoporosis and many other diseases when they are used as antioxidants in dietary supplements (Pandey & Rizvi, 2009)

The plants having polyphenols can be a good targets in research as anti-cancer, anti-microbial and anti-oxidative agents. Ginger has been under research as anti-inflammatory and antithrombotic agent at high levels. Mostly aqueous or ethanolic extracts are used and have been showing good results. (M. Stranahan, Martin, & Maudsley, 2012) green and black tea have also shown very effective biological activities. They also possess a lot of active compounds like catechins, caffeine, theaflavins, flavonoids and many others that possess good therapeutic activities. (Perva-Uzunalić et al., 2006)

Zingiber officinale, the rhizome of *Zingiber Officinalis*, is generally the most utilized types of *Z. Officinale* family. It's a typical food additive for different sustenance and

refreshments. *Z. Officinale* has a long history of restorative use going back 2500 years. *Z. Officinale* has been generally utilized from time immemorial for changed human sicknesses in diverse parts of the globe, to help processing and treat stomach surprise, looseness of the bowels, and queasiness. Some sharp constituents present in *Z. Officinale* and different zingiberaceous plants have strong cell reinforcement and mitigating exercises, and some of them display growth preventive movement in test carcinogenesis. The anticancer properties of *Z. Officinale* are ascribed to the vicinity of certain sharp vallinoids, viz. [6]-Z. Officinaleol and [6]-paradol, and also some different constituents like shogaols, zingerone and so on. Various systems that may be included in the chemo-preventive impacts of *Z. Officinale* and its parts have been accounted for from the research facility thinks about in an extensive variety of trial models. (Shukla & Singh, 2007)

Camellia sinensis is a rich source of polyphenols, which have increased scientist interest because of their intense cell reinforcement activities. Polyphenols, range in more than 8000 structures, are enriched in human diet and are reported to show significant effects against cardiovascular diseases, various types of cancers and antibiotic activity (Gormaz *et al.*, 2016). Till date, polyphenols from green tea (GTP) have pulled in significant consideration on account of their skin UV-protective impacts. GTP has been appeared to have amazing preventive impacts against photo-toxicity in murine models and additionally in people. (Vayalil*et al.*, 2004). Catechins are the important flavanols present in GTP, having medicinal activity. (Curin & Andriantsitohaina, 2005) among all catechins and the highest anticancer activity is shown by (-)-epigallocatechin-3-gallate (EGCG) (Stoner & Mukhtar, 1955). Black tea contain “theaflavin”, that has been reported to have various anticancer activities especially against intestinal cancer. (Caderni, 2000)

Molecular docking is commonly used practice in drug designing to understand drug-receptor binding. Compounds are first screened for their specific activities and then docked against respective protein target (Vijesh, Isloor, Telkar, Arulmoli, & Fun, 2013). Epidemiological studies, *in vivo* and *in vitro* studies open doors to find the mechanism of action and link between specific polyphenol and the proteins linked with them in the apoptotic pathways (Mukhtar, 2004). Active polyphenols found in these medicinal plants are epigallocatechin gallate, chlorogenic acic, oleuropein and miquelianin (Pleško, 2015). This is quite easy and effective way to predict some results without going into wet lab. Identify the target proteins and the expected ligands that bind with them and show good binding energy and after performing docking, the potential compounds can be analyzed and confirmed further using wet lab experiments.

Whenever a drug candidate is selected, it is tested a lot before coming to market. Testing is done by three methods; in vivo, in vitro and in silico analysis. In vitro testing is done in cell lines and this is the most critical step. Choosing a cell line, reviving it, growing it and then testing accordingly requires practice. All pharmacological companies must do in vitro testing before doing any animal model or human model trials (Dong et al., 2015). *HuH-7* is a hepatocellular carcinoma cell line that is functional with a p53 mutation. (Vecchi, Montosi, & Pietrangelo, 2009). Mostly HeLa, HepG2 and Huhh-7 cell lines are used depending upon availability and requirements. They don't show any preference over other, so any of them can be used to perform in vitro testing (Meex, Andreo, Sparks, & Fisher, 2010)

Food borne pathogens and diseases caused by them are increasing day by day. Bacteria are converting to super bugs because of being multi-drug resistant. A lot of research is going on to find out natural sources that can compete with MDR bacteria and inhibit their growth.

Polyphenols are a very rich and good source to inhibit various MDR bacterial strains (Taguri, Tanaka, & Kouno, 2004). Polyphenols extracted from medicinal plants, especially from green tea have proved to be good candidates in inhibition of MDR bacteria's growth. Various strains have been tested like; methicillin-resistant *Staphylococcus aureus* (MRSA) and multi-drug resistant *E. coli. Pseudomonas aeruginosa*, *Salmonella Typhi* and many other MDR bacterial strains and polyphenols have shown significant results on most of them. (Radji, Agustama, Elya, & Tjampakasari, 2013) a lot of research has been done related to antimicrobial activity of *Camellia sinensis* and significant results have been obtain against various strains. (Farooqui et al., 2015)

Oxidative substances, free radicals and other reactive species are a great cause of induction of oxidative stress in body. Antioxidants help in terminating the attack and reducing disease risk. Polyphenols present in medicinal plants may act as good antioxidant agents (Meena, Pandey, Pandey, Arya, & Ahmed, 2012). Plants rich in phenolic contents have showed a very high antioxidant activity. Medicinal plants can be a good source of releasing oxidative stress because of their high phenolic contents (Ramani, Sudini, Boddupalli, & Anisetti, 2012). When phenols are given as food supplements, they show good scavenging activity by various assay like DPPH, hydrogen peroxide and other reactive substances (Mišan et al., 2011)

Broad range of biologically active compounds and range of reported biological activities of *Z. Officinale* and *C. Sinensis* extracts make them strong candidate as medicinal plants to be tested against more types of cancers, microbes and antioxidants.

The current study was designed for comparative analysis of various biological activities of Ginger, green tea polyphenols and black tea polyphenols because a lot of data is available for all of the above mentioned herbs' anticancer and antimicrobial activity. First of all, polyphenols

that were expected to give desired anti-cancer activity, were docked against respective protein targets and suggested results showed that they have good binding and inhibition. To confirm the *in silico* results, *in vitro* analysis was done. For anti-cancer, the liver cell line was focused i.e., *HuH-7*, for antimicrobial five different bacterial strains were taken, i.e. *E. coli*, MRSA, *Shigella sonnei* , *Pseudomonas aeruginosa* and *Salmonella Typhi* while anti-oxidant activity was checked using hydrogen peroxide. All extracts gave good and expected results but best activities were shown by aqueous extracts of ginger and green tea.

Aims of Research:

The objectives of the study included:

- To determine and analyze active compounds in *Z. Officinale* and *C. Sinensis* extracts and check their mechanism of action through protein-ligand docking.
- To evaluate the comparative biological activities of *Z. Officinale* and *C. Sinensis* extracts. Focused activities were anticancer activity, anti-microbial activity and anti-oxidant activity.

CHAPTER 2

LITERATURE REVIEW

2.1. Medicinal Plants

It's a traditional therapy to use medicinal plants to cure different diseases since ancient days. Man has battled through agony and got mindfulness about restorative plants. Activity of therapeutic plants has been recognized by the science and they are incorporated into current pharmacotherapy (Efferth and Greten, 2014).

Herbs are for the most part characterized as any types of a plant or plant item including leaves, stems, blossoms, roots and seeds. Natural prescription is presently being utilized for treatment of different ailments in numerous nations. On the other hand, further research is required to recognize bioactivities of home grown fixings and elucidate atomic instruments of their activities for sane utilizations of herbs and home grown fixings. (Katiyar, Elmets, Agarwal & Mukhtar, 1995)

As per WHO, 80% of the total populace in creating nations depends on home grown meds (Kumar, 2014). 25% of the current meds are gotten from plants and about the same rate is the manufactured simple of phytochemicals (Kunle *et al.,* 2012). The information of therapeutic plants, along these lines, has profited the drug specialists and doctors to battle the difficulties of strength of man. Pakistan is honored with interesting botanical differences and diverse climatic zones.

WHO has arranged a technique to advance and improvement of customary pharmaceutical, which incorporates recognizable proof of therapeutic plants, creating participation amongst conventional and cutting edge drug specialists and development of herbs

to keep their decimation (Naseri, 2004). Plants e.g. Ginseng, Ginkgo, Saw palmetto and Ispaghol are well known for the treatment of a few issue (Alzaher, 2014). Distributed information about the restorative plants is expanding each day.

2.2. Medicinal Plants and Their Activities

Chronic disorders demonstrate the expanded levels of free radicals and (ROS) which are exceedingly receptive and can modify cell constituents (Alfadda, and Sallam, 2012). Plants are regular repositories of cell reinforcements and they are demonstrated to have critical cancer prevention agent exercises.

We can check numerous organic exercises of therapeutic plants refered to in the writing. Some of these exercises are recorded as under: Antimicrobial (Vashist and Jindal, 2012), Anti-malignancy (Sakarkar and Deshmukh, 2011), Anti-diabetic (Patel *et al.,* 2012), Anti-atherosclerosis (Ismail *et al.,* 2012), Immunomodulatory (Akram *et al.,* 2014), Reno-insurance (Musabayane, 2012), Hepatoprotection, (Kumar *et al.,* 2011).

To concentrate on the impacts of therapeutic plants against different ailments numerous creature models have been utilized. Creature models for Diabetes, Autoimmune encephalitis, Bowel infection, Hyperlipidemia, Arthritis, Hepatic and renal poisonous quality, Cataract and numerous viral ailments and so forth can be pointed in the writing (Rafieian-Kopaei, 2011).

WHO is meaning to incorporate restorative plants into social insurance in the nations where utilization of plants for medicinal designs is rehearsed (Nikam *et al.,* 2012). These nations ought to support research programs intended to find cutting edge drugs from phytochemicals. Enhanced division advancements and investigation strategies ought to be rehearsed to screen hostile to irresistible operators from plants.

A gainful utilization of plants can be in the generation of protein based treatments and immunizations on the grounds that few propose that pharmaceutical evaluation proteins are produced from plant sources. We have the greatest case of the outflow of HBV surface antigen in plants for the creation of subunit immunization and its fruitful testing in creatures and people (Guan *et al.*, 2014). Endeavors are being made to express infection like particles in plants (Thuenemann *et al.*, 2013).

Table 2.1: Reported Medicinal Plants and Their Uses

Medicinal Plant	**Common Name**	**Usage**	**Reference**
Medicago sativa	Alfalfa	lower cholesterol	Rose, 2002
Euphorbia hirta	Asthma-plant	Treat bronchitic asthma and laryngeal spasm	Katoh *et al.*, 1981
Astragalus propinquus	Astragalus	traditional Chinese medicine to strengthen the immune system	Astragalus, 2015
Berberis vulgaris	Barberry	skin ailments, scurvy and gastro-intestinal ailments	Barberry Shrub, 2005
Momordica charantia	Bitter gourd	reduce the blood glucose level	Baldwa *et al.*, 1977
***Vaccinium* blueberrie**	Blueberries	antioxidant	Prior *et al.*, 1998
Apium graveolens	Celery	diuretic	Gafner, 2004
Cinchona		source of alkaloids	Sadtler, 1918
Syzygium	Clove	treat toothache	Dabek & Martin,

aromaticum			1987
Vaccinium macrocarpon	Cranberry	urinary disorders, diarrhea, diabetes, stomach ailments, and liver problems	Kemper, 2006
Digitalis lanata	Digitalis	treating cardiac disease	Gibson, 1976
Eucalyptus globulus	Eucalyptus	analgesic	M. K, 2013
Zingiber officinale	Ginger	nausea	Szymanski, 2016
Citrus limon	Lemon	treating coughs and sore throat	Saitta *et al.*, 1997
Lavandula angustifolia	Lavender	mental health purposes	Lambin & Fagan, 2012
Papaver somniferum	Opium poppy	pain relief	Hillestad, 1980
Thymus vulgaris	Thyme	treat bronchitis and cough	Gagnier *et al.*, 2006
Triticum aestivum	Wheatgrass	antioxidant and anti-inflammatory compounds	Frank, 1994

A few reports recommend the utilization of unrefined plant extricates as cure as a large portion of the plants have low cytotoxicity. In any case, this sort of methodology can't get endorsement from association like sustenance and medication organization (FDA) of America. Along these lines, there ought to be a part of detachment, refinement and portrayal of dynamic item from the plants. Besides, different studies have additionally indicated hepatotoxicity

brought about by these plants separates in the rough shape (Fakurazi *et al.*, 2012). In this way, techniques ought to be made to address these issues also.

2.2.1. Medicinal Plants as Anti-Cancer Agents

Cancer, also called as an intimidating lump or lethal neoplasm, is basically a collection of various diseases that ultimately lead to a lump because of uncontrolled cell division with the possibility to attack or spread to different parts of the body. Not all tumors are carcinogenic; kind tumors don't spread to different parts of the body. There are more than 100 distinctive well reported, studied and diagnosed cancers that influence humans. Tobacco use is the reason for around 22% of cases. Another 10% is because of heftiness, a less than stellar eating routine, absence of physical action, and utilization of liquor. Different components incorporate certain contaminations, introduction to ionizing radiation, and ecological poisons. (Anand *et al.,* 2008)

It is surely understood that skin tumors (papilloma, keratoacanthomas, and squamous cell carcinomas) can be affected in mice by a complete carcinogenesis convention (cancer-causing agent given tediously) or by the consecutive utilization of a subthreshold measurements of a cancer-causing agent (start stage) trailed by dreary treatment with a frail or no carcinogenic tumor promoter (advancement stage). The start stage requires just a solitary utilization of either a direct or a circuitous cancer-causing agent at a subthreshold measurements and is basically irreversible, though the advancement stage is realized by redundant medications after start and is reversible for a timeframe however later gets to be irreversible. (Slaga, 1986)

Gastrointestinal (GI) tumors, for example, liver, gastric and colorectal diseases are a standout amongst the most widely recognized types of malignancies and represented around 25% of all growths from the appraisal by the United States (US) National Cancer Institute. Liver

tumor is the fifth most regular disease and the third most normal reason for growth related passing's on the planet. China has the most astounding rate of liver disease on the planet and represents more than 55% of all instances of essential liver growth. Albeit liver disease is extraordinary in the US, it is the speediest expanding reason for growth r elated passing's and has dramatically multiplied amid the previous two decades. Gastric (stomach) growth is the fourth normal disease and the second most basic reason for cancer related deaths on the planet. Like liver malignancy, gastric tumor has an extensive high frequency and death rates in China (half demise on the planet from China). Colorectal disease (CRC) is a standout amongst the most widely recognized and driving reason for growth related mortality in the Western world, positioned third in pervasiveness and lethality. CRC is normally determined further down the road to have most patients exhibiting after the age of 50. (Li *et al.*, 2014)

Non-melanoma skin tumors, including basal and squamous cell carcinoma, speak to the most widely recognized harmful neoplasms in people, especially in Caucasians. The improvement of compelling chemo preventive operators that can lessen or control the danger of UV-impelled skin malignancy is required to address this squeezing general wellbeing issue. (Mantana, 2015)

Lately concentrate on utilization of non-conventional ways to deal with treat sicknesses has been restored everywhere throughout the world. The proof gathered till now demonstrates tremendous capability of therapeutic plants utilized as a part of customary frameworks. The utilization of natural concentrates and healthful supplements either as option or complimentary solution to the ordinary chemotherapy for treatment of growth is all around archived in Ayurveda which is an option medicinal framework that has been rehearsed basically in the Indian subcontinent for a long time. *Andrographis paniculata* (Acanthaceae), additionally referred to

usually as "kalmegh," is a surely understood therapeutic plant of Ayurveda and has been utilized for quite a long time as a part of Asia. (AJAYAKUMAR, 2004)

Lately, there has been an extraordinary enthusiasm for the utilization of dietary supplements as reciprocal and option drugs that are gotten from normally happening botanicals for the counteractive action of UV photo damage, including skin tumor hazard. Herbal supplements, particularly dietary botanicals, having calming, immunomodulatory, and cell reinforcement properties are among the most encouraging gathering of intensifies that can be abused as perfect chemo-preventive operators for skin tumor counteractive action. (Wang, Agarwal, Bickers & Mukhtar, 1991)

In spite of the fact that the rates of new malignancy cases and growth mortality are falling by and large as anticipation practices have enhanced, the quantity of individuals determined to have tumor inside of the following couple of decades is still anticipated that would twofold. The American Cancer Society (ACS) predicts that in the United States around 552,000 passing's will happen from malignancy this year, with lung growth being the main source of disease mortality, trailed by colorectal, bosom, and prostate tumors. These four sorts of malignancy record for more than one portion of every single disease passing in the U.S. Despite the fact that these are so be ring measurements, it is currently realized that no less than 33% of every malignancy case could be maintained a strategic distance from through the modification of certain wellbeing practices (ACS, 2002; World Cancer Research Fund, 1997).

In this way, there is an expanding interest for novel demonstrative and restorative treatments that use non-conventional sources. It is in this area that bioactive proteins, including lectins, might assume an imperative part. Epidemiological studies show that the utilization of a

plant-based eating routine is emphatically connected with a diminished danger of adding to specific sorts of tumor. (De Mejía & Prisecaru, 2005)

This might be because of the way that plants contain various physiologically dynamic segments, or phytochemicals, that can change the biochemical pathways connected with growth start, advancement, or movement. Among the phytochemicals that are as a rule seriously concentrated on for their part in tumor chemoprevention are the lectins. Plant lectins are a significant gathering of naturally dynamic glycoproteins that have been appeared to have tumor chemo preventive action *in vitro*, in vivo and in human contextual analyses. Lectins are as of now being utilized as remedial operators in malignancy treatment contemplates. The point of this article is to assess the part of plant lectins as potential growth chemo preventive specialists. The reader is alluded to a late production for specific data on soybean lectins. (De Mejía & Prisecaru, 2005)

2.2.2. Medicinal Plants as Anti-microbial agents

Medicinal plants have been used as anti-microbial agents since ages. Screenings were done of a few plants of significance in the Ayurvedic arrangement of conventional solution utilized as a part of India to treat enteric infections. . (Locher *et al.,* 1995)

Aqueous and methanol extracts from various parts of nine conventional Zulu restorative plants, of the Vitaceae from KwaZulu-Natal, South Africa were assessed for remedial potential as calming and hostile to microbial operators. Of the twenty-nine rough concentrates examined for prostaglandin combination inhibitors, just five methanol extracts of *Cyphostemma natalitium*-root, *Rhoicissus digitata*-leaf, *R. rhomboidea*-root, *R. tomentosa*-leaf/stem and *R. tridentata*-root indicated critical hindrance of cyclo-oxygenase (COX-1). The concentrates of R. digitata-leaf

and of R. rhomboidea-root showed the most noteworthy restraint of prostaglandin blend with 53 and 56%, individually. The outcomes propose that *Rhoicissus digitata* leaves and of *Rhoicissus rhomboidea* roots may can possibly be utilized as mitigating operators. All the screened plant removes demonstrated a few degrees of hostile to microbial action against Gram-positive and Gram-negative microorganisms. (Lin *et al.*, 1999)

Chosen plants having a past filled with use in Polynesian customary medication for the treatment of irresistible illness were researched for hostile to viral, against contagious and hostile to bacterial movement in vitro. This study has affirmed a portion of the ethnobotanical reports of Hawaiian therapeutic plants having healing properties against diseases utilizing natural tests as a part of vitro. (Rani and Khullar, 2004)

Around 26 distinctive polyhedral details of this plant are specified in Ayurveda as a well-known solution for the treatment of different issue. Andrographis paniculata is a yearly shrub becomes inexhaustibly in India and developed widely in China and Thailand. The ethereal parts of the plant (leaves and stems) are utilized to remove the dynamic phytochemicals. The plant concentrate is known not triterpenes, flavonoids and stigma sterols. Broad examination of the most recent couple of decades has uncovered that the natural concentrate is valuable as against inflammatory, antiviral, antithrombotic, anticancer immune-stimulatory, hypoglycemic and hypotensive operation. (AJAYAKUMAR, 2004)

In the indigenous social insurance conveyance arrangement of Ethiopia, various plant species are utilized to treat illnesses of irresistible starting point. Despite the quantity of species, if any of such claims could be confirmed deductively, the potential importance for the change of the medicinal services administrations would be considerable. The target of this study was,

hence, to decide the nearness of hostile to microbial action in the unrefined concentrates of a portion of the regularly utilized therapeutic plants and also to distinguish the class of mixes in the plants that were subjected to such screening. The plants containing a greater amount of these metabolites exhibited more grounded hostile to microbial properties focusing on the requirement for further examinations utilizing fractionated separates and filtered concoction parts. (Geyid *et al.,* 2005)

Medicinal plants have numerous conventional cases including the treatment of afflictions of irresistible starting point. In the assessment of conventional cases, logical research is critical. The goal of the study was to decide the nearness of antibacterial action in the unrefined concentrates of a portion of the generally utilized therapeutic plants as a part of Malaysia, *Andrographis paniculata, Vitex negundo, Morinda citrifolia, Piper sarmentosum,* and *Centella asiatica*. In this preparatory examination, the leaves were utilized and the rough concentrates were subjected to screening against five strains of microscopic organism's species, *Methicillin Resistant Staphylococcus aureus* (MRSA), *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli,* utilizing standard convention of Disk Diffusion Method (DDM). The antibacterial exercises were surveyed by the nearness or nonattendance of restraint zones and MIC values. *M. citrifolia, P. sarmentosum* and *C. asiatica* methanol extricate and *A. paniculata* (water extricate) have potential antibacterial exercises to both gram positive *S. aureus* and Methicillin Resistant *S. aureus* (MRSA). (Zaiden *et al.,* Al, 2005)

Improvement of gastric malignancy relies on upon complex communications between a few bacterial, host hereditary and ecological components, for example, Helicobacter pylori, polymorphisms of cytokine qualities and nitrosamines. Gastric tumor still remains a noteworthy wellbeing issue in spite of late advances of methods and advances of endoscopy. Despite the fact

that chemotherapy altogether enhances survival in patients with built up gastric growth , extra operators powerful against gastric disease are still expected to increment accessible regimens and development the viability of chemotherapy (Ishiguro *et al.*, 2007).

2.2.3. Medicinal Plants as Anti-Oxidants

Research showed that about two-third of world's plants possess medicinal activities and most prominent one is Anti-oxidant activity. This help in reducing oxidative stress hence preventing a lot of chronic diseases like cardiovascular diseases, various types of cancers, diabetes and many others. Antioxidant potential of these medicinal plants have been found eve equivalent to the synthetic anti-oxidants (Krishnaiah, Sarbatly, & Nithyanandam, 2011). Key compounds in most of the medicinal plant is found to be phytochemicals that are further divided into sub-categories. These versatile phytochemicals possess number of pharmacological properties and the main things about them is that they are least toxic to body (Bhatt, Rawat, & Rawal, 2012).

Factors affecting a plant's anti-oxidant properties are climate, area, seasonal change, environmental conditions, geographical regions, degree of ripeness and other treatments and processing. So plants from different area may show different levels of anti-oxidant potential. Most studied plants species for anti-oxidant properties are Laminacae, Apiaceae and Zingiberaceae (Škrovánková, Mišurcová, & Machů, 2012). Practice of using medicinal plants as herbs, without proper testing, is increasing day by day which is not a healthy practice. Proper assay method, toxicity testing and extraction methods should be followed prior to any use of them (Bhatt, Rawat, & Rawal, 2012; Škrovánková, Mišurcová, & Machů, 2012).

2.2.4. Medicinal Plants as Anti-Viral Agents

Therapeutic plants having hostile to viral potential against numerous infections have been accounted for including HIV, HSV, Human flu infection, HBV, HCV, pox infection and SARS infection (Liu and Du, 2012). Some plant separates have indicated hostile to viral impacts against the strains which are impervious to customary antiviral specialists (Rajasekaran *et al.,* 2014). Components required in hostile to viral movement may fluctuate among various infections. Some plant extricates show immune-stimulatory capacities e.g. Heracleum greatest animated interlukin-6 affirming its immune-stimulatory capacity (Verma *et al.,* 2015). Other than immune-stimulatory work a few plants may contain phytochemicals which show antiviral properties e.g. Pandanin which is a lectin detached from Pandanus amaryllifolius had antiviral impact against HSV (Dhawan, 2012). Some unrefined concentrates of some different plants have additionally indicated antiviral properties.

2.3. Plants Used For Analysis

There are a lot of medicinal plants used for medicinal purpose and most of them are from Laminacae, Apiaceae, Theaceae and Zingiberaceae family. (Škrovánková, Mišurcová, & Machů, 2012).

2.3.1. *Zingiber officinalis*

This aromatic herb is known by various names throughout the world, but one universal name that is designated by a Swedish botanist Carl Linnaeus, is *Zingiber officinale*. This logical name can be utilized to characterize a native plant found and widely used in Asia: ginger.

Ginger, the rhizome of *Zingiber officinalis*, a standout amongst the most generally utilized types of the ginger family, is a typical fixing for different sustenance and refreshments. Ginger has a long history of restorative use going back 2500 years. Ginger has been generally utilized from time immemorial for changed human sicknesses in diverse parts of the globe, to help processing and treat stomach surprise, looseness of the bowels, and queasiness.

Table 2.2: Taxonomy of ***Zingiber officinalis***

Name:	Zingiber officinalis
Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Liliopsida
Order:	Zingiberales
Family:	Zingiberaceae
Genus:	Zingiber Mill.
Species:	*Zingiber officinale*

Some sharp constituents present in ginger and different zingiberaceous plants have strong cell reinforcement and mitigating exercises, and some of them display growth preventive movement in test carcinogenesis. The anticancer properties of ginger are ascribed to the vicinity of certain sharp vallinoids, viz. [6]-gingerol and [6]-paradol, and also some different constituents like shogaols, zingerone and so on. Various systems that may be included in the chemo-

preventive impacts of ginger and its parts have been accounted for from the research facility thinks about in an extensive variety of trial models. (Shukla & Singh, 2007)

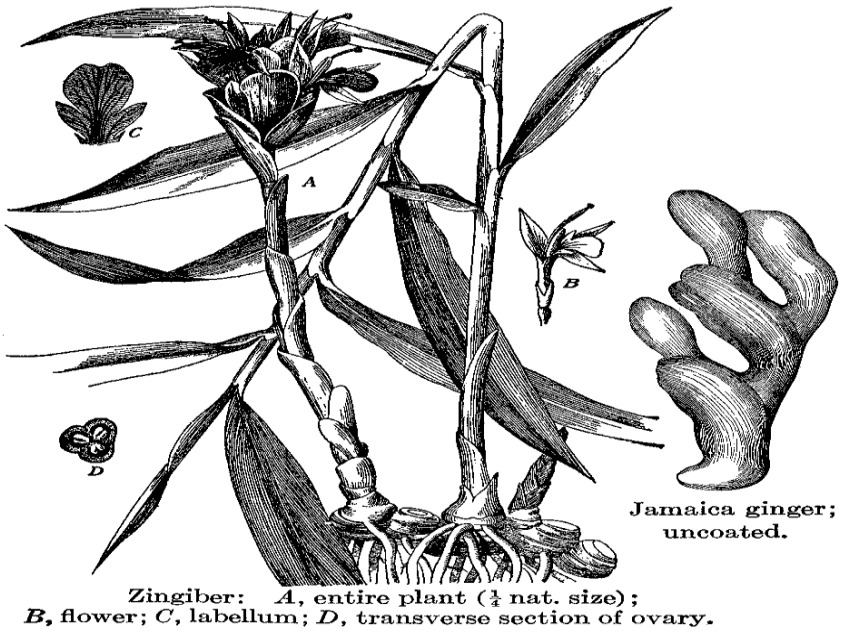


Figure 2.1: Illustration presentation of *Z. officinale* flora in Pakistan

GINGER (*Zingiber officinale* Roscoe) has a long history of restorative use.1,2 In customary Chinese and Indian drug, ginger has been utilized to treat an extensive variety of diseases including stomachaches, looseness of the bowels, queasiness, asthma, respiratory disarranges, toothache, gingivitis, and arthritis.2,3 Today, ginger and its concentrates are suggested by home grown experts fundamentally for dyspepsia and the counteractive action of movement sickness.4 various late studies have recharged enthusiasm for ginger for the treatment of unending provocative conditions. (Grzanna, Lindmark, & Frondoza, 2005)

These studies revealed an impact of ginger on the generation of cytokines that are incorporated and discharged at destinations of irritation. These atoms have turned out to be exceptionally encouraging focuses for the treatment of endless incendiary disorders.11 The

preclinical discoveries on instruments by which ginger creates its belongings are supplemented by late clinical trials that backing the conventional perspective that ginger has pain relieving and calming properties.12–15 Results from clinical trials, observational studies, and case writes about the therapeutic utilization of ginger can be found on the Internet.12 This audit contains a record of the logical information on ginger gathered in the course of recent years. These information firmly bolster the generally held perspective that ginger is an important therapeutic item for the treatment of endless provocative condition. (Grzanna, Lindmark, & Frondoza, 2005)

2.3.2 *Camellia sinensis*

Camellia sinensis is the types of plant whose leaves and leaf buds are utilized to create the well-known refreshment tea. It is of the type Camellia a sort of blooming plants in the family Theaceae. White tea, green tea, oolong, pu-erh tea and dark tea are all reaped from this species, yet are prepared contrastingly to accomplish distinctive levels of oxidation.

Table 2.3: Taxonomy of *Camellia sinensis*

Name:	*Camellia sinensis*
Kingdom:	Plantae
Division:	Tracheophyta
Class:	Magnoliopsida
Order:	Ericales
Family:	Theaceae
Genus:	Camellia

Species:	*Camellia sinensis*

Kukicha is additionally collected from *Camellia sinensis*, however utilizes twigs and stems as opposed to takes off. Basic names incorporate tea plant, tea bush, and tea tree (not to be mistaken for Melaleuca alternifolia, the wellspring of tea tree oil).

Tea is especially rich in polyphenols, including catechins, theaflavins and thearubigins, which are thought to add to the wellbeing benefits of tea. Tea polyphenols go about as cancer prevention agents in vitro by rummaging receptive oxygen and nitrogen species and chelating redox-dynamic move metal particles. They might likewise work in a roundabout way as cell reinforcements through 1) restraint of the redox-touchy translation variables, atomic component B and activator protein-1; 2) hindrance of "expert oxidant" catalysts, for example, inducible nitric oxide synthase, lipoxygenases, cyclooxygenases and xanthine oxidase; and 3) affectation of stage II and cancer prevention agent compounds, for example, glutathione S-transferases and superoxide dismutase. (Higdon & Frei, 2003)

Figure 2.2: Illustration presentation of *C. sinensis* flora in Pakistan

The way that catechins are quickly and broadly metabolized stresses the significance of exhibiting their cancer prevention agent movement in vivo. Creature thinks about offer a one of a kind chance to evaluate the commitment of the cancer prevention agent properties of tea and tea polyphenols to the physiological impacts of tea organization in various models of oxidative anxiety. (Higdon & Frei, 2003)

Tea is developed in around 30 nations and, besides water, is the most generally expended drink on the planet. Tea is made as either green, dark, or oolong; dark tea speaks to roughly 80% of tea items. Epidemiological concentrates, however uncertain, propose a defensive impact of tea utilization on human tumor. Exploratory investigations of the ant mutagenic and ant carcinogenic impacts of tea have been directed essentially with green tea polyphenols (GTPs). GTPs display ant mutagenic action in vitro, and they repress cancer-causing agent actuated skin, lung, forestomach, throat, duodenum and colon tumors in rodents. What's more, GTPs hinder TPA-impelled skin tumor advancement in mice. (Stoner & Mukhtar, 1995)

Albeit a few GTPs have ant carcinogenic action, the most dynamic is (–) -epigallocatechin-3-gallate (EGCG), the significant constituent in the GTP portion. A few components have all the earmarks of being in charge of the tumor-inhibitory properties of GTPs, including upgrade of cancer prevention agent (glutathione peroxidase, catalase and Quinone reductase) and stage II (glutathione-S-transferase) catalyst exercises; hindrance of synthetically instigated lipid peroxidation; restraint of light and TPA-incited epidermal ornithine decarboxylase (ODC) and cyclooxygenase exercises; hindrance of protein kinase C and cell multiplication; mitigating action; and improvement of crevice intersection intercellular correspondence (Stoner & Mukhtar, 1995)

As of late, polyphenols from green tea (GTP) have pulled in significant consideration on account of their skin photo-protective impacts. Green tea (*Camellia sinensis*) contains polyphenols, which have increased awesome enthusiasm because of their intense cell reinforcement exercises. GTP has been appeared to have amazing preventive impacts against photo-toxicity in murine models and additionally in people. Oral bolstering of GTP to bald mice as a sole wellspring of drinking water took after by UV illumination brought about critical insurance against UV-prompted cutaneous edema and restraint of cyclooxygenase action and their metabolites. Topical utilization of GTP to mouse skin before UV introduction diminished the UV-instigated hyperplastic reaction, edema, myeloperoxidase movement and concealment of contact excessive touchiness. Studies have as of late demonstrated that treatment of (-)-epigallocatechin-3-gallate (EGCG), a polyphenolic constituent of green tea, even without UV presentation specifically restrains the statement of MMP, for example, MMP-9, MMP-2, MT1-MMP and neutrophil elastase at pharmacologically achievable concentrations. (Vayalil, Mittal, Hara, Elmets & Katiyar, 2004).

CHAPTER 3

MTERIALS AND METHODS

3.1 Molecular Docking

Docking was done using an automated software called “Swiss Dock”. The purpose of this was to find the binding energy and binding sites of the ligand to suggest its mechanism of action. Most active compound in both the species was picked from literature review and its chemical structure is taken from available data. Their expected targets were also taken using literature survey. 3D structure in pdb format of CDK-1 and RTK proteins were taken from PDB. Chimera UCSF along with DOCKPrep was used to edit the structure and removal of extra water molecules and unnecessary ligands. Both the ligand and target proteins were added in the software “Swiss Dock” and run for docking

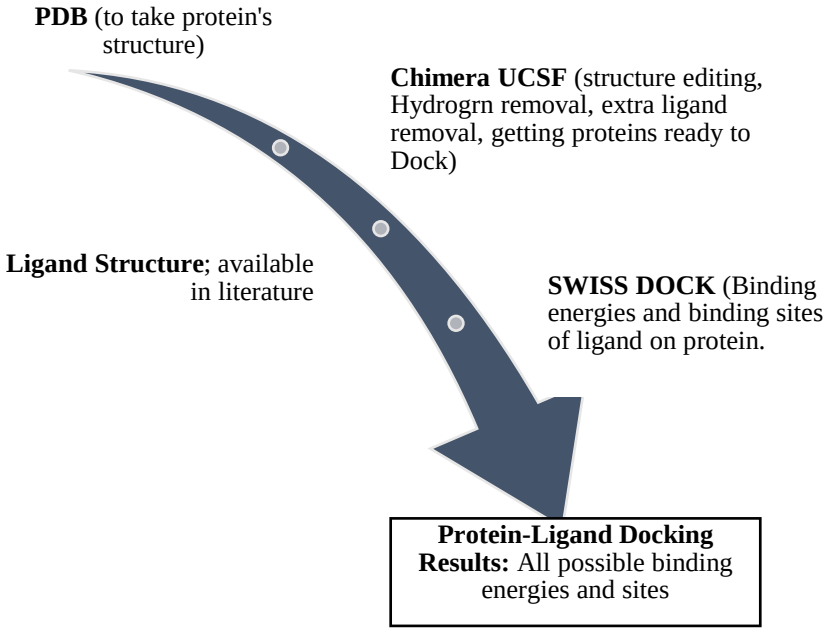


Figure 3.1: Schematic representation of docking process

Proteins chosen were Receptor Tyrosine Kinase (RTK) and Cyclin D-1. RTK is transcribed by two long regions and it has two chains. One is "immunoglobulin-like domains 1 and 2 of the receptor tyrosine kinase MuSK", PDB id; 2iep which is a Cyclin C-2 symmetrical Homo 2-mer - A2. The other is "MuSK Tyrosine Kinase", PDB id; 1luf which is asymmetric monomer. CD-1 is "Crystal structure of HHM", PDB id; 3ay5, effective in cell cycle and it is an asymmetric monomer.

All possible binding sites of the ligand along with their binding energy are found out through Docking. Protein's extracellular and intracellular domains were found out using CCTOP and were checked for their binding and active site in both of these domains. Binding and active site are available on UniProt. The best hits were chose for further analysis.

3.2. Plant Material

Zingiber officinale (ginger) and *Camellia sinensis* (tea) were collected from Kashmir, Pakistan. Roots and leaves of the plants were dried under shade at room temperature and were ground to fine powder. Passed through sieve of 80 mesh and very fine powder was obtained.

3.3. Extraction

Grinded fine powder of plant material was subjected to extraction using methanol and water as two different solvents ensuring extraction at different polarities. Extraction was done using two different methods i.e., Maceration and Soxhlets Extraction.

3.3.1 Maceration

Weighed amount of plant material i.e. 10 gram was soaked in the solvent (100 ml) for 24 hours covered with aluminum foil to avoid the escape of any phytochemical, on a shaker at 37ºC. Solvents were filtered. Those filtered solvents were then evaporated to dryness using the rotary

evaporator. The temperature of the evaporator was set according to the solvents i.e., for water 80ºC and for methanol, it was 50ºC. The concentrated extracts were then dried in an incubator (Mermet GmbH, Germany) at 37°C for 72 hours as previously described by Yang *et al.*, (2013). The extracts were stored at 4ºC in Eppendorf tubes covered in aluminum foil.

3.3.2 Soxhlet Extraction

Second method used for extraction is soxhlet extraction. It is a very efficient method as it doesn't allow any impurity. It has four major steps for extraction. A percolator is a boiler to circulate solvent, a thimble which has thick filter paper to retain solid sample, a distillation flask to carry the solvent and a siphon mechanism that empties the thimble from the solvent. The soxhlet apparatus has six assemblies which were all used simultaneously to extract 6 types of plant samples. Each sample was placed in each assembly, temperature was set and left overnight for extraction. Extracted solvent was then collected, dried on rotary evaporator and dried in incubator. Extracts were stored at 4ºC, covered.

Figure 3.2: Extraction using Soxhlet apparatus

3.4. Culturing *HuH-7* Cell Line

Human Hepatocellular carcinoma cell line (*HuH-7*) was taken from cell culture bank at ASAB, NUST. Growth and maintenance media used for cell line was Dulbecco's Modified Eagle Media (DMEM) (High Glucose) (Gibco®, Life Technologies).

3.4.1. Media Preparation

DMEM prepared media from Gibco®, Life Technologies was used. Color of fresh pure media is dark pink. pH of media was 7.2-7.4, measured using the pH meter. Heat inactivated, sterile 10% Fetal Bovine Serum (FBS) was also added in 10% of the total media. An antibiotic cocktail comprising penicillin and streptomycin (Sigma Aldrich, USA) was added in ratio of 1% of the total prepared media. Almost 5ml media was shifted to a $25cm^2$ tissue culture flask and incubated at 37ºC and in an incubator overnight to check for contamination.

3.4.2. Revival of Cryopreserved Cell Lines

Cryovials taken from cell bank at ASAB, containing cell suspension and cryoprotectant solution. It was revived by thawing the vial first and then by adding 5mL fresh media in 15ml falcon, centrifuged at 4ºC at 1000 rpm for 5 minutes. Pallet contained cells while supernatant was discarded as it contained cryoprotectant. Cells were suspended in 1ml fresh DMEM containing 10% FBS, called the growth media. Later it was seeded into two $25cm^2$ tissue culture flasks having 5ml fresh media. Flasks were checked at regular intervals to record cell growth and confluency under an inverted microscope.

3.4.3. Sub-Culturing of Cell Line

Cell culturing was done in 25cm^2 tissue culture flask. Confluency was observed using an inverted microscope. Cells were split thrice in 1:2, every time when the confluency reached 80%. Splitting method used was the standard method for cell culturing and splitting. Consumed media was discarded from the flask. 2ml autoclaved Phosphate Buffered Saline (PBS) (0.01M) was added. After PBS washing, 1ml Trypsin-EDTA (Gibco, Life technologies) was added to the flask. The flask was left at 37ºC for 5 minutes. Cells detachment was made sure by mild tapping on the flask with palm and fingers. When the cells were released in the suspension and desired number was achieved, 2ml fresh media was added in this. Media was transferred in two 1.5ml Eppendorf tubes and centrifuged at 500*g for 5 minutes in a centrifuge machine (5810R, Eppendorf, Germany). Pellet was suspended in 1ml DMEM containing 10% FBS while supernatant was discarded. The suspended pellet from both the Eppendorf tubes was transferred in two 25cm^2 tissue culture flasks. Prepared DMED was added in both the flasks, 5ml each and the flasks were placed at 37ºC in a 5% CO_2 incubator. The flasks were observed for confluency under an inverted microscope at 10X magnification power.

3.4.4. Cryopreservation

Cryopreservation was done to be on safe side and avoid any cell loss or instability. A cryoprotectant solution was used which was made using 9 parts Fetal Bovine Serum (FBS) and 1 part Dimethyl sulfoxide (DMSO) (10%, Merck, Germany). Total of 10mL solution was made. The cell suspension left after splitting the cells was mixed with the cryoprotectant solution. Afterwards 1.5mL of the mixture of cell suspension and cryoprotectant solution was dispensed in

cryovials. The vials were placed on ice for 15-20 minutes, then at -20ºC for 2 hours and then shifted to -80ºC for long storage. This gradual shift is to make cells used to of the temperature.

3.5. MTT Assay (Methylthiazoletetrazolium)

3.5.1. Sample Preparation

A stock solution of each extract was prepared in fresh media DMEM containing 10% FBS, keeping the concentration 20mg/ml. Stock solution was then filter sterilized using 0.2µm syringe filter.

3.5.2 Assay

MTT solution was made using 5mg/ml in phosphate buffer saline (PBS). 0.2 µm syringe filters were used to filter the solution and stored at 0-4ºC. 100µl cells were added in each well of a 96 well plate and incubated for 24 hours. Varying concentrations of extracts were added and incubated for another 24 to 72 hours at 37ºC and a lane was left without any extract as control and a lane with plain media as blank. 20µl of MTT Reagent was added to each well, including controls and blank. Incubation was done for 3 hours until purple precipitates were visible. Then 100µl of DMSO was added to all wells, including controls and blanks. Plate was covered and left at room temperature for an hour. Absorbance was recorded at 570nm in a microplate reader. Average values from duplicate wells were taken and blank value was subtracted. Cell viability was calculated and turned to percentage viability via formula. A graph was plotted against percentage viability and increasing extracts concentration.

3.6. Antibacterial Activity

3.6.1 Sample Preparation

A stock solution of each extract was prepared in fresh media DMEM containing 10% FBS, keeping the concentration 20mg/ml. Stock solution was then filter sterilized using 0.2µm syringe filter.

3.6.2 Collection of Bacterial Strains

Five clinical drug resistant bacterial strains of *E. coli*, MRSA, *Shigella sonnei*, *Pseudomonas aeruginosa* and *Salmonella Typhi* were taken from stocks made by nanotechnology lab of Department of Industrial Biotechnology, ASAB. The strains were isolated form patients in Armed Forces Institute of Pathology (AFIP), Rawalpindi. Strains were isolated using swabs sample from pus. Standard protocol of sample treatment defined by AFIP was used. Differential media was used for pure colonies. After checking the antibiotic resistance profile, pure strains were preserved in 50% glycerol stock solutions and stored at -80°C.

3.6.3 Preparation of Media

Media used was Nutrient Agar (Merk) in distilled water (28 g/L), sterilized by autoclaving it according to manufacturer's instructions. Petri plates were autoclaved and sterilized media was poured in them in a laminar flow hood cabinet to avoid any contamination. Plates were sealed and placed in an incubator overnight at 37ºC to assure the sterility.

3.6.4 Inoculation of Bacterial Strains

Stored bacterial strains were streaked on the Nutrient agar media and left in an incubator at 37ºC overnight to let the colonies grow. Inoculation was done using standard procedures that include picking up colonies with a sterile inoculation loop and streak them on petri plates, already checked for contamination. The streaked petri plates were left for 16 hours in an incubator at 37ºC.

3.6.5. Antibacterial Disc Diffusion Assay

Extracts were checked for their Bactericidal ability using disc diffusion assay. The assay was performed using standard protocol set for Antibacterial Assay. It includes a laminar flow cabinet which was made sure sterile by turning on UV for 15 minutes prior to any experimentation. Next a saline solution was made of 0.9% and poured in sterile tubes in the hood. A single pure colony was picked with an inoculation loop from petri plates grown for 16 hours and dipped in saline solution to prepare the bacterial inoculum for the assay. Petri plates were ready with nutrient agar media in them and checked for contamination. 100µl of the bacterial inoculum was picked using micropipette and poured on the petri plate. A spreader was used and plates were rotated 360º to ensure even spreading. Whattman Filter Paper Grade 4 was used to prepare filter discs of 6mm diameter, sterilized by autoclave and placed in petri plates at points that were already marked for each dilution of extract. Same procedure was used for all 5 bacterial strains and plates were made in triplicates. About 10 µl of each dilution of extract was then dispensed on the plates and allowed for absorption by filter paper discs. Deionized water was used as a negative control for disc diffusion assay. After dispensing plates for disc diffusion assay, the plates were then incubated at 37ºC overnight.

3.7. Hydrogen Peroxide Scavenging Capacity Assay

3.7.1 Extract Preparation

Extracts of *Z. Officinale* and *C. Sinensis* were analyzed for their ability to scavenge hydrogen peroxide. Phosphate buffer of pH 7.4 was used to make 40mM solution of hydrogen peroxide. Different concentrations of extracts were made in distilled water and were added to 0.6 mL of 40mM hydrogen peroxide solution.

3.7.2 Scavenging Assay

The protocol followed was the one followed by Ruch *et al.,* in 1989. The prepared extracts were placed for 10 minutes incubation at room temperature and absorbance was checked at 230nm against a blank solution containing plain phosphate buffer solution. The percentage of hydrogen peroxide scavenging of extracts of both *Z. Officinale* and *C. Sinensis* and the standard BHA were calculated using formula:

$$\% \text{ Scavenged } [H_2O_2] = [(AC - AS)/AC] \times 100$$

Where AC is the absorbance of the control and AS is the absorbance in the presence of the sample of *Z. Officinale* and *C. Sinensis* extracts or standards.

3.8 Statistical Analysis

Docking results were obtained and interpreted using Swiss Dock, JSmol (visualizer), Chimera UCSF and CCTOP. Further results obtained by *in vitro* analysis were interpreted using Microsoft Excel 2013 edition. Graphs were made and compared with each other.

CHAPTER 4

RESULTS

4.1 Molecular Docking

Two active chemicals present in target medicinal plants were selected using literature and were docked against the potential protein targets. Structures were obtained from PDB.

4.1.1 Receptor Tyrosine Kinase with Epigallocatechin gallate

Epigallocatechin gallate was checked against RTK. It has two chains and both were docked with ligand. The binding site is at position 608 and the active site of protein is at 724 position. Chain A is from 1-280 amino acids while second chain is from 560-888 amino acids. PDB id of chain A is 2iep and chain B is 1luf. Their UniProtKB reference is Q62838.

Structure of Epigallocatechin gallate is taken from available literature. This is used as a ligand present in *C. Sinensis* and have tendency to bind with Receptor Tyrosine Kinase.

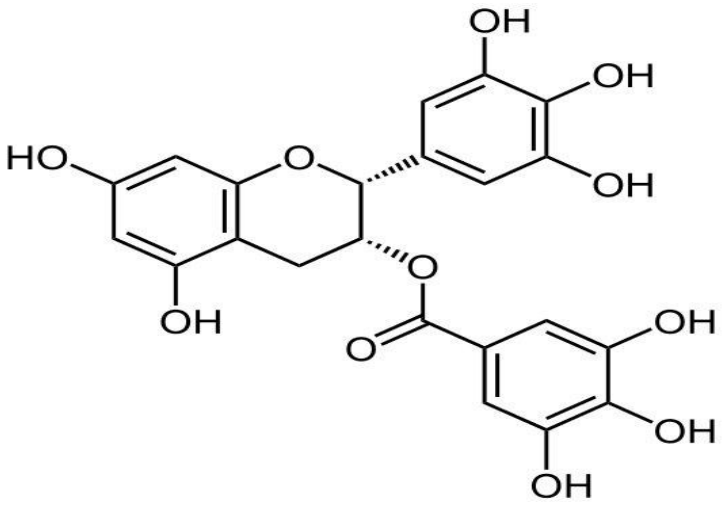
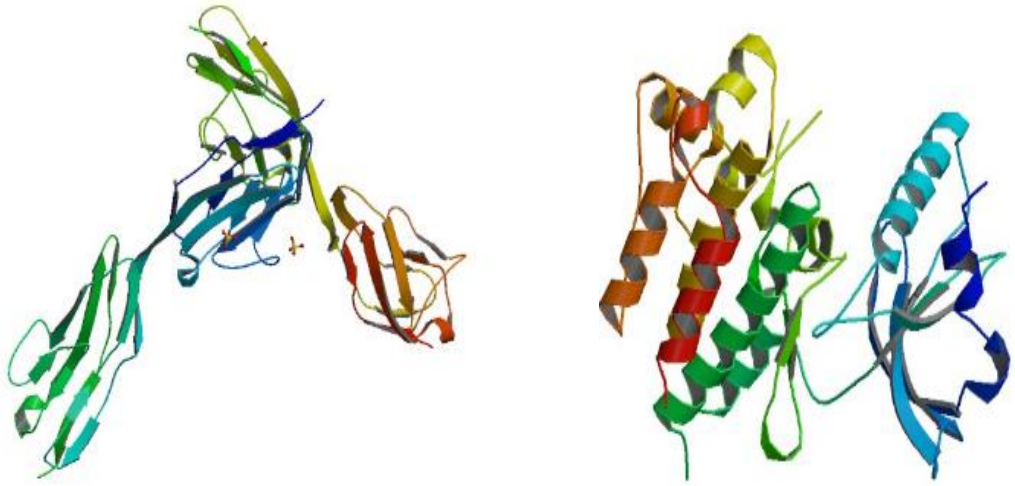
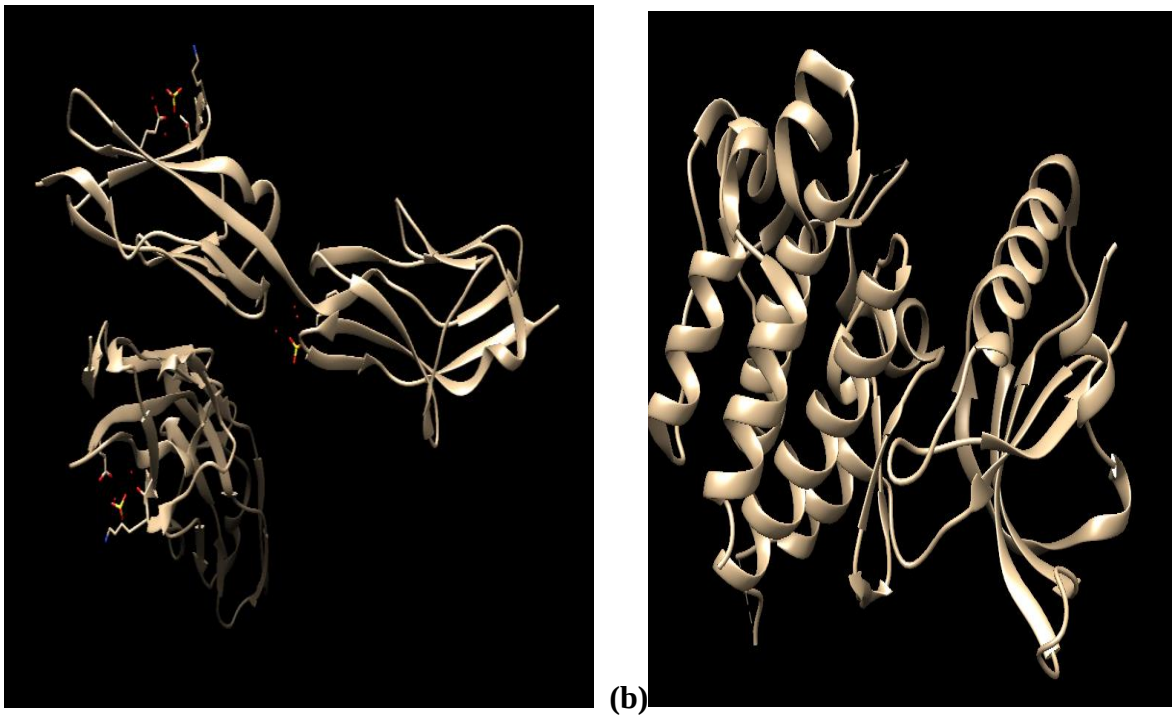


Figure 4.1: chemical structure of egcg having chemical formula of $C_{22}H_{18}O_{11}$



(a) **(b)**

Figure 4.2: PDB view of RTK chains (a) Chain A (translated by AA 1-288) (b) Chain B (Translated by AA 560-888)



(a) **(b)**

Figure 4.3: Chain views of RTK using Chimera UCSF (a) Dimeric Chain A (b) Monomeric Chain B

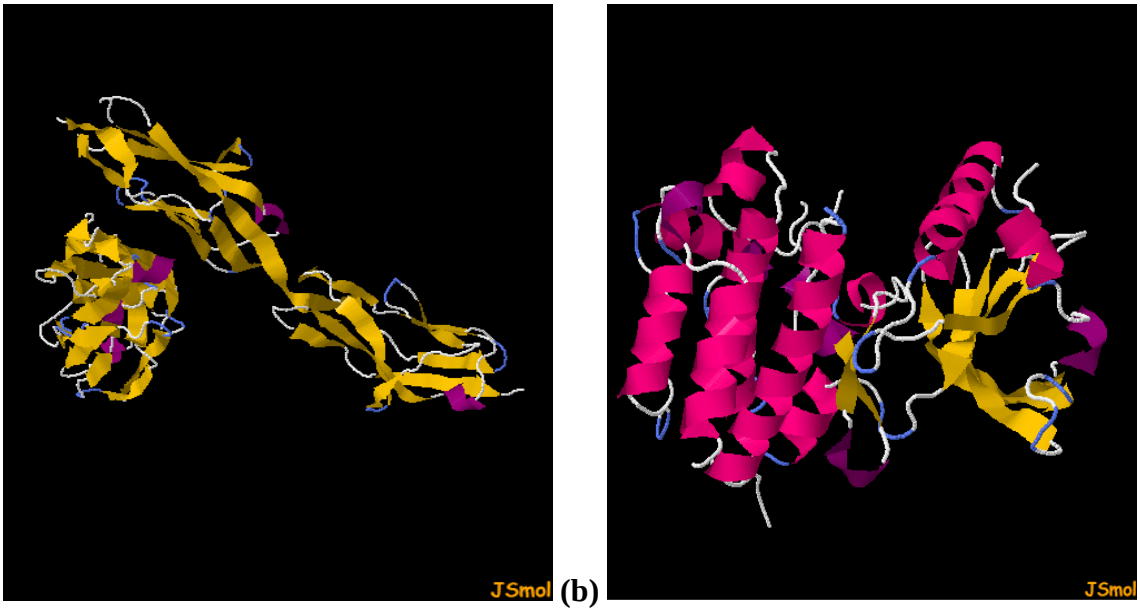


Figure 4.4: protein after docking. JSmol viewer. (a) Chain A (b) Chain B

Table 4.1: binding energies of best hits of RTK both chains.

Cluster	**Element**	**FullFitness (kcal/mol)**	**Estimated ΔG (kcal/mol)**	**Binding site**
RTK chain A (AA 1-200) (PDB id: 2iep)				
0	0	-2446.55	-7.31	125
0	1	-2444.41	-7.69	124
2	2	-2439.67	-7.19	200
RTK chain B (AA 560-860) (PDB id: 1luf)				
2	0	-1438.70	-8.33	660
2	1	-1437.45	-8.36	746
2	2	-1434.66	-7.85	657

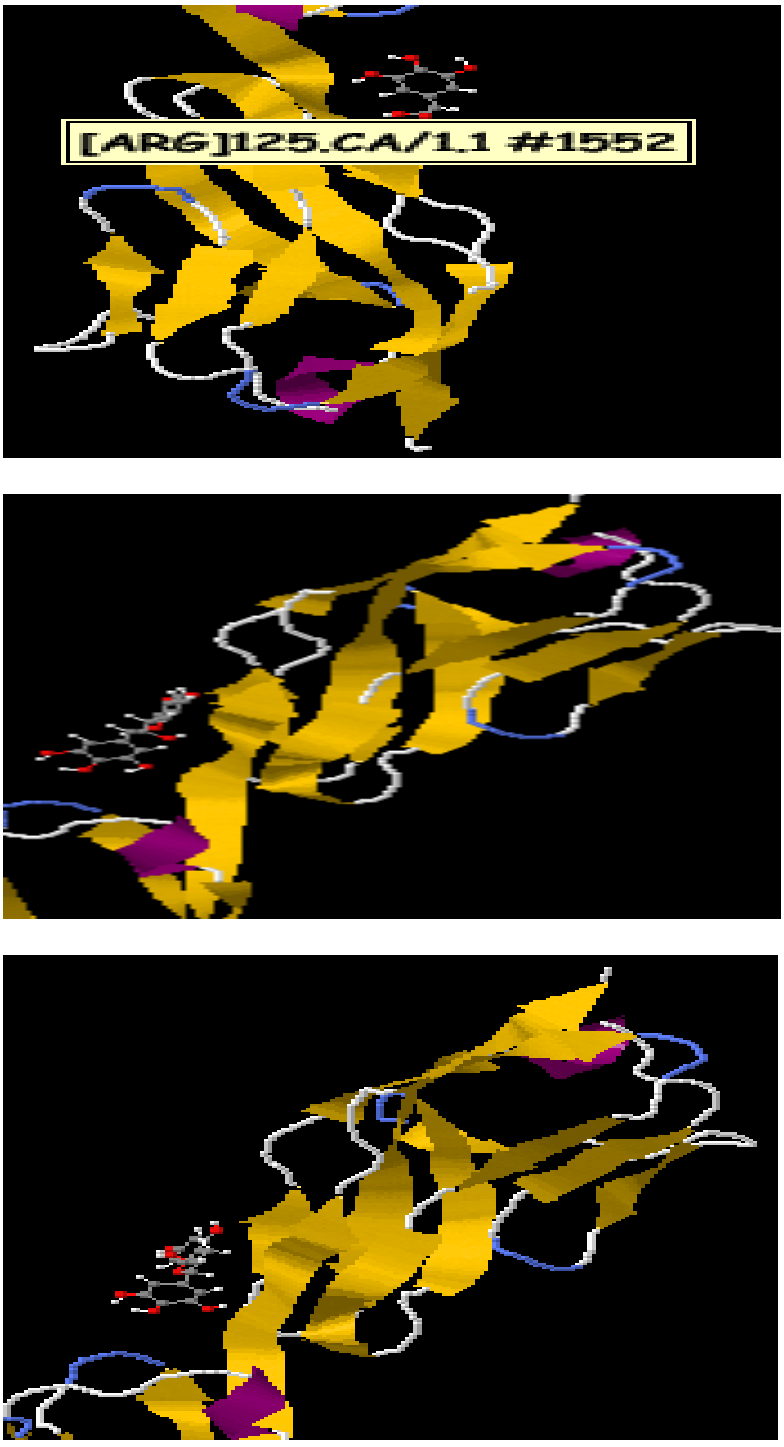
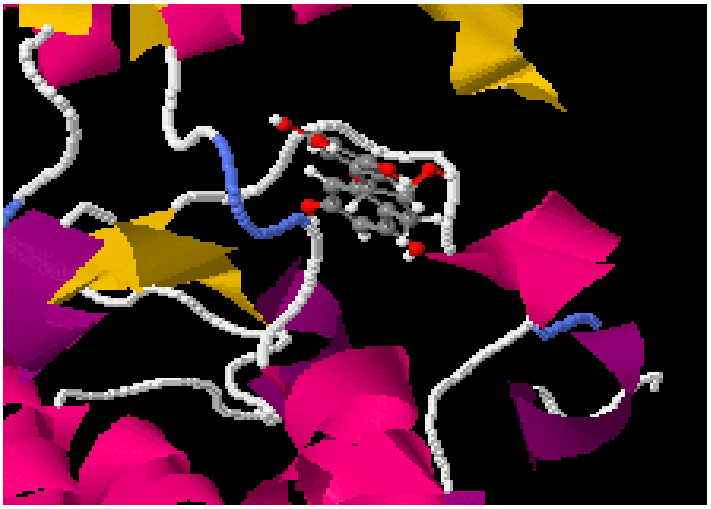
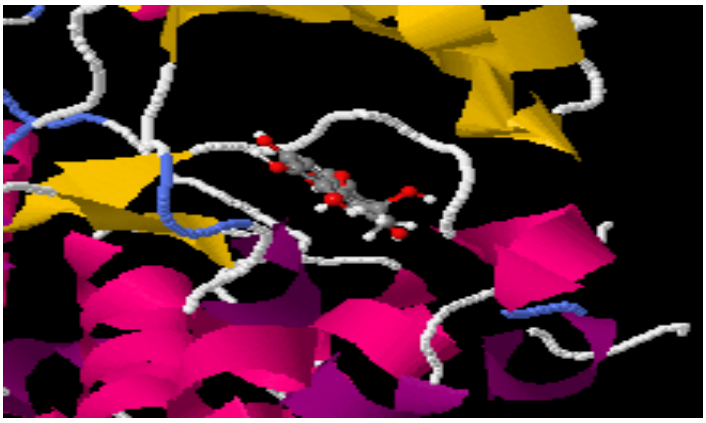


Figure 4.5: different cluster elements of RTK Chain A, clusters having highest energy (kcal/mol) (a) Cluster 0, element 0, energy -7.3, hit is at ARG 125 position. (b) Cluster 0, element 1, energy -7.69, hit is at THR 124 position (c) Cluster 2, element 2, energy -7.19, hit is at TYR 200 position.

(a)



(b)



(c)

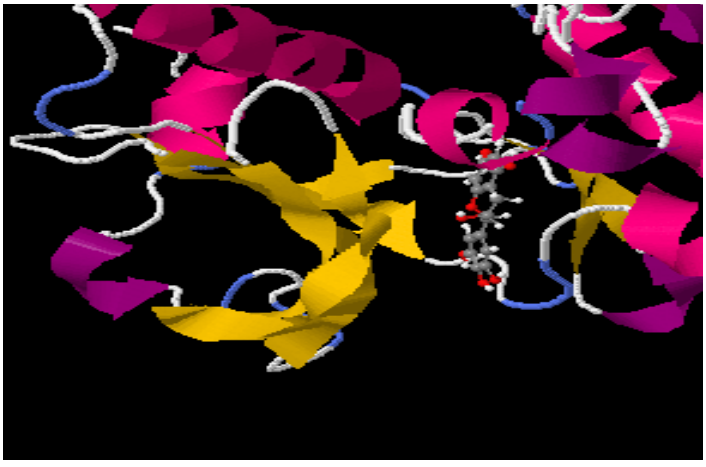


Figure 4.6: different cluster elements of RTK Chain B, clusters having highest energy (kcal/mol) (a) Cluster 2, element 0, energy -8.33, hit is at GLY 660 position. (b) Cluster 2, element 1, energy -8.36, hit is at SER 746 position (c) Cluster 2, element 2, energy -7.85, hit is at MET 657 position.

4.1.2 Cyclin D-1 with [6] – Gingerol

[6] - gingerol was checked against CD-1. Its length is 398 amino acids and mostly proteins are active but most active residue is at position 2. The PDB id is 3ay5 and UniProtKB reference is O95273.

Structure of [6] - gingerol is taken from available literature. This is used as a ligand present in *Z. officinale* and have tendency to bind with Cyclin D-1.

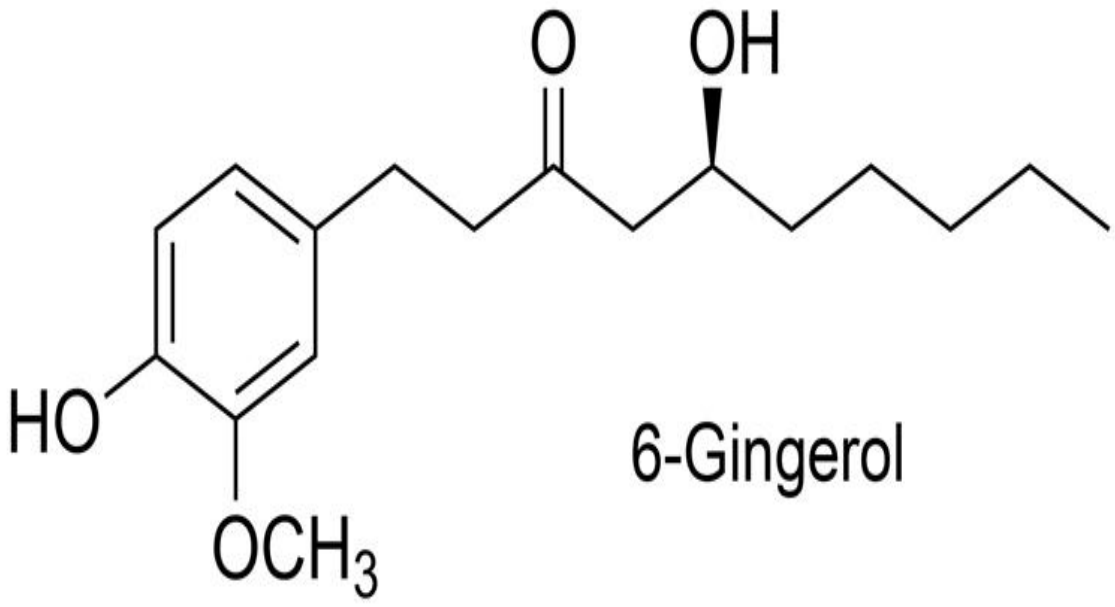


Figure 4.7: Chemical structure of [6] - gingerol having chemical formula of $C_{17}H_{26}O_4$

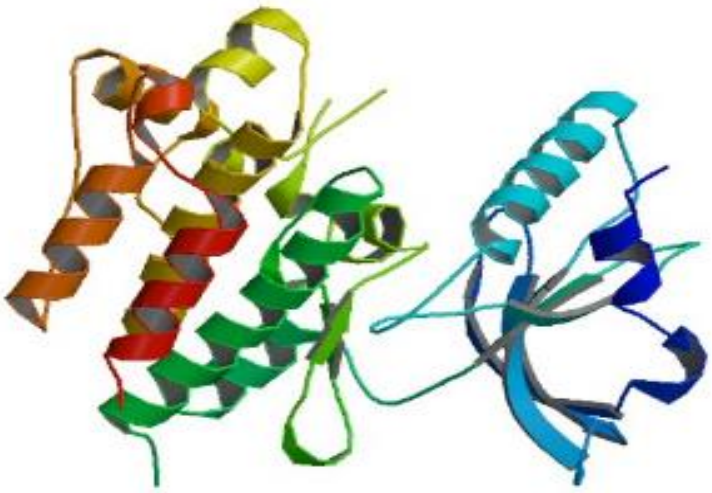


Figure 4.8: PDB view of Cyclin D-1

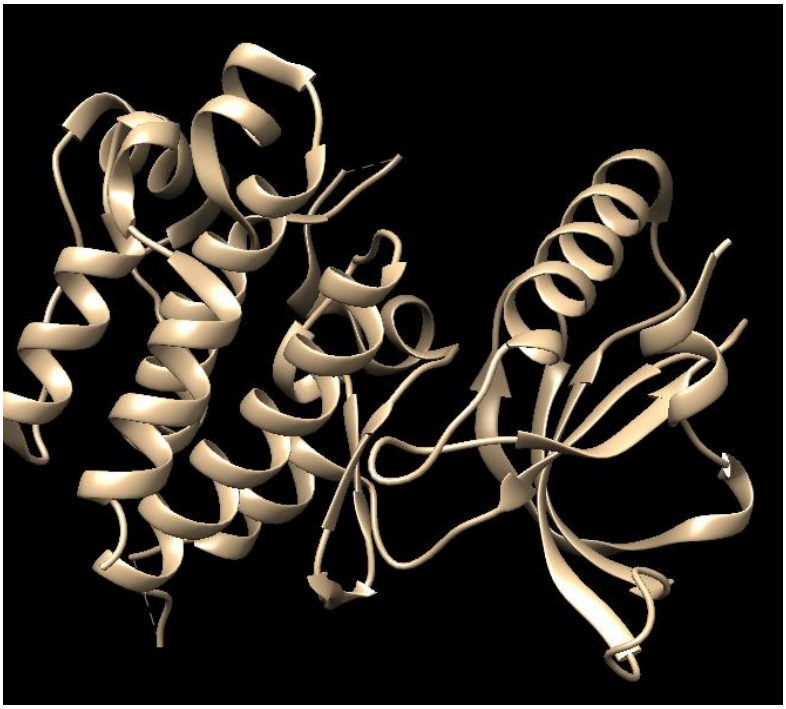


Figure 4.9: Chain views of CD-1 using Chimera UCSF

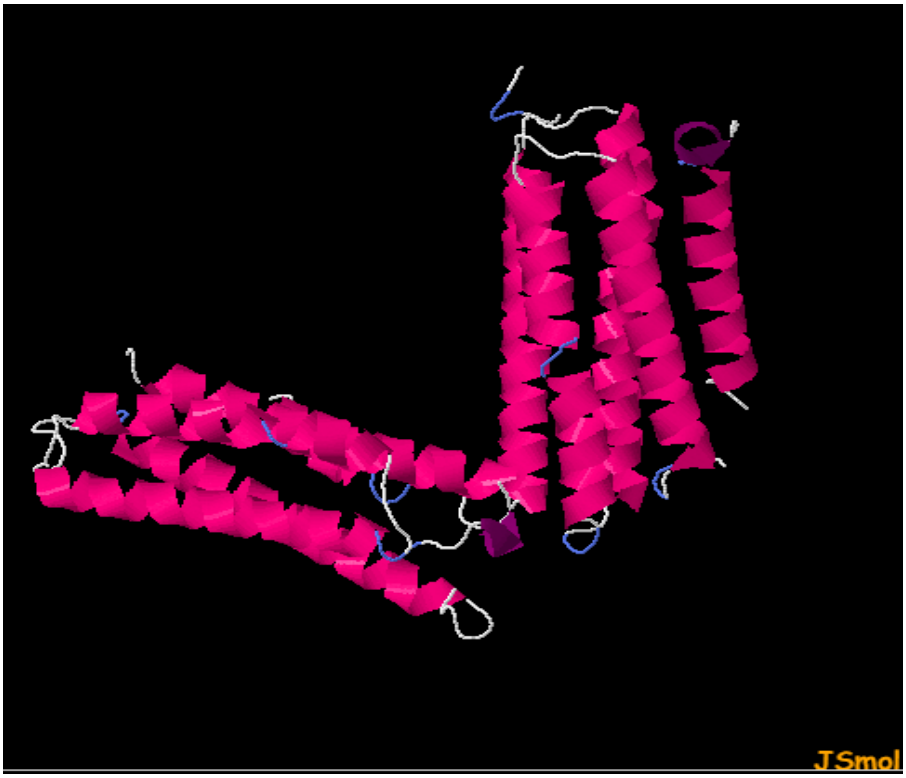
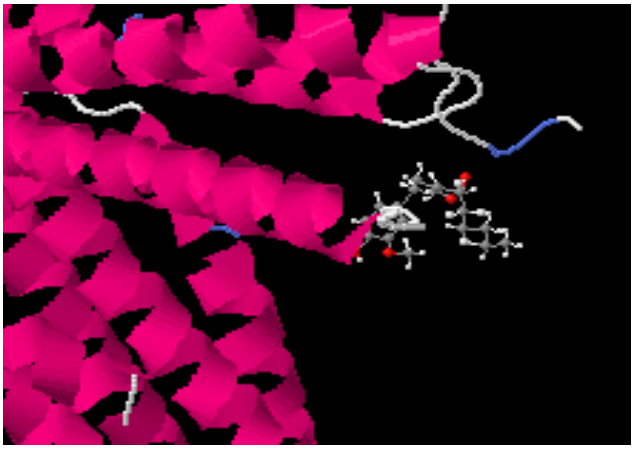


Figure 4.10: protein after docking. JSmol viewer

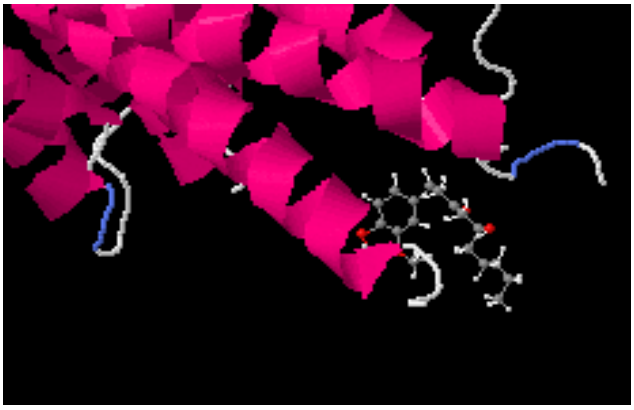
Table 4.2: binding energies of best hits of Cyclin D-1.

Cluster	**Element**	**FullFitness (kcal/mol)**	**Estimated ΔG (kcal/mol)**	**Binding site**
0	0	-2375.65	-7.50	197
0	1	-2375.45	-7.32	198
0	4	-2371.87	-7.58	233

(a)



(b)



(c)

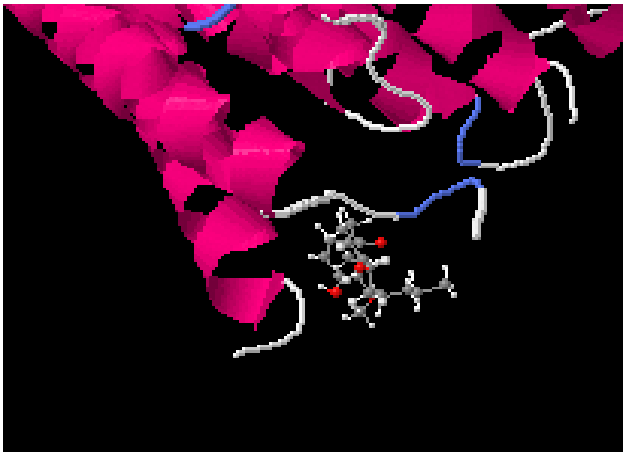


Figure 4.11: different cluster elements of Cyclin D-1, clusters having highest energy (kcal/mol) (a) Cluster 0, element 0, energy -7.50, hit is at GLU 197 position. (b) Cluster 0, element 1, energy -7.32, hit is at SER 198 position (c) Cluster 0, element 4, energy -7.58, hit is at TRP 233 position.

4.2. MTT (methyl thiazol tetrazolium) Assay

Toxicological effects of different extracts of different plants extracted with different methods were determined through MTT proliferation assay. Succinic dehydrogenase in living cells reduces MTT to purple formazan crystals. Formazan crystals are solubilized in DMSO and absorption in the visible range correlates with cell viability. Tables 4.1-4.4 gives the cell inhibition with respect of concentration. Figures 4.12-4.13 demonstrate cell proliferation of *HuH-7* cell line graphically on various concentrations.

4.2.1: MTT Assay of Macerated Extracts:

In case of extracts obtained from Maceration, highest toxicity for the cancerous cells was observed. Aqueous extracts of ginger showed maximum activity while aqueous extract of green tea exhibited the second good result. Best activity was observed at 10mg/ml.

Table 4.3: Showing the percentage inhibitory effect of different extracts by maceration in a dose dependent manner on HuH -7 cell line. Abbreviations used are G-ginger, BT- black tea, GT- green tea, M- methanol and W-water.

Conc. (mg/ml)	G+W	G+M	BT+W	BT+M	GT+W	GT+M
0.625	65.38	28.46	79.23	54.61	78.46	84.615
1.25	20.38	16.53	51.53	32.69	40.38	66.15
2.5	10.76	12.69	38.46	33.07	36.15	39.23
5	1.92	11.15	32.69	24.61	23.84	29.61
10	1.92	10.76	28.46	20.38	21.92	27.69
20	0.76	10.76	21.53	17.23	16.92	22.3

With increase in concentration of extracts, the interaction of extracts with cells increases leading to increased cell death and less live cells bind with MTT. Hence, the binding decreases with increasing concentration of extracts.

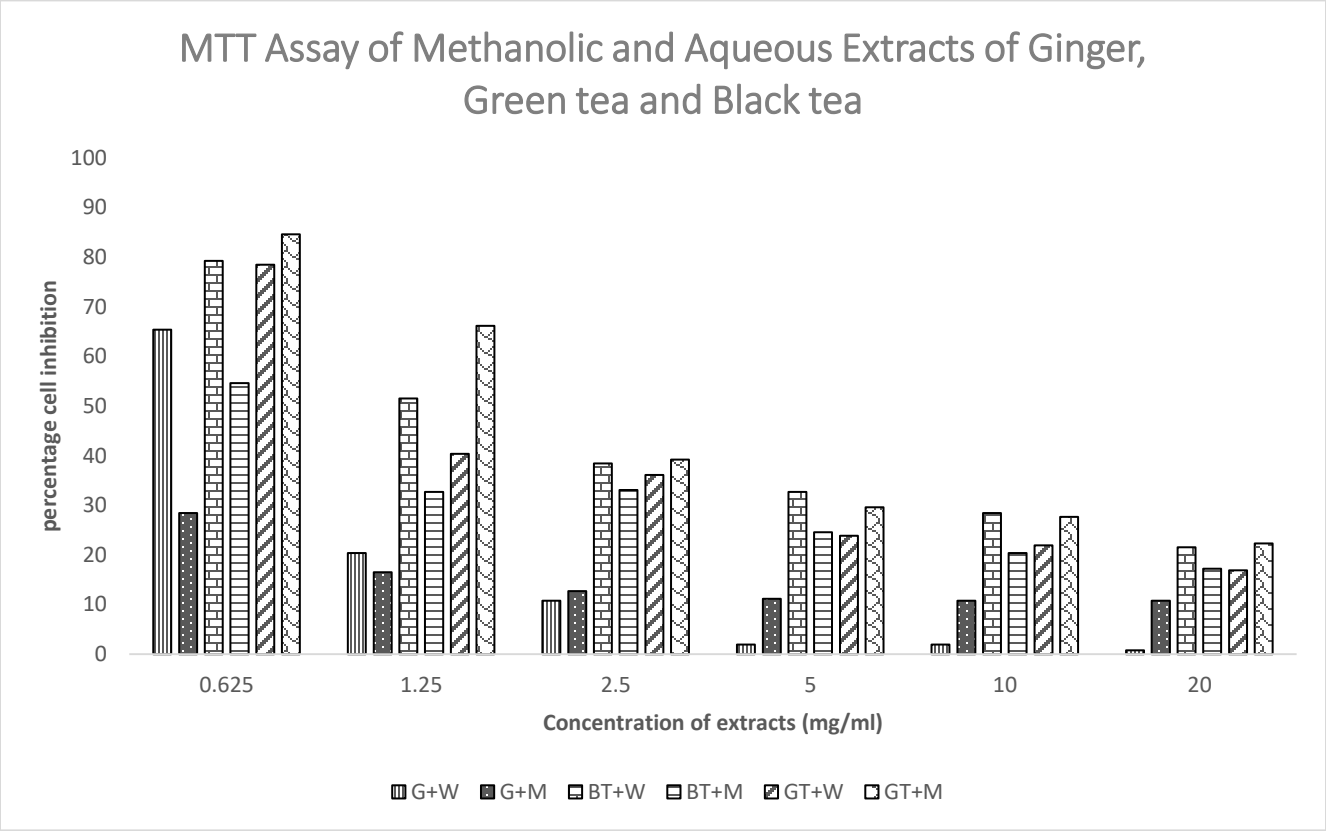


Figure 4.12 Showing the percentage inhibitory effect of different extracts by maceration in a dose dependent manner on HuH-7 cell line. Concentration of extracts (mg/ml) is taken along X-axis while percentage cell inhibition is taken along Y-axis. Abbreviations used are G-ginger, BT-black tea, GT- green tea, M- methanol and W-water.

4.2.2: MTT Assay of Extracts Obtained from Soxhlet extraction:

In case of extracts obtained from Soxhlet extraction, toxicity for the cancerous cells was observed but it was low as compared to the extracts obtained using Maceration. The results were likewise i.e. aqueous extracts of ginger showed maximum activity while aqueous extract of green tea exhibited the second good result. Activity increased in a dose dependent manner.

Table 4.4 Showing the percentage inhibitory effect of different extracts by Soxhlet extraction in a dose dependent manner on HuH -7 cell line. Abbreviations used are G-ginger, BT- black tea, GT- green tea, M- methanol and W-water.

Conc. (mg/ml)	G+W	G+M	BT+W	BT+M	GT+W	GT+M
0.625	51.18	68.483	95.26	90.83	91.83	91.78
1.25	59.55	63.66	80.25	76.69	69.74	90.55
2.5	44.865	45.81	79.06	60.34	68.16	84.91
5	37.099	39.09	67.85	59.63	53.08	82.93
10	33.01	38.07	66.11	52.21	52.76	75.59
20	21.16	31.67	62.16	43.52	47.7	74.64

With increase in concentration of extracts, the interaction of extracts with cells increases leading to increased cell death and less live cells bind with MTT. Hence, the binding decreases with increasing concentration of extracts.

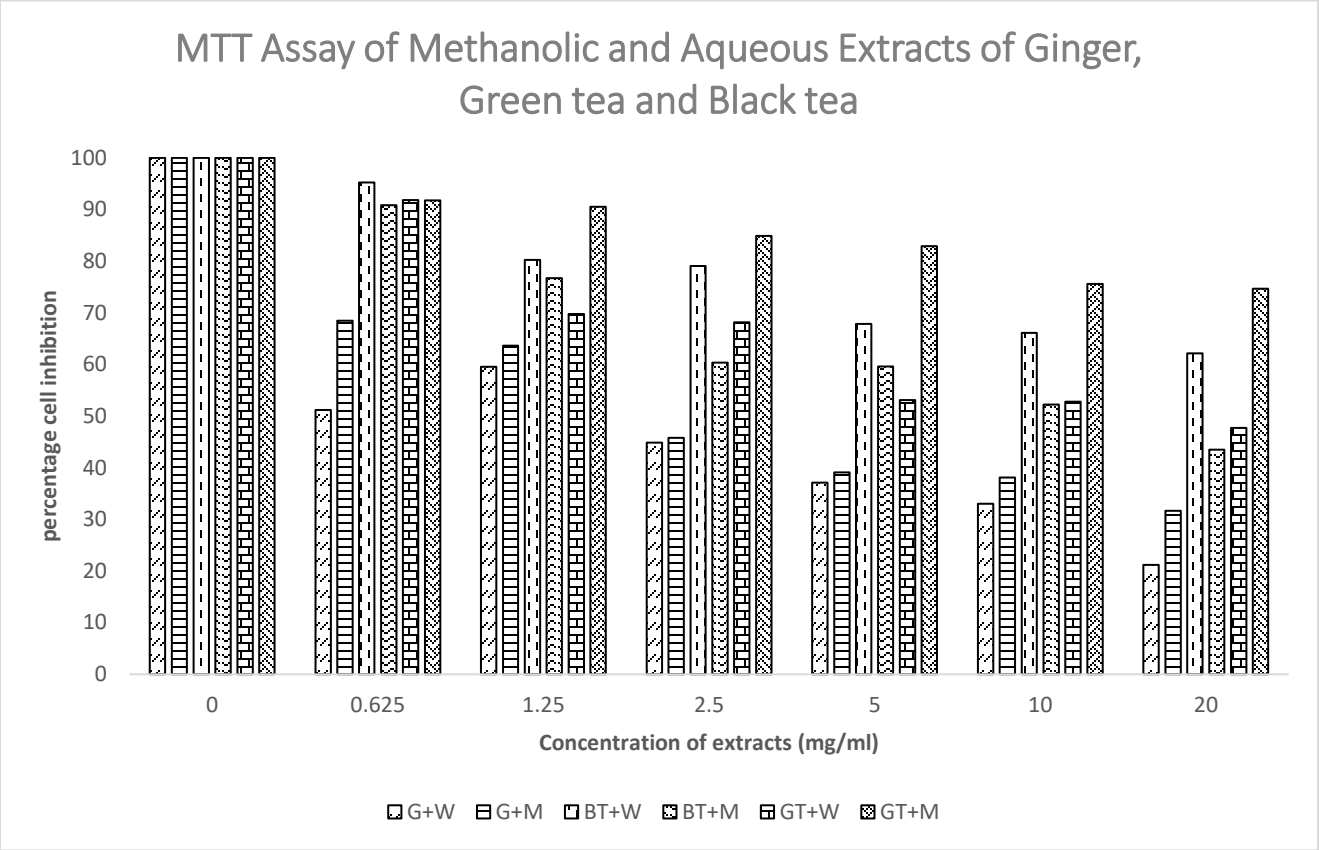


Figure 4.13 Showing the percentage inhibitory effect of different extracts by Soxhlet extraction in a dose dependent manner on HuH -7 cell line ($p<0.05$). Concentration of extracts (mg/ml) is taken along X-axis while percentage cell inhibition is taken along Y-axis. Abbreviations used are G-ginger, BT-black tea, GT- green tea, M- methanol and W-water.

4.3. Antibacterial activity

Disc diffusion method was used to measure Antibacterial activity of extracts. Clearing zones were measured in centimeter (cm). Values of clearing zone are given in appendix. Antibacterial activity of different extracts of *Z. Officinale* and *C. Sinensis* plants against five multi-drug resistant strains of bacteria naming *E. coli,* MRSA, *Shigella sonnei*, *Pseudomonas aeruginosa* and *Salmonella Typhi* is given in numerically in tables 4.3-4.7 and graphically in figures 4.3-4.7. Antibiotic susceptibility of all these strains were already measured against erythromycin and penicillin and they were found to be resistant. Different concentrations of extracts were used and they show good antibacterial activity in a dose dependent manner. Increasing concentration show an increase in the inhibition zone.

Maximum diameter of zone of inhibition was found to be approximately 3.133cm, showing the efficacy of the extracts against the multi-drug resistant strains. Minimum diameter of zone of inhibition was 1cm. Two strains didn't show any zone of inhibition and were totally resistant against the extracts. They were *Salmonella Typhi* and *Pseudomonas aeruginosa*. Also zero zone of inhibition was observed around filter paper discs treated with de-ionized water, which was used as a negative control. Thus, it can be said that the zone of inhibition observed in 3 strains and not observed in 2 strains and the discs having deionized water is purely because of the extract's activity.

4.3.1 *E.Coli:*

MDR *E. coli* was tested against various concentrations of plant extracts. Zone of inhibition was measured. All extracts exhibit strong inhibitory properties but the best was exhibited by methanol extract of ginger and aqueous extract of green tea. Inhibition increased with a dose dependent manner. Results are illustrated in table 4.3 and figure 4.3.

Table 4.5 Difference in clearance zone values (cm) of all extracts against *E. coli* at different concentrations. Abbreviations used are G-ginger, BT- black tea, GT- green tea, M- methanol and W-water.

Conc. mg/ml	G+W	G+M	BT+W	BT+M	GT+W	GT+M
2.5	0.33	0.566	0.2	0.266	0.633	0.266
5	0.733	0.966	0.433	0.66	1.1	0.66
10	0.9	1.466	0.7	1.066	1.473	1.333
20	1.133	1.66	1	1.266	1.533	1.533

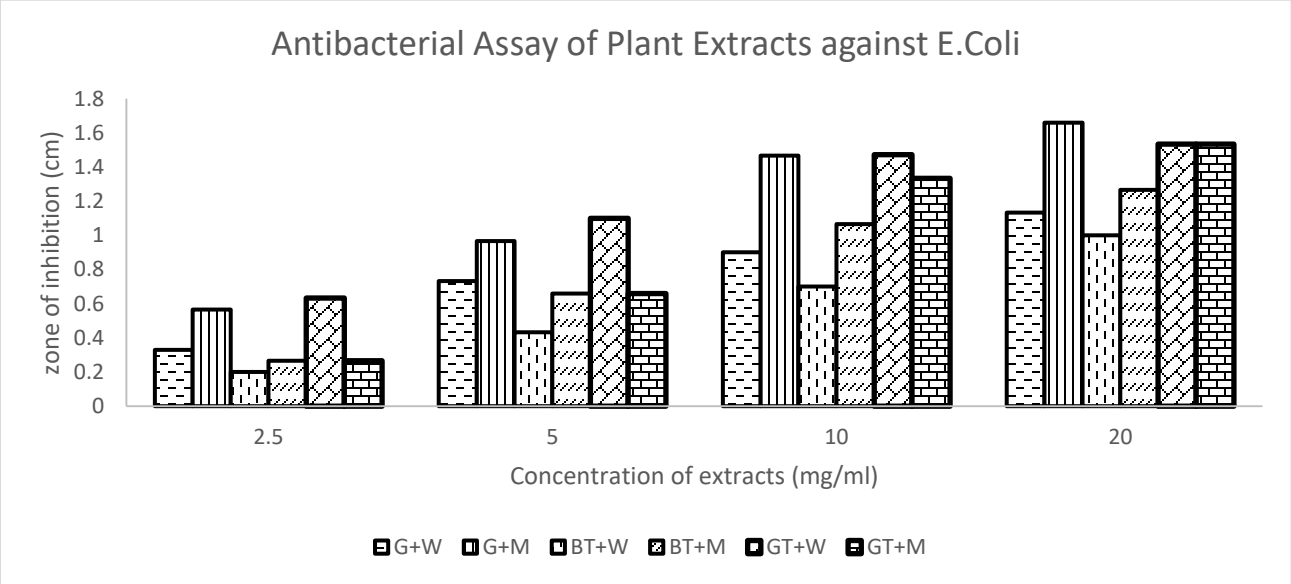


Figure 4.14 Difference in clearance zone values (cm) of all extracts against *E. coli* at different concentrations ($p>0.05$). Concentration of extracts (mg/ml) is taken along X-axis while zone of inhibition (centimeter) is taken along Y-axis. Abbreviations used are G-ginger, BT- black tea, GT- green tea, M-methanol and W-water.

4.3.2 MRSA:

MDR MRSA was tested against various concentrations of plant extracts. Zone of inhibition was measured. All extracts exhibit strong inhibitory properties but the best was exhibited by aqueous extract of green tea. Inhibition increased with a dose dependent manner. Results are illustrated in table 4.4 and figure 4.4.

Table 4.6. Difference in clearance zone values (cm) of all extracts against MRSA at different concentrations. Abbreviations used are G-ginger, BT- black tea, GT- green tea, M- methanol and W-water.

Conc. mg/ml	G+W	G+M	BT+W	BT+M	GT+W	GT+M
2.5	0.433	0.483	0.3	0.33	0.566	0.4
5	0.8	0.8	0.6	0.7	0.96	0.7
10	1.633	1.7	1.133	2.1	2.533	2.033
20	2.3	3.1	2.233	3.2	3.6	3.03

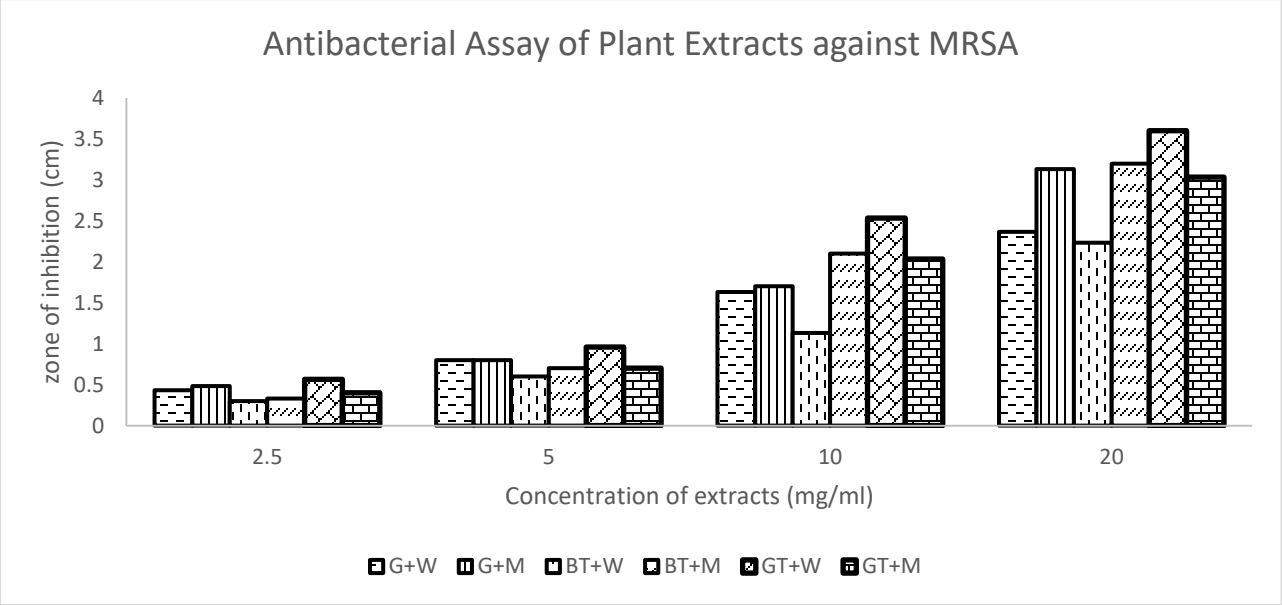


Figure 4.15. Difference in clearance zone values (cm) of all extracts against MRSA at different concentrations ($p>0.05$). Concentration of extracts (mg/ml) is taken along X-axis while zone of inhibition (cm) is taken along Y-axis. Abbreviations used are G-ginger, BT- black tea, GT- green tea, M- methanol and W-water.

4.3.3 *Shigella sonnei*

MDR *Shigella sonnei* was tested against various concentrations of plant extracts. Zone of inhibition was measured. All extracts exhibit strong inhibitory properties but the best was exhibited by aqueous extract of green tea. Inhibition increased with a dose dependent manner. Results are illustrated in table 4.5 and figure 4.5.

Table 4.7. Difference in clearance zone values (cm) of all extracts against *Shigella sonnei* at different concentrations. Abbreviations used are G-ginger, BT- black tea, GT- green tea, M- methanol and W-water. (Values were taken in triplicates and the average values from all those experiments are given below)

Conc. mg/ml	G+W	G+M	BT+W	BT+M	GT+W	GT+M
2.5	0.833333	1.5	0.866667	1.066667	1.1	0.9
5	1.333333	2.133333	1.566667	1.533333	1.566667	1.466667
10	1.833333	2.566667	2.066667	1.9	2.2	1.8
20	2	2.633333	2.233333	2.033333	2.266667	2.133333

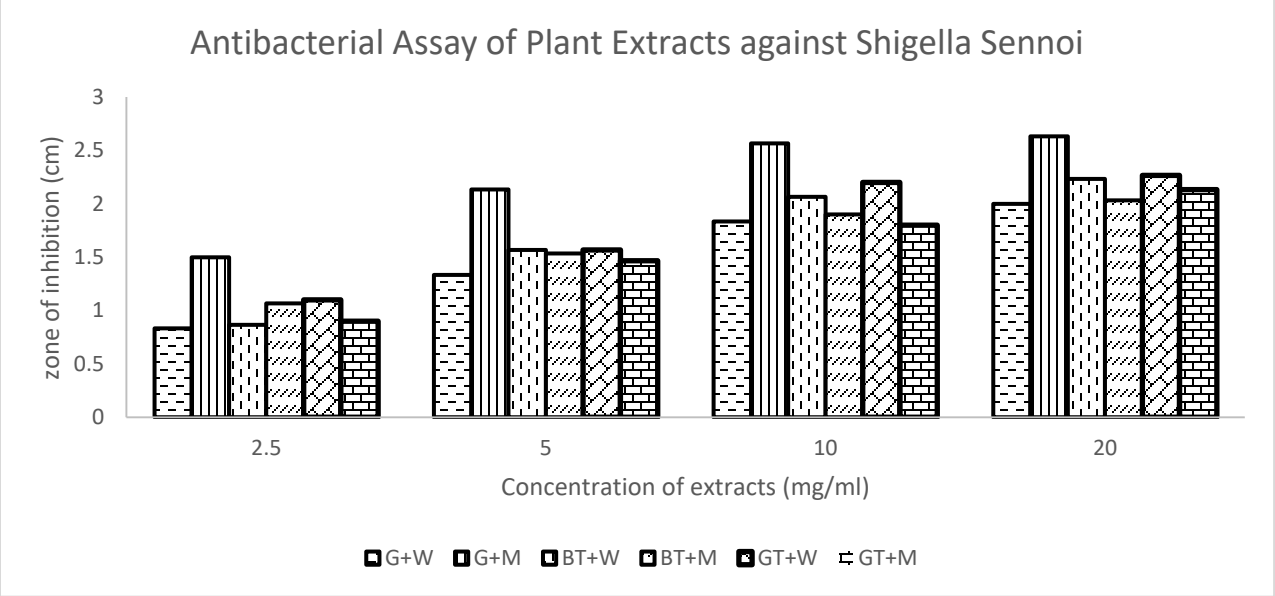


Figure 4.16. Difference in clearance zone values (cm) of all extracts against *Shigella sonnei* at different concentrations. Concentration of extracts (mg/ml) is taken along X-axis while zone of inhibition (cm) is taken along Y-axis. Abbreviations used are G-ginger, BT- black tea, GT- green tea, M- methanol and W-water.

4.3.4 *Pseudomonas aeruginosa*:

MDR *Pseudomonas aeruginosa* was tested against various concentrations of plant extracts. No inhibition was shown at all by any of the extract. This strain is resistant against the polyphenols in all these extracts. Results are illustrated in table 4.6 and figure 4.6.

Table 4.8. Difference in clearance zone values (cm) of all extracts against *Pseudomonas aeruginosa* at different concentrations. Abbreviations used are G-ginger, BT- black tea, GT- green tea, M- methanol and W-water.

Conc. mg/ml	G+W	G+M	BT+W	BT+M	GT+W	GT+M
2.5	0	0	0	0	0	0
5	0	0	0	0	0	0
10	0	0	0	0	0	0
20	0	0	0	0	0	0

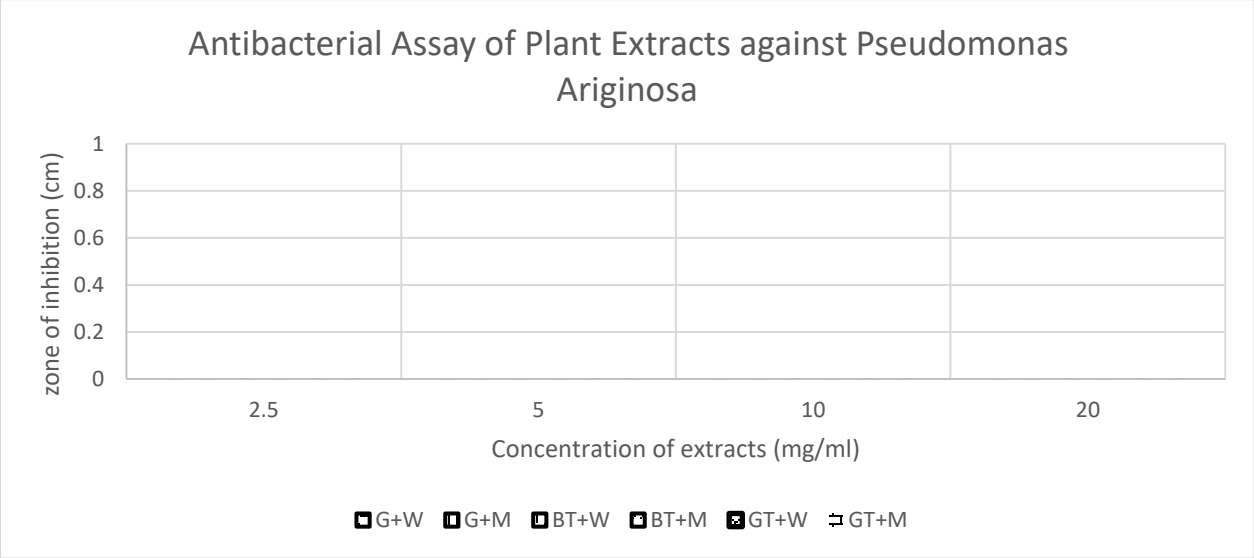


Figure 4.17 Difference in clearance zone values (cm) of all extracts against *Pseudomonas aeruginosa* at different concentrations. Concentration of extracts (mg/ml) is taken along X-axis while zone of inhibition (cm) is taken along Y-axis. Abbreviations used are G-ginger, BT- black tea, GT- green tea, M- methanol and W-water.

4.3.5 *Salmonella Typhi*:

MDR *Salmonella Typhi* was tested against various concentrations of plant extracts. No inhibition was shown at all by any of the extract. This strain is resistant against the polyphenols in all these extracts. Results are illustrated in table 4.7 and figure 4.7.

Table 4.9. Difference in clearance zone values (cm) of all extracts against *Salmonella Typhi* at different concentrations. Abbreviations used are G-ginger, BT- black tea, GT- green tea, M- methanol and W-water.

Conc. mg/ml	G+W	G+M	BT+W	BT+M	GT+W	GT+M
2.5	0	0	0	0	0	0
5	0	0	0	0	0	0
10	0	0	0	0	0	0
20	0	0	0	0	0	0

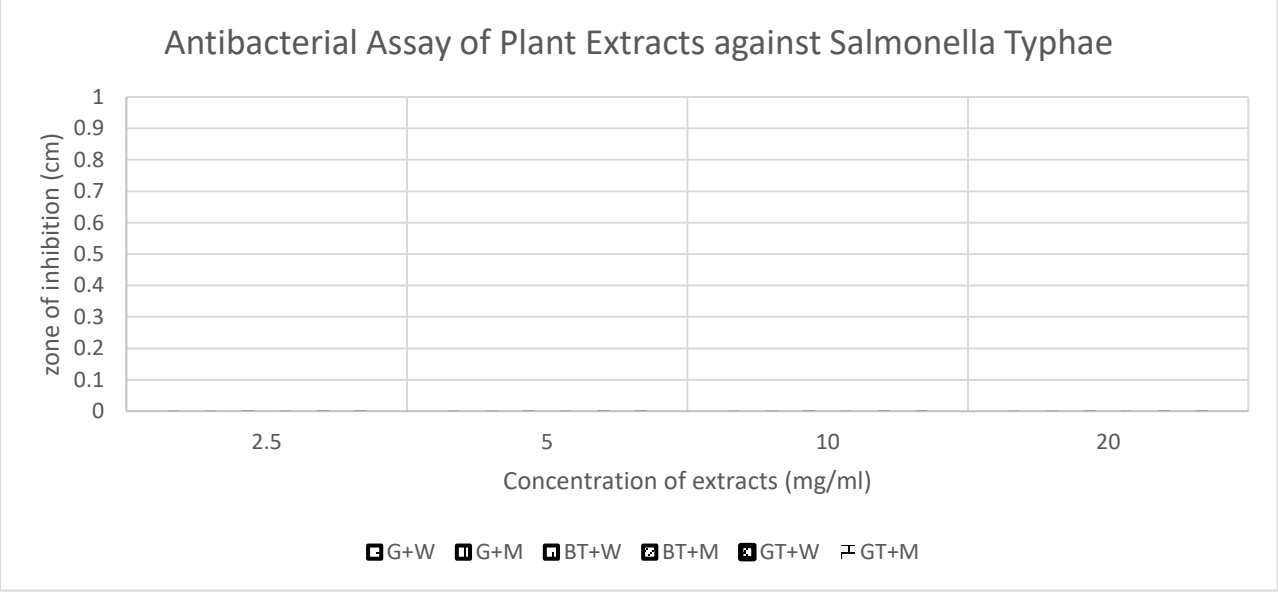


Figure 4.18 Difference in clearance zone values (cm) of all extracts against *Salmonella Typhi* at different concentrations. Concentration of extracts (mg/ml) is taken along X-axis while zone of inhibition (cm) is

taken along Y-axis. Abbreviations used are G-ginger, BT- black tea, GT- green tea, M- methanol and W-water.

4.4 Scavenging Capacity

Aqueous and methanol extracts of *Z. Officinale* and *C. Sinensis* were analyzed for scavenging ability on hydrogen peroxide. Both extracts show positive results in hydrogen peroxide in an amount dependent manner as compared to the standard BHA. Different concentrations of water and methanol extracts of *Z. Officinale* and *C. Sinensis* exhibited 14.483-31.55% scavenging activity on hydrogen peroxide. While same amount of BHA showed 40.96% hydrogen peroxide scavenging activity.

Scavenging activity values on hydrogen peroxide were decreased for different concentrations of extracts of *Z. Officinale* and *C. Sinensis* decreases than that of BHA in the order of BHA (%40.96) > aqueous extract of *C. Sinensis* (green tea) (%31.55)> Methanol extract of *Z. Officinale* (%28.67) > methanol extract of *C. Sinensis* (green tea) (%22.99) > aqueous extract of *Z. Officinale* (%21.16) > methanol extract of *C. Sinensis* (black tea) (%16.77)> aqueous extract of C. Sinensis(black tea) (%16.16).

Table 4.10: Showing the antioxidant activity of various extracts compared to the standard BHA using Hydrogen peroxide. Abbreviations used are G-ginger, BT- black tea, GT- green tea, M- methanol and W-water.

Conc. mg/ml	G+W	G+M	BT+W	BT+M	GT+W	GT+M	BHA
0.625	14.483	23.18	11.26	11.83	26.83	18.78	35.78
1.25	15.44	24.55	12.25	12.69	27.74	19.58	36.55
2.5	16.44	25.81	13.06	13.34	28.16	20.91	37.91
5	19.66	26.09	14.85	14.63	29.08	21.93	38.55
10	20.07	27.13	15.44	15.17	30.13	22	39.26

20	21.16	28.67	16.16	16.77	31.55	22.99	40.96

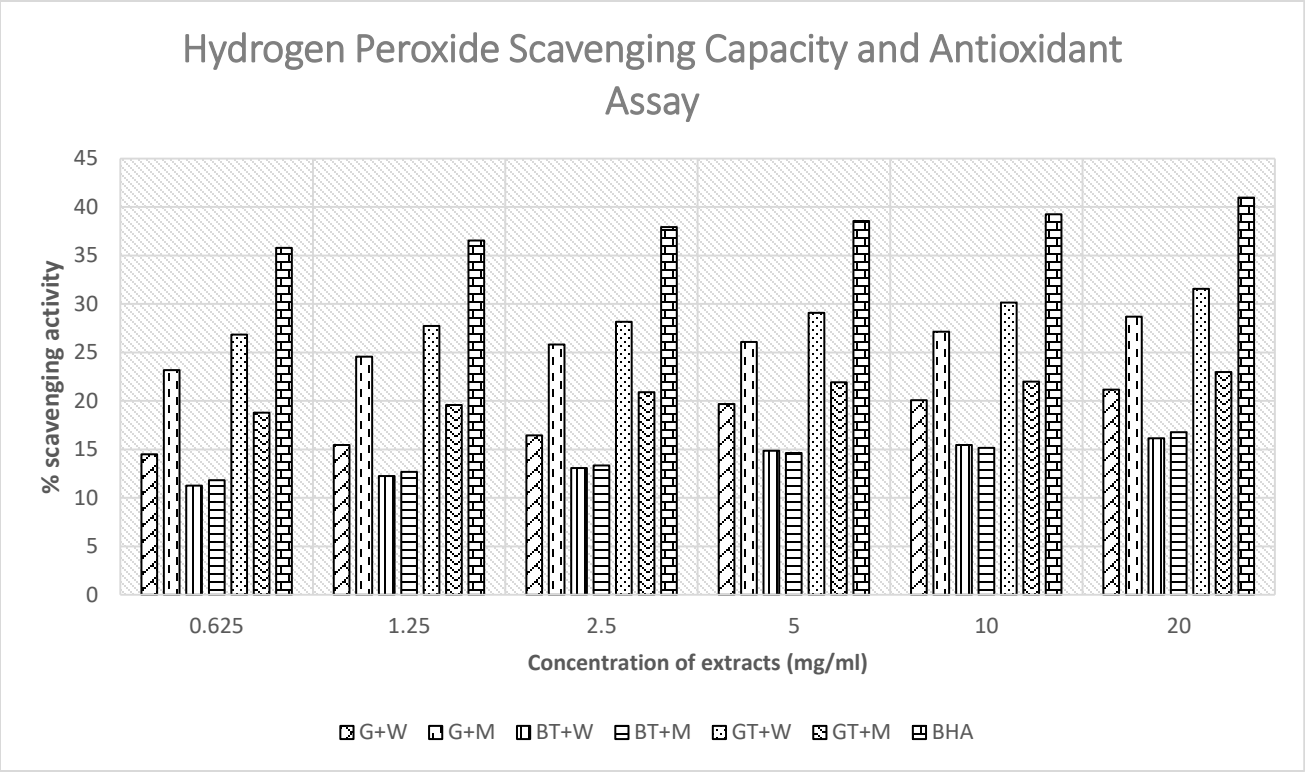


Figure 4.19: Showing the antioxidant activity of various extracts compared to the standard BHA using Hydrogen peroxide. Concentration of extracts (mg/ml) is taken along X-axis while % scavenging activity is taken along Y-axis. Abbreviations used are G-ginger, BT- black tea, GT- green tea, M- methanol and W-water.

Hydrogen peroxide is not a very toxic or harmful chemical itself as it's not very reactive, but it may release hydroxyl radical in the cells making it toxic for the cells. (Halliwell, 1991). So, removal of H_2O_2 is quite important when it comes to antioxidant defense mechanism in cell and food systems.

CHAPTER 5

DISCUSSION

Medicinal plants are herbal way to cure diseases and is always preferred over synthetic drugs. Healthcare system has largely been dependent on plants and plant derived phytochemicals and medicines since ages. Baby boomers used self-prescribed herbal and supplementary remedies and most of them result in successful treatment (Cohen, Ek, and Pan 2002). It is still not a healthy practice, proper research should be done before taking anything as a medicine just to make sure that these chemotherapeutics' effects and binding in body. A lot of plants have been studied in detail and their biological activities are well documented. The main activities focused upon are anti-cancer, antimicrobial, anti-oxidant and anti-diabetic and various other biological activities have been reported (Engdal, Klepp, and Nilsen 2009). Focus has been increasingly directed towards medicinal plants day by day. Herbal plants are more focused because of their effective action and lesser toxicity (Bent & Ko, 2004). Phytochemicals present in medicinal plants are rapidly metabolized and are very good for body as they have been proved very functional in animal models for various types of cancers. Published data and research on these phytochemicals is increasing day by day (Keria *et al.*, 2013)

This study focus on two very commonly used plants in daily routine i.e. *Zingiber officinale* and *Camellia sinensis* are studied for their potential biological activities. Ginger (*Zingiber officinale* Roscoe, Zingiberaceae) is largely used in food and for herbal tea (Surh *et al.*, 1999). Oleoresins present in its rhizome contain a number of bioactive compounds for example [6]-gingerol (1-[4′-hydroxy-3′- methoxyphenyl]-5-hydroxy-3-decanone. This is the compound that makes it pungent and gives most of the pharmacological activities (Kaul and Joshi 2001)

U.S. Food and Drug Administration (FDA) has recommended as a food additive that makes it a safe plant (Morgan and Penovich 1978).

On the other hand, tea *(Camellia sinensis*, Theaceae) has variety of sub-types, of which two were chosen i.e. green tea and black tea. During the current study, about 30 countries in the world cultivate tea and it is the most consumed drink after water (Hu *et al.*, 2013). Black tea exhibits 80% of tea consumption while rest is green tea and oolong tea. Studies show that tea contain such bioactive compounds like Catechins, Theaflavins and others that helps in fighting cancer. Mostly the studies are carried out using green tea polyphenols (GTPs) and they are found to be very effective (Ho *et al.,* 2013). (-)-Epigallocatechin-3-gallate has proven itself to be an active chemical in binding with receptor tyrosine kinase and fighting against variety of cancers like lung, skin, forestomach, colon, intestinal, skin and many others (Hu *et al.*, 2013).

In silico analysis performed before proceeding to *in vitro* analysis suggested that there are compounds in both the plants which are showing very strong binding with the target proteins and can be used as potential drug candidates. Active site in RTK is found at 724 and binding site is at 608 and the highest binding energy is found at site 660 and 746. This means that the binding lies somewhere near the active region of protein and this may be said the mode of action of egcg is competitive inhibition. On the other hand, in case of CD-1, highest binding by ligand [6] – gingerol is shown at position 197, 198 and 233 while the active site is at position 2. So this can be the type of non-competitive inhibition. Both the ligands have shown significant results and can be used as potential drug candidates.

Detailed literature review and in *silico analysis* have shown that these two plants have a lot of active phytochemicals that are good for pharmaceutical research. These phytochemicals

have great anti-mutagenic and anti-carcinogenic activities and are explored and compared extensively for their biological activities (Kucuk, 2002). Plants were taken, processed, extracts were made and compared for their anti-cancer results. *HuH-7* cell line was used to evaluate the anti-cancer activity. Different dilutions of methanol and aqueous extracts were checked after different incubations. MTT show the potential of cell inhibition of all the extracts. Ginger showed higher anti-cancer activity then rest of the two. In previous studies, ginger showed 10X stronger activity then chemotherapy and other herbs till now (Barker, 2016) and second highest cell inhibition was shown by aqueous extract of green tea.

Anti-microbial activities of medicinal plants is reported and well documented. For prevention of resistant microbes, combinations of different herbs of individual herbs are very effective (Patel, Thaker, & Patel, 2011). Ginger extracts possess good inhibition against *E. coli* and *Salmonella typhi* (Indu *et al.,* 2006), while tea flush extracts and tea leaves extracts show great anti-microbial activity against *Bacillus subtilis, Escherichia coli, Proteus vulgaris* and *Pseudomonas fluorescens* (Chou, 1999). Aqueous and methanol extracts were prepared for ginger, green tea and black tea extracts and checked for their anti-microbial activity against MDR *E. coli*, MRSA, *Salmonella Typhi* and *Pseudomonas aeruginosa*. Anti-microbial activity was very strong against MDR *E. coli* and MRSA while no activity was shown against the rest of two. Also, aqueous extract of ginger showed strongest activity and aqueous extract of green tea showed second highest activity.

Previous studies have revealed that some medicinal plants have shown great beneficial effects because of possessing antioxidant activity and can be counted as strong drug candidates (Lee *et al.,* 2003). Green tea possess greatest anti-oxidant activity when it checked for its Total Antioxidant Activity (TAA) (ARK & JR, 2016). Extracts were also checked for their antioxidant

activity using Hydrogen peroxide. The results were very good and green tea extract showed quite good TAA. Second highest TAA was exhibited by ginger.

Overall, extracts of roots of *Zingiber officinale* and leaves of two sub-types of *Camellia sinensis* were obtained using two different extraction methods and two different solvents. *In silico* analysis suggested their active potential in drug designing. To further confirm this hypothesis and result, their biological activities were assessed i.e. anti-cancer, antimicrobial and antioxidant activities, it is clear that they all are potential candidates for herbal medicines. Extracts that show higher activity were the ones obtained using Maceration method. Anticancer activity was evaluated using MTT Assay and according to literature phenols in the extracts i.e. catechins present in green tea, theaflavins in black tea and 6-gingerol in ginger acts as the substances to react with MTT. Highest anticancer activity observed was in aqueous extract of ginger and then aqueous extract of green tea. The inhibition of cancerous cell was significant in these two extracts. Also bactericidal activities were observed against MDR MRSA and *E. coli* and the results were almost the same. These two extracts showed highest activity against these strains. While anti-oxidant activity against hydrogen peroxide observed was higher in aqueous solution of green tea then of ginger. Obtained results showed that catechins and 6-gingerol are potential candidates for drug designing. Although the complete mechanism of action is yet to be discovered but aqueous solution of ginger and green tea have been proved to have strong biological activities and effective as anticancer, antimicrobial and antioxidant candidates.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

The study was focused on comparative analysis of biological activity of *Z. Officinale* and *C. sinensis*. *In silico* results have suggested the compounds that may have strong activity and can be used as strong drug candidates. Both these compounds are present in green tea and ginger in high amount and traces of egcg are found in black tea and no trace of [6] – gingerol found. Also the binding energy exhibited by egcg, a chemical found in green tea was the highest. This high energy of green tea suggested that green tea is more active as compared to ginger.

After going through various activities, aqueous extracts of ginger and green tea have appeared as good drug candidates. Anticancer evaluation proved that ginger and green tea possess strong activity to control tumor. This makes them a good anti-cancer drug candidate. Also through analysis for various MDR bacterial strains, they can be used a potential disinfectants. Green tea clearly has more strong activities in most cases as compared to ginger. Black tea showed some activities but it is comparatively not as good for medicinal purpose as ginger and green tea. *In vitro* results also validate the *in silico* results.

After complete analysis of data and results obtained in this study, it is recommended to purify the phytochemicals like catechins and 6-gingerol from these plants as they are the active compounds. Characterization should be done on basis of Total Phenolic Contents (TPC) using HPLC or NMR. After detailed *in vitro* and *in silico* analysis, this research can be move forward to *in vivo* models that may help in discovery of new drugs with low side effects and vast therapeutic potentials.

CHAPTER 7

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