Comparative analysis of phytochemical constituents, antibacterial and antioxidant activity of green tea (*Camellia sinensis)*

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My inspiration, my mother

My sister and mentor

&

To my father for being the man he is

DECLARATION

I hereby declare that the research work embodying the results reported in this thesis entitled **"Comparative analysis of phytochemical constituents, antibacterial and antioxidant activity of green tea (*Camellia sinensis*)"** submitted by the undersigned has been carried out under the supervision of Ms. Jebunnesa Chowdhury, Assistant Professor, Biotechnology Programme, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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Abstract

Camellia sinensis, the tea plant belongs to the Theaceae family. Tea industry is the second highest foreign exchange earner in Bangladesh. Increased consumption of green tea is due to its antioxidant properties. Research suggests green tea also possesses medicinal properties including antimicrobial, antifungal, and anti-carcinogenic activities. Hence, this study was carried out using locally produced organic green tea. Preliminary screening of the extracts was conducted to identify the presence of alkaloids, flavonoids, steroids, saponins, tannins, phenols, cardiac glycosides and gums. Comparative analysis of the antimicrobial activity of the extracts was investigated against *Escherichia coli*, *Enterotoxigenic Escherichia coli* (ETEC), *Shigella flexneri*, *Methicillin-resistant Staphylococcus aureus* (MRSA) and *Staphylococcus aureus*. Ampicillin, ciprofloxacin and cefoxitin discs were used as positive control to measure the antimicrobial activity and synergistic activity of tea extracts and antibiotics. It was observed that repeated subculturing had changed the virulence pattern of MRSA to a sensitive strain. S. *aureus* alone exhibited sensitivity to all tested concentrations of each extract. Methanolic and ethanolic extracts showed highest activity index in all test strains. Aqueous extract had inhibitory effect only on S. *aureus*. Extracts did not demonstrate synergisitic activity with any of the drugs. Antioxidant activity of extracts was determined by DPPH free radical scavenging activity. Ethanolic extract showed the highest antioxidant activity followed by methanolic and aqueous extract. It is expected that the antimicrobial activities and presence of metabolites identified in this study will serve as a pivotal ground for further research regarding the many medicinal attributes of green tea.

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List of Abbreviations

BRAC	Bangladesh Rural Advancement Committee
icddr,b	International Center for Diarrheal Disease Research, Bangladesh
rpm	Rotation per minute
^{o}C	Degree Celsius
g	Gram
mg	Milligram
mm	Milliliter
µg	Microgram
S. aureus	*Staphylococcus aureus*
MRSA	Methicillin-resistant *Staphylococcus aureus*
E. coli	*Escherichia coli*
ETEC	*Enterotoxigenic Escherichia coli*
S. flexneri	*Shigella flexneri*
HEPA	High Efficiency Particulate Air
v/v	Volume by volume
DMSO	Dimethyl Sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
mM	Milli Moles
FRSA	Free Radical Scavenging Activity
NaCl	Sodium Chloride

Chapter 1: Introduction

1. Introduction

Tea is the oldest and cheapest health beverage in the world next to water (Rahman *et al*., 2013). Today, green tea is produced in over 20 countries in tropical, sub-tropical and temperate regions. It is the most widely consumed beverage after water, due to its health, sensory, stimulant, relaxing and cultural properties (Ahmed and Stepp, 2012). The beneficial effects of green tea are owing to its polyphenolic compounds. Among the tea polyphenols, flavonoids, especially catechins, are the leading functional components, which accounts for 30% of the dry weight of green tea leaves. Fresh green tea leaves are very rich in catechins, which include mainly epicatechin (EC), epicatechin-3- gallate (ECG), epigallocatechin (EGC), epigallocatechin-3-gallate (EGCG), and gallocatechin (GC). EGCG is the most abundant catechin in green tea which accounts for at least 65% of the total catechin (Zaveri, 2005).

Tea is a specialty of Asia, occupying around 2.7 million hectares of cultivable land in the world. Cultivated from antiquity in China, tea is now readily available in more than 30 countries like Japan, Bangladesh, India, Kenya, Ethiopia, etc. Produced from young leaves of *Camellia sinensis* L. (Kuntz), tea is one of the most popular beverages worldwide. Processed green tea is a product prepared and cured from the leaves, leaf buds and internodes of this shrub. Of the total amount of tea produced and consumed in the world, 78% is black, 20% is green, and 2% is oolong. Black tea is consumed primarily in western countries and in South Asian countries such as Bangladesh, India and Sri Lanka, whereas green and oolong teas are consumed mainly in East Asian countries such as China, Japan, and Taiwan (Chan *et al*., 2015).

Preliminary studies showed the existence of secondary metabolites in green tea. Studies have further reported the presence of antioxidants and antimicrobial properties in green tea (Khisa *et al*., 2001). Hence, the current work was dedicated towards the investigation on the antimicrobial and antioxidant activities of locally produced green tea.

Widespread indiscriminate usage of commercial antibiotics is the leading cause of multiple drug resistance in human pathogenic microorganisms. Due to the emergence of antibiotic resistant strains, it is becoming increasingly necessary to develop therapeutic alternatives derived from natural resources such as green tea.

1.1 Background

Commercial cultivation of tea started from establishing the Malnicherra Tea Estate in Sylhet in 1857 (Khisa *et al*., 2001). At present tea industry has spread over 172 tea estates which comprise of 48,587 hectares of land and a total number of 158 gardens (Khisa *et al*., 2001). 93% of this plantation area being in Sylhet of which, 62% is of Moulvibazar district. Thus, 96% of annual tea production is contributed by Sylhet, 63% of which is from the district Moulvibazar (Khisa *et al*., 2001). Among thirty countries that contribute to global tea production, Bangladesh annually produces 2% of the world production and 3% of global export. Average earning from world export is around 1,500 to 2,000 million BDT, share in national export is 1.2% and contribution towards GDP is 0.81% (Khisa *et al*., 2001). This agricultural, export oriented industry currently offers direct employment to around 1, 20,000 poor people, 50% of who are women (Ahammed, 2012).

Major studies in Bangladesh are done in agricultural areas surrounding the economic importance of tea. Especially due to the rising health awareness in the growing urban population, green tea has created a local as well as global demand due to its various health benefits. Isolation of the antimicrobial and antioxidant property could create a new arena in research and development of pharmaceutical products; thereby, creating an impact on the national growth and economy.

1.2 The Tea Plant

1.2.1 Taxonomic Hierarchy (Integrated Taxonomic Information System, n.d.)

Kingdom	Plantae – plantes, Planta, Vegetal, plants
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta – land plants
Superdivision	Embryophyta
Division	Tracheophyta – vascular plants, tracheophytes
Subdivision	Spermatophytina – spermatophytes, seed plants
Class	Magnoliopsida
Superorder	Asteranae
Order	Ericales
Family	Theaceae – tea
Genus	Camellia L. – tea
Species	*Camellia sinensis* (L.) Kuntze – tea

1.2.2 Description of the tea plant

Camellia sinensis is an evergreen tree or shrub with yellow-white flowers and long, serrated leaves. Flowers are axillary, solitary, or up to three in a cluster. They are 2.5-3.5 cm in diameter and have six to eight petals. The outer petals are sepaloid and the inner petals are obovate to broadly obovate. There are numerous stamens 0.8-1.3 cm in length. Flowering of *Camellia sinensis* occurs from October through February and fruiting occurs from August to October Young leaves have short white hairs on their underside and young branches are grayish yellow and glabrous. Current year branchlets are purplish red. Terminal buds are silvery gray and sericeous. Petioles are 4-7 mm in length, pubescent, and glabrescent. Leaf blades are elliptic, oblong-elliptic, or oblong. Seeds are brown, subglobose, and 1-1.4 cm in diameter (Ahmed and Stepp, 2012).

proper growth. Cultivation may occur from sea level to 2,200 meters. Bangladesh provides these favorable climatic conditions for tea cultivation especially over the eastern region in Sylhet, Habigonj, Moulvibazar and Chittagong (Khisa *et al*., 2001). Higher altitudes are often associated with higher tea quality. Soils must be well-drained, sandy, thoroughly aired, deep and nutritious with a healthy layer of humus and low pH (Ahmed and Stepp, 2012). Drought, water logging, excessive heat, and frost are harmful for the growth of tea plants and may result in a lower quality product in terms of chemistry, taste, aroma, and bioactivity.

Tea plants are self-incompatible hence they cross-pollinate and are propagated sexually by seeds or asexually by vegetative cuttings of clonal propagules. Grafting may also be used for vegetative propagation and seedlings may be used as rootstocks. Tea plants cultivated from seeds result in heterozygous products compared to plants propagated by vegetative cuttings (Ahmed and Stepp, 2012). Tea plants are raised in controlled nursery conditions for the first two to four years. During this immature period they are not harvested. Once the tea plants mature, they are transplanted to the field and are ready for harvest (Ahmed and Stepp, 2012).

1.3 Processing of green tea

The quality of green tea is affected by the numerous steps involved in processing (Figure 2) (Ahmed and Stepp, 2012). Green tea is non-fermented, steamed and dried to inactivate the polyphenol oxidase enzyme, to prevent catechin oxidation. This preserves the polyphenols in their monomeric forms. Fresh tea leaves are heated for 10-15 minutes in a process known as fixing to prevent oxidation and fermentation of polyphenol oxidase, catalase, peroxidase and ascorbic acid oxidase in order to deactivate them; thereby, retaining their natural green color. Steaming generally preserves more color, polyphenolic content and antioxidant bioactivity than pan fixing (Ahmed and Stepp, 2012). Once the enzyme has been deactivated, it will not produce any theaflavines or thearubigines. Hence, green tea does not develop the red

liquor that is characteristic of black tea. On the contrary, black tea is produced by prolonged fermentation of tea leaves producing thearubigins and theaflavins which contribute to the red colour of black tea. Oolong tea is semi- fermented and contains a mixture of the monomeric polyphenols and theaflavins (Graham, 1992). All three types of tea contain 3–6% of caffeine despite the different processing methods (Chu, 1997).

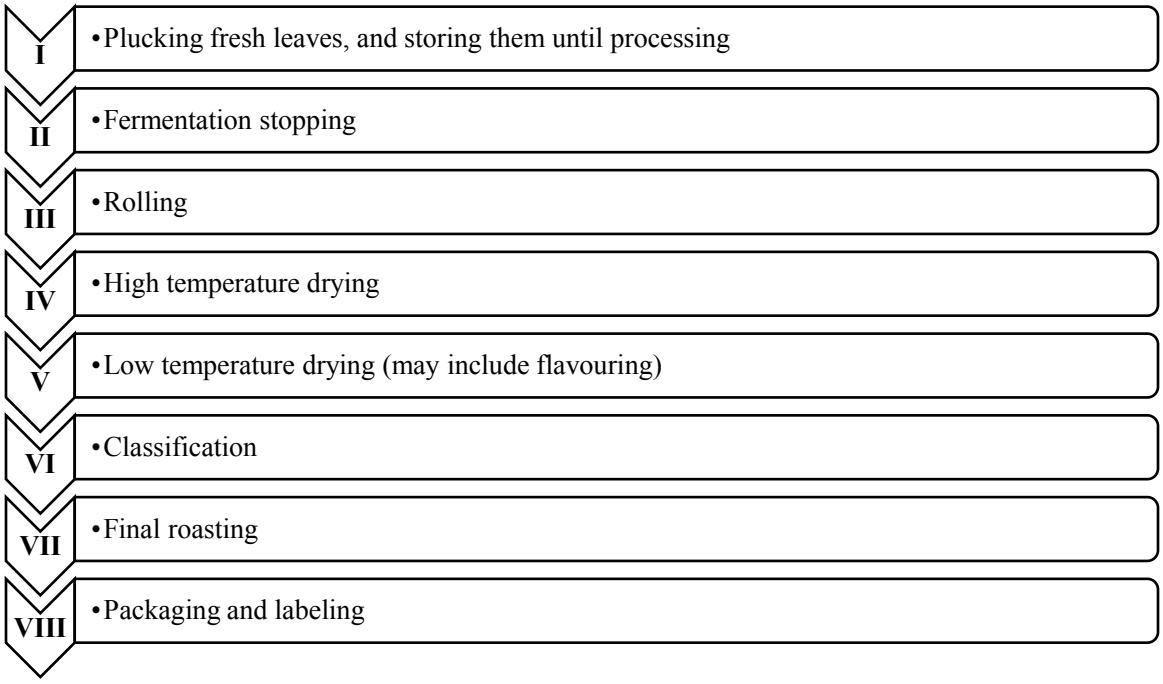

Fig. 2: Steps involved in the processing of green tea (Ahmed and Stepp, 2012).

1.4 Phenolic Compounds

Phenolic compounds are secondary metabolites synthesized by plants as a response to ecological stress and physiological pressures like pathogens, insect attacks, UV radiation and wounding. These renowned phytochemicals are acknowledged for their contribution in healthy human diet and disease prevention. There is a detailed classification of plant phenolic compounds in Figure 3. Overview of the phytochemicals that were analyzed in this study is provided.

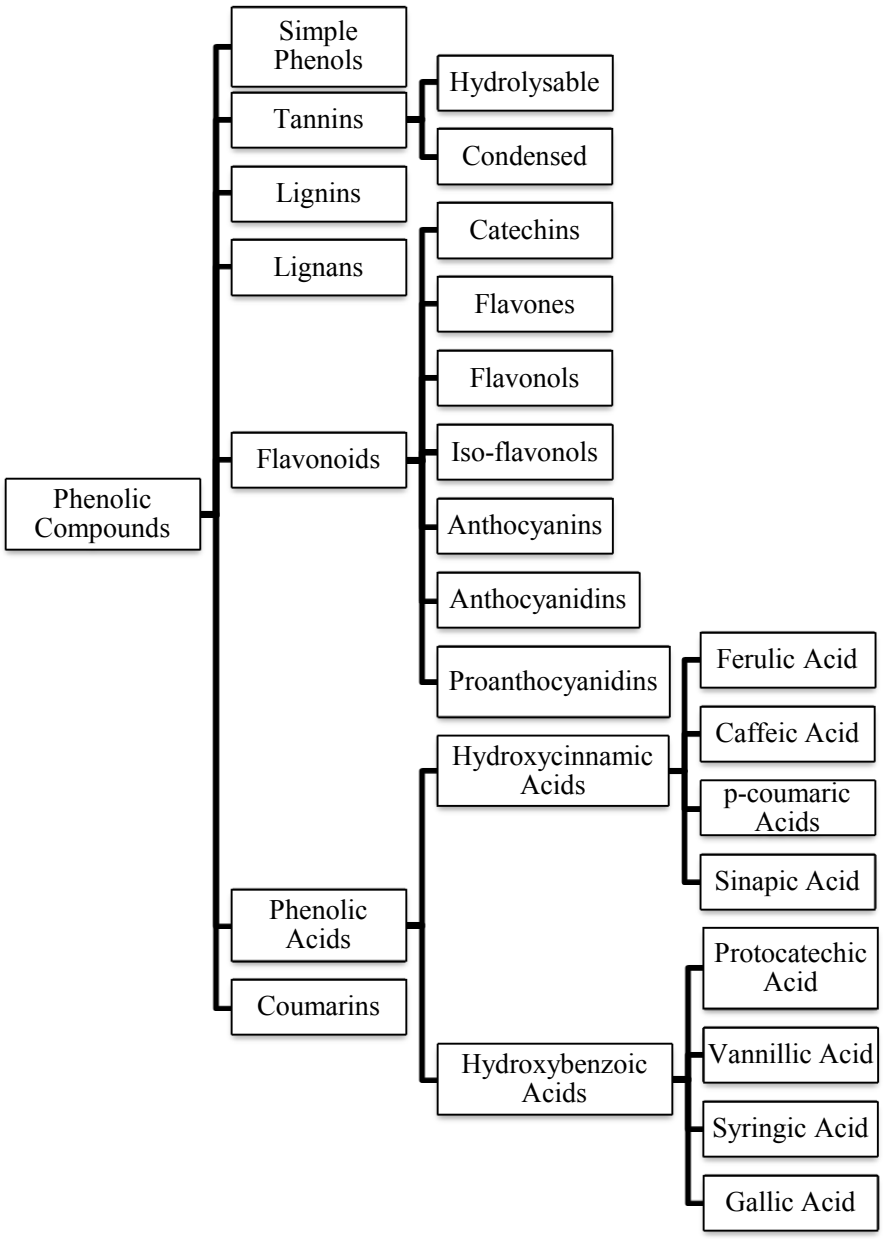

Fig. 3: Detailed classification of plant phenolic compounds (Khoddami *et al*., 2013)

1.4.1 Phenols

Phenolics are aromatic benzene ring compounds with one or more hydroxyl groups produced by plants mainly for protection against stress. Phenolics play important roles in plant development, particularly in lignin and pigment biosynthesis. They also provide structural integrity and scaffolding support to plants. Importantly, phenolics

secreted by wounded plants, repel or kill many microorganisms, and some pathogens can counteract or nullify these defences or even subvert them to their own advantage (Bhattacharya *et al*., 2010).

1.4.2 Alkaloids

Alkaloids are a heterogeneous class of naturally occurring secondary metabolites. They are organic nitrogen-containing bases which have diverse and significant physiological effects on humans and animals. Distinguished alkaloids include morphine, strychnine, quinine, ephedrine, and nicotine. Alkaloids are found primarily in plants and are especially common in certain families of flowering plants (Britannica, n.d.).

1.4.3 Cardiac Glycoside

Cardiac glycosides are glycosides of mostly C^{23}-steroidal compounds. They are called cardiac glycosides because they modify heart action and are used to treat congestive heart failure. Cardenolides inhibit the Na^{+}-K^{+}-ATPase pump in mammals. There are about 400 known cardenolides which are derived from steroidal precursors, such as cholesterol, via the intermediacy of pregnenolone or progesterone intermediates (Life, n.d.).

1.4.4 Saponins

Saponins are glucosides with foaming characteristics. The foaming ability of saponins is caused by the combination of a hydrophobic sapogenin and a hydrophilic sugar part. Saponins are used in beverages, such as beer, to produce stable foam. Studies have illustrated the beneficial effects on blood cholesterol levels, reduce risk of cancer, bone health and stimulation of the immune system (Phytochemicals Information, n.d.). Rising consumer demands for natural products coupled with physicochemical properties and mounting evidence on their biological activity (such as anticancer and anticholesterol activity) has led to the emergence of saponins as commercially significant compounds with expanding applications in food, cosmetics, and pharmaceutical sectors (Güçlü-Ustündağ and Mazza, 2007).

1.4.5 Flavonoids

Flavonoids are some of the most common phenolics, widely distributed in plant tissues, and often responsible alongside the carotenoids and chlorophylls for their blue, purple, yellow, orange and red colors. The flavonoid family is elaborated in Figure 6. All flavonoids are derived from the aromatic amino acids, phenyalanine and tyrosine, and have three-ringed structures (Khoddami *et al*., 2013).

1.4.6 Steroids

In plants, steroids play an important role as constituents of cell membranes, insect deterrents, and growth hormones. Steroids extracted from plants have been used in traditional medicine and some of these are still valuable drugs (Zeelen, 1995).

1.4.7 Tannins

Tannins or tannic acid are water-soluble polyphenols. Results indicate that tannins reduce the mutagenic activity of a number of mutagens. The anticarcinogenic and antimutagenic potential of tannins may be related to their antioxidative property, which is important in protecting cellular oxidative damage, including lipid peroxidation. The antimicrobial activities of tannins are well documented. The growth of many fungi, yeasts, bacteria, and viruses was inhibited by tannins. Tannins can thus be used to increase the shelf-life of certain foods accelerate blood clotting, reduce blood pressure, decrease the serum lipid level, produce liver necrosis, and modulate immune responses (Chung *et al*., 1998).

1.4.8 Gums

Gums are water-soluble, non-starch polysaccharides that are used in practical applications primarily to thicken or gel aqueous systems and to control water. They may also function as adhesives, crystallisation inhibitors, emulsifying agents, emulsion stabilisers, encapsulating agents, film formers, foam stabilisers, suspending agents, suspension stabilisers etc (BeMiller, 2014).

1.5 Assessment of Antimicrobial Activity of *Camellia sinensis* Extracts Against Bacteria

Bioassays play an important role in the evaluation of a particular bioactivity. A bioassay which is applied to large numbers of initial samples to determine whether or not they have any bioactivity such as antibacterial or antioxidant assay is referred to as a prescreen assay. Hence, the tests must be simple, rapid, reliable, reproducible, sensitive, meaningful and, most importantly, predictive. The *in vitro* assessment of antimicrobial susceptibility is done by Agar-Well Diffusion Assay. Brief overview of the selected bacteria on which the antibacterial effects were observed:

1.5.1 *Escherichia coli*

It is a gram negative bacterium. Although they are commensal gut bacteria, some pathogenic strains produce enterotoxins that cause very serious foodborne diseases and gastrointestinal infections. On entering the urinary tract, E. *coli* can cause urinary tract infections (Tortora *et al*., 2010).

1.5.2 *Staphylococcus aureus*

It is named thus for its yellow-pigmented colonies *(aureus* means golden) and occurs in grapelike clusters. It is one of the most common gram positive facultative anaerobic bacteria responsible for food poisoning (Wikipedia, n.d.). **Methicillin-resistant *staphylococcus aureus* (MRSA)** is a leading problem as it is a multi-drug resistant superbug.

1.5.3 *Shigella flexneri*

This is the causative agent of shigellosis. A disease found only in humans. Abdominal cramps and fever are some of the symptoms of infection. The bacteria rarely invade the bloodstream. It is quickly developing resistance against antibiotics such as ampicillin, chloramphenicol, streptomycin, trimethoprim-sulphamethoxazol and tetracycline (Replogle *et al*, 2000).

1.5.1 *Enterotoxigenic Escherichia coli* (ETEC)

It is one of several strains of E. *coli*. ETEC is not invasive but produces an enterotoxin that causes a watery diarrhea that resembles a mild case of cholera (Tortora *et al*., 2010).

1.6 Synergistic Activity of Tea and Antibiotics

Combination chemotherapy, the use of two or more antimicrobial agents, is being employed in medical practice with increasing frequency. The rationale for using a combination of drugs is the expectation that the effective combinations might lower the incidence of bacterial response, reduce host toxicity of the antimicrobial agents (due to lower dosage requirements), or enhance the agents' bactericidal activity. Enhanced bactericidal activity is known as synergism. Synergistic activity is evident when the sum of the effects of the chemotherapeutic agents used in combination is significantly greater than the sum of their effects when used individually. This result is readily differentiated from an additive effect, which is evident when the interaction of two drugs produces a combined effect that is no greater than the sum of their separately measured individual effects (Cappuccino and Natalie, 2013).

1.7 Common Extraction Techniques

Extraction is generally influenced by several parameters such extraction time, temperature, solvent-to-sample ratio, the number of repeat extractions of the sample, as well as solvent type (Khoddami *et al*., 2013). The quality of polyphenol extracts and their antioxidant activity depend not only on the quality of the starting material (geographic origin, climatic conditions, harvesting date, storage conditions), but also on the technological processes involved in manufacturing. Extraction must take place under mild and efficient conditions in order to minimize the alteration of tea flavanols, which are especially sensitive to epimerisation and oxidative oligomerisation (Wang and Helliwell, 2000; Roginsky and Alegria, 2005; Wang *et al*., 2006). The choice of extraction solvents such as water, acetone, ethyl acetate, alcohols (methanol, ethanol and propanol) and ratio of their mixtures will influence

the overall recovery of phenolic compounds. While increasing time and temperature promote analyte solubility, plant phenolics are normally degraded or undergo undesirable reactions such as enzymatic oxidation by prolong extraction and high temperatures (Khoddami *et al*., 2013).

Soxhlet, heated reflux extraction and maceration are conventional procedures commonly used to recover phenolics from solid samples. The Soxhlet and heated reflux methods are normally performed at high temperatures for several hours while maceration is performed over days at ambient temperatures (Khoddami *et al*., 2013). Though these methods are simple, require relatively cheap apparatus and result in moderately high rates of extraction, they work less efficiently in terms of the time required for extraction, the yield and quality of extract produced. While there are many positive aspects of Soxhlet extraction, there are substantial disadvantages such as: (1) large volumes of organic solvents are required, which are environmental pollutants and health hazards; (2) long extraction hours and (3) interference with, and degradation of, targeted components due to light, air, high temperatures, prolonged exposure to high temperatures and undesired enzymatic reactions (Khoddami *et al*., 2013).

Organic solvent extraction is the main method used to extract phenolics and separate active ingredients from plant materials. Hence, it is extensively used in the process of isolation of phytoconstituents from plants. Conventional techniques include maceration, decoction, infusion, enzymatic extraction, and continuous hot (Soxhlet) extraction (Kalakoti *et al*., 2014), Liquid-Liquid Extraction (LLE), Simultaneous Distillation-Solvent Extraction (SDE) (Gu *et al*., 2009).

1.7.1 Recent Extraction Techniques

Substantial developments have occurred in recent years to develop modernized methods that were found to be more advantageous and efficient over orthodox techniques of extraction, because of their sophisticated design and improved working

performance (Kalakoti *et al*., 2014). These are namely, (1) Ultrasound-Assisted Extraction (UAE), (2) Microwave-Assisted Extraction (MAE), (3) Microwave-Assisted High Pressure Extraction, (4) Ultrasound/Microwave Assisted Extraction (UMAE), (5) Supercritical Fluid Extraction (SFE), (6) Subcritical Water Extraction (SCWE), (7) High Hydrostatic Pressure Extraction (HHPE), (8) Pulsed Electric Field (PEF) processing, (9) Accelerated Solvent Extraction (ASE) , (10) Sequential Alkaline Extraction method (Khoddami *et al*., 2013) and many more.

1.8 Medicinal Value of Green Tea

History of medicine dates back to ancient civilizations (Ahmed and Stepp, 2012). Plants have been used as a source of medicine throughout history and continue to serve as the basis for many pharmaceuticals used today. Despite the emergence of modern pharmaceutical industry from botanical medicine, synthetic approaches to drug discovery have become standard. Plants continue to serve as a valuable source of therapeutic compounds due to the vast biosynthetic capacity they possess (Schmidt *et al*., 2008). It is considered that the complexity and the vast number of secondary metabolites will constitute a resource beyond the capacity of current synthetic chemistry throughout time (Koch, *et al*., 2005). Recently, there has been a renewed interest in natural products due to the failure of alternative drug discovery methods (Lahlou, 2013).

Many synthetic drugs cause numerous side effects that are unacceptable as general methods of treatment save for terminal diseases like cancer. The metabolites discovered in medicinal plants may avoid the side effects of synthetic drugs, because they must accumulate within living cells. Biosynthesis of natural products involves repeated interaction with modulating enzymes, and the biological function of many of them involves protein binding and interaction. Thus, the ability of plant metabolites to interact with other molecules is an indispensable prerequisite to making an effective drug. This is considered to be biologically validated. The fact remains that many

natural products demonstrate advanced binding characteristics compared to synthetics (Lutz, 2003).

The popularity of green tea is increasingly globally due to its countless beneficial effects in cancer, weight loss, diabetes, neurological and cardiovascular diseases. Awareness of these beneficial effects in the consumer society has led to the increased consumption of green tea by patients and people alike (Zaveri, 2005). It is medicinally beneficial because the non-fermented leaves retain a higher concentration of natural vitamins and polyphenols than fermented tea (Rahman *et al*., 2013).

Green tea extracts have been used as natural antibacterial and antiviral agents. Many studies revealed that green tea catechine have antibacterial and antiviral activity by its effectiveness against any type of diarrhea and typhoid, also it inhibits the reproduction and growth of many bacteria, which some types of *Salmonella*, *Clostridium*, *Bacillus*, *Helicobacter pylori*, and *Staphylococcus aureus*. Regarding its antiviral action, it affects against the Influenza virus, especially in its earliest stage, as well as against Herpes simplex virus and Adenoviral infection. These findings may aid in drug discovery against such pathogens which could prove to be revolutionary for Bangladesh where infectious diseases are a staggering concern.

Evidence suggests that tea consumption is beneficial for a wide range of clinical illness such as cancer, hypercholesterolemia, atherosclerosis, stroke, Parkinson's disease, Alzheimer's disease, diabetes, inflammation, microbial diseases, aging etc (Zaveri, 2005). Tea polyphenols are free radical scavengers, metal chelators, inhibitors of transcription factors and enzymes. Therefore green tea extracts have been used as natural antioxidants. According to the free radical theory of aging (Harman, 1994), increased free radical generation and oxidative stress are the basis for phenotypic changes that lead to age-associated functional deterioration and neurodegeneration (Zaveri, 2005). The human body has a complex system of natural enzymatic and non-enzymatic antioxidant defenses which counteract the harmful effects of free radicals and other oxidants. Free radicals are responsible for causing a

large number of diseases associated with reactive oxygen species (ROS), such as cancer, cardiovascular and neurodegenerative diseases (Zaveri, 2005).

Protection against free radicals can be enhanced by ample intake of dietary antioxidants. Substantial evidence indicates that foods containing antioxidants and possibly in particular the antioxidant nutrients may be of major importance in disease prevention. Antioxidants may be of great benefit in improving the quality of life by preventing or postponing the onset of degenerative diseases (Zaveri, 2005).

1.9 Objectives

There is ample potential in the study of green tea. It is known to contain numerous secondary metabolites which create plenty of room for research and clinical trials of pharmaceutical agents and novel compounds which may serve to combat drug resistance problems. In this study, three different types of extracts; ethanolic, methanolic and aqueous, from samples of *Camellia sinensis* were collected and investigated. The objectives of the present study are as follows:

- Establishment of a suitable extraction process for green tea (*Camellia sinensis*) and collection of extracts
- Detection of the presence of certain metabolites using standard phytochemical assays in the extracts
- Study of antimicrobial activity of green tea extracts on five selected microorganisms
- Study of synergistic activity of green tea extracts with specific antibiotic discs
- Study of antioxidant activity in green tea extracts

Chapter 2: Materials and Methods

2. Materials and Methods

This study was performed in the Biotechnology Laboratory in the Department of Mathematics and Natural Sciences, BRAC University.

2.1 Specimen Collection and Sample Preparation

Commercially available Orthodox Green Tea was used in this survey which is 100% organic and grown in northern Bangladesh. Orthodox tea refers to whole-leaf tea that is either hand-processed or rolled with machinery to mimic hand-rolling (Orthodox Tea, n.d.). In order to obtain maximum extract the tea leaves were ground in a mortar and pestle prior to extraction to increase surface area of the tea during the process of extraction.

2.2 Method of Extraction

Soxhlet extraction was carried out to obtain pure crude extracts of *Camellia sinensis* using two different solvents for the extraction procedure and hot aqueous extraction procedure was carried out for the collection of aqueous extract.

2.2.1 Ethanolic Extraction

250 ml of absolute ethanol was poured in the round-bottom flask of the soxhlet apparatus and brought to a boil at 70^{o}C then lowered to 65^{o}C. The end of the thimble was secured with cotton wool to prevent the ground sample from falling through. Then 60g of ground sample was weighed in an electronic balance (Appendix IIII) and heated under reflux at 65^{o}C for 3 to 4 hours. Throughout the period of extraction, temperature was kept close to the boiling point of ethanol which ensures the solvent is constantly on a boil so that the evaporation and condensation process may proceed unhindered without raising the temperature exceedingly which may reduce the yield of phenolics in the extract.

2.2.2 Methanolic Extraction

250 ml of absolute methanol was added to the round-bottom flask of the soxhlet apparatus (Appendix IIII) and brought to a boil at 65^{o}C then dropped to 60^{o}C. After securing the end of the thimble with cotton wool, 60g of tea sample was measured in an electronic balance (Appendix IIII) and heated under reflux at 60-65^{o}C for 3 to 4 hours. Temperature was maintained within this range during the course of the extraction to secure the reflux process without running the risk of burning the extract collecting in the round-bottom flask.

Following extraction, the collected alcoholic extracts were poured into petri dishes and left uncovered at room temperature in a closed, clean, dry fume-hood overnight to allow the excess solvent to evaporate and produce sticky viscous extracts.

2.2.3 Hot Aqueous Extraction

25g of tea sample was measured into a 500ml conical flask to provide adequate room for shaking. 250 ml of boiling distilled water was poured over the ground tea sample and stirred. After sealing the mouth of the flask with foil and parafilm to avoid contamination, it was placed in a shaker incubator for continuous swirling at 120 rpm, 30^{o}C for 24 hours. The boiling water was allowed to cool throughout the extraction process to mimic tea brewing. Afterwards, the mixture was filtered through Whatman No. 1 filter paper (Chan *et al*., 2011). The filtrate was then subjected to centrifugation (Appendix IIII) at 8000 rpm to allow the sedimentation of minuscule ground tea leaves that were sieved through during filtration. The supernatant was collected in petri dishes using a micropipette and the residual water was evaporated in a water bath at 95^{o}C. Once the excess water had evaporated a thick viscous extract was obtained.

2.3 Storage and Preservation of Extracts

Once the desired consistency was achieved, the extracts were stored in 25 ml McCartney bottles which were thoroughly cleaned with 70% ethanol prior to storage.

Bottles were tightly stoppered and wrapped in foil to protect any photosensitive compounds that maybe present in the extracts from degradation. After proper labeling, the McCartney bottles were stored at $4^{o}C$ for preservation and prevention from external contaminants.

2.4 Preparation of Stock Solution for Phytochemical Assays

0.5gm of crude extract was dissolved in 100 ml of respective solvent to make a stock solution of 5µg/µl. This served as the working solution for all the phytochemical tests without any further dilutions.

2.5 Phytochemical Screening of *Camellia sinensis* extracts

Qualitative preliminary screening of all three extracts was carried out for identifying the presence or absence of any phytochemicals present in the extracts. The crude extracts were initially tested for the presence of phenolic compounds following the detection of several secondary metabolites such as alkaloids, steroidal compounds, flavonoids, saponins, tannins and cardiac glycosides (Shanmugam *et al.,* 2010). As per the literature review, this nature of qualitative assay has not been reported in Bangladesh for the study of green tea. All three extracts were tested individually. All tests were conducted in triplicates. Two types of negative control were used; only reagent and only sample. In some cases, one or both controls were applied.

2.5.1 Test for Phenolic Compounds

1% ferric chloride solution and 1% potassium ferrocyanide were mixed in equal amounts. 3 drops of this freshly prepared solution was added to 2ml of extract. The formation of a blue-black color is considered positive.

2.5.2 Test for Alkaloids

There are a various number of tests for alkaloids, of which four were used to detect the presence or absence of alkaloids in the tea extracts.

2.5.2.1 Hager's Test

1 ml of extract was carefully mixed with 3 drops of freshly prepared Hager's reagent (Appendix II) in a test tube. The formation of yellow precipitates showed a positive result and the presence of alkaloids in the extract.

2.5.2.2 Wagner's Test

1 ml of extract was mixed together in a test tube with 3 drops of Wagner's reagent (Appendix II). The formation of brown precipitate showed the presence of alkaloids.

2.5.2.3 Test with Marquis' Reagent

The addition of Marquis' reagent (Appendix II) yields a precipitate in any sample in the presence of alkaloids.

2.5.3 Test for Steroidal Compounds (Salkowaski's Test)

2ml extracts were dissolved in 2ml chloroform in a test tube. 4ml concentrated sulfuric acid (97%) was carefully poured from the inner wall of the test tube. Formation of a red colour in the upper layer and sulphuric acid layer turning yellow with green fluorescence is indicative of positive for steroids.

2.5.4 Test for Flavonoids (Lead Acetate Test)

1ml of freshly prepared 10% lead acetate was added to 1ml of extract. Formation of a precipitate indicates the presence of flavonoids.

2.5.5 Tests for Saponins (Froth Test)

0.5g of each type of extract was dissolved in 10ml distilled water. The test tube was stoppered and then shaken vigorously for 30 seconds. It was then allowed to stand for 30 minutes. Formation and retention of honey-comb froth on the surface for 30 minutes is considered positive for saponins.

2.5.6 Tests for Tannins (Ferric Chloride Test)

Few drops of freshly prepared 10% ferric chloride were added to 1ml sample extract. Formation of blue-black precipitate indicates the presence of tannins.

2.5.7 Test for Cardiac Glycoside (Killer-Killani Test)

2ml glacial acetic acid was added to 5ml of extract followed by 1 drop of ferric chloride and 1 ml concentrated sulphuric acid. A positive result shows a brown ring at the interface.

2.5.8 Test for Gums (Molisch's Test)

To 5ml extract, Molisch's reagent (Appendix II) and concentrated sulphuric acid were added drop-wise. Positive result shows a red-violet ring.

2.6 Antibacterial Activity of Different Extracts of *Camellia sinensis*

The *in vitro* assessment of antimicrobial susceptibility is done by Agar-Well Diffusion Assay. The antimicrobial activity of the extracts of *Camellia sinensis* against the targeted microbes was studied. Standard antibiotic discs were used as positive control and to further examine the synergistic activity of tea and antibiotics. For each organism, nine plates were inoculated having three replicates of each extract. Each test was replicated thrice for validation and accuracy of the results. Ciprofloxacin was used as positive control for *E. coli* and ETEC. Ampicillin served as positive control for MRSA and S. *aureus*; lastly, cefoxitin was positive control for S. *flexneri*.

2.6.1 Aseptic Conditions

All antibacterial assays were conducted inside the biosafety hood under complete aseptic conditions. The laminar air flow chamber with HEPA filters was cleaned with 70% ethanol.

2.6.2 Test Organism

The pathogens used in this experiment were obtained from clinical isolates from icddr,b and preserved in the Biotechnology Laboratory, Department of Mathematics and Natural Sciences, BRAC University**.** In this study, four bacterial strains *Escherichia coli*, *Enterotoxigenic Escherichia coli, Shigella flexneri*, Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus aureus* were used**.**

2.6.3 Maintenance and preparation of inoculum

Each organism was cultured on nutrient agar (Appendix I) and only fresh cultures were used. Cell suspensions were freshly prepared the day of the test using fresh cultures only. Loop full of the test organisms were taken from fresh cultures incubated 24 hours prior to the test and dissolved in individual autoclaved test tubes, each containing 10ml of 0.9% NaCl to make the respective bacterial cell suspensions. The test tubes were vigorously mixed using a vortex (Appendix IIII) to ensure complete dissolution of the bacterial cells within the saline. The cell suspensions were of equal concentration to make sure that there are an equal number of cells in every cell plate. The concentration of each cell suspensions were checked until the turbidity equals or exceeds that of a 0.5 McFarland Standard. The suspensions were adjusted to obtain turbidity optically comparable to that of the 0.5 McFarland. A spectrophotometer was used to measure an absorbance of 0.110 at 600 nm which is the standard turbidity of 0.5 McFarland Standard.

2.6.4 Preparation of Extract Solution for Antibacterial Activity Test

All three types of the collected crude extracts of *Camellia sinensis* were dissolved in 0.25% (v/v) autoclaved dimethyl sulphoxide (DMSO) to make two different concentrations of extract solutions for the antibacterial activity tests. 1.2gm of crude extract was dissolved in 15ml of 0.25% DMSO to prepare 80µg/µl of extract. This was further diluted to 60µg/µl by using 7.5ml of the 80µg/µl stock and further diluting it with 12.5ml DMSO.

2.6.5 Inoculation of Media

Antimicrobial activity was observed by preparing lawn culture after turbidity of bacterial suspension was adjusted to equivalent of 0.5 McFarland on nutrient agar plates. The lid was left slightly ajar to allow inoculum to be absorbed before carrying out agar-well diffusion method.

2.6.6 Agar-Well Diffusion and Application of Disks for Bioassay of Different Concentrations of Extracts

Test plates were previously labeled into four quadrants; one for positive control, second for crude extract, third for 80µg/µl of extract and the fourth for 60µg/µl of extract. Three wells of about 0.6mm diameter were aseptically cut out from the inoculated plates using the back of sterile micropipette tips allowing 30mm between adjacent wells and the edge of the petri dishes. 60 µl of each extract were then added into the wells. This process was repeated for each extract as every plate contained three concentrations of a single extract. Antimicrobial discs were applied as positive control to the first quadrant of the inoculated plates no more than 15 minutes following inoculation. Flame sterilized bend-forceps were used to impregnate the discs on the agar by tapping them gently to ensure complete contact with the agar surface. Care was taken not to relocate the disks once it has come in contact with the agar surface as the drug diffuses almost instantaneously. Plates were kept in an upright position in an incubator to allow the extracts to diffuse into the agar. The plates were incubated for 24 hours at 37^{o}C and observed for zone of inhibition (mm) around the wells. The extracts producing significant zone of inhibition were subsequently tested for synergistic activity with standard antibiotic discs.

2.6.7 Measuring Activity Index

The inhibitory effects of the methanolic, ethanolic and aqueous extracts were calculated and compared by measuring the activity index using the following formula (Rahman, 2014):

$$\text{Activity Index} = \frac{\text{Zone of inhibition of extract}}{\text{Zone of inhibition of antibiotic}}$$

2.7 Synergistic Activity of Tea and Antibiotics

In the current study, a disc-agar diffusion technique was performed to demonstrate the synergistic activity of green tea with the respective antibiotics. This technique uses the Kirby-Bauer antibiotic susceptibility test procedure. Bacterial cultures were swabbed on their respective nutrient agar plates from their cell suspensions. Antibiotic discs were soaked in extracts for a few minutes. The plates were divided into three equal sections and labeled as having only antibiotic disk in one segment, antibiotic disc soaked in extract in the second segment and only the respective extract in the last section. Here, both the antibiotic disc and the extract serve as positive controls for comparison of synergistic or additive activity. The two discs representing the drug combination are placed on the inoculated agar plate separated by a distance (in mm) that is equal to or slightly greater than half the sum of their individual zones of inhibition when obtained separately. The plates were placed upright and incubated at 37^{o}C for 24 hours and synergistic or additive activity was recorded by measuring the zones of inhibition (mm).

2.8 Antioxidant Assay: DPPH Scavenging Activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a stable free radical due to the delocalization of its spare electron over the molecule so that the molecule does not dimerize like most other free radicals. The delocalization of electron gives rise to the characteristic deep violet color of DPPH solution. When a solution of DPPH is mixed with that of a hydrogen donating substrate, DPPH gets reduced, resulting in the loss

of this violet color. In methanolic or ethanolic solutions of DPPH, the absorbance of this violet colour is generally measured at 517 nm (Alam *et al*., 2013). The antioxidant activity of the extracts was assessed using DPPH free radical scavenging activity by modified method (Prabhune *et al*., 2013).

2.8.1 Preparation of Standard Solution

Ascorbic acid was used as standard by dissolving ascorbic acid in methanol to give the concentration of 10, 50, 100, 500 and 100µg/ml.

2.8.2 Preparation of Test Sample

Stock solutions of each extract of sample was prepared by dissolving 10 mg extract in 10 ml of respective solvent to make concentration of 1mg/ml. This was further diluted into different concentrations of 10, 50, 100, 500 and 100µg/ml which were used in the assay.

2.8.3 Preparation of DPPH Solution

0.1 mM concentration of DPPH was freshly prepared by dissolving 4 mg of DPPH in 100 ml methanol. This was wrapped in aluminum foil beforehand in order to protect the reagent from light.

2.8.4 Measuring Absorbance of Control

For the control, 1ml DPPH was added to 3ml methanol and the absorbance was immediately measured at 517 nm wavelength. This is the control absorbance or blank containing all reagents except the test sample.

2.8.5 Measuring Absorbance of Standard

In each test tube 3ml of each concentration of ascorbic acid was added along with 1ml of DPPH solution. After 30 minutes dark incubation at room temperature, the absorbance was measured at 517nm keeping methanol as blank.

2.8.6 Measuring Absorbance of Sample Extracts

3ml of each concentration of sample was added to 1ml of DPPH solution in each test tube. After 30 minutes dark incubation at room temperature, the absorbance was measured at 517nm keeping methanol as blank.

2.8.7 Determination of Antiradical Activity

Each experiment was carried out in triplicate and results are expressed as mean % antiradical activity. The free radical scavenging activity (FRSA) (% antiradical activity) was calculated using the following equation (Sanja *et al*., 2009):

$$\%\ \text{Scavenging Activity} = \frac{\text{Control Absorbance} - \text{Sample Absorbance}}{\text{Control Absorbance}}\ \text{X}\ 100$$

Chapter 3: Results

3. Results

3.1 Phytochemical Screening

Specific tests were conducted to screen for the presence of the following phytochemicals in three different extracts of *Camellia sinensis*. The presence of phenolic compounds was first screened. On obtaining consistent positive results; Hager's Test, Marquis' Test, Wagner's Test, Lead Acetate Test, Salkowaski's Test, Froth Test, Ferric Chloride Test and Molisch's Test were performed and their results are recorded in Table 1. The study indicates the presence of phenolics, alkaloids, flavonoids, steroids, saponins, tannins and cardiac glycosides in the crude forms of ethanolic, methanolic and aqueous extracts of *Camellia sinensis*; however, gums were present only in the crude aqueous extract (Fig. 4).

Table 1: Results of phytochemical tests performed

Chemical Group Test	**Specific Test**	**Observation**		
		Ethanolic Extract	**Methanolic Extract**	**Aqueous Extract**
Phenolics	—	positive	positive	Positive
Alkaloids	a) Hager's Test	negative	negative	negative
	b) Marquis' Test	positive	positive	Positive
	c) Wagner's Test	positive	positive	Positive
Flavonoids	Lead Acetate Test	positive	positive	Positive
Steroids	Salkowaski's Test	positive	positive	Positive
Saponins	Froth Test	positive	positive	Positive
Tannins	Ferric Chloride Test	positive	positive	Positive
Gums	Molisch's Test	negative	negative	Positive
Cardiac Glycosides	Killer-Killani Test	positive	positive	Positive

* All tests were performed in triplicate

3.1.1 Test for Phenolic Compounds

1% ferric chloride solution and 1% potassium ferrocyanide were mixed in equal amounts. 3 drops of this freshly prepared solution was added to 2ml of extract. The formation of a blue-black color was considered positive.

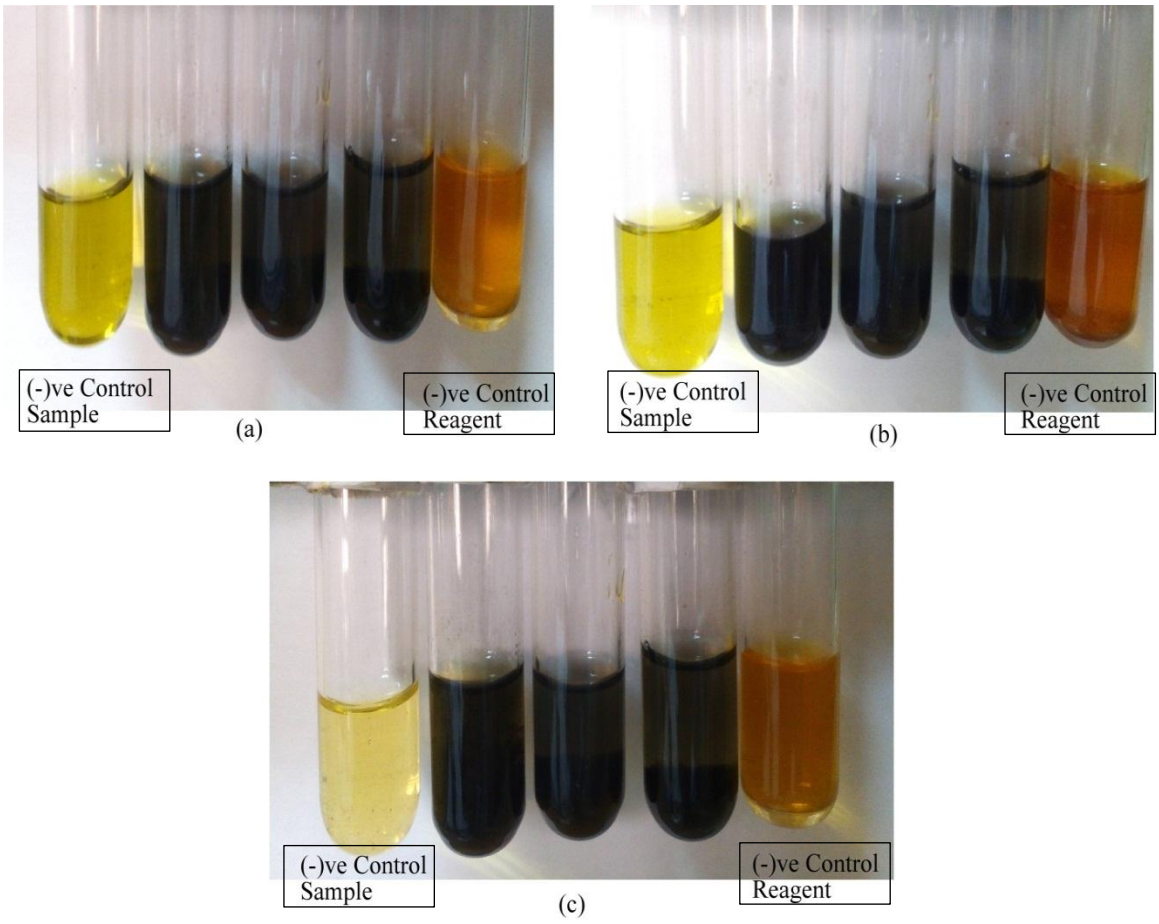

Fig. 4: Test for the preliminary detection of phenolic compounds in (a) Ethanolic; (b) Methanolic; (c) Aqueous extracts of *Camellia sinensis*;

* All tests were performed in triplicate

3.1.2 Test for Alkaloids

3.1.2.1 Hager's Test

1 ml of extract was carefully mixed with 3 drops of freshly prepared Hager's reagent in a test tube. No colour change was observed.

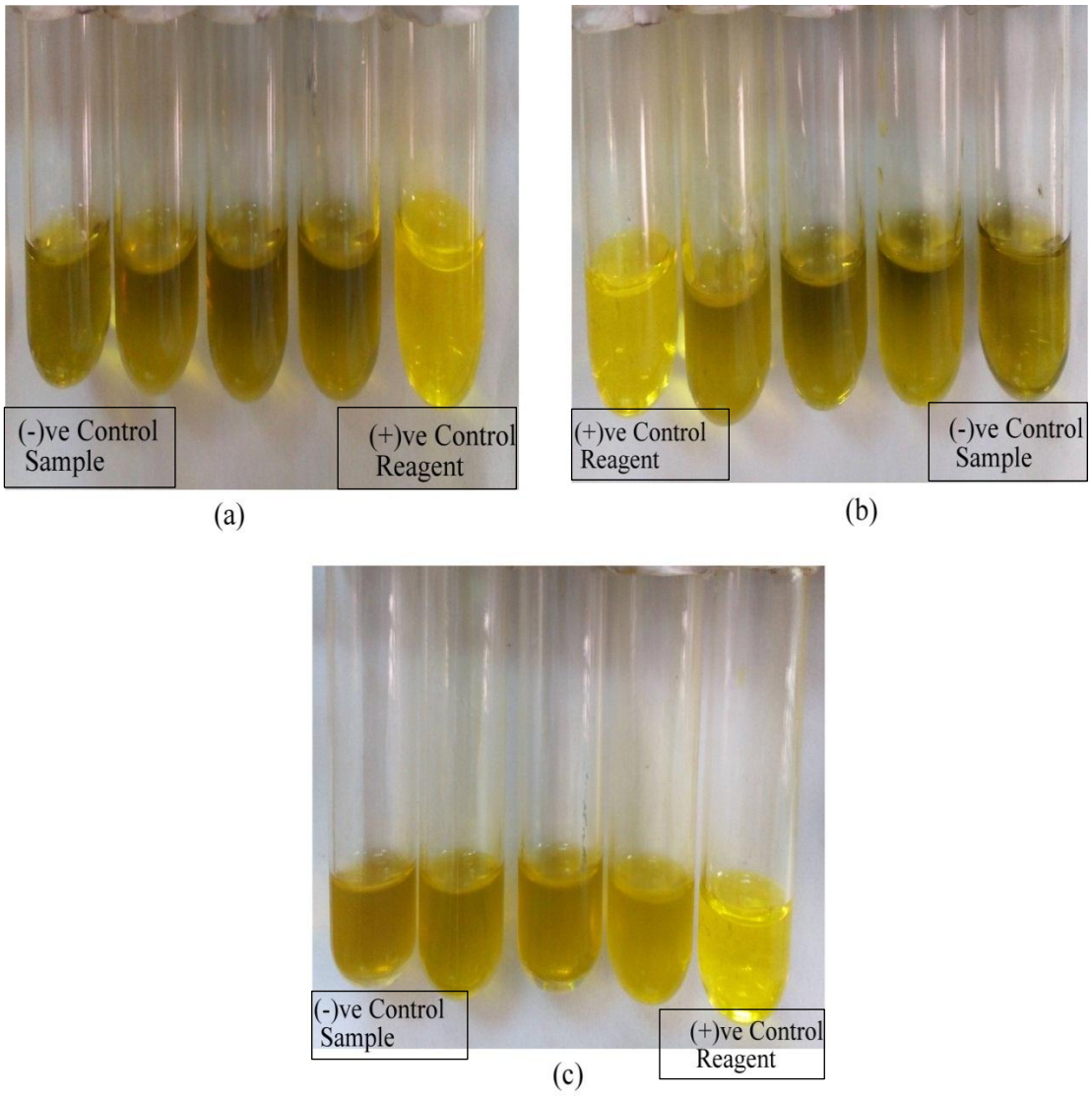

Fig. 5: Hager's Test for the detection of alkaloids in (a) Ethanolic; (b) Methanolic; (c) Aqueous extracts of *Camellia sinensis*;

* All tests were performed in triplicate

3.1.2.2 Wagner's Test

1 ml of extract was mixed together in a test tube with 3 drops of Wagner's reagent. The formation of brown precipitate showed the presence of alkaloids.

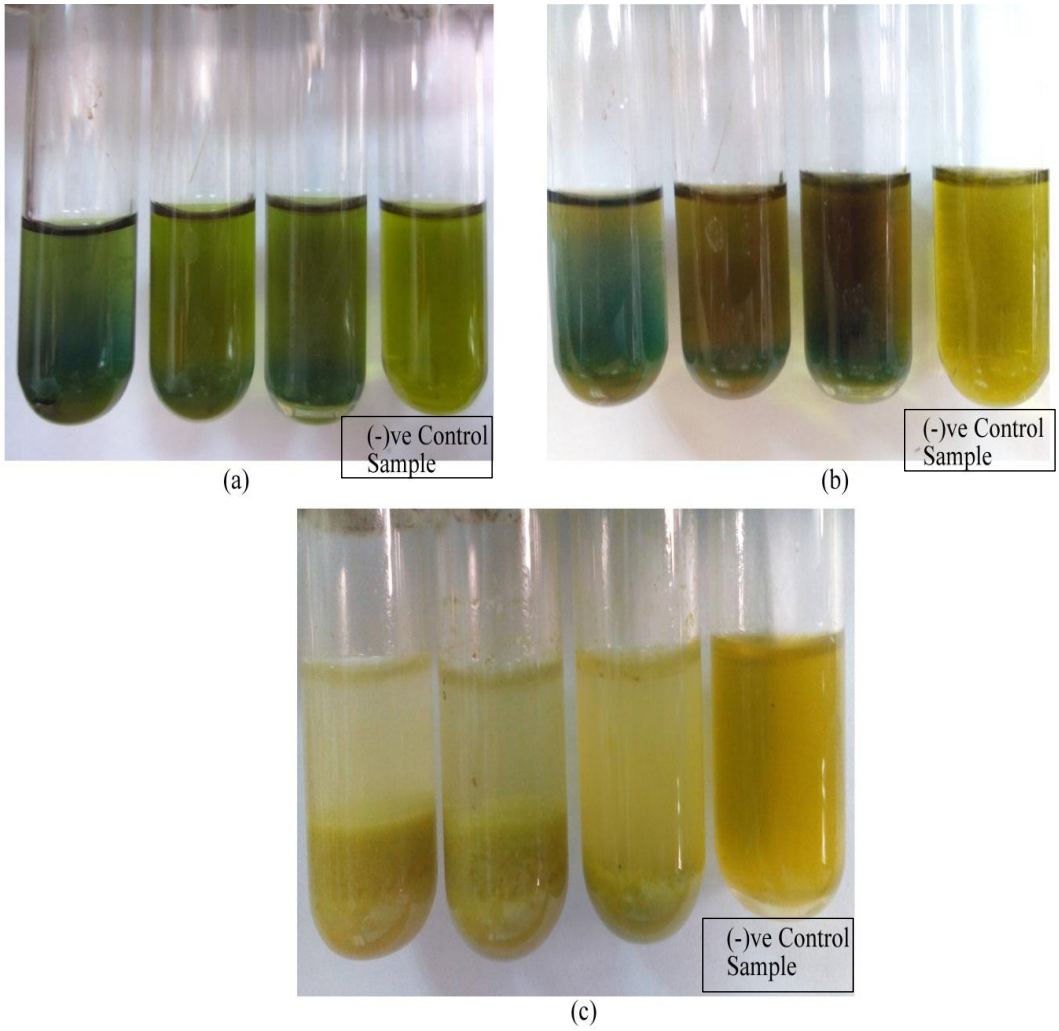

Fig. 6: Marquis' Test for the detection of alkaloids in (a) Ethanolic; (b) Methanolic; (c) Aqueous extracts of *Camellia sinensis*;

* All tests were performed in triplicate

3.1.2.3 Test with Marquis' Reagent

The addition of Marquis' reagent yields a reddish precipitate in the samples indicating the presence of alkaloids.

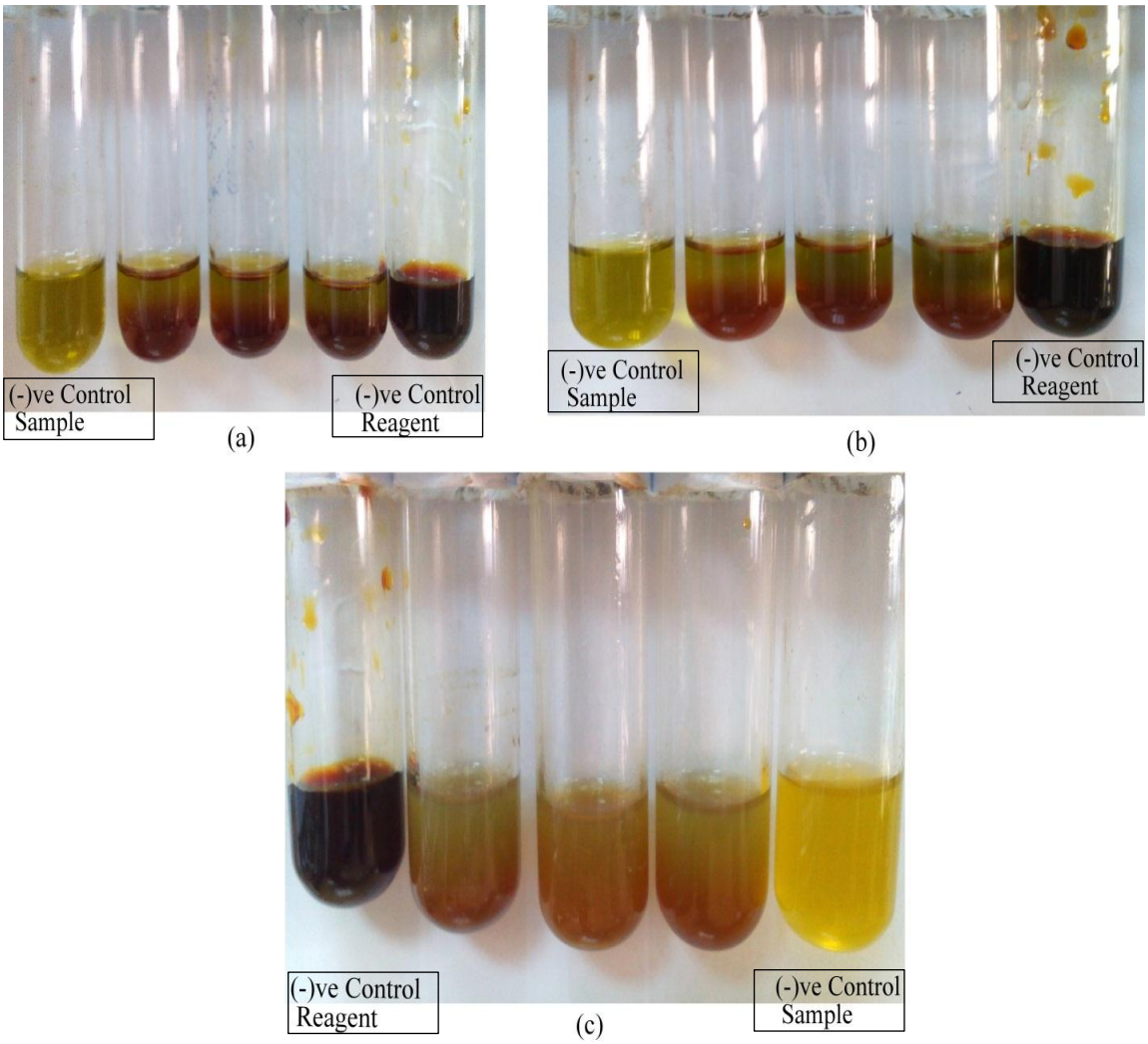

Fig. 7: Wagner's Test for the detection of alkaloids in (a) Ethanolic; (b) Methanolic; (c) Aqueous extracts of *Camellia sinensis*;

* All tests were performed in triplicate

3.1.3 Test for Flavonoids (Lead Acetate Test)

1ml of freshly prepared 10% lead acetate was added to 1ml of extract. Precipitate indicates the presence of flavonoids.

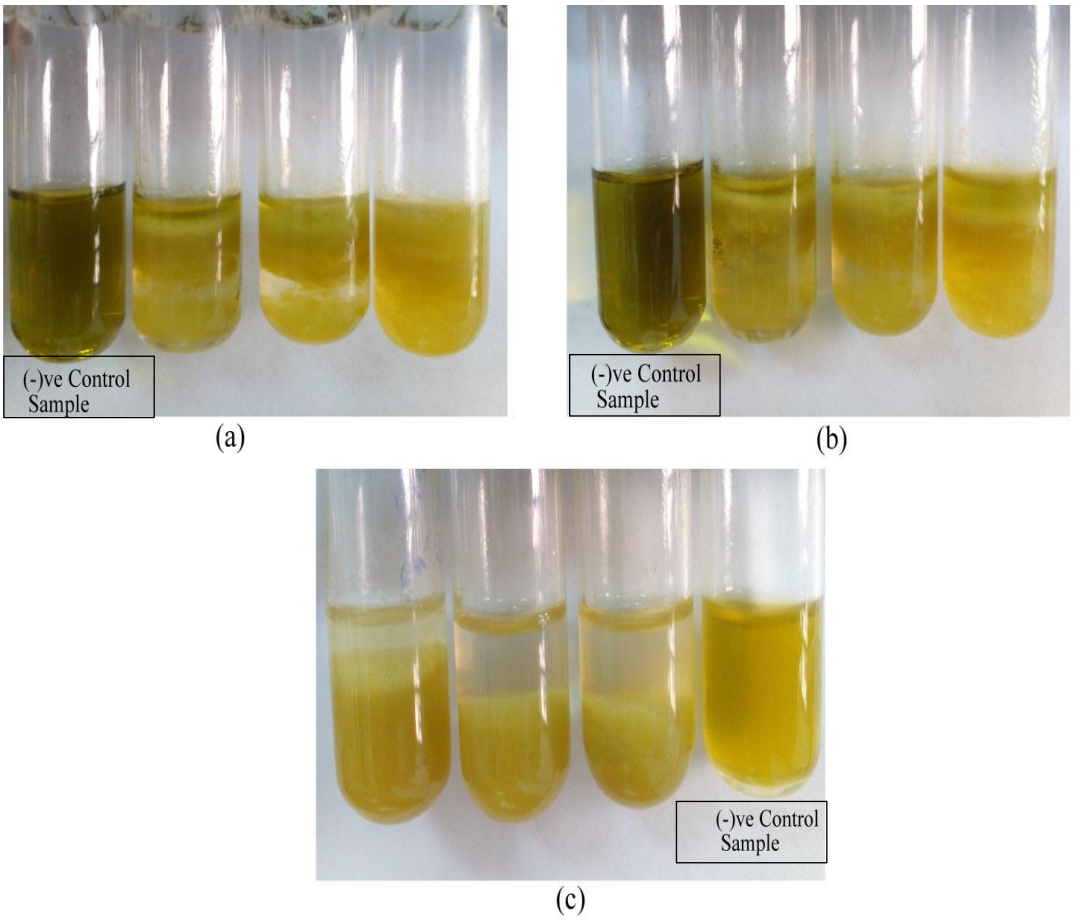

Fig. 8: Lead Acetate Test for the detection of flavonoids in (a) Ethanolic; (b) Methanolic; (c) Aqueous extracts of *Camellia sinensis*;

* All tests were performed in triplicate

3.1.4 Test for Steroids (Salkowaski's Test)

2ml extracts were dissolved in 2ml chloroform in a test tube. 4ml concentrated sulfuric acid (97%) was carefully poured from the inner wall of the test tube. Formation of a red colour in the upper layer and sulphuric acid layer was observed.

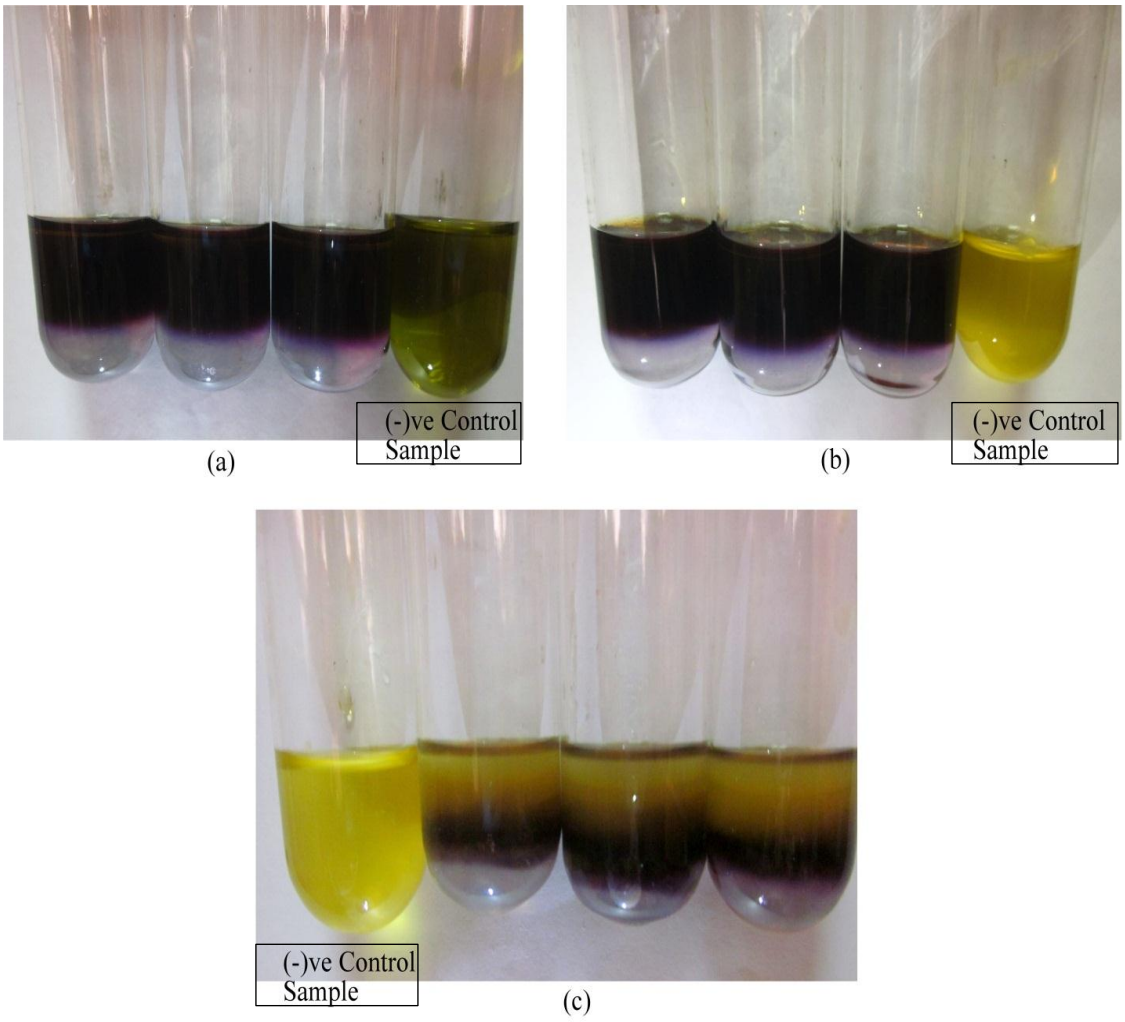

Fig. 9: Salkowaski's Test for the detection of steroids in (a) Ethanolic; (b) Methanolic; (c) Aqueous extracts of *Camellia sinensis*;

* All tests were performed in triplicate

3.1.5 Test for Saponins (Froth Test)

0.5g of each extract was dissolved in 10ml distilled water. The test tube was stoppered and then shaken vigorously for 30 seconds. Honey-comb froth remained on the surface for 30 minutes.

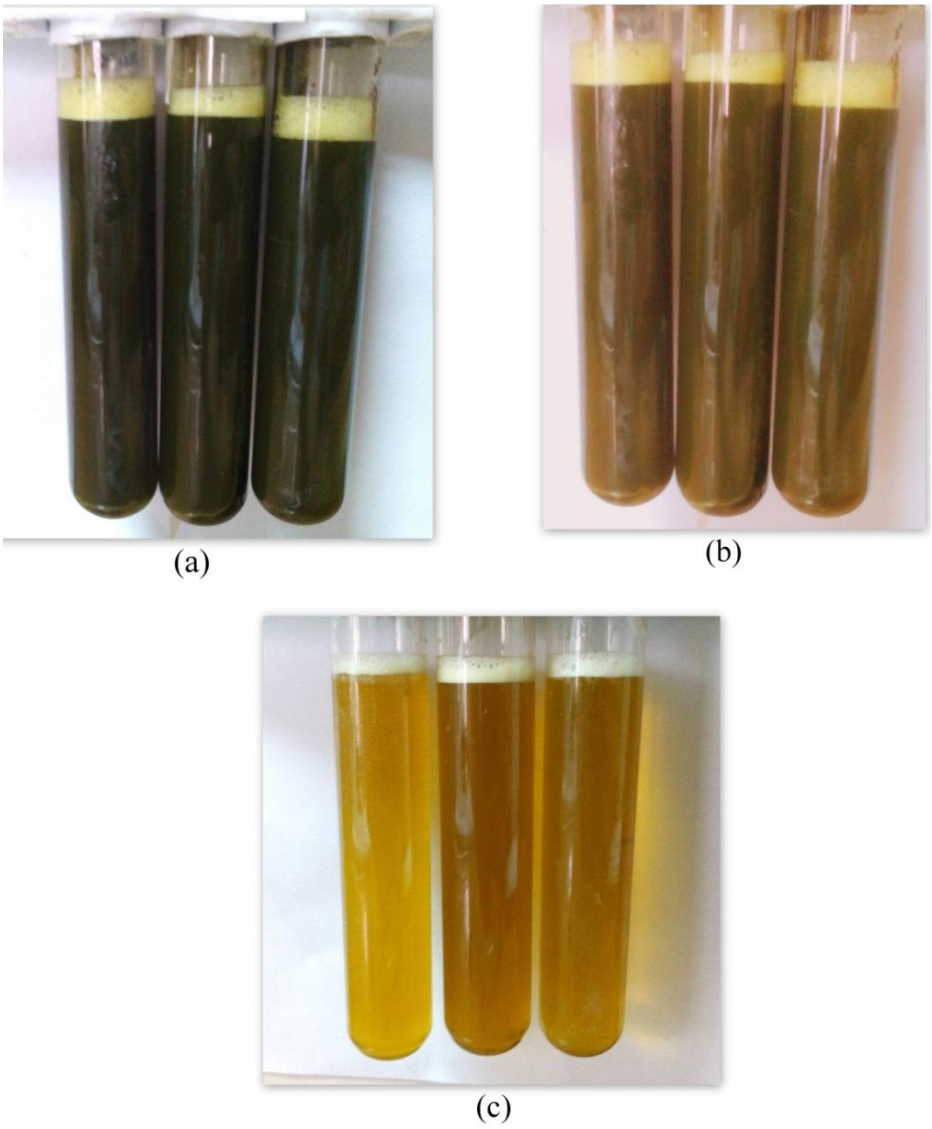

Fig. 10: Froth Test for the detection of saponins in (a) Ethanolic; (b) Methanolic; (c) Aqueous extracts of *Camellia sinensis*;

* All tests were performed in triplicate

3.1.6 Test for Tannins (Ferric Chloride Test)

Few drops of freshly prepared 10% ferric chloride were added to 1ml sample extract. Formation of blue-black precipitate indicates the presence of tannins.

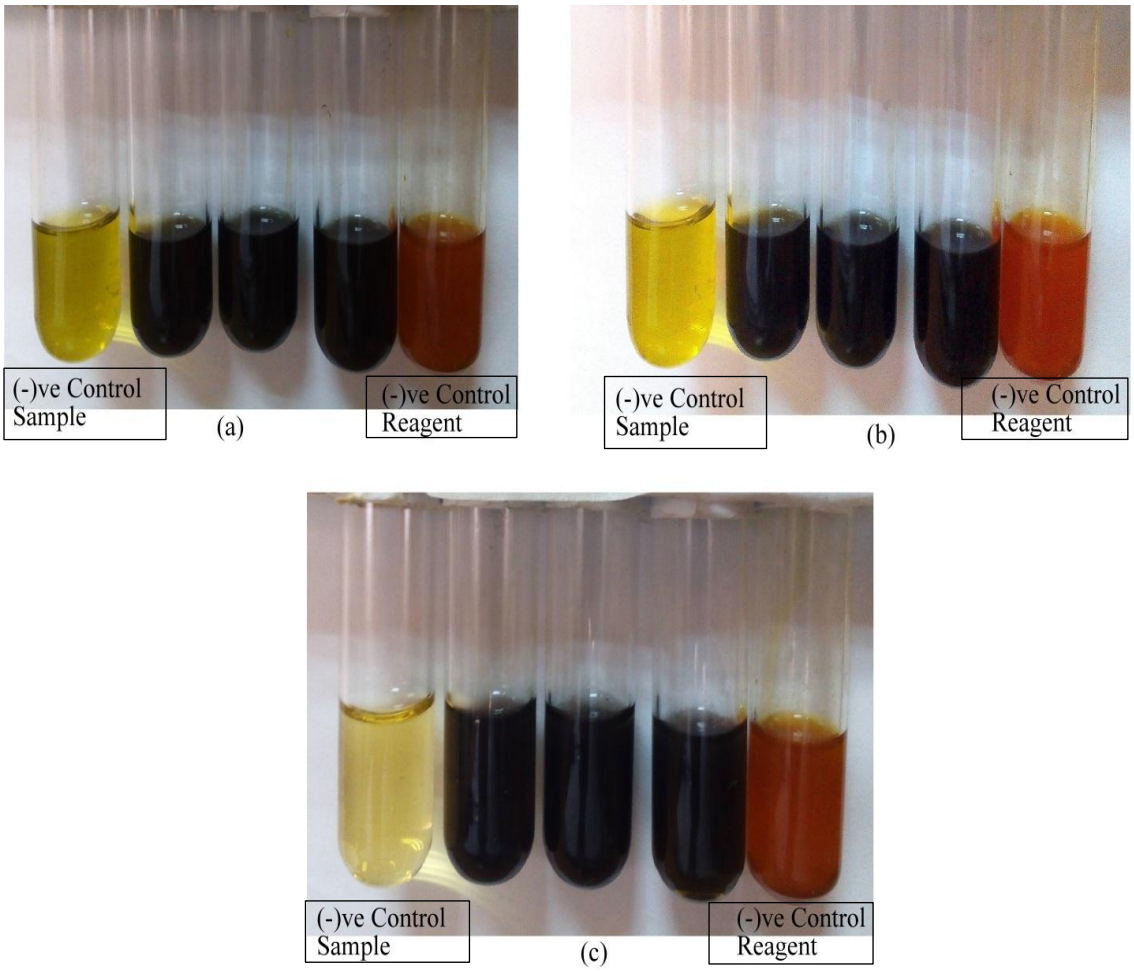

Fig. 11: Ferric Chloride Test for the detection of tannins in (a) Ethanolic; (b) Methanolic; (c) Aqueous extracts of *Camellia sinensis*;

* All tests were performed in triplicate

3.1.7 Test for Gums (Molisch's Test)

To 5ml extract, Molisch's reagent and concentrated sulphuric acid were added drop-wise. Positive result shows a red-violet ring only in the aqueous extract.

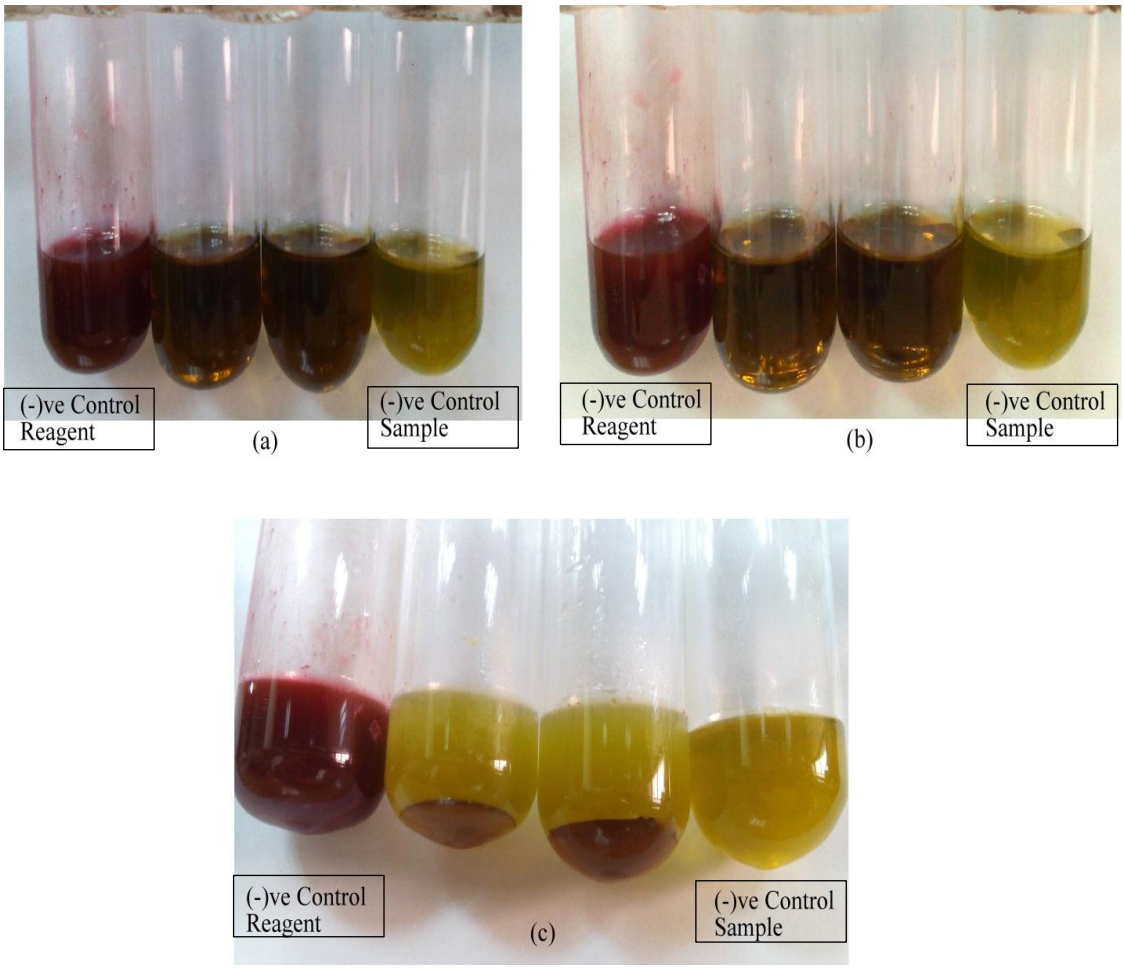

Fig. 12: Molisch's Test for the detection of gums in (a) Ethanolic; (b) Methanolic; (c) Aqueous extracts of *Camellia sinensis*;

* All tests were performed in triplicate

3.1.8 Test for Cardiac Glycosides (Killer-Killani Test)

2ml glacial acetic acid was added to 5ml of extract followed by 1 drop of ferric chloride and 1 ml concentrated sulphuric acid. A positive result shows a brown ring at the interface.

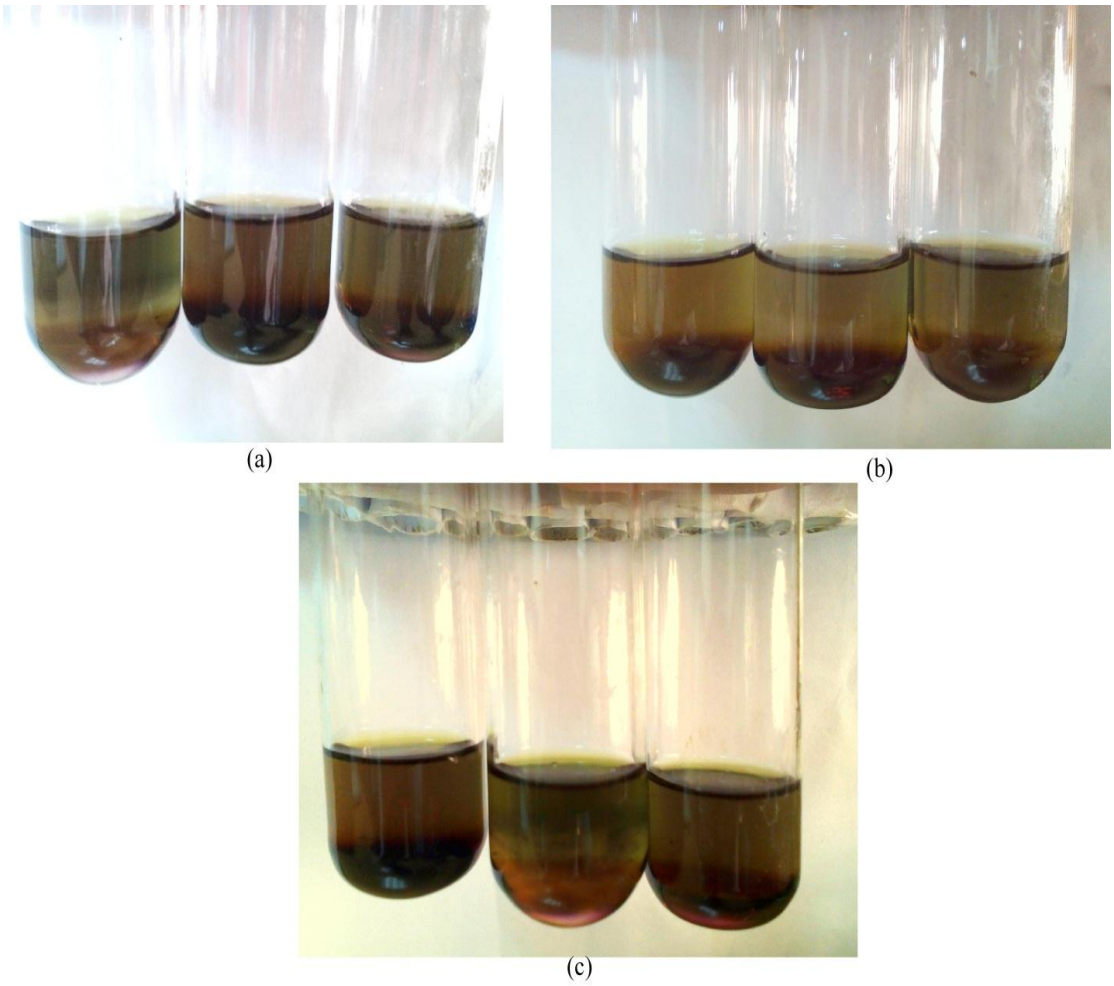

Fig. 13: Killer-Killani Test for the detection of cardiac glycosides in (a) Ethanolic; (b) Methanolic; (c) Aqueous extracts of *Camellia sinensis*;

* All tests were performed in triplicate

3.2 Antibacterial Activity of Different Concentrations of Methanolic, Ethanolic and Aqueous Extracts of *Camellia sinensis*

In vitro antimicrobial screening of ethanolic, methanolic and aqueous extracts of *Camellia sinensis* were carried out using crude extracts and subsequently diluted concentrations of 80µg/µl and 60µg/µl to compare the extent of antimicrobial activity that the extracts possess. Ciprofloxacin was used as positive control for *Enterotoxigenic Escherichia coli* and *Escherichia coli*. Ampicillin served as positive control for Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus aureus* whereas Cefoxitin served as positive control for *Shigella flexneri*. Results of the antimicrobial assay of ethanolic (Figures 14 and 17), methanolic (Figures 15 and 18) and aqueous (Figures 16 and 19) extracts are shown in Tables 2, 3 and 4 respectively.

Results show that aqueous extract shows no antimicrobial activity except against *S. aureus*. Crude ethanolic extract exhibits highest antimicrobial activity against *E.coli* and *S.flexneri*. Against ETEC, MRSA and *S.aureus* crude methanolic and ethanolic extracts exhibit similar antimicrobial activity. However, 80µg/µl concentration of ethanolic extract had higher antimicrobial activity than 80µg/µl concentration of ethanolic extract for all test strains. 60µg/µl did not prove to be an effective concentration to prevent microbial growth with the exception of ethanolic and methanolic extracts against *S. flexneri* and *S. aureus* alone.

Table 2: Effect of different concentrations of ethanolic extract of *Camellia sinensis* on the mean diameter inhibition of test organisms

Test Organism	Average Zone of Inhibition (mm)			
	Positive Control	**Crude Extract**	**80µg/µl**	**60µg/µl**
Escherichia coli	21.7	19.88	14.3	0
Enterotoxigenic Escherichia coli	24.3	14.8	4	0
Shigella flexneri	22.8	21.8	20.3	16.6
Staphylococcus aureus	23.3	21	18.2	16
Methicillin-resistant *Staphylococcus aureus*	23.2	15.72	11.8	0

*All measurements are means of individual data obtained from triplicate tests

Table 3: Effect of different concentrations of methanolic extract of *Camellia sinensis* on the mean diameter inhibition of test organisms

Test Organism	Average Zone of Inhibition (mm)			
	Positive Control	**Crude Extract**	**80µg/µl**	**60µg/µl**
Escherichia coli	20.5	16.3	4.5	0
Enterotoxigenic Escherichia coli	24.7	15.8	7.9	0
Shigella flexneri	22.3	20.7	18.8	15.8
Staphylococcus aureus	22.5	21.5	19	16
Methicillin-resistant *Staphylococcus aureus*	22.6	15.1	0	0

*All measurements are means of individual data obtained from triplicate

Table 4: Effect of different concentrations of aqueous extract of *Camellia sinensis* on the mean diameter inhibition of test organisms

Test Organism	Average Zone of Inhibition (mm)			
	Positive Control	**Crude Extract**	**80µg/µl**	**60µg/µl**
Escherichia coli	21.5	0	0	0
Enterotoxigenic Escherichia coli	24.3	0	0	0
Shigella flexneri	22.7	0	0	0
Staphylococcus aureus	22.8	17.3	9	0
Methicillin resistant *Staphylococcus aureus*	22.9	0	0	0

*All measurements are means of individual data obtained from triplicate

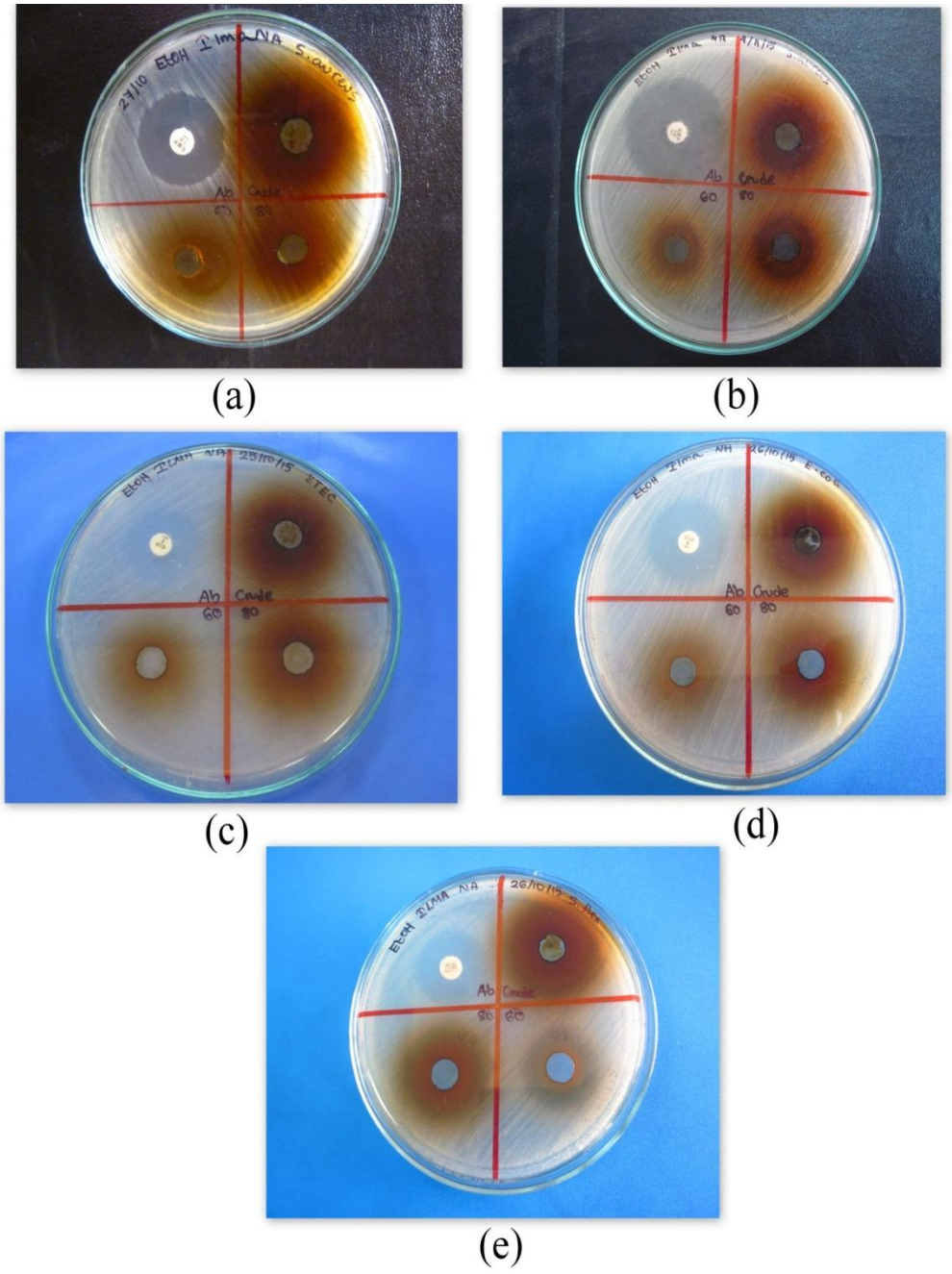

Fig. 14: Antibacterial activity of different concentrations of ethanolic extract of *Camellia sinensis* against (a) Methicillin Resistant *Staphylococcus aureus*; (b) *Staphylococcus aureus*; (c) *Enterotoxigenic Escherichia coli* ; (d) *Escherichia coli*; (e) *Shigella flexneri;*

*All tests were performed in triplicate

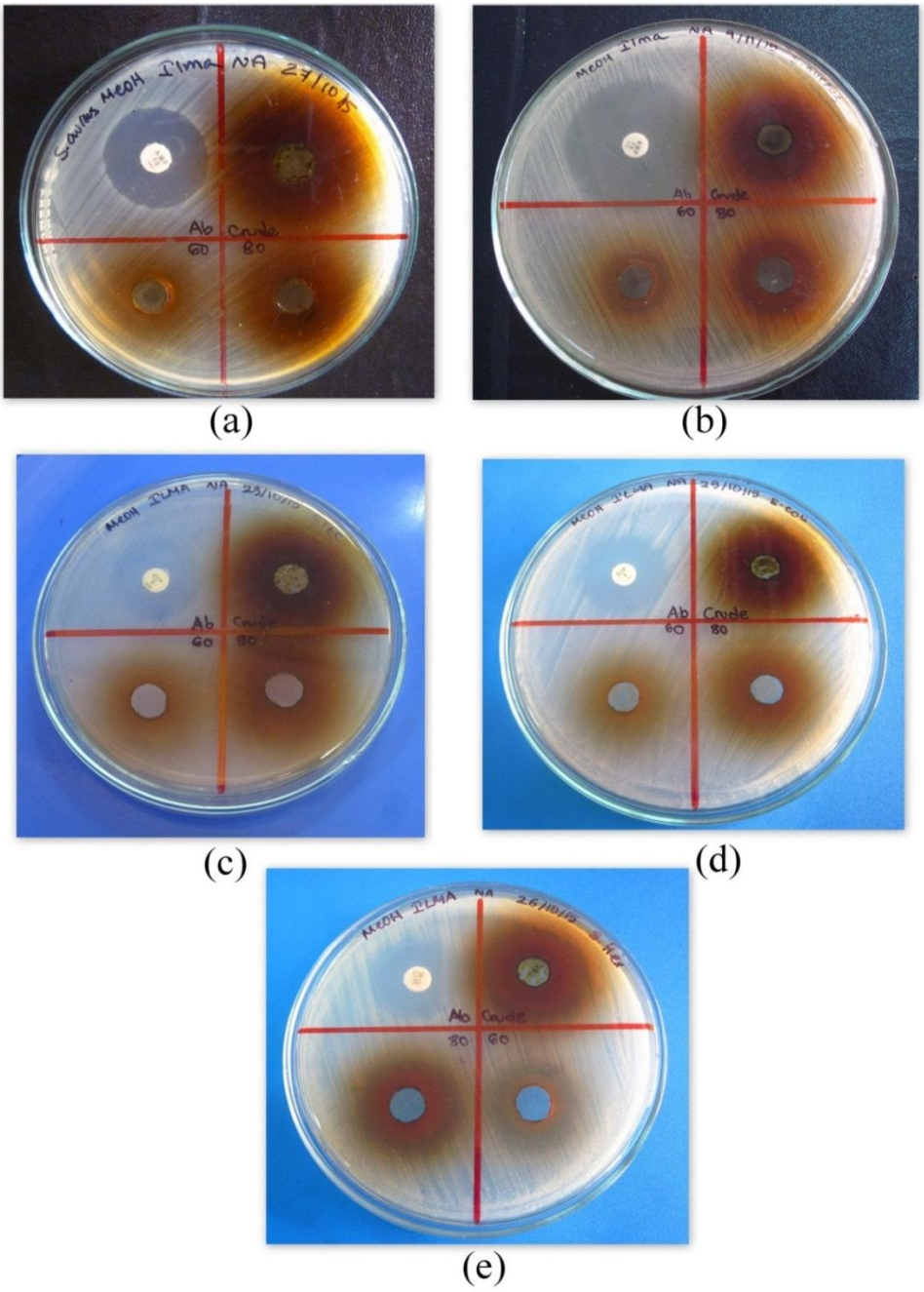

Fig. 15: Antibacterial activity of different concentrations of methanolic extract of *Camellia sinensis* against (a) Methicillin Resistant *Staphylococcus aureus*; (b) *Staphylococcus aureus*; (c) *Enterotoxigenic Escherichia coli* ; (d) *Escherichia coli*; (e) *Shigella flexneri;*

*All tests were performed in triplicate

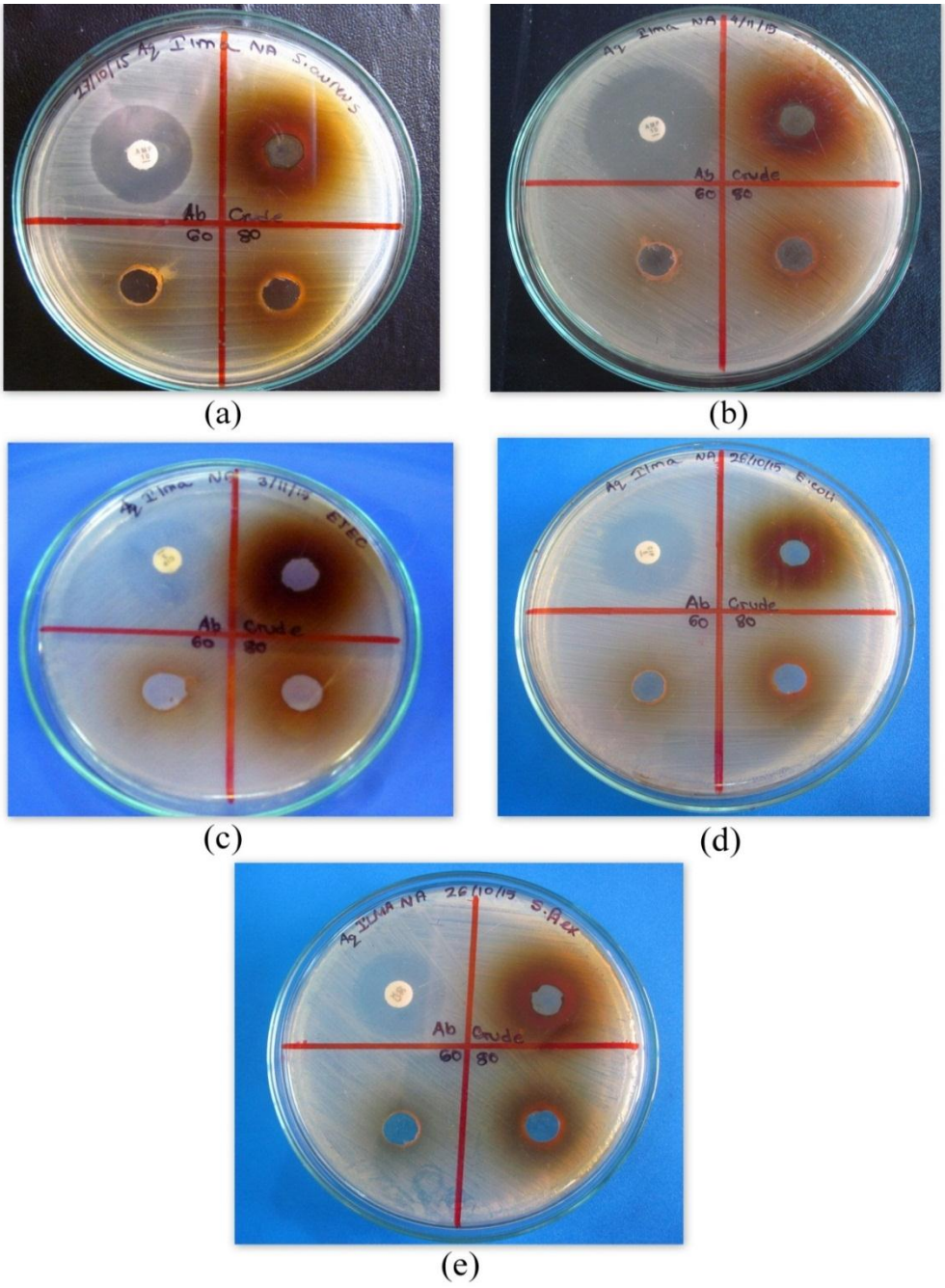

Fig. 16: Antibacterial activity of different concentrations of aqueous extract of *Camellia sinensis* against (a) Methicillin Resistant *Staphylococcus aureus*; (b) *Staphylococcus aureus*; (c) *Enterotoxigenic Escherichia coli* ; (d) *Escherichia coli*; (e) *Shigella flexneri;*

*All tests were performed in triplicate

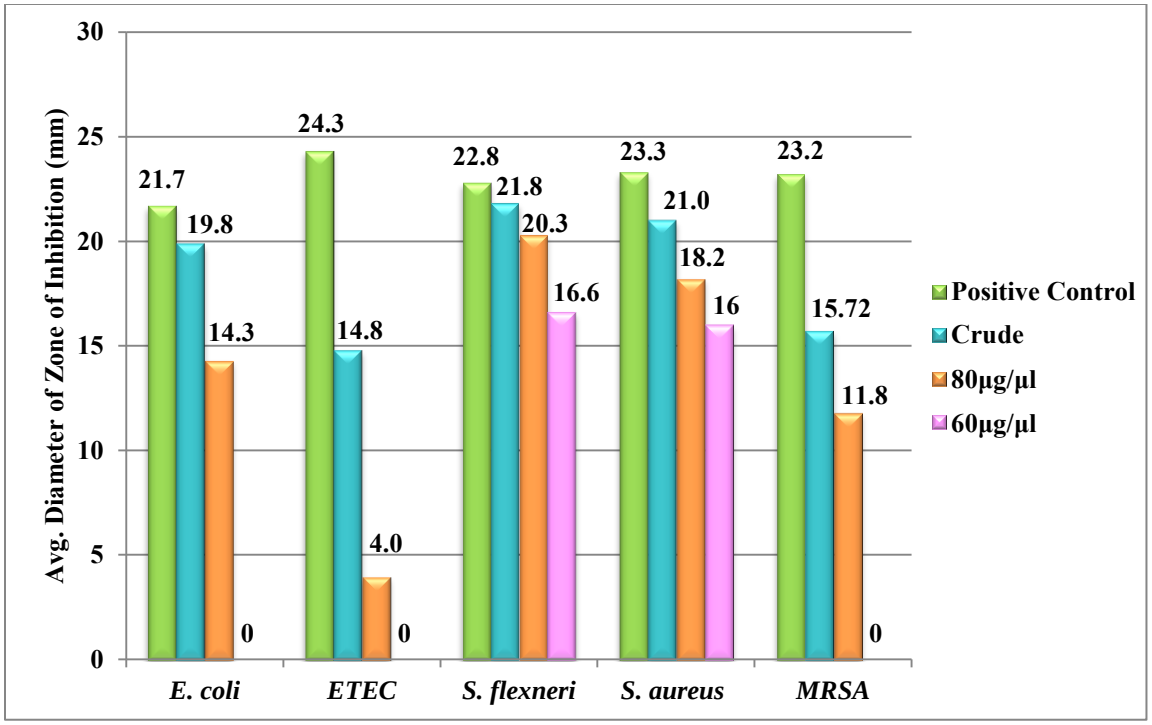

Fig. 17: Graphical representation of antimicrobial activity of different concentrations of ethanolic extracts on test strains;

*All measurements are means of individual data obtained from triplicate

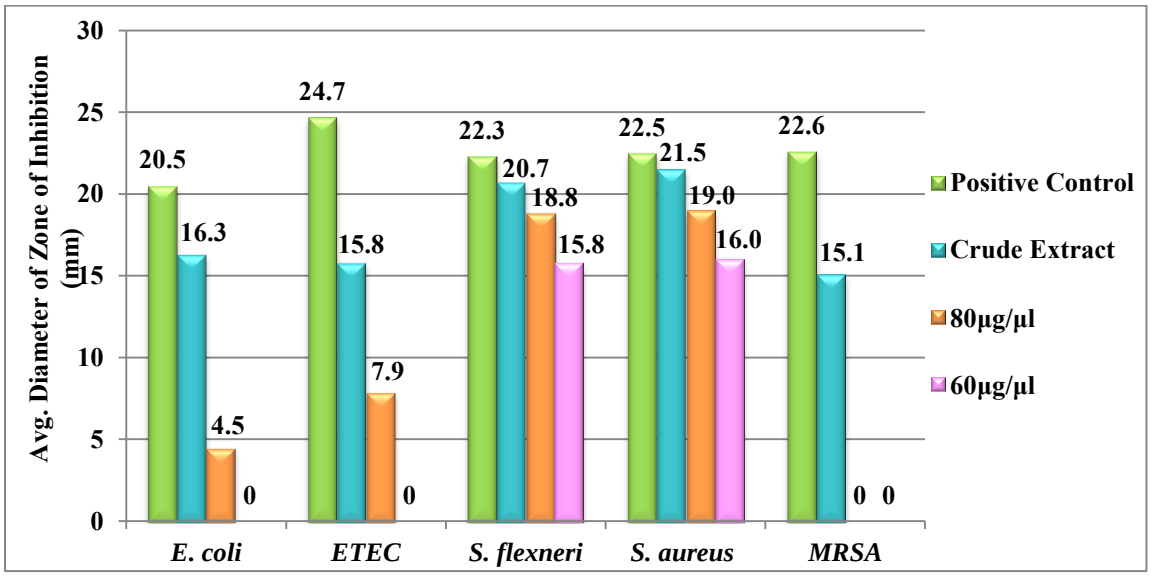

Fig. 18: Graphical representation of antimicrobial activity of different concentrations of methanolic extracts on test strains;

*All measurements are means of individual data obtained from triplicate

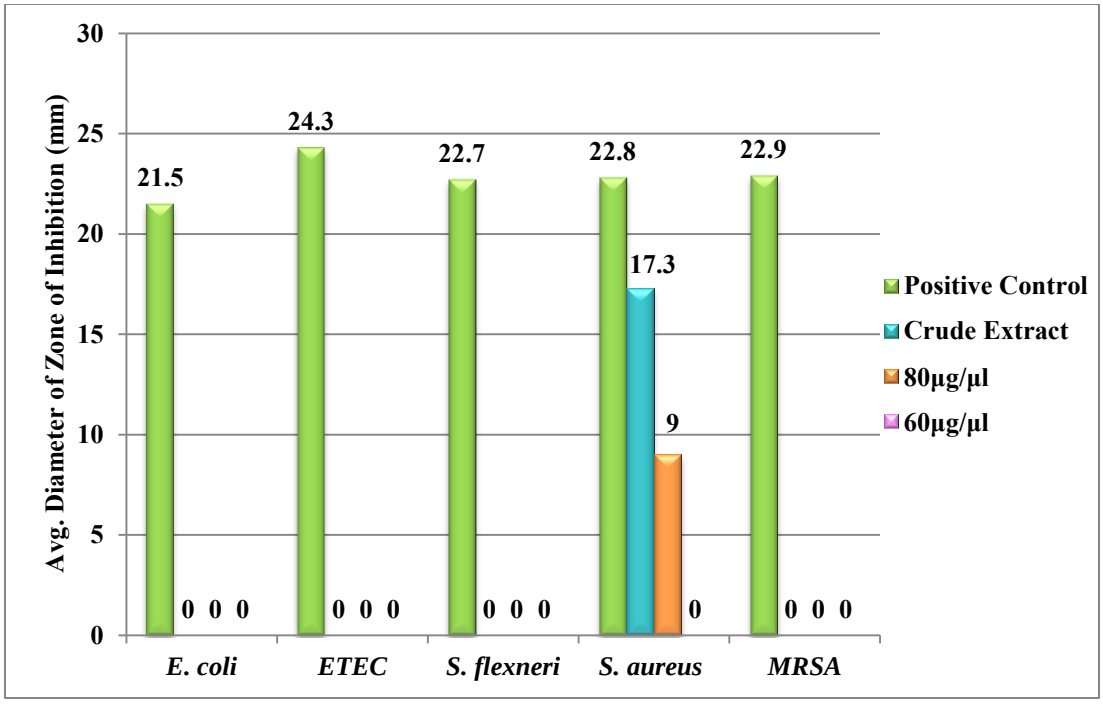

Fig. 19: Graphical representation of antimicrobial activity of different concentrations of aqueous extracts on test strains;

*All measurements are means of individual data obtained from triplicate

3.2.1 Activity Index of Different Concentrations of *Camellia sinensis* Extracts

The activity index is a ratio of the average diameter of zone of inhibition to the average diameter of zone of inhibition of antibiotic. The activity index of the sample gives a quantifiable data for the identification of the level of antibacterial activity possessed by the sample. Activity index were plotted against ciprofloxacin (Figure 20), ampicillin (Figure 21) and cefoxitin (Figure 22). The highest activity index was produced by crude ethanolic extract in all test strains except ETEC and *S. aureus* for which crude methanolic extract gave slightly higher activity index. The highest recorded value was for crude ethanolic extract against *S. flexneri*.

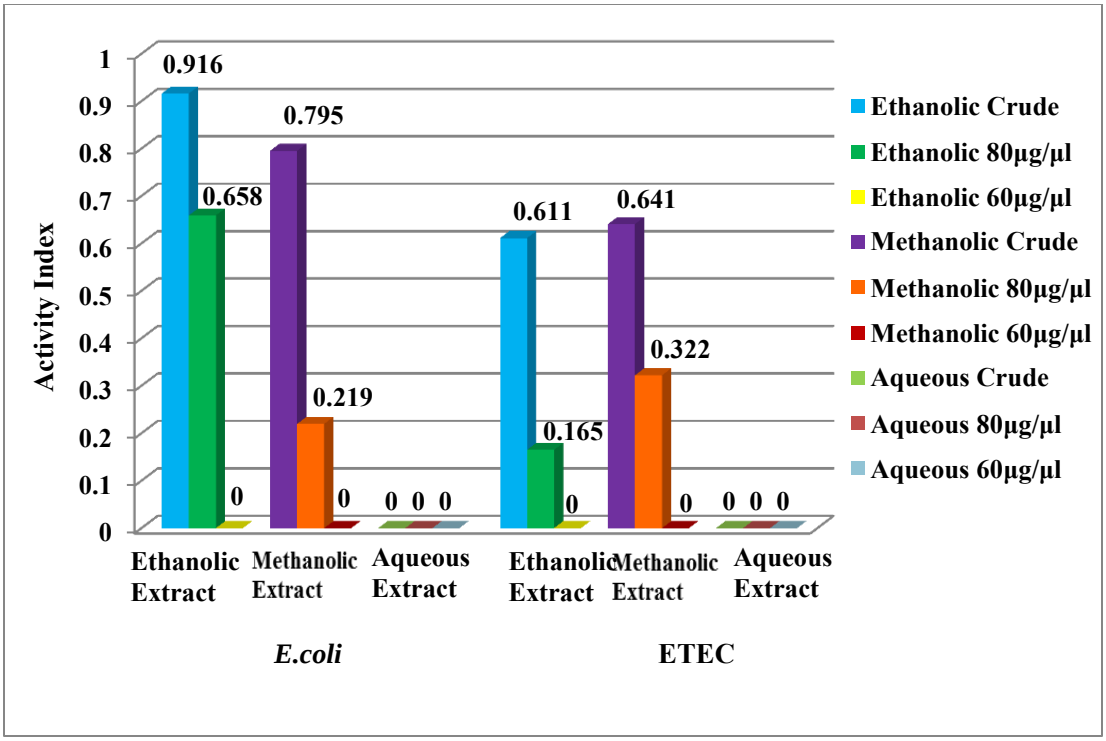

Fig. 20: Graphical analysis of activity index of *Camellia sinensis* extracts to Ciprofloxacin

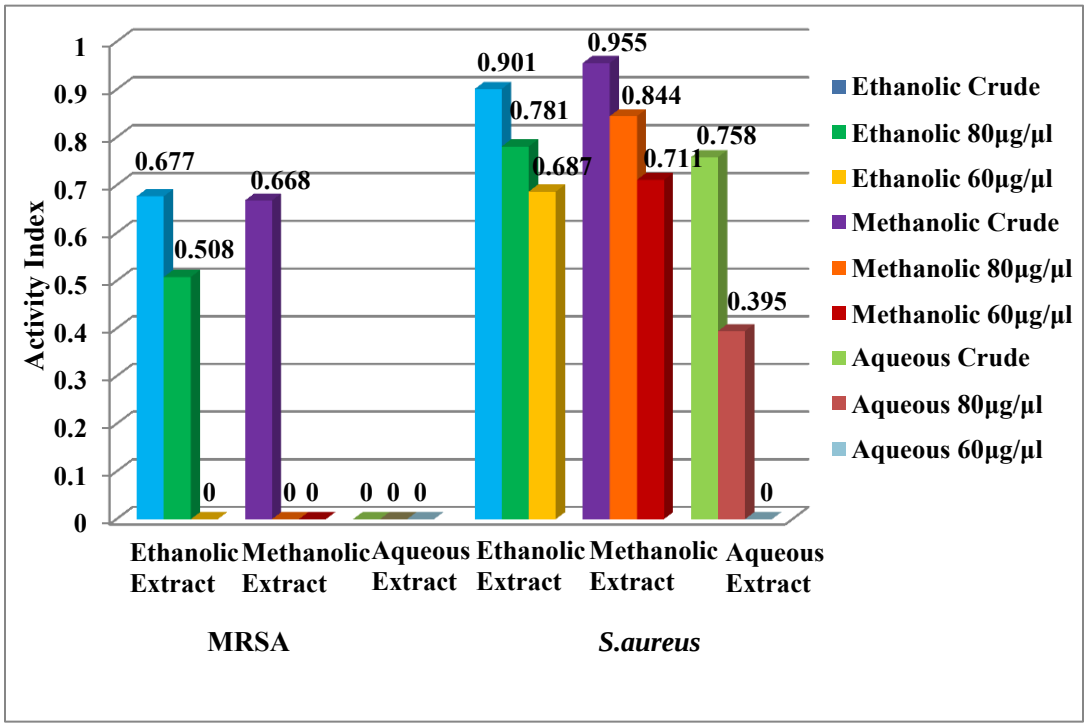

Fig. 21: Graphical analysis of activity index of *Camellia sinensis* extracts to Ampicillin

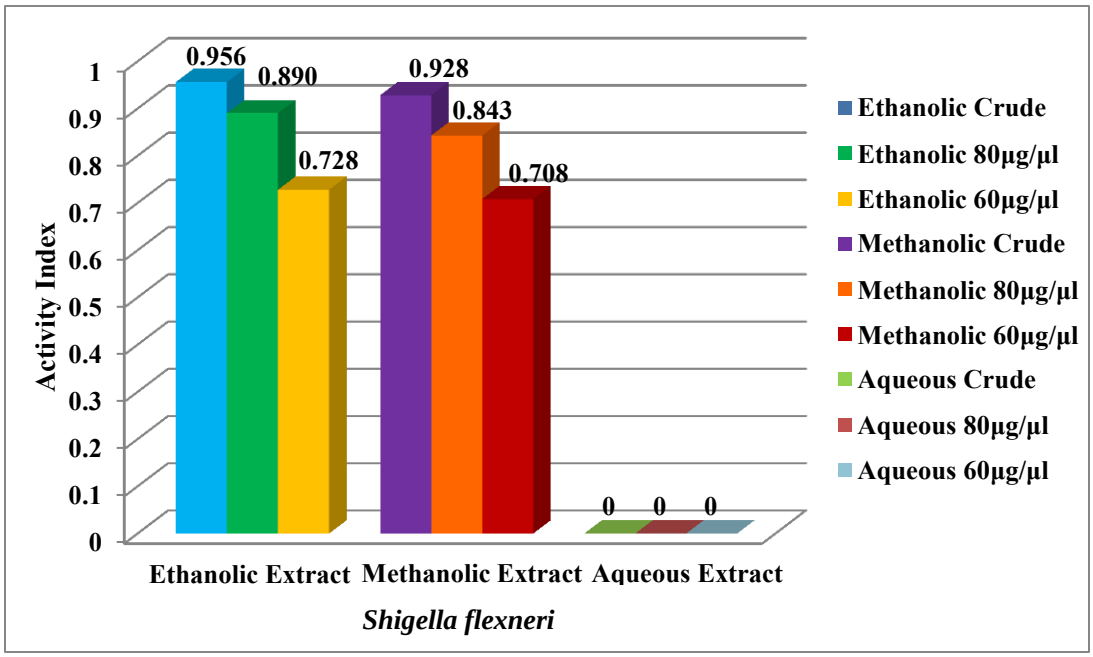

Fig. 22: Graphical analysis of activity index of *Camellia sinensis* extracts to Cefoxitin

3.2.2 Synergistic Activity of Extracts and Selected Antibiotics against Test Organisms

Synergism is tested using a combination of drugs to increase efficiency and reduce dosage as well as side effects. Synergistic activity of the crude extracts were tested with three broad-range antibiotics namely ciprofloxacin, ampicillin and cefoxitin. The crude extracts did not show any synergistic activity with any of the drugs. Results of ethanolic extract are shown in Table 5 and Figure 23. Table 6 and Figure 24 show results of methanolic extract and the results of aqueous extract are displayed in Table 25 and Table 7.

Table 5: Average diameter of clear zone for synergistic activity of crude ethanolic extract and antibiotic

Test Organism	Average Zone of Inhibition (mm)		
	Antibiotic	Crude Extract	Extract & Antibiotic
Escherichia coli	21.7	19.8	14.3
Enterotoxigenic Escherichia coli	24.3	14.8	4.0
Shigella flexneri	22.8	21.8	20.3
Staphylococcus aureus	23.3	21.0	18.2
Methicillin-resistant *Staphylococcus aureus*	23.2	15.7	11.8

*All measurements are means of individual data obtained from triplicate

Table 6: Average diameter of clear zone for synergistic activity of crude methanolic extract and antibiotic

Test Organism	Average Zone of Inhibition (mm)		
	Antibiotic	Crude Extract	Extract & Antibiotic
Escherichia coli	25.0	20.2	17.4
Enterotoxigenic Escherichia coli	24.3	20.3	19.0
Shigella flexneri	23.3	15.7	20.6
Staphylococcus aureus	35.0	33.3	22.0
Methicillin-resistant *Staphylococcus aureus*	21.7	16.2	16.5

*All measurements are means of individual data obtained from triplicate

Table 7: Average diameter of clear zone for synergistic activity of crude aqueous extract and antibiotic

Test Organism	**Average Zone of Inhibition (mm)**		
	Antibiotic	**Crude Extract**	**Extract & Antibiotic**
Escherichia coli	24.7	13.9	0
Enterotoxigenic Escherichia coli	25.0	12.4	0
Shigella flexneri	23.7	16.5	13.9
Staphylococcus aureus	35.0	34.0	14.0
Methicillin-resistant *Staphylococcus aureus*	22.42	18.2	4.0

*All measurements are means of individual data obtained from triplicate

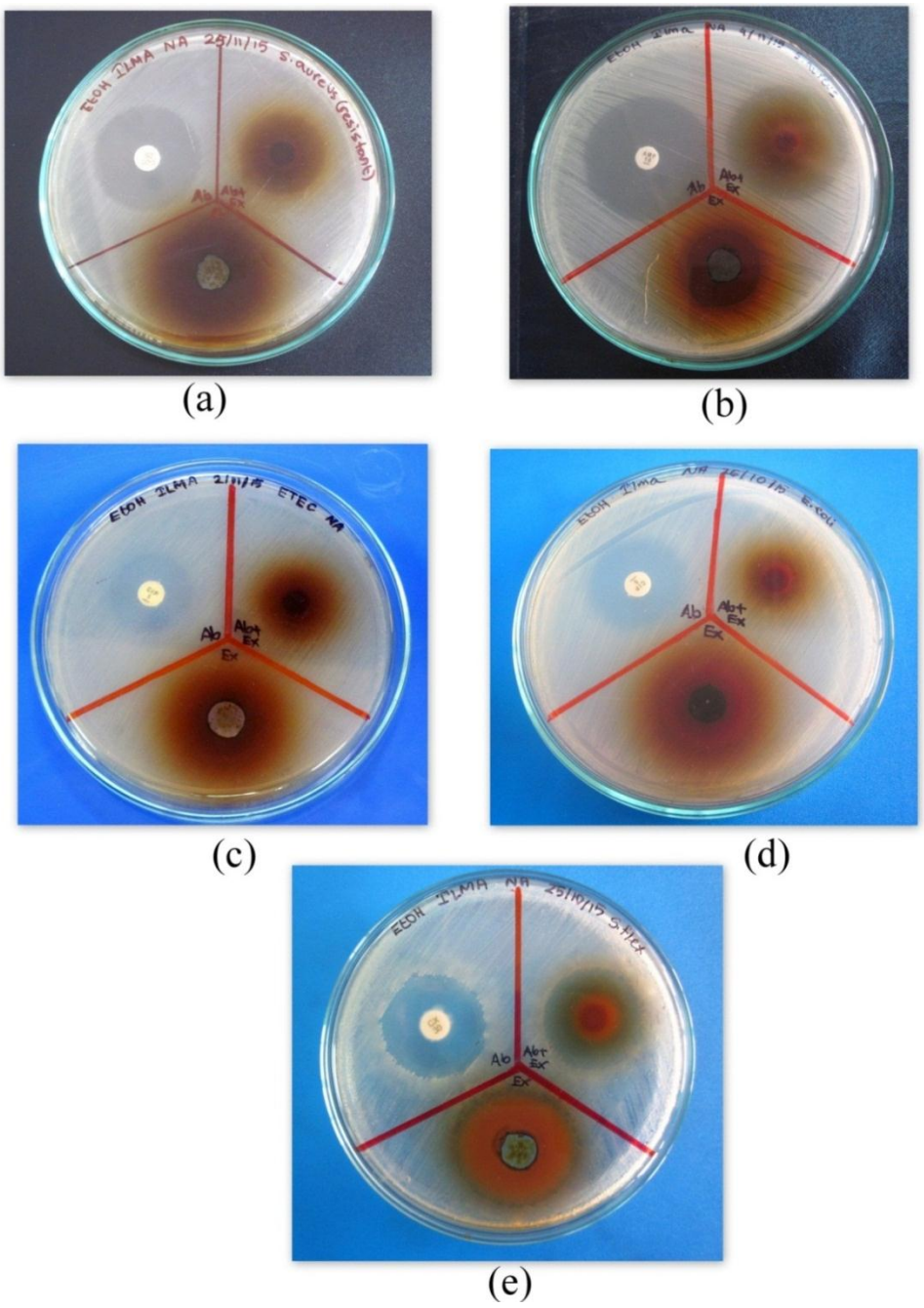

Fig. 23: Synergistic activity of crude ethanolic extract of *Camellia sinensis* against (a) Methicillin-resistant *Staphylococcus aureus*; (b) *Staphylococcus aureus*; (c) *Enterotoxigenic Escherichia coli*; (d) *Escherichia coli*; (e) *Shigella flexneri*

*All tests were performed in triplicate

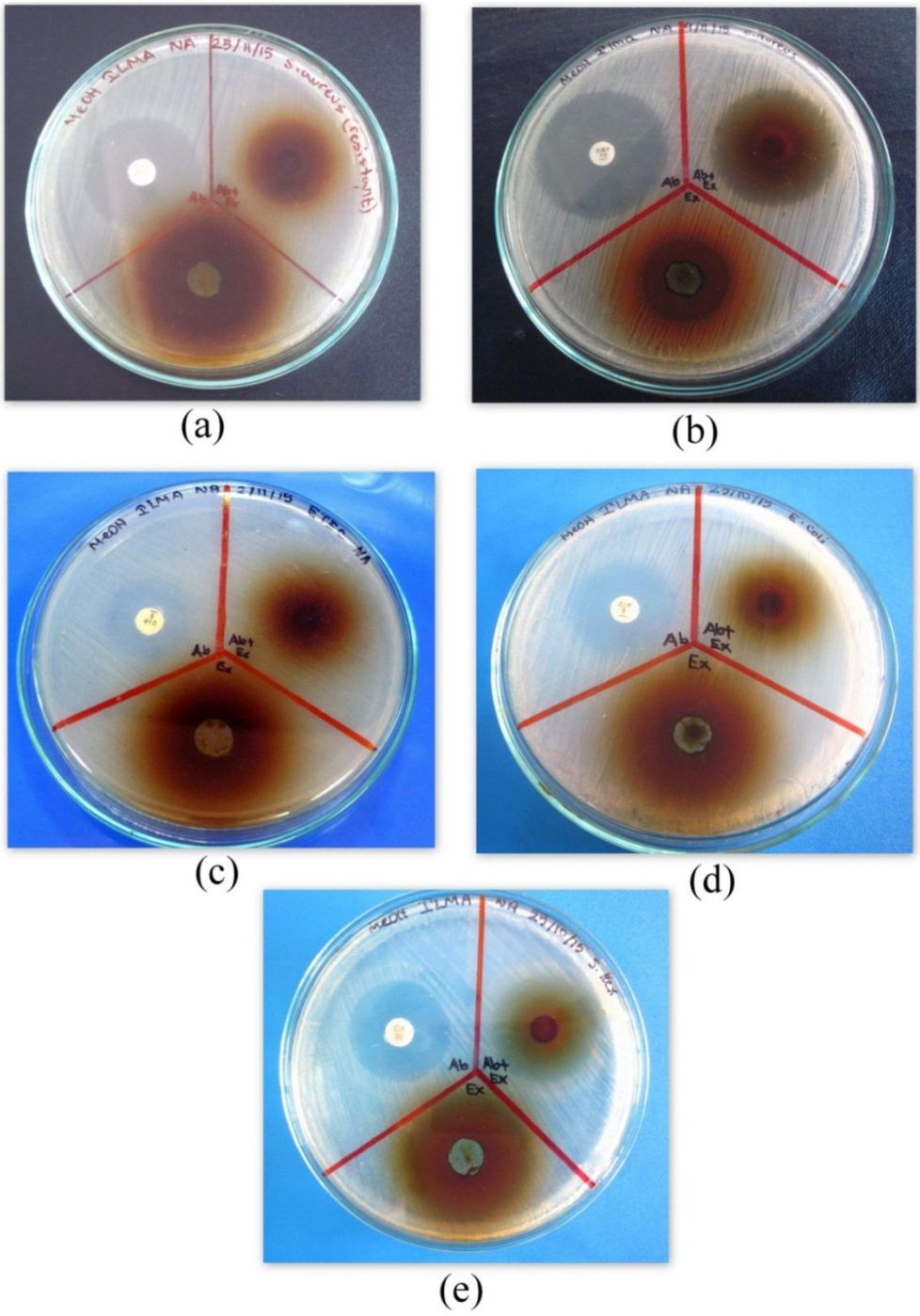

Fig. 24: Synergistic activity of methanolic extract of *Camellia sinensis* against (a) Methicillin-resistant *Staphylococcus aureus*; (b) *Staphylococcus aureus*; (c) *Enterotoxigenic Escherichia coli*; (d) *Escherichia coli*; (e) *Shigella flexneri*

*All tests were performed in triplicate

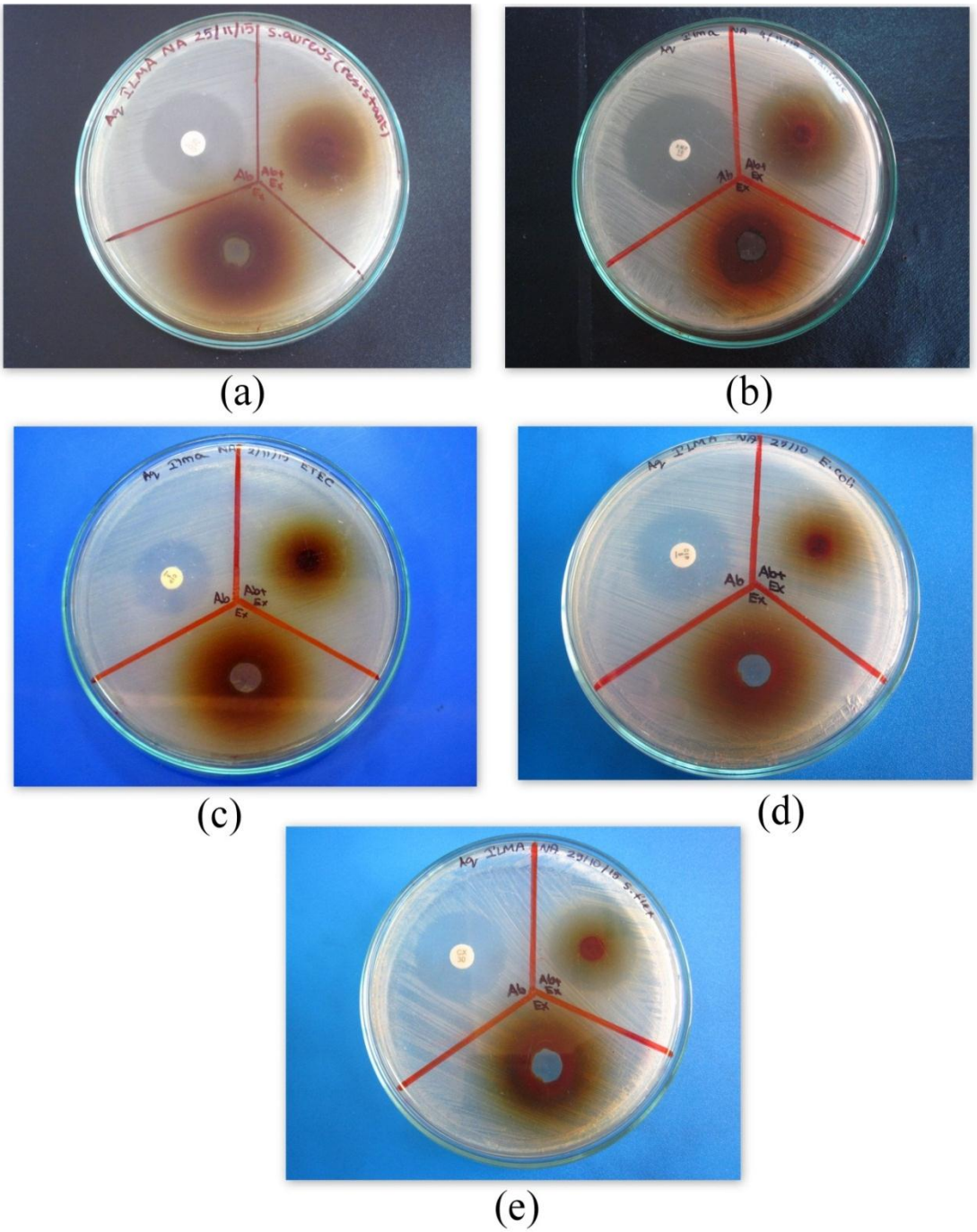

Fig. 25: Synergistic activity of aqueous extract of *Camellia sinensis* against (a) Methicillin-resistant *Staphylococcus aureus*; (b) *Staphylococcus aureus*; (c) *Enterotoxigenic Escherichia coli*; (d) *Escherichia coli*; (e) *Shigella Flexneri*

*All tests were performed in triplicate

3.3 Study of Antioxidant Activity in Three Different Extracts of Green Tea

Green tea is a rich dietary source of antioxidants. It is a widely consumed for its well-known antioxidant properties. Evaluation of DPPH free radical scavenging activity of green tea extracts was conducted. The current study establishes the strong presence of antioxidants in locally grown and produced commercially available green tea. Compared to the standard ascorbic standard which gave an average free radical scavenging activity (FRSA) of 80.275% at 10µg/ml and 94.115% at 1000µg/ml, ethanolic extract showed the highest antioxidant activity with 70.713% at 10µg/ml and 77.287% at 1000µg/ml. Methanolic extract followed suit with 65.241% at 10µg/ml and 71.402% at 1000µg/ml and lastly aqueous extract exhibited the least amount of antioxidant activity, having 46.988% at 10µg/ml and 57.655% at 1000µg/ml. Absorbance of the extracts are shown in Figure 26 and the antioxidant activity are shown in Figure 27.

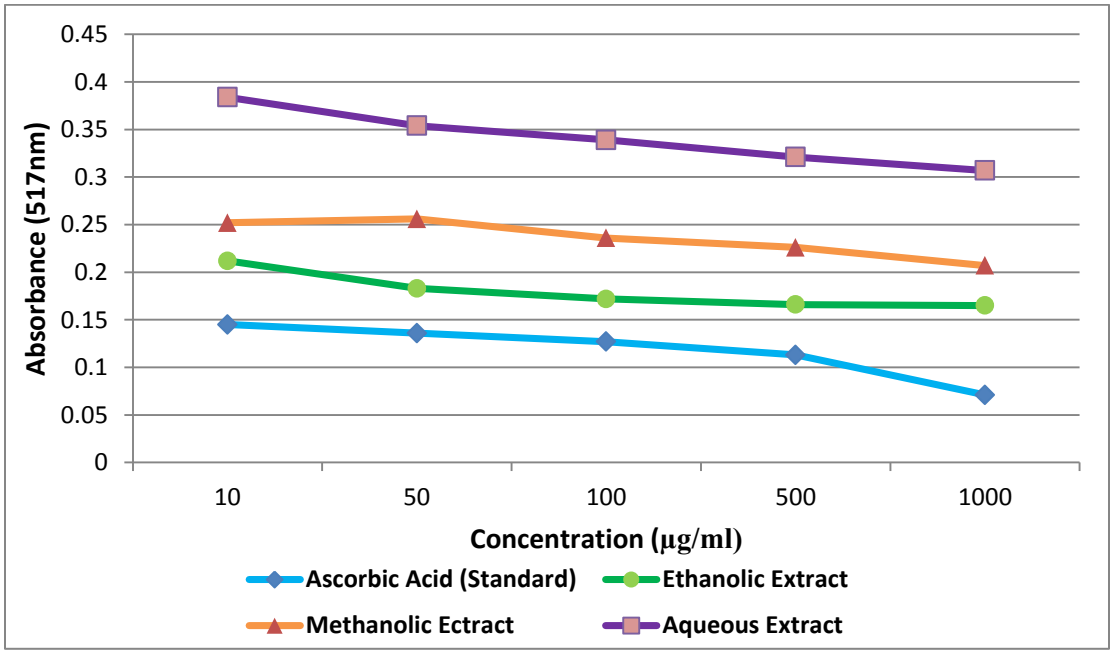

Fig. 26: Absorbance of different concentrations of three types of extracts of *Camellia sinensis;*

* All measurements are means of individual data obtained from triplicate

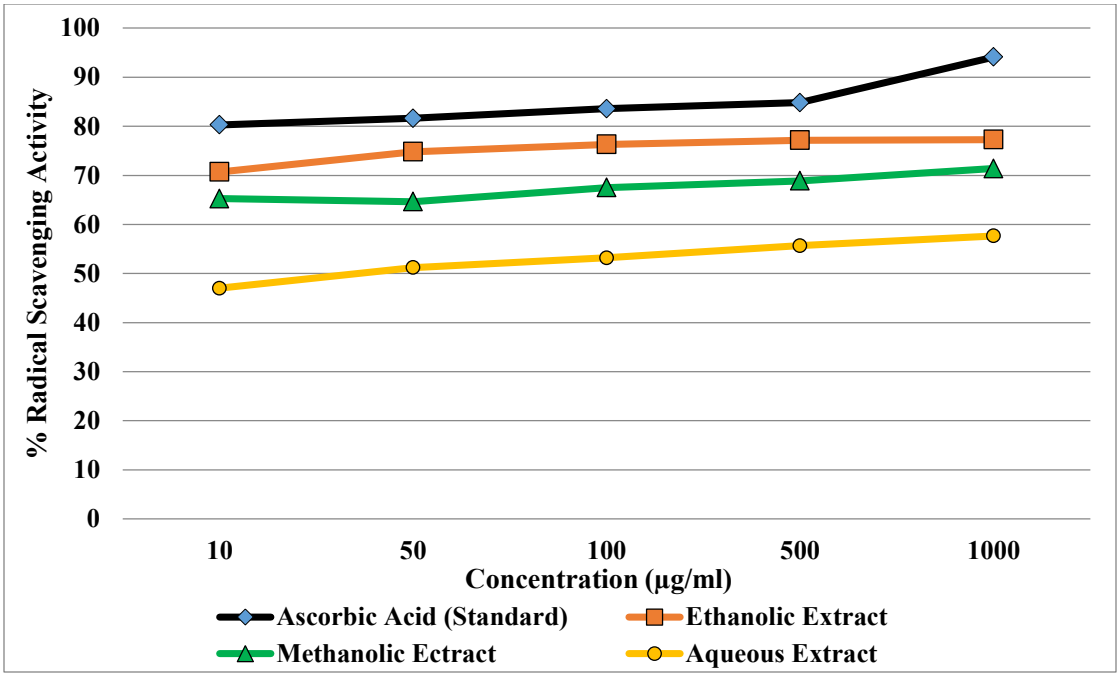

Fig. 27: Results of free radical scavenging activity using DPPH

* All measurements are means of individual data obtained from triplicate

Chapter 4: Discussion

4. Discussion

The results of preliminary phytochemical screening confirmed the presence of major classes of phytochemicals in the extracts of green tea. These polyphenols, produced either from phenylalanine or from its precursor shikmic acid, are important dietary antioxidants because they possess an ideal structural chemistry for free radical scavenging activity. Various *in vitro* studies have shown their antioxidant potential in the prevention of numerous diseases (Matkowski *et al*., 2008).

Treating hospital community acquired infections caused by multi-drug resistant bacteria has become a major problem worldwide. In the past few decades, MRSA has become an increasingly persistent pathogen in both hospitals and communities (Radji *et al*., 2011; Kollef *et al*., 2006). Treatment of these infections is often very difficult due to cross-resistance of these bacteria with a large group of antibiotics. Hence, studies conducted to explore new sources of natural compounds with antibacterial activity against multi-drug resistant pathogens, such as this, hold great significance.

The current study shows the inhibitory effect of green tea extracts on some strains of bacteria (*E. coli, S. aureus,* MRSA*, S. flexneri and* ETEC). In the current study, crude ethanolic and methanolic extracts of green tea exerted the greatest inhibitory activity on all test strains including MRSA, while aqueous extract exhibited the least. Ethanolic extract exercised antibacterial properties even at lower concentrations of 80µg/µl, yielding larger clear zones than methanolic or aqueous extracts of the same concentration. These findings are significant because *S. aureus* is one of the major causes of both nosocomial and community-acquired infections globally. The anti-bacterial activity of green tea extract is comparable to standard antibiotic. The results of this study are consistent with other studies that reported that green tea extract showed activity against both MRSA and *Staphylococcus aureus* (Hamilton-Miller and Shah, 2002).

The inhibitory effect of tea depends on the preparation and concentration of extracts as well as the tested microbes. It was found that the biological activity of the extracts increased with concentration. The effects of ethanolic and methanolic extracts could be due to the thermostability of some bioactive chemical constituents which may have been enhanced by an increase in the solubility of active ingredients in hot alcohol making more constituents available in the resulting extract. According to Yassien, bioactive substances in green tea are more soluble in alcohol than water. In our study, we also found similar results as methanolic and thanolic extracts of green tea showed antimicrobial activities against the test organisms.

Despite the strong presence of flavonoids in aqueous extract in the phytochemical assay, the aqueous extracts of green tea showed no antibacterial activity save for against *S. aureus*. This is consistent to the findings of not in agreement to previous findings where aqueous extracts of green tea proved effective against a variety of gram-positive and gram-negative bacteria using similar extraction techniques (Chan *et al*., 2011). A primary reason for this may be the extraction process itself because studies have shown that the temperature of water is an important factor when extracting tea (Lin *et al*., 2008; Su *et al*., 2006). It has been reported that higher temperatures reduce the polarity of water, increasing the efficiency of extraction and ability to dissolve less polar compounds (Hassas-Roudsari*et al*., 2009). Raising the temperature of water also reduces its surface tension and viscosity, thereby increasing the rate of diffusion and mass transfer during extraction (Chan *et al*., 2011).

Green tea catechins, particularly EGCG and ECG, have antibacterial activity against both Gram-positive and Gram-negative bacteria (Bancirova, 2010); (Toda *et al*., 1989); (Hamilton-Miller, 1995). However, Chan *et al*. reported that all extracts showed inhibitory effects on Gram-positive but not on Gram-negative bacteria (Chan *et al*., 2011). Antibacterial activity of green tea is owing to effects of catechins which damage the bacterial cell membrane, inhibit fatty acid synthesis and enzyme activity. Tea catechins have less effect on gram negative bacterial cell membranes because the

outer membrane of gram negative bacteria is negatively charged (Ikigai *et al*., 1993). Many of the antibacterial effects are due to the catechins binding to the bacterial lipid bilayer cell membrane which then causes damage to the membrane (Sirk *et al*., 2008, 2009). Bacterial cell membrane damage inhibits the ability of the bacteria to bind to host cells (Sharma *et al*., 2012), and to each other to form biofilms, which are significant in pathogenesis (Blanco *et al*., 2005). Bacterial membrane damage also disables the bacteria to secrete toxins (Sugita- Konishi *et al*., 1999; Shah *et al*., 2008). Inhibition of fatty acid synthesis by green tea has also been found to inhibit bacterial production of toxic metabolites (Sakanaka and Okada, 2004). In the current study, tea extracts showed better response against gram-positive bacteria.

Tannins possess strong, broad spectrum antibacterial properties (Turkmen *et al*., 2007; Amarowicz *et al*., 2008; Min *et al*., 2008; Doss *et al*., 2009). Tannins affect bacterial growth by inhibiting extracellular microbial enzymes, depriving them of the substrates required for microbial growth or by the inhibition of oxidative phosphorylation (Scalbert, 1991). All three extracts showed positive for tannins in phytochemical assay.

The properties of green tea which inhibit bacterial growth are owing to their polyphenols, mainly the flavonoids especially catechins. Catechins can inhibit the activity of efflux pumps like Tet(K) efflux pump and reverse tetracycline resistance in *staphylococci* (Roccaro *et al*., 2004).

It has been reported that EGCG can reverse methicillin resistance of MRSA by inhibiting the synthesis of PBP2 (Yam *et al*., 1998). β-Lactam resistance in *S. aureus* is associated with the *mecA* gene which encodes a penicillin binding protein (PBP) called PBP2a. PBPs are transpeptidases that are used in the synthesis of the peptidoglycan layer of the bacterial cell wall. Researchers have found that green tea catechins inhibit the synthesis of PBP2 in MRSA which leads to the reversal of resistance to β-lactam drugs (Yam *et al*., 1998).

Despite the promising antibacterial results, no synergism was established between the tea extracts with the standard class of drugs. Although studies have reported synergistic activity of green tea with tetracycline against *S. aureus*, with oxacillin, penicillin, ampicillin against MRSA (Hu *et al*., 2001, 2002; Zhao *et al*., 2001b; Stapleton *et al*., 2006) and with chloramphenicol, ciprofloxacin, and cefotaxime against isolates of *E. coli* (Fanaki *et al*., 2008; Cui *et al*., 2012a; Passat, 2012). In this study the green tea catechins did not work synergistically with ciprofloxacin, ampicillin or cefoxitin against any of the test strains. The zone of inhibition was at best as large as the zone of antibiotic but not greater than the sum of both individual antibacterial agents.

Plant phenolics are important secondary metabolites with antioxidant properties due to their redox potential, which plays an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Mishra *et al*., 2011). Plant phenolics are one of the main compounds working as primary antioxidants or free radical scavengers. They work as singlet oxygen scavengers, reducing agents and hydrogen atom donators (Karaman *et al*., 2010); (Rice-Evans *et al*., 1996).

Among the examined extracts, alcoholic extracts of green tea exhibited the highest free radical scavenging activity. This is in accordance with other findings (CHEMIK 2011, 65, 10, 968-973). The strong antioxidant activities of green tea catechins are due to the three adjacent hydroxyl(OH) groups on the B-ring as in EGCG, GCG, EGC, and GC (Figure 28) which are better at scavenging free radicals than the two adjacent OH groups as in ECG, CG, and EC (Sharma *et al*., 2011).

DPPH after quenching by antioxidant (Paital, 2014). Compared to the standard ascorbic standard, ethanolic extract showed the highest antioxidant activity followed by methanolic extract and lastly aqueous extract exhibited the least amount of antioxidant activity.

Chapter 5: Conclusion

5. Conclusion

With the emerging menace of multi-drug resistant organisms and the lack of effective antimicrobial agents, cost of treatment is rising higher by the day. Despite being a global problem, this is an especially burning issue in developing countries like Bangladesh where infectious diseases are extremely common and antibiotics are readily available over-the counter drugs. In light of growing concerns the exceptional potential of green tea should be widely exploited.

After examining the results of the study it can be concluded that the method of extraction and the type of solvent used determine the efficacy of antibacterial and antioxidant properties of green tea extracts. Among the examined extracts the best antibacterial and antioxidant activities were exhibited by the alcoholic extracts while the aqueous extract demonstrated the least efficiency. The results of this study suggest that *Camellia sinensis* extracts are rich in phenolic compounds and have potent antimicrobial and antioxidant activity. Hence, green tea can be used as a natural source of antioxidants to prevent the progression of many diseases.

It can be concluded that the extracts of *Camellia sinensis* are effective against both gram-positive and gram-negative bacteria, including multi-drug resistant clinical isolates.

Attempts can be made to identify the individual polyphenolic compounds that are responsible for various pharmacological effects. Investigations can be further conducted to develop green tea as an alternative therapy to treat infectious diseases especially multi-drug resistant pathogens. However, further *in vivo* examination is required to determine the dose, toxicity and bioavailability of the active compounds of green tea in order to analyze its efficacy as a therapeutic agent.

Moreover, the majority of green tea extracts exhibited synergistic activity with antibiotic combinations against test organisms with the exception of the current study. Hence, it is recommended to further study this synergistic activity *in vivo*.

It has been established in the current study that green tea is rich in antioxidants and ethanolic extract possesses the highest antioxidant activity in comparison to ascorbic acid which is a standard antioxidant. Aqueous extract was prepared in a way so as to mimic tea brewing and although it has the least antioxidant activity with respect to ascorbic acid, the antioxidant potential is still significantly high. At the lowest concentration of 10µg/ml the aqueous extract of green tea shows 46.9% free radical scavenging activity.

Further studies can be carried out in order to quantify the phytochemical constituents in green tea extracts via HPLC and column chromatography. Though the consumption of green tea is increasing in the general population, black tea is still widely consumed. Hence, further studies can be conducted to analyze the antioxidant activity of black tea.

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Appendix-I

Media Composition

Only Nutrient Agar media was used in this study. Composition of the media used is provided below. Media was autoclaved at 121^{o}C for 15 min at 121psi. Preparation of nutrient agar media was done by adding 28 g of nutrient agar powder in 1000 ml of distilled water.

1. Nutrient Agar (HiMedia, India)

Ingredients	Amounts (g/L)
Peptic digest of animal tissue	5.0
Beef extract	1.50
Sodium chloride	5.0
Yeast extract	1.50
Agar	15.0

Appendix-II

Reagents and Chemicals

1. **1% Ferric chloride**
   1 g of Ferric chloride in 100 ml distilled water

2. **1% potassium ferrocyanide**
   1 g of Ferric chloride in 100 ml distilled water

3. **Hager's Reagent**
   1% Picric Acid: dissolve 1g of picric acid in 100ml distilled water

4. **Wagner's reagent**
   Iodo-potassium iodide: dissolve 2g of iodine and 6g of potassium iodide in 100ml distilled water

5. **Marquis' Reagent**
   Formaldehyde-sulphuric acid: add 10ml formaldehyde solution to 50ml sulphuric acid

6. **10% Lead Acetate**
   1 g of lead acetate in 100 ml distilled water

7. **10% Ferric chloride**
   1 g of Ferric chloride in 10 ml distilled water

8. **5% Ferric chloride**
   1 g of Ferric chloride in 20 ml distilled water

9. **Molisch's Reagent**
   1-Naphol: dissolve 15g of 1-napthol in 100ml of alcohol or chloroform

10. **DPPH (2, 2-diphenyl-1-picrylhydrazyl)**
    0.1 mM DPPH: 4 mg of DPPH in 100 ml methanol

Appendix-III

Instruments

Soxhlet	Heating Mantle Made in India
Electronic Balance	RADWAG Wagi ELEktroniczne Model: WTB 200
Shaking Incubator	Model: JSSI-1000C JS RESEARCH INC. Made in Rep. of Korea
Table Top Centrifuge	Model: DSC-200A-2 Digisystem Laboratory Instruments Inc. Made in Taiwan
Water Bath WiseBath^{R}	Wisd Laboratory Instruments DAIHAN Scientific Co., Ltd Made in Korea
Refrigerator (4^{0}C)	Model: 0636 Samsung
Vortex Mixer	Model: VM-2000 Digisystem Laboratory Instruments Inc. Made in Taiwan
Fume Hood	-
Dry Oven	Model: LDO-060E DAIHAN LABTECH CO. LTD Made in Korea
Autoclave (WiseClave^{R})	Wisd Laboratory Instruments Made in Korea
Laminar Air Flow Cabinet	SAARC Engineering
Incubator	Model: DSI 3000 Digisystem Laboratory Instruments Inc. Made in Taiwan
Spectrophotometer	UVmini-1204 SHIMADZU CORP.