PRELIMINARY PHYTOCHEMICAL SCREENING, QUALITY ASSESSMENT AND MINERAL CONTENT DETERMINATION OF SELECTED BRANDS OF GREEN TEA MARKETED IN ZARIA, NIGERIA.

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JULY, 2017

DECLARATION

I hereby declare that the work in this dissertation titled "PRELIMINARY PHYTOCHEMICAL SCREENING, QUALITY ASSESSMENT AND MINERAL CONTENT DETERMINATION OF SELECTED BRANDS OF GREEN TEA MARKETED IN ZARIA, NIGERIA" was performed by me in the Department of Pharmaceutical and Medicinal Chemistry, under the supervision of Dr. Aminu Musa and Prof. I. Yakasai. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work has been presented for another degree or diploma in any institution.

_____ _____ _____

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CERTIFICATION

This dissertation titled "PRELIMINARY PHYTOCHEMICAL SCREENING, QUALITY ASSESMENT AND MINERAL CONTENT DETERMINATION OF SELECTED BRANDS OF GREEN TEA MARKETED IN ZARIA, NIGERIA" by YUSUF ABDULHAMID IBRAHIM meets the regulations governing the award of the degree of M.Sc. Pharmaceutical and Medicinal Chemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literature presentation.

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ABSTRACT

Green tea in its purest and most unadultered form has always influenced human health for generations. Though green tea is not officially recognized as a medical agent, it is one of the most researched plant-based remedies with possible benefits including antioxidant activity, skin protection, oral health, good vision, help combat obesity, antiviral potential and many more. This study was aimed at assessing the preliminary phytochemical screening, quality assesment and mineral content determination of selected brands of green tea marketed in Zaria. Quality assessment was conducted on twelve selected brands of green tea marketed in Zaria. Identification test, uniformity of weight, moisture content, total ash values, water soluble ash and acid insoluble ash were determined using official methods. Preliminary phytochemical screening of the samples was conducted according to standard method. A rapid, simple, precise and accurate UV spectrophotometry method for analysis of Epigallocatechin gallate (EGCG) in green tea at 274 nm was developed and validated based on ICH guideline. Calibration curve was constructed within the range of 2.0 to 10.0 µg/ml, and the developed method was employed to quantify EGCG in the various brands of green tea. The EGCG content ranged from 0.75 – 0.82 mg/g, with three samples having significantly higher values ($p < 0.05$) than the rest. Essential trace metals (Fe, Zn and Cu) and other heavy metals (Pb and Cd) were analysed in the green tea samples. All the green tea brands passed uniformity of weight test as their percentage deviations were less than 7.5 %. The moisture content of the samples range from 1.6 – 14.35 %, with the percentages in eight samples been significantly higher ($p < 0.05$) than the NAFDAC limit (8 %). The total ash values of the samples range from 5 to 12.50 %; seven of the samples were significantly different ($p < 0.05$) from the IARC-WHO limit (4 – 8 %). Similarly, for the water soluble ash range was 1.75 – 6 %, only one of the samples was found to have significantly higher

percentage ($p < 0.05$) than the IARC-WHO limit (5 %). The acid insoluble ash range from 1.00 to 4.25 %, with seven of the samples havng significantly higher percentage ($p < 0.05$) than the IARC-WHO limit (1 %). The phytochemical analysis showed the presence of tannins, saponins, cardiac glycosides, flavonoids, alkaloids, and terpenoids. The wavelength of maximum absorbance of EGCG in water was obtained at 274 nm. The calibration curve obeyed Beers-Lambert law in the concentration range from 2.0 – 10.0 µg/ml with correlation coefficient of 0.998. The precision, accuracy, (% relative error), % recovery, limit of detection and limit of quantitation were determined (1.7 %, 1.99 %, 95.77 %, 0.68 µg/ml and 2.07 µg/ml) respectively. Heavy metals (Fe, Zn, Cu, Pb and Cd) were detected in almost all the samples, with Fe having the highest concentration (164.04 mg/g) recorded and Cd having the lowest concentration (0.02 mg/g) recorded. There was significant difference ($p < 0.05$) in metal content in all the brands, however, for some brands the metals were below detection limit of the analytical method. Thus, it can be concluded that consumption of all the green tea brands analyzed may be beneficial to human health as a result of the phytochemical and essential metal content, and may also be safe because the toxic metals are within the permissible limits.

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ABBREVIATIONS

EGCG	Epigallocatechin gallate
EC	Epicatechin
EGC	Epigallocatechin
ECG	Epicatechin gallate
WHO	World Health Organization
UV	Ultraviolet
AAS	Atomic Absorption Spectrometry
AIDS	Acquired Immune Deficiency Syndrome
HIV	Human Immunodeficiency Virus
RT	Reverse Transcriptase
IR	Infra-red
M.C	Moisture content
CV	Coefficient of variation
Er	Relative error
DL	Detection limit
QL	Quantitation limit
BLD	Below detectable limit
BP	British pharmacopoeia
GMP	Good manufacturing practice
ICH	International conference on harmonization
NAFDAC	Nigeria agency for food and drug administration
SEM	standard error of the mean
PPM	Part per million

CHAPTER ONE

INTRODUCTION

1.1 General Introduction

The human race faces a growing number of ailments and diseases that are of serious concern to normal sustenance. These include cancer, diabetes, hypertension, bacterial and fungal infections, dental caries, skin diseases like acne and many more. Control and cure of these diseases require sources that can overcome these health concerns with minimal potential to cause adverse effects. The use of herbal drugs have for a variety of reasons been increasing globally. Some of these reasons include the perception that orthodox drugs are increasingly over prescribed, expensive and toxic (Bateman *et al.*, 1998). Another important reason is the cost of herbal drugs and the wide-spread perception that they are safer and more efficacious than orthodox drugs (Bateman *et al.*, 1998; Rai and Mehrotra, 2005). Globally, people are turning to nature by using the natural herbal products in treatment of different diseases. There has been a rising interest in naturally derived biologically active compounds that may have potential therapeutic uses in medicine and dentistry (Groppo *et al.*, 2008; Newman, 2008). Medicinal plants are of great importance to the health of individuals and communities. The medicinal value of these plants lie are due to the presence of some chemical substances (secondary metabolites) that produce a definite physiological action in the human body and these chemical substances are called phytochemicals. These could be non-nutritive chemicals that have protective or disease preventive property (Subhashini *et al.*, 2010). Some of the active constituents in green tea are powerful antioxidants called polyphenols. Tea is reported to contain nearly 4,000 bioactive

compounds of which one third compress or consists of polyphenols (Tariq *et al.,* 2010). Among the polyphenols in tea, is a family of compounds called flavonoids. Catechins (a group of flavonoids) are the basic phenolic compounds in green tea responsible for antioxidant activities (Horzic *et al.,* 2009). Tea, in particular green tea, is considered to be a good source of antioxidants due to the larger proportion of Epigallocatechin gallate (EGCG) that it contains (Gramza *et al.,* 2004). Within the body during normal metabolic processes and respiration, reactive oxygen species are created and damage macromolecules such as DNA and lipids with the body's antioxidant defence system becoming overwhelmed. For this reason it is very beneficial and indeed necessary to consume compounds with antioxidant activity to reduce the effects of reactive oxygen species. Tea catechins can act as antioxidants by donation of a hydrogen atom, as an acceptor of free radicals, interrupting chain oxidation reactions, or by chelating metals (Gramza *et al.,* 2004). Polyphenols in tea are capable of trapping reactive oxygen species due to their vicinal dihydroxy or trihydroxy structure which prevent damage to macromolecules by chelating metal ions. Many of green tea's health promoting abilities are attributed to EGCG (Khan and Mukhtar, 2007). A number of studies have confirmed the health benefits of green tea, although much focus has been put on EGCG (Raymond *et al.,* 2005).

Both deficiency and excess of essential micronurients may cause undesirable effects. Effect of the toxic metals on human health may produce serious consequences. Trace elements refer to "elements that occurs in natural and perturbed environments in small amounts (Stohs and Bagchi, 1995). However, this tiny fraction exerts a tremendous influence on all body functions. Most of them mediate vital biochemical reactions by acting as a cofactor or catalyst for many enzymytic reactions. They also act as centers of building stabilizing structures such as enzymes

and proteins. Although trace elements account for only 0.02 % of the total body weight, they play significant roles, as active centers of enzymes or as trace bioactive substances (Wada, 2004). Metals such as iron, zinc, copper, and selenium are essential components of enzymes where they attract or subtract molecules and facilitate their conversion to specific end products. Trace metals control important biological processes by facilitating the binding of molecules to their receptor sites on cell membrane, by alternating the structures or ionic nature of membrane to prevent or allow specific molecules to enter or leave a cell and in inducing gene expression resulting in the formation of proteins involved in life processes (Nielsen, 1990). The accumulation of metals or deficiency of these elements may stimulate an alternate pathway which might produce diseases. Interaction among the trace elements may also act as a scaffold upon which the etiopathogenesis of many nutritional disorders lie (Stohs and Bagchi, 1995). There is no clear definition of what a heavy metal is, density is in most cases taken to be the defining factor; heavy metals are thus commonly defined as those having a specific density of more than 5 g/cm^3 (Jarup, 2003; Raikwar *et al.*, 2008). Heavy metals are among the most important source of contaminant in the environment (Tangahu *et al.*, 2011). They are released into the environment by both natural and anthropogenic sources (Jung, 2008). Some metals are redox active, that is, they undergo redox cycling reactions whilst others are redox inactive. Metal concentrations in soil range from less than 1 mg/kg (ppm) to high as 100,000 mg/kg, whether due to the geological origin of the soil or as a result of human activity (Blaylock and Huang, 2000). Medicinal plants can be contaminated by heavy metals via uptake from the soil or by direct deposition of contaminants from the atmosphere onto plant surfaces. Moreover, contamination of heavy metals represents one of the most pressing threats to water and soil

resources as well as human health (Yoon *et al.,* 2006). Transition metals cause oxidative injury in plant tissues (Schutzendubel and Polle, 2001). Toxicity of phytochemicals, plant-based extracts, dietary supplements, and medicinal plants in general, is of medical importance and must be considered in phyto therapy and other plant uses (Chen and Vieira, 2010). There are three main mechanisms that have been proposed to account for heavy metal contamination in medical herbal products, viz: contamination during cultivation (Annan *et al.,* 2013), accidental cross contamination occurring during processing and/or the deliberate introduction of heavy metals as a therapeutic ingredient (Mohammad *et al.,* 2012). The accumulation of heavy metals in medicinal plants have also been reported to be affected by climatic factors, plant species, air pollution and other environmental factors (Lasisi *et al.,* 2005). Plants absorb metals from soil and they predominantly accumulate in the roots, and portions are transferred to other parts of the plant (Cheng, 2003). Plants can immobilize heavy metals through absorption and accumulation by roots, adsorption onto roots, or precipitation within rhizosphere. Metal concentrations in leaves are usually much higher than those in grain (Yoon *et al.,* 2006). However, some plant species are able to accumulate fairly large amounts of heavy metals without showing stress, which represents a potential risk for animals and humans (Oliver, 1997).These plants that easily absorb high levels of metals from the surrounding soil are called hyper accumulators. Medicinal plants have been cited as a potential source of heavy metal toxicity to both man and animals (Dwivedi and Dey, 2002).

The World Health Organization has therefore recommended that medicinal plants which form the raw materials to finished products for most herbal remedies, should be checked for the presence of heavy metals (WHO, 1998a). In addition, it becomes necessary to improve quality

standards for herbal medicines by examining and revising the maximum allowable values of heavy metals present in herbal/ medicinal plants (Cao *et al.,* 2009).

1.2 Statement of the Research Problem

Green tea is widely consumed as a beverage and herbal supplement due to its antioxidant activity. As a result, many brands of green tea products are available in the markets world-wide including Nigeria. However, the quality of most of these green tea products cannot be ascertained due to inadequate control and regulation in the country. Studies have shown that green tea can be contaminated with toxic metals and that the quantities of phenolic compounds found in them vary depending on their quality (Gramza *et al.,* 2004; Vilma *et al.,* 2011).

1.3 Justification for the Research

The proliferation of different brands of green tea products in the market, some of which have no NAFDAC registration number, necessitatis quality assessment and evaluation of heavy metals toxicity levels of these tea products in order to determine their nutritional value and safety.

1.4 Aim and Objectives

1.4.1 Aim

To carry out preliminary phytochemical screening, quality assessment and mineral content determination of selected brands of green tea marketed in Zaria.

1.4.2 Objectives

The objectives of the study are to:

- carried out a survey of the brands of green tea marketed in Zaria and collect some selected samples among the various brands.

- conduct preliminary analysis of phytochemical constituents of the selected brands.
- develop and validate UV-spectroscopic method for the assay of EGCG in the selected samples of the green tea.
- analyzed and compare the metal contents in the different brands.

1.5 Research Hypothesis

The selected green tea brands marketed in Zaria contain antioxidant phytochemicals and essential metals in the right proportion.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Green tea

Green tea is derived from the leave of the plant *Camellia sinensis* (L.) Kuntze, which is an angiosperm dicotyledonous plant. *C. sinensis*,belonging to the family Theaceae and is an evergreen tree or shrub that attains a height of 10-15 m. Green tea is basically a rain fed crop which grows in humid climate of temperate, tropical and sub tropical regions (Kemmler, 1986). The leaves are light green, short stalked, coriaceous, alternate, lanceolate, serrate margin, glabrous or pubescent beneath, varying in length from 5 - 30 cm and about 4 cm width. Mature leaves are bright green colored, smooth and leathery while young leaves are pubescent (Akhtar *et al.,* 2010). Its ancestry begins in China; credited with introducing tea to the world. The ever green tea plant is in fact native to southern China, North India, Myanmar and Cambodia (Hicks, 2001). However, the different types of tea are classified according to the degree of fermentation that takes place during processing; green tea being unfermented; oolong tea semi-fermented and black tea fully fermented (Chan *et al.,* 2011). These basic types of tea have different quality characteristics, including appearance, aroma, taste, and color. The manufacturing process of tea is designed to either preclude, or permit tea polyphenolic compounds to be oxidized by naturally occurring polyphenols oxidase in the tea leaves. Green tea is produced by inactivating the heat-labile enzyme polyphenol oxidases in the fresh leaves by either applying heat or steam, which prevents the enzymatic oxidation of catechins, the most abundant flavonoid compounds present in green tea extracts (Velayutham *et al.,* 2008). The tea leaves are then rolled, dried and

packaged. The process of black tea production is more complicated. The leaves are also subjected to withering during which the first fermentation processes occur. Next, they are rolled up and undergo fermentation. Fermented leaves are roasted, until the dark - brown or black color, thereafter a suitable aroma appears. Blocking the activity of enzymes (polyphenols oxidase and glycosidase) as well as stopping the fermentation is the aim of roasting (Chu and Juneja, 1997). Semi fermented tea Oolong is partially fermented tea with considerably shorter fermentation time than the black one (Milind *et al.,* 2012). Green tea's secret lies with its rich source of catechins (polyphenols), particularly EGCG. The EGCG is a powerful antioxidant; besides inhibiting the growth of cancer cells, it kills cancer cells without harming normal tissues (Sinija and Mishra, 2008). The catechins have been found to possess antibacterial and antiviral properties (Archana and Jayanthi, 2011). However, the results from epidemiological and clinical studies of the relationship between green tea consumption and human health are mixed. For example, conflicting results between human studies may arise in part, from ignoring socioeconomic and lifestyle factors as well as by inadequate methodology to define tea preparation and intake (Mckay and Blumberg, 2002; Rietveld and Wiseman, 2003; Il'yasova *et al.,* 2003).

2.2 Chemical Constituents of Green Tea

The chemical composition of green tea varies with climate, season, horticultural practices, and age of the leaf (Pastore, 2005). A cup of green tea (2.5 g of green tea leaves/200 ml of water) may contain 90 mg of EGCG (figure 2.1), 22 mg of Epigallocatechin (EGC; figure 2.2), 18 mg of Epicatechin gallate (ECG figure 2.3), 11 mg of Epicatechin (EC) (Wu and Wei 2002; Blumberg *et al.,* 2015). It was reported that green tea contain over four times the concentration

of antioxidant catechins than black tea, about 70 mg catechins per 100 ml compared to 15 mg per 100 ml of black tea. Other polyphenols present in green tea are flavanols and their glycosides, as chlorogenic acid, coumarylquinic acid (Balentine, 1997). Amino acid degradation is involved in the biogenesis of the tea aroma (Balentine, 1997). Metal analysis of green tea reveals that it is a rich source of mineral elements which are essential for health these include zinc, manganese, iron, magnesium, silver, copper, titanium, aluminum, sodium, potassium as well as nickel, and chromium. These metal ions promote the antioxidant property of green tea (Hui, 1992; Chu *et al.,* 1997; Kok and Tascioglu, 1998; Fernández-Cáceres *et al.*, 2001; Gramza *et al.*, 2004).

2.2.1 Phytochemical constituent of green tea

Phytochemicals (from the Greek word phyto, meaning plant) are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further than those attributed to macronutrients and micronutrients (Hasler and Blumberg, 1999; Blumberg *et al.,* 2015). They protect plants from diseases and damages; contribute to the plant's color, aroma and flavor. In general, the plant chemicals that protect plant cells from environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attack are refers to as phytochemicals (Gibson *et al.,* 1998). More than 4,000 phytochemicals have been cataloged and are classified by protective function, physical characteristics and chemical characteristics and about 150 phytochemicals have been studied in detail (American Cancer Society, 2000). These compounds are known as secondary plant metabolites and have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of

hormone metabolism and anticancer property (Higdon and Frei, 2003). There are more than thousand known and many unknown phytochemicals. It is well-known that plants produce these chemicals to protect themselves, but research demonstrates that many phytochemicals can also protect human against diseases (Narasinga, 2003). Some of these secondary metabolite are as follows;

2.2.1.1 Flavonoids

Flavonoids are polyphenolic compounds that are ubiquitous in nature. More than 4,000 flavonoids have been recognized, many of which occur in vegetables, fruits and beverages like tea, coffee and fruit drinks (Pridham, 1960). The flavonoids appear to have played a major role in successful medical treatments of ancient times, and their use has persisted up to now (Hicks, 2001). Flavonoids have gained recent attention because of their broad biological and pharmacological activities; they have been reported to posses multiple biological property including antimicrobial, cytotoxicity, antiinflammatory as well as antitumor activities but the best described property of almost every group of flavonoids is their capacity to act as powerful antioxidants which can protect the human body from free radicals and reactive oxygen species (Atmani *et al.,* 2009). The major flavonoids present in green tea are the catechins.

Epigallocatechin gallate: Epigallocatechin gallate (EGCG), also known as epigallocatechin-3-gallate, the ester of epigallocatechin and gallic acid, is a type of catechin belonging to the Flavonoids. EGCG is the most abundant catechin in green tea infusions and it is considered as one of the most active molecules known for their antioxidant properties (Higdon and Frei, 2003). Investigations have shown that green tea contains a larger quantity of catechins than the

black tea (Katavic *et al.,* 2007). This polyphenolic compound and several related catechins are believed to be responsible for the health benefits associated with the consumption of green tea. Increasing evidence has suggested that EGCG exhibit anti-inflammatory, antioxidant and immunosuppressive effects (Wang, 2006). Other health benefits ascribed to green tea and EGCG include antioxidant effects, cancer chemoprevention, antimicrobial, improved cardiovascular and enhancing weight loss. A meta-analysis of 20 randomized controlled trials evaluating the effect of green tea catechins on lipid levels reported that green tea intake ranged from 145-3000 mg/day. EGCG doses ranging from 68 - 1037.8 mg/day. Subgroup analysis found that 200 mg/day of EGCG or more was necessary to decrease total cholesterol and low-density lipoprotein (LDL) cholesterol levels. Lower intake levels had no effect (Kim *et al.,* 2011a). In addition to health benefits, some potential harm may be associated with EGCG intake levels. Preliminary research in animals showed that oral doses ranging from 500 -1500 mg/kg of EGCG caused increases in biochemical markers for liver injury as well as moderate to severe hepatotoxicity (Lambert *et al.,* 2010; Wu *et al.,* 2011). Based on allometric scaling, an equivalent human dose would be 30 - 90 mg/kg (Lambert *et al.,* 2010). Several cases of green tea-related hepatotoxicity have also been reported. However, causality is difficult to assess in many of these reports (Sarma *et al.,* 2008). Wang, (2006) pretreated mice with EGCG before concanavalin-A injection, and then measured alanine amino transferase levels in plasma, inflammatory infiltration and hepatocyte apoptosis in liver. He found that mice pretreated with EGCG exhibited much less increased alanine amino transferase levels in plasma, reduced inflammatory infiltration and hepatocyte apoptosis in liver compared with control mice. Park, (2006) compared the antifungal activities of EGCG with six antifungal agents, amphotericin B,

fluconazole, flucytosin, itraconazole and miconazole and concluded that EGCG has comparable antifungal activities on some strains of these antifungal agents.

Fig 2.1; Structure of EGCG

EGCG has a Molecular Formula of $C_{22}H_{18}O_{11}$ and molecular weight of 458.40g/mol. Chemical and trade names of epigallocatechin gallate include; epigallocatechin 3-gallate; (–)-epigallocatechin-3-*O*-gallate; 3,4,5-trihydroxybenzoic acid, (2*R*-*cis*)-3,4-dihydro-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-2*H*-1benzopyran-3-yl ester; (2*R*, 3*R*)-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-1(2*H*)-benzopyran-3,5,7-triol 3-(3,4,5-trihydroxybenzoate). EGCG in its pure form is an odorless, white, faint pink or cream-colored powder or crystals (Alexis Corporation, 2000; Merck, 1997; Sigma Aldrich, 2000). EGCG has a melting point of 218 °C (Merck, 1997). It is

soluble in water (clear, colorless solution at 5 mg/ml), acetone, ethanol, methanol, pyridine, and tetrahydrofuran (Sigma-Aldrich, 2000).

Epicatechin gallate: Epicatechin gallate (ECG) is a flavan-3-ol, a type of flavonoid present in green tea (Shiota *et al.,* 1999). ECG, as well as many other flavonoids, has been found to act as non-selective antagonist of the opioid receptors, albeit with somewhat low affinity (Katavic *et al.,* 2007). The tea component ECG is reported to showed; *in vitro* reverse methicillin resistance in bacteria like *Staphylococcus aureus*. If confirmed this means the combined intake of a tea extract containing this component might also enhance the effectiveness of methicillin treatment against some resistant bacteria *in vivo* (Shiota *et al.,* 1999).

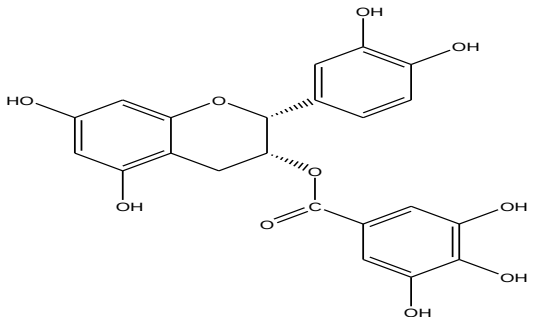


Fig 2.2; Structure of ECG

Epicatechin: Epicatechin (EC) is a natural plant phenol antioxidant found in green tea. It is a strong antioxidant and insulinomimetic which reduces lipid peroxidation, inhibits platete aggregation, control vasodilation by regulating nitric oxide and is thought to improve cardiac efficiency (Katavic *et al.,* 2007). Epicatechins have apparent activity against human cancer: they reportedly may promote apoptosis (Okabe *et al.,* 1999; Kinjo *et al.,* 2002), arrest metastasis by

inhibiting metalloproteinases, impair angiogenesis and reverse multidrug resistance (Demeule *et al.,* 2000).

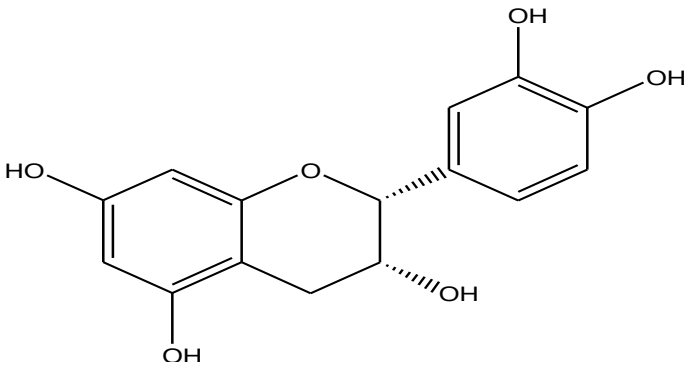


Fig 2.3; Structure of EC

2.2.1.2 Tannins

In medicine, especially in Asian (Japanese and Chinese) natural healing, tannin-containing plant extracts are used as astringents, against diarrhea, as diuretics, against stomach and duodenal tumours (De Bruyne *et al.,* 1999), and as anti inflammatory, antiseptic, antioxidant and haemostatic (Dolara *et al.,* 2005). Tannins are used in the dyestuff industry as caustics for cationic dyes (tannin dyes), and also in the production of inks (iron gallate ink). In the food industry tannins are used to clarify wine, beer, and fruit juices. Furthermore, these tannins may be use as textile dyes, as antioxidants in the fruit juice, beer, and wine industries, and as coagulants in rubber production (Gyamfi and Aniya, 2002). Tannins have attracted scientific interest, especially due to the increased incidence of deadly illnesses such as AIDS and various cancers (Blytt *et al.,* 1988). The search for new lead compounds for the development of novel

pharmaceuticals has become increasingly important, especially as the biological action of tannin-containing plant extracts has been well documented (Palavy and Priscilla, 2006; Mueller-Harvey, 1999). Gallic tannins and Catecholic tannins are predominantly present in green tea (Tariq and Reyaz, 2012).

2.2.1.3 Alkaloids

Alkaloids are natural products that contains heterocyclic nitrogen atoms and are basic in character. The name is alkaloid derives from the word "alkaline" and it was used to describe any nitrogen-containing base (Mueller- Harvey and McAllan, 1992). Alkaloids are important for the protection and survival of plant because they ensure their survival against micro-organisms (antibacterial and antifungal activities), feeding deterrants from insects and herbivores and also against other plants by means of allelopathically active chemicals (Molyneux *et al.,* 1996). Alkaloids have many pharmacological activities including antihypertensive effects, anti-arrhythmic effect, antimalarial activity, and anti-cancer actions (Wink *et al.,* 1998). Some alkaloids; caffeine and nicotine, morphine are used as stimulant and are used as analgesics and quinine is used as anti-malarial drug (Rao *et al.,* 1978). Purine alkaloids (caffeine and theobromine) are present in green tea (Huai *et al.,* 2012).

2.2.1.4 Terpenoids

Many of the terpenoids are commercially interesting because of their use as flavours and fragrances in foods and cosmetics such as menthol and sclareol and/or because they are important for the quality of agricultural products, such as flavour of fruits and fragrance of flowers like linalool (Harborne and Tomas-Barberan, 1991). In addition, terpenoids can have

medicinal properties such as anti-carcinogenic (e.g. perilla alcohol), anti-ulcer, antimicrobial or diuretic (e.g. glycyrrhizin) activity (Langenheim, 1994; Dudareva *et al.*, 2004). S-limonene, S-linalool and S-α-terpineol were the major terpenoids components in green tea (Zhu *et al.*, 2017).

2.2.1.5 Saponins

Saponins are a group of secondary metabolites found widely distributed in the plant kingdom. They form a stable foam in aqueous solutions such as soap, hence the name "saponin". Chemically, saponins as a group include compounds that are glycosylated steroids, triterpenoids, and steroid alkaloids (Bohlmann *et al.,* 1998). Saponins have also been observed to kill protozoans and molluscs, to be antioxidants, to impair the digestion of protein and the uptake of vitamins and minerals in the gut, to cause hypoglycaemia, and to act as antifungal and antiviral (Traore *et al.,* 2000). Triterpene saponins are predominantly present in green tea (Rao and Gurfinkel, 2000).

2.2.1.6 Cardiac glycosides

The cardiac glycosides are an important class of naturally occurring drugs which actions include both beneficial and toxic effects on the heart, and have played an outstanding role in the therapy of congestive heart failures (Wang *et al.,* 2008). They are organic compounds containing a glycoside (sugar) that act on the contractile force of the cardiac muscle. Because of their potency in disrupting the function of the heart, most are extremely toxic. These glycosides are found as secondary metabolites in several plants, and in some insects, such as the milkweed butterflies, that consume the plants (Singh and Rastogi, 1970). Anthraquinone glycosides are present in green tea (Rao and Gurfinkel, 2000).

2.3 Some Important Medicinal Properties of Green Tea

2.3.1 Antioxidant

Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species; such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals, and peroxynitrite. An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage. Catechins are hypothesized to help protect against these diseases by contributing, along with antioxidant vitamins (i.e., vitamins C and E) and enzymes (i.e., superoxide dismutase and catalase), to the total antioxidant defense system (Halliwell and Gutteridge, 1985). Autoxidation, the most common process leading to oxidative deterioration of food lipids, is a free radical chain reaction that proceeds through three distinct stages of initiation, propagation and termination. The antioxidant activity of green tea polyphenols is primarily attributed to the combination of aromatic rings and hydroxyl groups that assemble their chemical structure and consequently binding and neutralization of lipid free radicals by these hydroxyl groups.

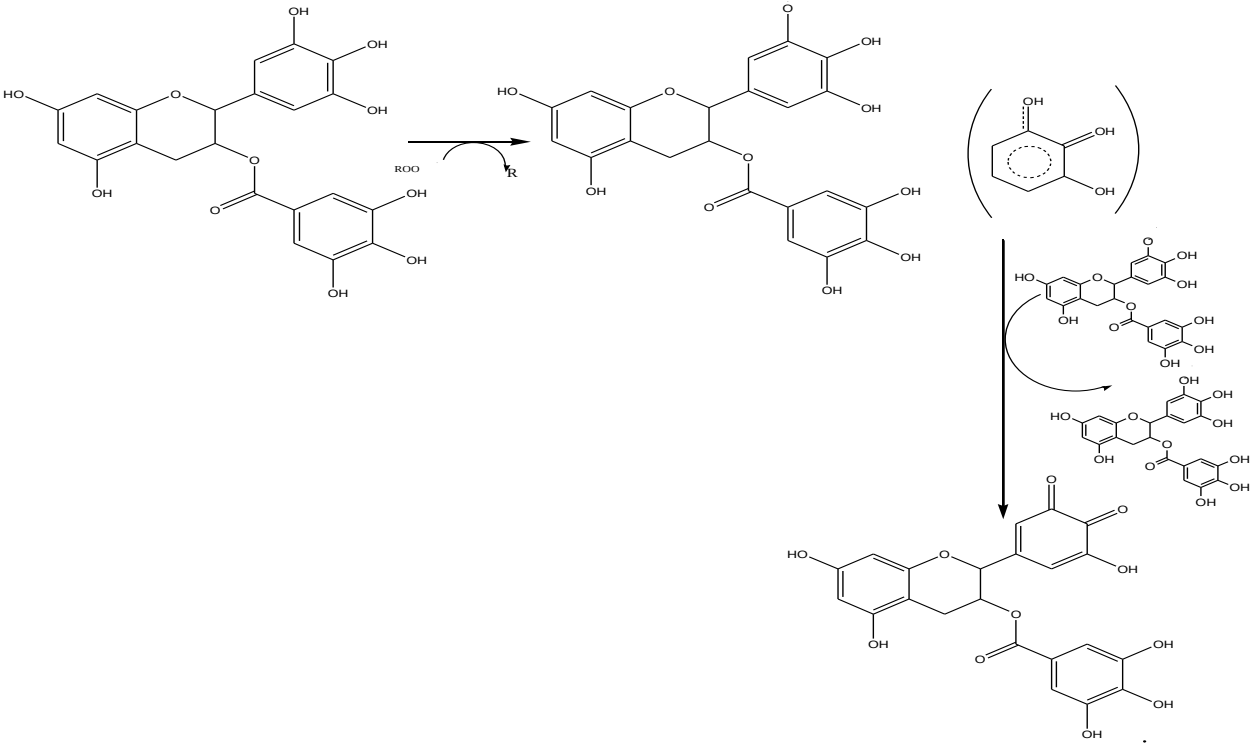


Fig 2.4; Reaction of EGCG with lipid peroxyl radicals

Numerous studies have demonstrated that polyphenols and tea catechins are exceptional electron donors and effective scavengers of physiologically relevant reactive oxygen species *in vitro*, including superoxide anions (Guo *et al.*, 1999; Michalak, 2006; Nakagawa and Yokozawa, 2002; Nanjo *et al.*, 1993; Velayutham *et al.*, 2008), peroxyl radicals, and singlet oxygen (Guo *et al.*, 1999; Michalak, 2006). Catechins also exhibit antioxidant activity via chelating redox active transition metal ions. Polyphenolic compounds, possess hydroxyl and carboxyl groups which are able to bind particularly iron and copper (Michalak, 2006). As shown in figure 2.4

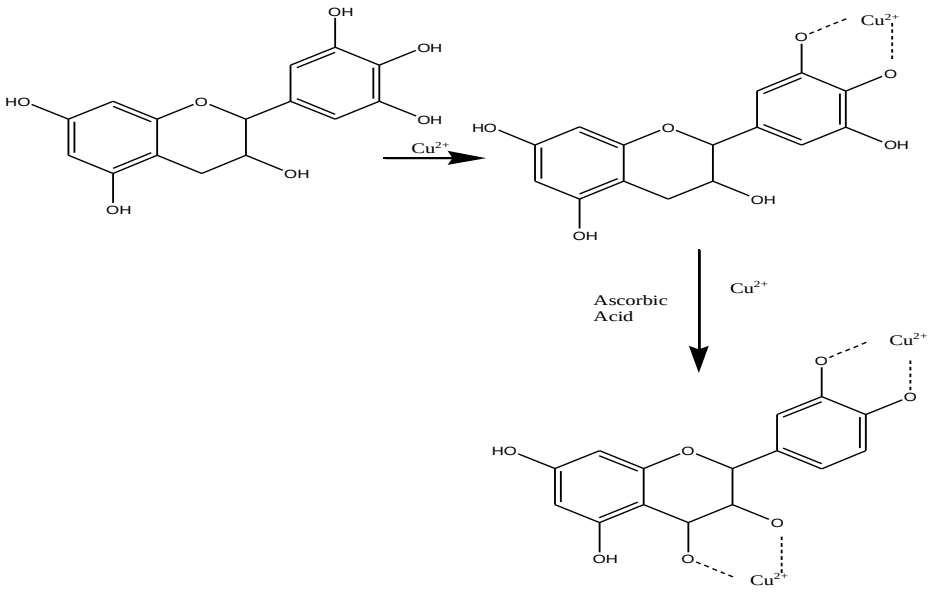


Fig 2.5; Reaction of polyphenols with Copper

There is another mechanism underlying the antioxidant ability of plant polyphenols, including tea catechins. Transition metal ions typically have the ability to initiate free radical chain oxidations by decomposing lipid hydroperoxides by the hemolytic cleavage of the O–O bond and producing lipid alkoxyl radicals. Phenolic antioxidants, including tea catechins, inhibit lipid peroxidation by binding these lipid alkoxyl radicals. This activity depends on the structure of the molecules, and the number and position of the hydroxyl group in the molecules (Milic *et al.*, 1998).

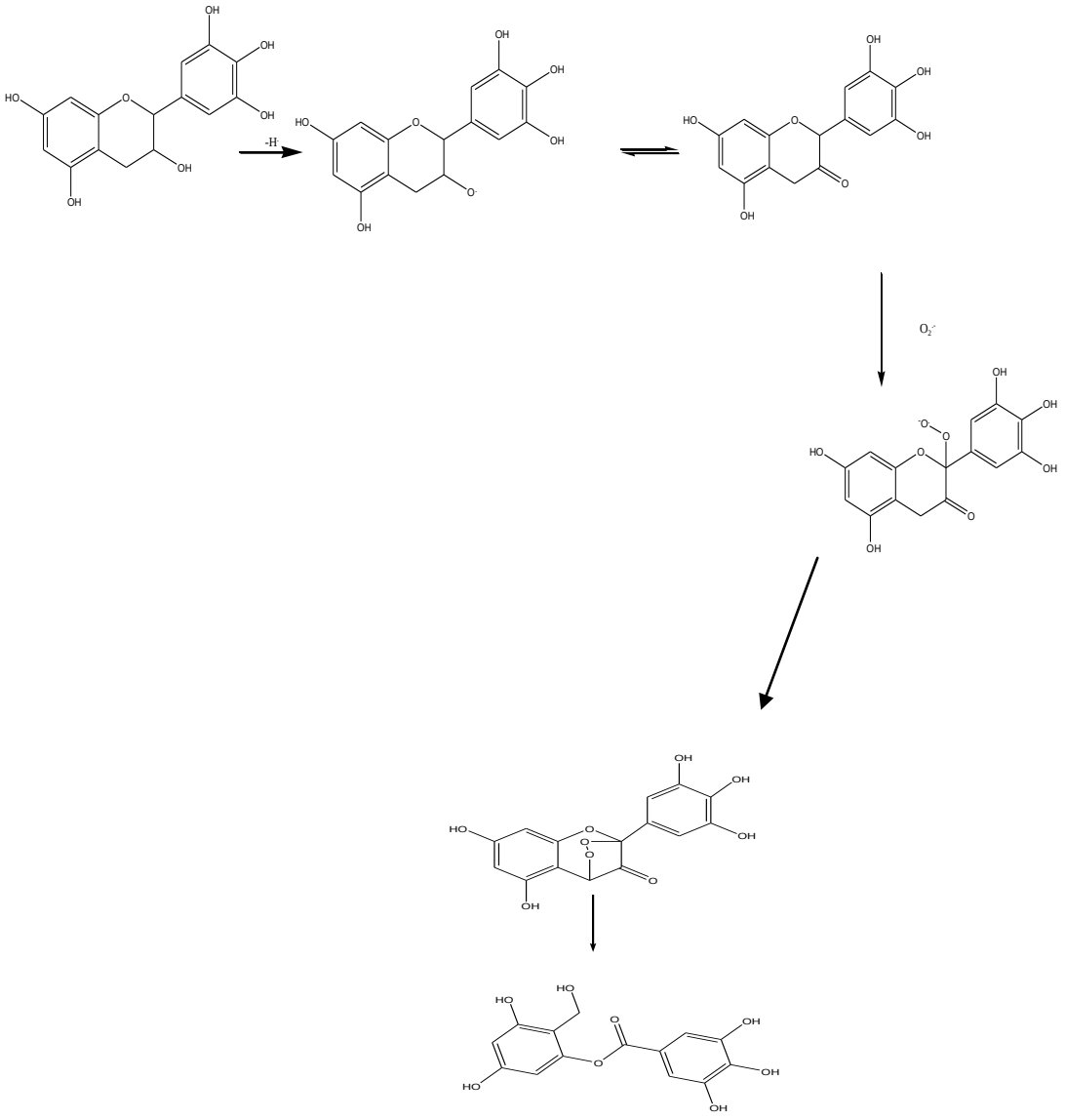


Fig 2.6; Hemolytic cleavage of the O-O bond

Green tea catechins also exhibit antioxidant activity through inhibiting prooxidant enzymes and inducing antioxidant enzymes (Velayutham *et al.*, 2008). Amongst the tea catechins, EGCG is the most potent one and has also been found to outperform vitamin C and β carotene 10 time in scavenging the allyl peroxyl radical (Vilma *et al.*, 2011). Antioxidant activity of EGCG is about

25-100 times more potent than vitamin C and E and is the single most studied catechins in relation to health contributing potential (Cabrera *et al*., 2006). The high antioxidant activity of green tea makes it more beneficial in protecting the body from oxidative damage due to free radical.

2.3.2 Obesity

Green tea has recently become the latest weapon in fighting over weight condition (Anand *et al.*, 2012). It appears to fight obesity by increasing the rate of caloric burning, reducing body fat levels and preventing excess weight gain. The consumption of green tea extract is associated with a statistically significant reduction in total and low density lipoprotein cholesterol level (Kim *et al.*, 2011). Green tea catechins enhance exercise induced abdominal fat loss in over weight and obese adults (Maki *et al.*, 2009). Green tea catechins and EGCG, have been shown to reduce adipocytes differentiation and proliferation, lipogenesis i.e., birth of new fat cells; fat mass, body weight, fat oxidation, plasma levels of triglyceride, free fatty acids, cholesterol, glucose, insulin, and leptin and increased beta-oxidation and thermogenesis (Wolfram *et al.*, 2006). It has also been reported that green tea sends glucose to muscle, where it is used for energy rather than to fat tissue, where it is stored (Murase and Harmizu, 2006). Although few epidemiological and clinical studies have shown the health benefits of EGCG on obesity and diabetes, the mechanisms of its actions are emerging based on various laboratory data. These mechanisms may be related to certain pathways, such as through the modulations of energy balance, endocrine systems, food intake, lipid and carbohydrate metabolism (Yang *et al.*, 2001).

2.3.3 Good vision

Green tea catechins are among a number of antioxidants such as vitamin C, vitamin E, lutein, and zeaxanthin thought capable of protecting the eye (Chu *et al.*, 2010). A study conducted at Chosun University College of Medicine in Korea discovered that the green tea antioxidant EGCG can protect human retina against UV damage. They concluded that the administration of EGCG increased the cell count and that the cell activity after UV irradiation in cultured human retinal pigment epithelial cell improved (Yang *et al.*, 2007). This suggests that EGCG provided protection against UV damage in cultured human retinal pigmented epithelial cells. In an animal trial it was found that green tea may protect against the formation of cataracts (Gupta *et al.*, 2009). The results suggest that green tea possesses significant anticataract potential and acts primarily by preserving the antioxidant defense system. Green tea may protect age related macular degeneration and glaucoma (Zhang and Osborne, 2006).

2.3.4 Oral health

Among oral disease like dental caries, periodontal disease, and tooth loss dental caries is a multi factorial infectious disease in which nutrition, microbiological infection, and host response play important roles. *Streptococcus mutans* is mainly responsible for causing dental caries. Green tea has proved to have anti- *Streptococcus mutans* activity (Naderi *et al.*, 2011). It has also been found that routine intake of green tea may also help in fighting against these oral diseases. It promotes healthy teeth and gums. Apart from polyphenols contents, green tea is a natural source of fluoride and an effective vehicle for fluoride delivery to the cavity. The mean fluoride concentration in green tea is approximately 2.1 ppm, which lies within the acceptable daily intake (Simpson *et al.*, 2001). This fluoride content may have a beneficial impact on caries and may carry out a wide range of biological activities including prevention of tooth loss and oral

cancer (Lee *et al.*, 2004; Okamoto *et al.*, 2004). In consequence, green tea has been considered as functional food for oral health and is widely used in toothpaste formulation (Pasture 2005). The greater the concentration of catechins in green tea, the better the health benefits(Pasture 2005). As such, consumption of green tea in comparison to other beverages may be widely recommended (Venkateswara *et al.*, 2011).

2.3.5 Antiviral potential

In a preliminary study, AIDS/HIV prevention research has shown that green tea antioxidant catechins especially EGCG have anti-HIV activity in each step of the HIV life cycle (Yamaguchi *et al.*, 2002). Green tea slows Reverse Transcriptase (HIV-RT) and inhibits replication of two strains of HIV (Fassina *et al.*, 2002). Also *in vitro* studies have revealed that adenovirus infection is inhibited by green tea catechins (Weber *et al.*, 2003). Green tea also suppresses the adenovirus, Epstein-Barr, herpes simplex, and influenza viruses (Matsumoto *et al.*, 2011). EGCG binds to the hemagglutinin of the influenza virus; blocking it from attaching to (and infecting) target receptor cells. EGCG also alters the virus cell membrane, which further inhibits its ability to infect other cells (Naderi *et al.*, 2011); green tea catechins and theanine are effective in preventing influenza (Matsumoto *et al.*, 2011).

2.3.6 Synergism with antibiotics

Antibacterial treatment of many infections is hindered due to resistance developed by the pathogenic micro-organism against several antibiotics. Smeeton (2011), reported green tea penicillin G interraction. Catechins, in green tea have antimicrobial activity. The synergistic antimicrobial activity with antibiotics could be useful in fighting emerging drug resistance problem especially among entero pathogens (Archana and Jayanthi, 2011). Green tea extract in

combination with probiotics significantly reduced the viable count of *Staphylococcus aureus* and *Streptococcus pyogens* (Ping *et al.*, 2008). Also synergy between green tea extract and levofloxacin against entero heamorrhagic *Escherichia coli* has been reported (Isogai *et al.*, 2001).

2.3.7 Anticarcinogenic activity

Green tea polyphenols may have cancer preventing potential, especially in the case of solar UV-induced cancer (Ahmad and Mukhtar, 2001). Zhang *et al.* (2012) reported that, a study with 10 women with advance breast cancer undergoing radiation therapy for the treatment, half were given radiation therapy plus EGCG three times daily. The results showed that EGCG may be an effective strategy to inhibit the spread of invasive breast cancer cells. In another study, the extracts of green tea and green tea polyphenols have exhibited inhibitory effects against the formation and development of tumors at different organ site in animals (Zhang *et al.*, 2012). These include animal models skin, lung, oral cavity, oesophagus, stomach, intestine, colon, liver, pancreas, bladder, mammary gland, cancer land, and prostate cancers (Yang and Wang, 2010). EGCG can inhibit tumorgenesis during the initiation, promotion and progression stages in animal models of carcinogenesis (Lambert and Elias, 2010). Green tea consumption has also been linked to the prevention of many types of cancer, including lung,colon, esophagus, mouth, stomach, small intestine, kidney, pancreas, and mammary glands (Koo and Cho, 2004).

2.3.8 Role in skin damages

Green tea is effective in the area of skin care, particularly in alleviating the symptoms of acne and eczema. When used in a combination with sunscreen, green tea enhances sun protection. Due to the presence of antioxidants, green tea is also effective in slowing down the process of

aging (Anand *et al.*, 2012). Green tea extract has proved to be effective for the treatment of patients who have suffered from skin damage following radiotherapy for cancer (Pajonk *et al.*, 2006). The *in vitro* and *in vivo* animal and human studies have suggested that green tea polyphenols are photo protective in nature, and can be used as pharmacological agents for the prevention of solar light-induced skin disorders including photo aging, melanoma and non-melanoma skin cancers (Wu and Wei, 2002).

2.4 Contamination of Herbal Products with Heavy Metals

Herbal plants represent an important class of various traditional medicine systems and, in recent years, they are increasingly used in the primary health care intervention in both developed and developing countries (WHO, 2002). According to World Health Organization estimates, nearly 70–80 % of the world population still primarily relies on nonconventional medications, mostly derived from herbal plants (Sahoo *et al.*, 2010; WHO, 2002). The widespread perception of the relative safety of herbal drugs coupled with the economic hardship experienced by most Nigerians has resulted in an increase in the use of herbal medicines. In an ethnographic survey of the use of herbal medicines in Nigeria, 39 % of the people used herbal medicines for cough, 62 % for measles, 38 % for ear infections and 24 % used topical creams and soaps for skin infections (Oyejide and Oke, 1995). In Nigeria, the first line of treatment for 60 % of children with high fever resulting from malaria is the use of herbal medicine at home (WHO, 2003). With such high usage of herbal medicines, more people in Nigeria may be exposed to the adverse health effect associated with them (Musa and Hamza, 2009). Despite the increasing interest in the therapeutic benefits of herbal products, there has been an increasing concern over the safety and toxicity of natural herbs and formulations available on the market(Musa and Hamza, 2009).

There has been a large volume of reports on incidences of toxicity and adverse effects linked to the use of herbal plants and their formulations in different parts of the world (Ernst, 2002).The toxicity of herbal plants may be related to contaminants such as pesticides, microbes, heavy metals, chemical toxins, and adulterants (Saad *et al.,* 2006); Garba *et al.* (2010) reported that lead (Pb) was detected above the WHO limit (0.01 mg/g) in all the herbal samples they analyzed in Makarfi, Nigeria, while, Rania *et al.* (2015) reported varied metals concentrations in herbal samples ranging from 0.1–1.11 ppm for cadmium, 1.0–23.52 ppm for lead, 1.44–156.24 ppm for copper, 12.65–146.67 ppm for zinc, and 81.25–1101.22 ppm for iron. The findings of the study suggest that most of the analyzed herbs contained unsafe levels of heavy metals that exceeded the FAO/WHO permissible limits. Plants are the main link in the transfer of heavy metals from the contaminated soil to humans. Heavy metals have a tendency to accumulate in the food chain. Heavy metals have low excretion rates through the kidney which could result in damaging effects on humans even at very low concentrations. Metals such as Zn, Cu and Fe are essential nutrients; they are important for the physiological and biological functions of the human body at trace concentrations. However, at higher concentration these essential metals can be harmful. On the other hand metals like lead and cadmium are toxic and have no physiological role in the body. In general, the geography, the geochemical soil characteristics, contaminants in the soil, water, air, and growth, transport, and storage conditions can significantly affect the properties and the quality of the herbal plants and their formulations (Saad *et al.,* 2006).

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Materials

3.1.1 Samples

EGCG standard powder from Sigma Aldrich

Twelve Green Tea Brands

3.1.2 Glass wares and accessories

2×100ml measuring cylinders (Pyrex England)

2×100ml and 2×250ml conical flasks (Pyrex England)

2×25ml and 2×50ml beakers (Pyrex England)

6×10ml test tubes (Pyrex England)

6×15ml centrifuge tubes (Pyrex England)

2×25ml, 2×50ml and 2×100ml volumetric flasks (Pyrex England)

20×Filter paper

3.1.3 Equipment

Electronic weighing balance (Metlers Pr 63 Switzerland)

Centrifugation machine (Gallen Kamp, Philip Harries Ltd, England)

Gallen Kamp Hot Air Oven (Philip Harries Ltd, England)

Thermometer (Mc Donald Scientific International, England)

UV Spectrophotometer (Helios Zeta, Model 164617)

Stop watch (Smith England clock system)

Melting point apparatus

Infra-red spectrophotometer (Cary 630 FTIR)

Atomic absorption spectrophotometer (Model 6800 Shimadzu Japan)

Capillary tube

Hot plate

Whatman filter paper No.42

Magnetic stirrer

3.1.4 Reagents

Analytical grade solvents were used

Nitric acid

Deionized water

Chloroform

Perchloric acid

3.2 Method

3.2.1 Collection of samples

Twelve slected brands of green tea samples were purchased from Sabon Gari Market Zaria, Nigeria. The samples were coded and parameters like, address of manufacturers, batch number, manufacturing and expiry dates were recorded.

3.2.2 Quality control of standard EGCG powder and various brands of green tea samples

Quality control method recommended by WHO (1998a) on herbal products was adopted in study;

3.2.2.1 Identification of standard powder

The standard powder (EGCG), was purchased from Sigma Aldrich Germany and identified using melting point and Infra-red (IR) spectroscopy as follows:-

Melting point

This was determined by filling a capillary tube with standard powder (EGCG), through careful tapping on a hard surface to a height of about 3 mm. The capillary tube containing the standard was then placed into the melting point apparatus and the machine was set at high temperature for rapid determination of melting point. Melting point process was observed through the magnifying lens. The obtained melting point range was compared with the standard melting point range.

Infra-red (I.R) spectroscopy

Standard powder (EGCG), was mixed with Nujol for FTIR analysis. The finger print region of the spectrum was then compared with that of the standard (EGCG) spectrum in the reported EGCG certified reference material of the powder.

3.2.2.2 Uniformity of weight

Twenty units (tea bags) from each green tea brand were randomly sampled and weighed individually and the percent mean deviation was calculated as shown in the result.

3.2.2.3 Determination of moisture content (M C)

A quantity of the tea sample (1 g) was placed in an oven at 103 ^{O}C for one hour and later cooled in a desiccator to prevent absorption of atmospheric moisture. This process was repeated until constant weight was obtained. Moisture content was calculated using the formula:-

$$\text{M C (\%)} = \frac{\text{Weight of air-dried sample} - \text{Weight of oven dried sample}}{\text{Weight of air-dried sample}} \times 100$$

3.2.2.4 Total ash value

Two gram of the sample was accurately weighed, in a previously ignited and tarred crucible. The sample was then Spread in an even layer and ignited by gradually increasing the heat, until it turned white, indicating the absence of carbon. The residue was cooled in a desiccator and weighed. The total ash value was calculated by using the relationship:-

$$\text{Ash value} = \frac{\text{Initial Weight} - \text{Final Weight}}{\text{Initial Weight}} \times 100$$

3.2.2.5 Water soluble ash

The total ash obtained was boiled with 25 ml of distilled water for 5 minutes. The insoluble matter was collected on an ash less filter –paper, washed with hot water and ignited to constant weight at low temperature. The water soluble ash was obtained from the difference between the

weight of the insoluble matter and the weight of total ash. The percentage of water soluble ash was calculated with reference to the air dried samples.

3.2.2.6 Acid Insoluble ash

The total ash obtained was boiled with 25 ml of dilute hydrochloric acid for 5 minutes. The insoluble matter was collected on tarred crucible, washed with hot acidulated water, ignited, cooled and weighed. The percentage acid insoluble ash was calculated with reference to the air dried samples.

3.2.3 Extraction method

Five green tea bags (10 g) were macerated using methanol for 3 days. It was filtered to afford a crude methanol extract.

3.2.4 Preliminary phytochemical screening

Preliminary phytochemical tests were carried out on methanol extract according to standard procedures as follows:

3.2.4.1 Test for carbohydrates (Molisch's test)

Small portion of the extract was dissolved in distilled water in a test tube, 3 drops of Molisch's reagent was added followed by concentrated sulphuric acid. The formation of a reddish colored ring at the interface indicates the presence of carbohydrates (Trease and Evans, 1996).

3.2.4.2 Test for saponins (Frothing test)

To a small portion of the extract in test tube, 10 ml of distilled water was added and then shaken continuously for 30 seconds. The solution was allowed to stand for 5 minutes; the formation of a persistent froth indicates the presence of saponins (Trease and Evans, 1996).

3.2.4.3 Test for flavonoids

- Sodium hydroxide

2 drops of 10 % Sodium hydroxide was added to the solution of extract, yellow coloration indicates the presence of flavonoids (Trease and Evans, 1996).

- Ferric chloride test

An amount of 2 to 3 drops of Ferric chloride solution were added to the solution of the crude extract. The color was observed (Trease and Evans, 1996).

3.2.4.4 Test for tannins

- Lead sub-acetate test

  To a small portion of the extract, 4 drops of acetate solution was added, the formation of a cream colored precipitate indicates presence of tannins (Trease and Evans, 1996).

3.2.4.5 Test for terpenoids

- Salkowki's Test

A small portion of the extract was dissolved in 2 ml of chloroform, 3 drops of concentrated sulphuric acid (Salkowki's reagent) was added at the side of the test tube. A red brown coloration at the interface indicates the presence of terpenoids (Trease and Evans, 1996).

3.2.4.6 Test for alkaloids

- Dragendoff's Test

The extract (0.2 g) was dissolved in 2 ml of 1 % aqueous hydrochloric acid with continuous stirring in a water bath. The mixture was filtered and few drops of Dragendoff's reagent was added, rose red precipitate indicates the presence of alkaloids (Trease and Evans, 1996).

- Mayer's Test

To 2 ml acidic solution of the extract in a test tube, few drops of Mayer's reagent were added, a cream precipitate indicates the presence of alkaloids (Trease and Evans, 1996).

3.2.4.7 Test for Cardiac Glycosides (Keller-Kiliani Test)

A small portion of the extract was dissolved in 1 ml glacial acetic acid containing traces of ferric chloride solution. The solution was then transferred into a dry test tube to which an equal volume of sulphuric acid was added, a brown ring obtained at the interface indicates the presence of a deoxy sugar (Trease and Evans, 1996).

3.2.5 Method development and determination of EGCG in green tea using UV spectrophotometer

3.2.5.1 Preparation of stock solution

A quantity of EGCG standard powder (10 mg) was dissolved in 30 ml of distilled water contained in 100 ml volumetric flask and was made up to mark with the same solvent to produce a 100 µg/ml solution.

3.2.5.2 Determination of wavelength of maximum absorption

1 ml was withdrawn from the stock solution into a 10 ml volumetric flask and made up to mark with distilled water to produce 10 µg/ml solution. This was then scanned in the spectrophotometer through a wavelengths range 240 – 400 nm to obtain the wavelength of maximum absorption (274 nm).

3.2.5.3 Sample preparation and analysis of EGCG

A quantity (50 mg) of powdered leaves of green tea was added to 40 ml of distilled water at room temperature and stirrered with magnetic stirrer for 1h and then filtered. The tea infusion was washed with 40 ml of chloroform in a separator funnel to remove caffeine, pigments and

other non-polar impurities. The washing with chloroform was repeated four times, the absorbance of the aqueous phase obtained was taken at maximum wavelength (274 nm).

3.2.5.4 Preparation of calibration curve

From the stock solution, different aliquots in the range 1.0 ml to 6.7 ml were transferred into series of 10 ml volumetric flask and the volume made up to the mark with distilled water to obtain solution of concentrations 2.0 to 10.0 µg/ml. 5 ml of each concentration was treated as described in the method and their respective absorbance's were determined at 274 nm against the reagent blank. A plot of absorbance against the corresponding concentration gave the calibration curve.

3.2.5.5 Method Validation

The method was validated with reference to linearity, precision, accuracy, percentage recovery, limit of detection and quantitative limit (ICH, 1996).

Linearity

This was established by least square method using Microsoft excel 2010.

Precision

The precision of this method was checked by replicate analysis of a standard solution (10 µg/ml). This was done by taking six replicate absorbance of the solution. The percentage coefficient of variation (% CV) for the replicate analysis of each determination was taken as a measure of precision.

$\% CV = S/X \times 100$……..equation 1

Where S is the standard deviation and X is the mean.

It consists of;

Intra-day precision (within day precision):

This essentially consists of determining six replicate absorbance of a 10 µg/ml solution for six times at one hour intervals.

Intra-day precision (between day precision):

This essentially consists of determining six replicate absorbance of a 10 µg/ml daily for three consecutive days.

Accuracy

The accuracy of this method was checked by standard addition method. Solution of EGCG (10 µg/ml) were prepared in five separate 10 ml volumetric flask containing 4 ml of 5 µg/ml of EGCG, by adding 0.3 ml of the EGCG stock solution to each of the flasks and making up to the mark with distilled water. Five replicate analysis of each of the resulting solutions was done for this determination (Musa *et al.*, 2014). Accuracy is expressed as percentage relative error (% Er).

$\% E_r = X-\mu/\mu \times 100$……..equation 2

Where X is the mean and µ is the expected value.

Recovery

The recovery of the method was determined by weighing 10 mg of green tea powder leaves into four labeled 10 ml volumetric flask each containing 4 ml of distilled water and labeled flask 1, 2, 3 and 4 respectively. Flask 1 was left unspiked while 2, 3, and 4 were spiked with 1, 2, and 3 mg of EGCG standard powder respectively and then mixed thoroughly. The content of the entire flask were made up to mark with distilled water and stirred for 1 hour using magnetic stirrer. Each mixture was defatted using chloroform as described in the method and filtered. The

absorbance of each filtered infusion was taken at the wavelength as described in the method. The absorbance of the unspiked flask (flask 1) was subtracted from absorbances obtained from flasks 2, 3 and 4. The concentration of EGCG recovered from each flask was extrapolated from the calibration curve. Thus, the percentage recovery was computed using the formula:

% Recovery = measured concentration/added concentration × 100……..equation 3

Detection limit

The detection limit (DL) was determined by studying the calibration curve using samples containing the EGCG in the range of DL. The standard deviation of y-intercepts of the regression line was use. DL is expressed as:

DL= 3.3 σ/S……..equation 4

Where σ is the standard deviation of y-intercepts of the regression line and S is the slope of the calibration curve.

Quantitation limit

The quantitation limit (QL) was determined using the expression:

QL = 10 σ/S……..equation 5

Where σ is the standard deviation of y-intercepts of the regression line and S is the slope of the calibration curve.

3.2.6 Determination of heavy metal content in Green Tea Sample using Atomic Absorption Spectroscopy

Nitric-Perchloric acid digestion was performed, following the procedure reported by the AOAC (1990). A quantity of green tea sample (1 g) was placed in a 250 ml crucible and 10 ml of

concentrated HNO_3 was added. The mixture was boiled gently for 30 minutes to oxidize all easily oxidizing matter. After cooling, 5 ml of 70 % $HClO_4$was added and the mixture was boiled gently until dense white fumes appeared. After cooling, 20 ml of distilled water was added and the mixture was boiled further to release any fumes. The solution was cooled, further filtered through Whatman No. 42 filter paper and transferred quantitatively to 25 ml volumetric flask by adding distilled water. The resulting solution was analyzed for Lead, Cadmium, Copper, Iron and Zinc using AAS.

3.2.7 Statistical Analysis

Data were presented in mean ± SEM. One way ANOVA was used to compare differences in mean and significance was set at $p < 0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Quality control of standard EGCG powder and various brands of green tea samples

4.1.1 Identification Test for EGCG Standard Powder

The melting point of EGCG standard powder was found to be 218 - 218.1 °C, while the infrared (IR) spectrum is presented in Figure 4.1a vis-à-vis the reference spectrum of EGCG (Figure 4.1b).

The IR spectrum of EGCG standard powder (Figure 4.1a) revealed the presence of characteristic peaks of EGCG as shown below;

Wavenumber (CM^{-1})	Spectral Assignments

3487.54 – 3365.57	O-H group attached to the aromatic ring
1683.71 – 1606.65	Strong for C=O group that linked the trihydroxybenzoate group and chromone group
1451.24	C-H group present in the chromone ring
1332.46	O-C=O group
1218.57	O-C=O group
1054.54	C-O-C group linked the chromone ring and trihydroxy benzoate ring
794.04	the three neighbouring aromatic C-H group in chromone ring

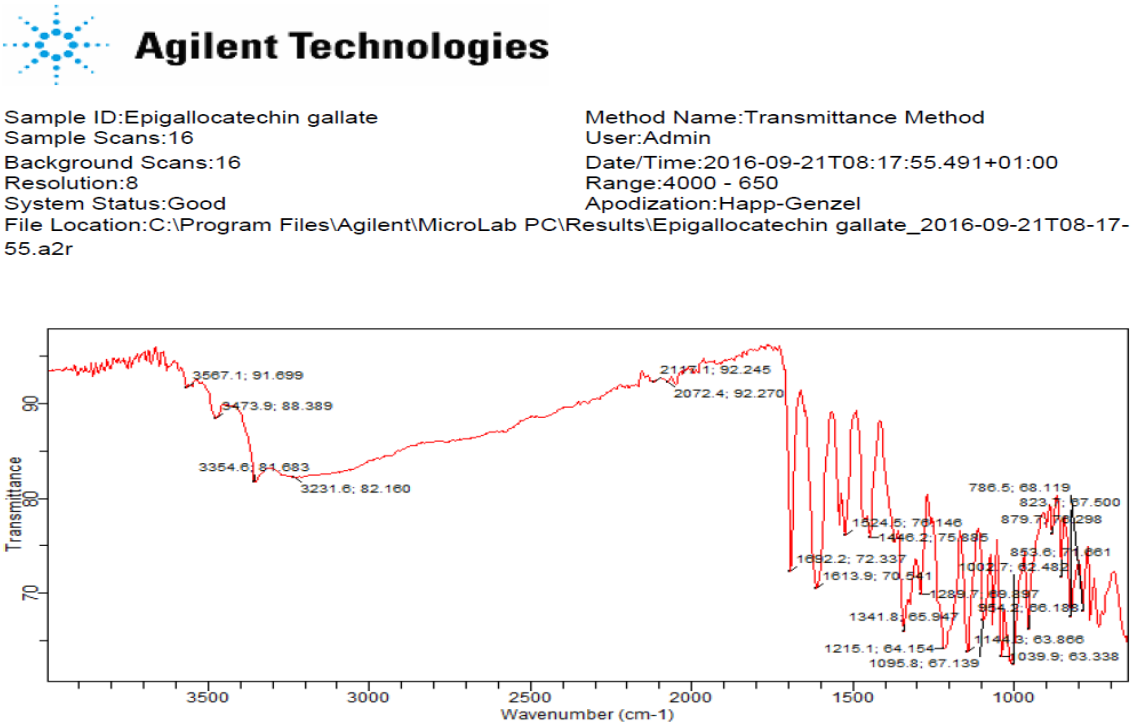


Figure 4.1a; IR spectrum of EGCG standard powder

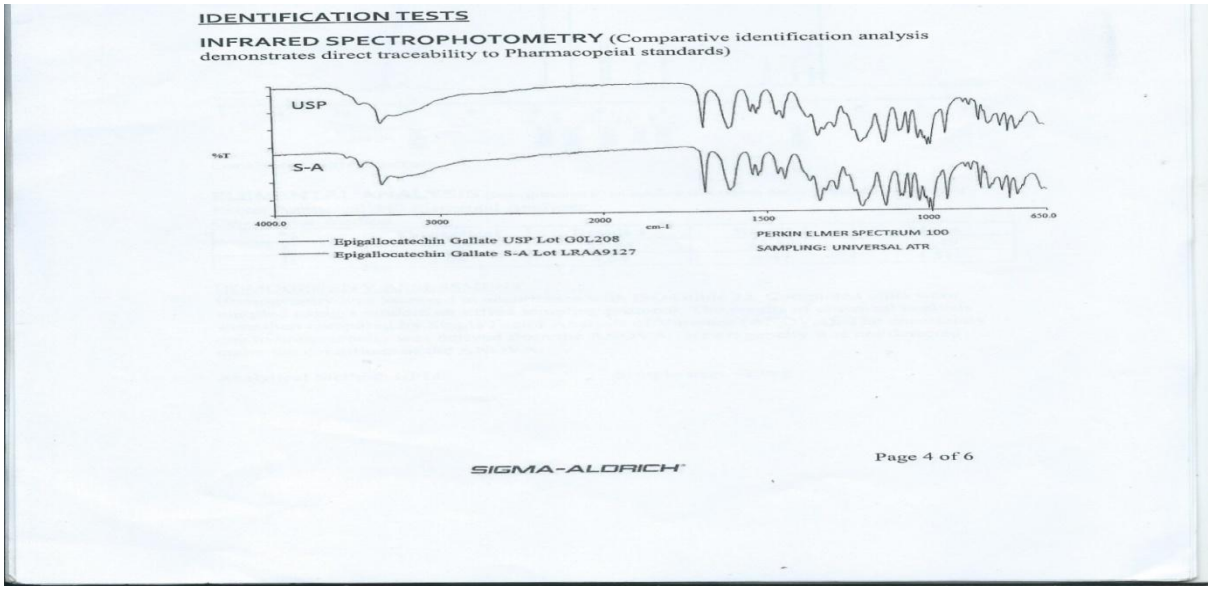


Figure 4.1b; Reference IR spectrum of EGCG

4.1.2 Uniformity of weight

The weight variation studies of various green tea brands and the percentage mean deviation are shown in Table 4.1

Table 4.1: Uniformity of weight studies of various green tea brands

S/No.	Samples	Mean weight (g) ±SEM	Mean deviation (%)
1	G-1	2.25 ± 0.04	1.40
2	G-2	2.28 ± 0.04	1.50
3	G-3	2.19± 0.03	1.60
4	G-4	2.30± 0.04	1.50
5	G-5	2.15± 0.03	1.30

6	G-6	2.11± 0.05	1.80
7	G-7	2.18± 0.03	1.30
8	G-8	2.20± 0.06	2.40
9	G-9	2.17± 0.03	1.20
10	G-10	2.16± 0.05	1.90
11	G-11	2.25± 0.04	1.40
12	G-12	2.18± 0.03	1.30

≥ 7.5 % (BP, 2009)

4.1.3 Moisture content

The results of moisture content for the various green tea brands are presented in Table 4.2

Table 4.2: Moisture content of various green tea brands

S/No.	Samples	Mean % ± SEM
1	G-1	8.38 ± 0.08
2	G-2	11.81 ± 0.09*
3	G-3	1.62 ± 0.00
4	G-4	9.92 ± 0.08*

5	G-5	9.07 ± 0.06*
6	G-6	13.20 ± 0.12*
7	G-7	8.84 ± 0.00
8	G-8	14.35 ± 0.33*
9	G-9	9.64 ± 0.03*
10	G-10	14.00 ± 0.00*
11	G-11	8.30± 0.08
12	G-12	9.07± 0.06*

*Significantly higher ($p < 0.05$) than the NAFDAC limit (8 %)

4.1.4 Total ash value

Total ash value results are shown in Table 4.3

Table 4.3: Total ash of various green tea brands

S/No.	Samples	Mean ± SEM %
1	G-1	6.50 ± 0.65
2	G-2	5.00 ± 0.35
3	G-3	11.75 ± 0.43*

4	G-4	6.88 ± 0.24
5	G-5	7.50 ± 0.35
6	G-6	7.75 ± 0.48
7	G-7	5.88 ± 0.43
8	G-8	12.50 ± 0.41*
9	G-9	11.30 ± 0.43*
10	G-10	7.38 ± 0.37
11	G-11	5.00± 0.35
12	G-12	12.50 ±0.41*

*Significantly different ($p < 0.05$) from the IARC-WHO limit (4 – 8 %)

4.1.5 Water soluble ash

Water soluble ash results are presented in Table 4.4

Table 4.4: Water soluble ash of various green tea brands

S/No.	Samples	Mean % ± SEM
1	G-1	2.25 ± 0.25

2	G-2	1.75 ± 0.25
3	G-3	4.50 ± 1.00
4	G-4	2.00 ± 0.50
5	G-5	2.50 ± 0.00
6	G-6	3.25 ± 0.75
7	G-7	2.75 ± 0.25
8	G-8	4.50 ± 0.50
9	G-9	6.00 ± 0.50*
10	G-10	4.00 ± 0.00
11	G-11	1.75± 0.25
12	G-12	2.00± 0.50

*Significantly higher ($p < 0.05$) than the IARC-WHO (5 %)

4.1.6 Acid insoluble ash

The results of acid insoluble are presented in Table 4.5

Table 4.5 Acid insoluble ash of various green tea brands

S/No.	Samples	Mean % ± SEM

1	G-1	1.50 ± 0.00
2	G-2	1.25 ± 0.25
3	G-3	4.25 ± 0.25*
4	G-4	2.00 ± 0.00*
5	G-5	2.25 ± 0.25*
6	G-6	1.50 ± 0.00
7	G-7	2.50 ± 0.50*
8	G-8	2.50 ± 0.50*
9	G-9	3.75 ± 1.25*
10	G-10	3.25 ± 0.25*
11	G-11	1.00± 0.00
12	G-12	1.50± 0.00

*Significantly higher ($p < 0.05$) than the IARC-WHO limit (1 %)

4.2 Phytochemical Screening of Methanolic Extracts of the Green Tea Brands

The results of the phytochemical screening after treating each green tea brand with respective reagents which result in color change are presented in Table 4.6

Table 4.6: Phytochemical screening of methanolic extract of the green tea brands

S/No.	Samples	Terpenoids	Cardiac Glycosides	Saponins	Tannins	Flavonoids	Alkaloids
1	G-1	+	+	+	+	+	+
2	G-2	+	+	+	+	+	+
3	G-3	+	+	+	+	+	+
4	G-4	+	+	+	+	+	+
5	G-5	+	+	+	+	+	+
6	G-6	+	+	+	+	+	+
7	G-7	+	+	+	+	+	+
8	G-8	+	+	+	+	+	+
9	G-9	+	+	+	+	+	+
10	G-10	+	+	+	+	+	+
11	G-11	+	+	+	+	+	+
12	G-12	+	+	+	+	+	+

+ = present

4.3 Method Development

4.3.1 Wavelength of maximum absorption

The wavelength of maximum absorption for the determination of EGCG using the proposed method was found to be 274 nm as shown below in Figure 4.2.

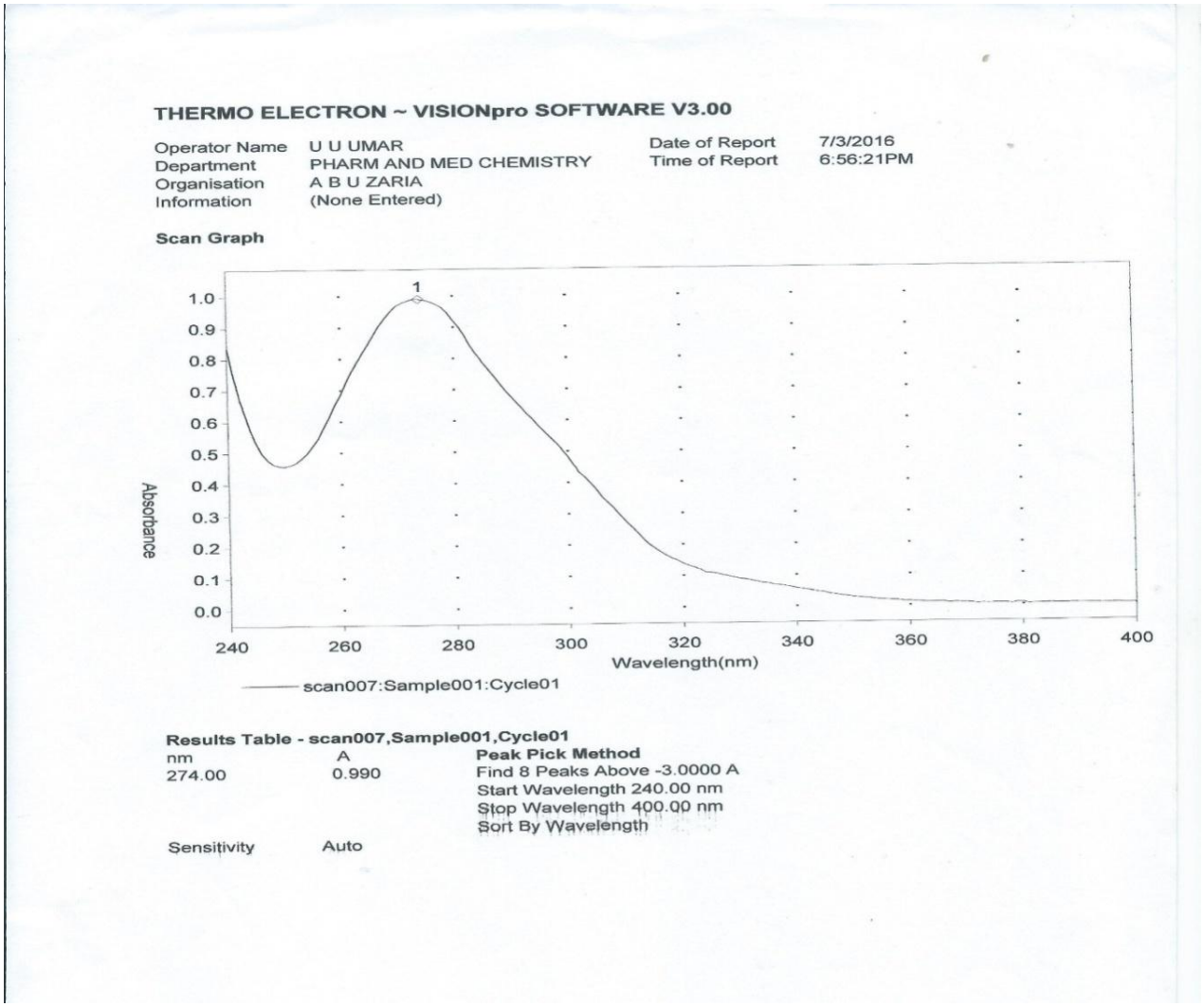


Figure 4.2; UV spectrum of EGCG in distilled water

4.3.2 Calibration Curve

The calibration curve (Figure 4.3) for the quantitative determination of EGCG using the developed method obeyed the Beer-Lambert's law in the range of 2 – 10 µg/ml. the linear relationship between absorbance (A) and concentration (C in µg/ml) gives the regression

equation A = Cy + x, and the coefficients of correlation (r) are shown in Table 4.7 and the calibration curve is presented in Figure 4.3.

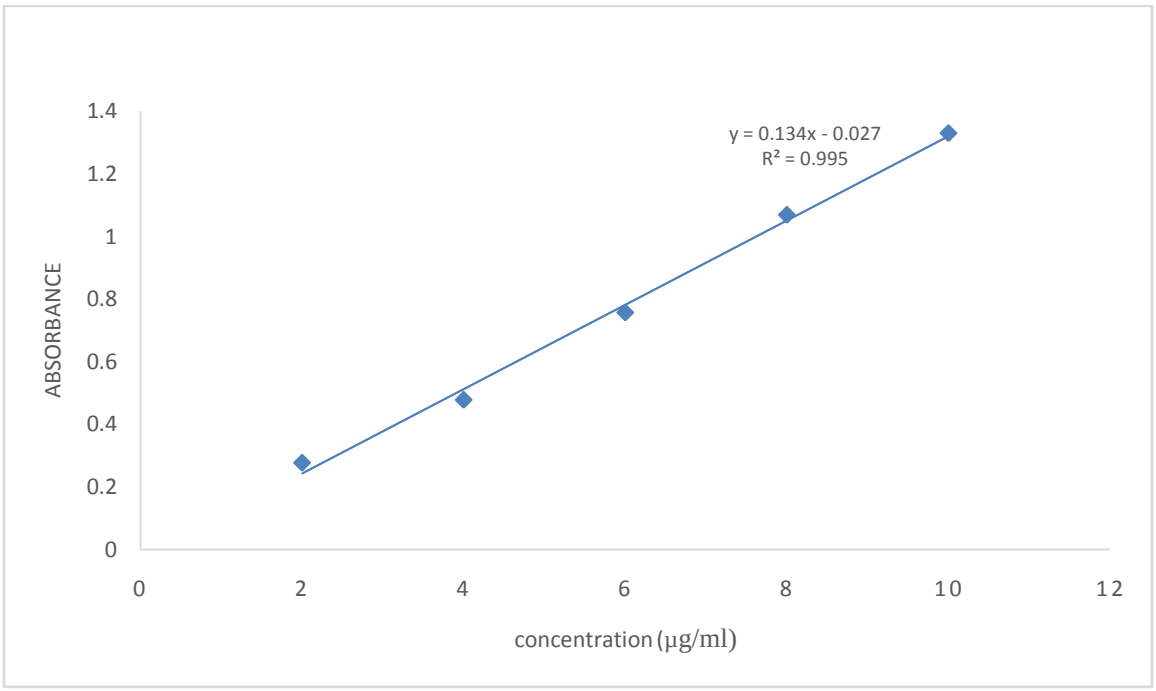


Figure 4.3; Calibration curve of EGCG showing linearity in Conc. of 2 – 10 µg/ml

Table 4.7: Regression analysis of the calibration curve for the proposed method

Parameter	Value
Absorbance maximum (nm)	274

Linearity range (µg/ml)	2-10
Linear regression coefficient (r)	0.998
Regression equation	A = Cy + x
Slope (y)	0.1349
Intercept (x)	0.0279

A = absorbance, C = concentration

4.3.3 Validation of method

The accuracy, precision, percentage recovery, detection limit and quantitation limit for the developed method are shown in Table 4.8.

Table 4.8: Validation parameters for the proposed method

Validation parameter	Value obtained
Precision : -	
Intra-day (% *CV*)	1.7
Inter-day (%*CV*)	2.88
Accuracy (% E_r)	1.99
Average % recovery	95.77
Limit of detection (µg/ml)	0.68
Limit of quantitation (µg/ml)	2.07

CV = Coefficient of variation, E_r = Relative error

4.4 Quantification of EGCG in the Various Brands of Green Tea

The concentration (µg/ml) of EGCG in each green tea brand using the developed method is shown in Table 4.9.

Table 4.9: Concentrations of EGCG (mg/g) in the green tea brands

S/No.	Samples	Mean % ± SEM
1	G-1	0.82 ± 0.00*
2	G-2	0.81 ± 0.01*
3	G-3	0.80 ± 0.00*
4	G-4	0.76 ± 0.00
5	G-5	0.75 ± 0.00
6	G-6	0.78 ± 0.00
7	G-7	0.78 ± 0.00
8	G-8	0.77 ± 0.00
9	G-9	0.76 ± 0.00
10	G-10	0.79 ± 0.01
11	G-11	0.75 ± 0.00
12	G-12	0.77 ± 0.00

*Significantly higher ($p < 0.05$) levels of EGCG

4.5 Elementals Analysis

The concentrations of the essential trace metals (Fe, Zn and Cu) and toxic metals (Pb and Cd) analyzed in the various tea brands are shown in Table 4.10.

Table 4.10: Metals concentration (mg/g) in the green tea brands

S/No	Samples	Metal Concentration (mg/g)				
		Fe	Zn	Cu	Pb	Cd
1	G-1	44.05 ± 1.28	BLD	BLD	BLD	0.10 ± 0.01*
2	G-2	69.26 ± 0.13	BLD	BLD	5.41 ± 0.01*	BLD
3	G-3	83.52 ± 0.29	BLD	BLD	2.71 ± 0.00*	0.04 ± 0.01*
4	G-4	36.27 ± 0.14	BLD	0.09 ± 0.00	6.77 ± 0.00*	0.08 ± 0.00*
5	G-5	67.18 ± 0.02	BLD	0.02 ± 0.03	6.05 ± 0.00*	0.03 ± 0.01*
6	G-6	99.61 ± 0.01	BLD	0.59 ± 0.01	4.93 ± 0.00*	BLD
7	G-7	63.39 ± 0.00	BLD	BLD	1.99 ± 0.01*	0.08 ± 0.00*
8	G-8	164.102 ± 0.01	BLD	BLD	4.69 ± 0.00*	BLD
9	G-9	145.07 ± 0.01	1.26 ± 0.30*	BLD	8.76 ± 0.00*	0.11 ± 0.01*
10	G-10	40.31 ± 0.01	BLD	0.12 ± 0.01	6.77 ± 0.00*	BLD
11	G-11	44.86 ± 0.07	24.96 ± 0.31*	0.09 ± 0.00	6.05 ± 0.00*	BLD
12	G-12	32.20 ± 0.10	1.26 ± 0.30*	BLD	4.93 ± 0.00*	BLD

FAO/WHO limits: Fe (Not established yet), Zn (50 mg/g), Cu (Not established yet), Pb (10 mg/g) and Cd (0.3)
BLD: Below limit of detection

*Significantly lower ($p < 0.05$) than the FAO/WHO limit

CHAPTER FIVE

5.0 DISCUSSION

5.1 Quality control of various brands of green tea samples

5.1.1 Identification Test of Standard EGCG

The melting point ranged (218 – 218.1 ^{o} C) shows that, the standard EGCG powder is pure. Merck (1997) reported 218 ^{o} C. The IR spectrum of EGCG standard powder (Figure 4.1a) was found to be super impossible (at both finger print and functional group regions) with that of certified reference material (Figure 4.1b).

5.1.2 Uniformity of weight

The percentage mean deviations were within the range of 1.20—2.40 % (Table 4.1).Thus, all the brands passed the uniformity of weight test as none deviated from the mean weight by more than 7.5 % (BP, 2009). For single-dose powder or granule (≥ 300 mg) to pass the weight uniformity test; no more than two of the powders or granules should differ from the average weight by 7.5 %, and no powder or granule should differ by more than double that percentage (15 %). This indicates that good manufacturing practice was adhered to during size reduction and packaging stages of the various brands of green tea.

5.1.3 Moisture content

From the results shown in table 4.2, only Gold blend brand had less than 8 % moisture content while others had above 8 % (8.34 – 14.35 %); the moisture content in eight (8) of the samples were significantly higher ($p < 0.05$) than the NAFDAC limit 8 % (NAFDAC SOP, 2000). This

could be due to lack of proper drying of the green tea before packing and hence, this could give room for microorganism growth. Odumosu *et al.* (2015) reported moisture content range of 7 – 10 % in green tea brands marketed in Jos, Nigeria while Kalra *et al.* (2013) reported 5.5 % in green tea samples from Old Delhi, India. High moisture content aids microbial activities, oxidation – reduction processes and fungal growth. The variation in moisture may be attributed to the degree of drying, and/or type and nature of tea involved (Kumar *et al.,* 2005).

5.1.4 Ash value

Ash value which comprises of total ash, water soluble ash and acid-insoluble ash, all indicate the level of purity and/or adulteration of herbal products (WHO, 2011). The ash content of a sample is an indication of its mineral content; it does not necessarily indicate high quality except when there is a favorable balance of the essential minerals (Ahmed *et al.,* 1989).The total ash measures ash derived from plant tissue itself and residue of extraneous matter such as sand and soil which adhere to the plant surface. Water soluble ash gives the difference in weight after treatment of total ash with water while acid-insoluble ash measures the amount of silica present especially as sand and siliceous earth (WHO, 2011).

The standard requirement for total ash value in tea is 4 - 8 % (IARC WHO, 1991). As shown in Table 4.3 eight of the samples had total ash values (5 - 7.75 %) within the permissible limit, while four of the remaining samples had values (11 - 12.50 %) that were significantly ($p < 0.05$) above the limit. Odumosu *et al.* (2015) reported total ash value of 9.75-17.5 %; Kalra *et al.* (2013) reported 8.5 % for green teas.

The standard requirement for water soluble ash value in tea is 1 – 5 % (IARC WHO, 1991). As shown in Table 4.4 the water soluble ash in eleven of the green tea samples were within the

permissible limit (5 %) with percentage ranging from 1.75 - 4.5 % while one sample (G-9) was found to exceed the permissible limit (6.00 %) significantly ($p < 0.05$). Odumosu *et al.* (2015) had ranges between 10.8 - 14.8 %; Kalra *et al.* (2013) reported 3.2 % was reported.

The standard requirement for acid insoluble ash 1 % (IARC- WHO, 1991). As shown in Table 4.5 all the green tea samples except G-11 (1 %) exceeded the permissible limit with range between 1.25 - 4.25 %; seven (7) of these samples were significantly different ($p < 0.05$) at higher values. Odumosu *et al.* 2015, reported a range 2.8 - 8.5 % and Kalra *et al.* (2013), reported 1.25 %.

5.2 Preliminary phytochemical screening

Preliminary phytochemical screening of the various brands of green tea showed the presence of tannins, saponins, cardiac glycosides, terpenoids, flavonoids and alkaloids (Table 4.6). A similar study was conducted by Kalra *et al.* (2013) and the same phytoconstituents were reported. Tariq and Reyaz (2012) on the other hand reported the absence of terpenoids, saponins, and glycosides in the *Camellia sinesis* leaves they analyzed. This may be due to geographical location and growing condition, of the various samples selected (Pastore, 2005). These phytochemicals (Table 4.6) may occur in low concentrations and usually have a pharmacological effect; prevent cancer formation, prevent inflammation and work as antioxidant (Tariq and Reyaz, 2012). However, these effects have been used in naturopathy in form of medicinal herbs and teas especially green tea. Their presence in green tea has been associated with health benefits and this in turn has been regarded as an indicator of the quality (Odumosu *et al.*, 2015).

5.3 Method Development

The calibration curves for quantitative determination of EGCG in the developed method obeyed Beer- Lamberts law with correlation coefficient (r) of 0.998. This clearly showed the direct proportional relationship between the absorbance (A) and the respective concentration (C) used for these determination.

The proposed method was found to be precise as the % CV for intra-day and inter-day were found to be 1.7 and 2.88 % respectively. With good technique and reliable methodology the % CV should be < 15 % (Harvey, 2000).The precision of the proposed method are better than the 2.07 (intra-day) and 6.65 (inter-day) % reported by Saito *et al.* (2007), and the 2.8 (intra-day) and 5.9 (inter-day) % reported by Fernando and Preethi, (2016) both of which are HPLC methods for determination of catechins in green tea. This suggests that the precision of the method is satisfactory. The accuracy of the proposed method expressed as the measure of the percentage relative error (% E_r) was found to be 1.99 %, which is within the range of 1 – 5 % (Harvey, 2000). The average percentage recovery for the method was found to be 95.77 % which is within the acceptable limits 85 – 105 % (ICH, 1996) and is better than the 93.8 % reported by Roman *et al.* (2013). The calculated detected limit (DL) of 0.68 µg/ml and quantitative limit (QL) of 2.07 µg/ml for the proposed method are comparable to the 0.7 µg/ml (DL) and 2.2 µg/ml (QL) to the ones reported by Fernando and Preethi, (2016) in their HPLC methods for determination of catechins in green tea. This shows that the proposed method is quite sensitive for analysis of EGCG in green tea.

5.4 Quantification of EGCG

The concentration of EGCG in the tea brands ranged from 0.75 – 0.82 mg/g, with G-1 brand recording the highest and G-11/ G-5 the lowest (Table 4.9). The concentrations of EGCG in three of the brands [G-1 (0.82 mg/g), G-2 (0.81mg/g) and G-3 (0.80 mg/g)] were significantly higher ($p < 0.05$) than those observed in the other samples. The high concentration of EGCG recorded in G-1, G-2, and G-3 suggest that these brands may be more beneficial for human consumption. Variation in EGCG concentration may be due to climate, season, horticultural practices, and age of the leaf (Pastore, 2005). The tea brand with the lowest content of EGCG per tea bag in this study is G-11/G-5 (32.25 mg/tea bag). Thus, consumption of eight tea bags (Chow *et al.,* 2003), of G-11/G-5 per day will give a total of 258 mg of EGCG which is in concordance with the ≥ 200 mg recommended by kim *et al.* (2011) necessary to decrease total cholesterol and low-density lipoprotein. On the other hand, G-1 brand with the highest EGCG content (36.9 mg/tea bag) will result in 295.2 mg of EGCG after consumption of eight tea bags. This will also decrease total cholesterol and low-density lipoprotein without resulting in any adverse effects since the safe dose of EGCG range from 800 to 1600 mg (Chow *et al.,* 2003; Ullmann *et al.,* 2003). Thus, all the green tea brands analyzed in this study can be considered to be beneficial and safe for human consumption.

Although consensus on an optimal EGCG dose or intake level currently exists; however, evidence suggests that higher green tea catechin levels and specifically EGCG are needed for therapeutic effects. Clinical research in humans has found that EGCG at doses of 800 mg and

1600 mg do not adversely affect the liver (Chow *et al.*, 2003; Ullmann *et al.*, 2003). It is safe for healthy individuals to take green tea polyphenol products in amount equivalent to the EGCG content in 8 – 16 cups of green tea once a day or in divided dose twice a day for 4 weeks. There is a > 60 % increase in the systemic availability of free EGCG after chronic green tea polyphenol administration at high daily bolus dose (800 mg EGCG or polyphenon E once daily) (Chow *et al.*, 2003).

5.5 Elementals analysis

Herbal remedies are widely used for the treatment of various illnesses. They often contain highly active pharmacological components including minerals and trace metals (Fabricant and Farnsworth, 2001). However, an increase in the intake of these trace metals above certain permissible limits can become harmful (Korfali *et al.*, 2013a; Korfali *et al.*, 2013b). Other non-essential metals (e.g lead and cadmium) are usually present in herbal medicines as either contaminants or adulterants.

5.5.1 Iron (Fe)

Fe was found in variable amount in all the green tea samples with a concentration range of 32.30 -164.04 mg/g (Table 4.10). Hussain *et al.* (2006) reported 0.25 - 2.7 ppm of Fe in investigation of heavy metals in commercial tea brands. Donkor *et al.* (2015) conducted a study on assessment of essential minerals and toxic trace metals in popularly consumed tea products in Ghana and reported 125.4 ppm as Fe concentration in the samples analyzed. The WHO limit for Fe in medicinal herbs has not been established yet (WHO, 2005; WHO, 2006). Fe is an essential element for plants and animals and its deficiency cause problem in metabolism. Fe is a

constituent of the active site of various reductive hydrogenases, most frequently being associated with sulfur containing ligands. Fe has several key functions in the human body including oxygen supply, energy production, and immunity. Deficiency of Fe in plants produces chlorosis disease (Sakolnik *et al.*, 1984). Fe together with hemoglobin and ferredoxin plays a central role in metabolism (Martin and Griswold, 2009). Fe facilitates the oxidation of carbohydrates, protein and fat to control body weight (Rajurkar and Perdeshi, 1997). Fe overdose is associated with symptoms of dizziness, nausea and vomiting, diarrhea, joints pain, shock, and liver damage. Fe toxicity has an adverse effect on various metabolic functions and cardiovascular system.

5.5.2 Zinc (Zn)

Zn was only detected in three of the twelve brands analyzed. The highest concentration (25.00 mg/g) of Zn was found in G-11 brand, while in G-9 and G-12 the same concentration (1.23 mg/g) was recorded (Table 4.10). However, Zn concentrations were significantly different ($p < 0.05$) at lower values. Thus, it can be concluded that the concentration of Zn was in the other brands was below detection limit of the method. The FAO/WHO permissible limit set for Zn in herbs is 50 ppm (WHO, 2005; WHO, 2006). Thus, the samples analyzed in this study are safe for consumption with respect to Zn. Hussain *et al.* (2006) reported lower concentration (0.25 – 1.5 ppm). Donkor *et al.* (2015) reported 31.3 ppm of Zn in the tea samples they analyzed. Zn is an essential trace element necessary for proper growth, blood clotting, thyroid function, and protein and DNA synthesis. Little information is available on Zn toxicity; however, high Zn intake beyond permissible limits produces toxic effects on the immune system, blood lipoprotein levels, and copper level (Fosmire, 1990). Furthermore, Zn is a very important metal for plant and

animals, in the blood about 85 % of the Zn combines with protein for transport after its absorption and its turnover is rapid in the pancreas (Hussain *et al.,* 2006).

5.5.3 Copper (Cu)

Small traces of Cu were found in five of the brands at a concentration range of 0.02 – 0.59 mg/g (Table 4.10). The levels of Cu in the other brands however, were below detection limit. Hussain *et al.* (2006) reported a concentration range of 0.25- 1.00 ppm. Donkor *et al.* (2015) reported 26.7 ppm of Cu in the samples they analyzed. The regulatory limits of the WHO/FAO have not been established yet for the Cu in herbs (WHO, 2005; WHO, 2006). China set limits for Cu in medicinal plants at 20 ppm, while in Singapore it is 150 ppm (Ulla *et al.,* 2012). Cu is essential enzymatic element for normal growth and development but can be toxic at excessive level. Thus, Cu play a significant role in a wide range of physiological processes including Fe utilization, free radical elimination, bone and connective tissues development, melanin production, and many others. Nevertheless, excessive intake of Cu can cause dermatitis, irritation of the upper respiratory tract, abdominal pain, nausea, diarrhea, vomiting, and liver damage (Ulla *et al.,* 2012). Excessive Cu levels can also cause oily skin, loss of skin tone (due to its ability to block vitamin C), and dark pigmentation of the skin, usually around the face. It can contribute to hair loss especially in women (Martin and Griswold, 2009). As with other heavy metals, some species can tolerate very high amount of Cu.

5.5.4 Lead (Pb)

Pb was detected in all the green tea samples with concentration ranging from 1.98 to 8.76 mg/g, which were significantly different ($p < 0.05$). Akbar was the only brand in which Pb was not detected (Table 4.10). However, in all the tea samples, Pb was found to be within permissible limit (10 ppm) for Pb content in herbs (WHO, 1998b). Thus, the tea samples analyzed can be considered safe for human consumption with respect to Pb. Hussain *et al.* (2006) reported a Pb concentration range of 0.25- 4.75 ppm. Donkor *et al.* (2015) reported 4.11 ppm as Pb concentration in their findings. It is believed that 95 % of Pb in herbs is due to foliar uptake in plants (Tjell *et al.*, 1979). Lead is known to be one of the highly toxic environmental pollutants. It can complex with various biomolecules and adversely affect their functions. It enters the human body in various ways including inhalation, drinking water, and absorption by skin. It accumulates in the body and has no known useful biological effect (Venkatesh, 2004). Lead exposure may have an adverse effect on the blood, nervous, immune, renal, skeletal, muscular, reproductive, and cardiovascular systems causing poor muscle coordination, gastrointestinal symptoms, brain and kidneys damage, hearing and vision impairments, and reproductive defects. Exposures to lead at early childhood and prenatal stage are associated with slowed cognitive development, learning deficits, and many other effects (Johnson, 1998; ATSDR, 2007). Pb can cause a number of diseases. For example, Pb can inhibit Cu dependent enzymes needed for neurotransmitters, causing hyperactivity. Gout can occur from Pb toxicity raising uric acid level and impairing kidney functions. Pb inhibits Zn, Cu, Fe, Calcium, and Manganese, causing their deficiencies. Pb can inactivate the thyroid hormone, because it interferes with the iodine uptake to the thyroid gland.

5.5.5 Cadmium (Cd)

Cd is another toxic metal having unknown functions neither in human body nor in animals or plants. Cd was found at a concentration range of 0.03 - 0.11 mg/g in six of the tea brands analyzed, which were significantly different ($p < 0.05$) at lower values. The Cd detected in the samples may have been introduced during packaging and/or the tea plant might have taken it (Cd) from air or soil. Donkor *et al.* (2015) reported 1.242 ppm as Cd concentration in the tea sample they analyzed. The permissible limit set by FAO/WHO for medicinal herbs and plants is 0.3 ppm (WHO, 2005; WHO, 2006). Thus, the results of this study indicate the probable safety of consuming these samples. High levels of Cd can results in serious toxicological effect on human health. Kidney is the critical target organ in exposed population. Excretion of Cd is very slow and it accumulates in human kidney for a relatively long time. This may result in an irreversible impairment of the renal tract (Li *et al.,* 2012; Maobe *et al.,* 2012). At high concentrations, Cd produces serious effects in the liver, and in vascular and immune system (Maobe *et al.,* 2012). Cd directly damages nerve cells, it inhibits the release of acetylcholine and activates cholinesterase enzyme, resulting in a tendency for hyperactivity of the nervous system. In cardiovascular system, Cd replaces Zn in the arteries, which contributes to arteries being brittle and inflexible. Cd exposure leads to a variety of adverse effects due to its extremely long biological half-life. The rate of Cd transfer from soil to plants is very high and the metal is considered to have carcinogenic effects on liver, pancreas and stomach (Waalkes, 2003).

Additionally, during tea infusion, both essential and non-essential elements are leached into the beverage (Szymczycha *et al.,* 2012). Despite this, not all metals contained in the leaves are extracted into the beverage since the degrees to which these metals are extracted into the

beverage are dependent on many factors like the temperature, infusion time, the water hardness and the leaf to water ratio (Astill *et al.*, 2001). Hence, these samples are safe for consumptions.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

Currently there has been an increased interest globally in the production and consumption of green tea, which is pharmacologically potent and has low or no side effects for use in protective medicine due to larger proportion of EGCG. Nevertheless, there is an urgent need to check the efficacy and safety for a green tea to be used as safe and effective drug. As such, quality control studies (WHO, 1998a) were performed on the standard powder (EGCG) from Sigma Aldrich Germany (melting point and IR spectroscopy) and collected samples marketed in Zaria (uniformity of weight, moisture content determination, total ash value, water soluble ash and acid insoluble ash). The results revealed good manufacturing practice was adhered to during productions of the various brands. This shows that the green tea brands were not adultered and are pure. Some brands have significantly higher values ($p < 0.05$). Preliminary screening of phytochemicals is a valuable step, in the detection of the bioactive principles present in medicinal plants and subsequently may lead to drug discovery and development. In this study, screening of the sampled green tea brands was performed for the presence of tannins, saponins, flavonoids, alkaloids, cardiac glycosides and terpenoids using standard methods. Also, a rapid, simple, precise, accurate and reproducible spectrophotometric method for determination of

EGCG in pure and herbal formulations was developed and validated in the present study according to ICH guideline, 1996. Moreover, all the validated parameters were within the acceptable limits. However, the developed method was successfully applied in this study to quantified EGCG in each brand of green tea, moreover, three of the brands have significantly higher values *($p < 0.05$*). Nitric-perchloric acid digestion was performed, following the produce reported by the AOAC, 1990. The resulting solution was analyzed for Fe, Zn, Cu, Pb and Cd using AAS (AA-6800 Shimadzu), with concentration ranges of 32.30 – 164.04, 1.23 – 25.00, 0.02 – 0.59, 1.98 – 8.76, and 0.08 – 0.11 mg/g respectively. Furthermore, all the detected metals were within the permissible limit (WHO, 2005; WHO, 2006), some have significantly higher values (*$p < 0.05$*), while in some brands these metals were below detection limit.

6.2 Conclusion

Thus, it can be concluded that consumption of all the green tea brands analyzed may be beneficial to human health as a result of the phytochemical, quality assessment showed that these samples were not adulterated and essential metal content, and may also be safe because the toxic metals are with the permissible limits.

6.3 Recommendations

Further studies should be carried out on mineral analysis of green tea infusion (like Na, K, Mg, Mn, Ca, etc.). Further studies should be carried out on the effect of temperature and storage time on metal extraction of green tea infusion. Antioxidant activity of these samples should also be studied. The regulatory bodies particularly NAFDAC should carry out periodic quality control of green tea products that are marketed in Nigeria.

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Appendices

Appendix 1; Label information of twelve different brands of green tea samples

S/No.	Samples	Batch No. (NAFDAC No.)	Mfg. Date	Exp. Date	Price/ Tea Bag (#)
1	G-1	TC/E/PR/238/109 (NIL)	Jul-2015	Jul-2018	700
2	G-2	712714 (NIL)	Jan-2015	Jan-2018	600
3	G-3	CV-01-13 (NIL)	Jan-2013	Jan-2018	800
4	G-4	MTE/ UR / 01 (NIL)	Jul-2015	Jul-2018	500
5	G-5	321914 (NIL)	Jun-2014	Jun-2017	800
6	G-6	N.LB.GT.Gar.B-15/80 (NIL)	Dec-2015	Dec-2018	800
7	G-7	NG/07/2015 (C1-0815)	Jul-2015	Jul-2018	700
8	G-8	D15151 (NIL)	May-2015	May-2018	600
9	G-9	TC/E/PR/1369/895 (NIL)	Dec-2015	Dec-2019	600
10	G-10	N.SB.GT.A.B-15/80 (B1-9602)	Dec-2015	Dec-2018	800
11	G-11	83271979 (NIL)	Jun-2015	Jun 2017	1000
12	G-12	TC/E/PR/776/337 (NIL)	Sept-2014	Sept-2018	500

Only two of the brands passed the NAFDAC requirements as shown above.

Appindix 2; Sample calculation of uniformity of weight studies/Percentage mean deviation (G-2 in gram).

Individual tea bag weight for 20 bags = 2.28+2.30+2.30+2.34+2.31+2.25+2.20+2.24+2.30+2.33 2.28+2.30+2.30+2.34+2.31+2.25+2.20+2.24+2.30+2.33

X = 2.28

X ± SEM = 2.28 ± 0.09

X= Mean

SEM = Standard error of mean.

Percentage mean deviation (%) = mean deviation × 100 as follows:-

X_1= 2.28+2.30+2.30+2.34+2.31+2.25+2.20+2.24+2.30+2.332.28+2.30+2.30+2.34+2.31+2.25+2.20+ 2.24+2.30+2.33

X_2= 2.28

X_1- X_2

X_1= weight of individual tea bag.

X_2= mean of the samples

0+0.02+0.02+0.06+0.03+0.03+0.08+0.04+0.02+0.050+0.02+0.02+0.06+0.03+0.03+0.08+0.04+ 0.02+0.05 = 0.7

Average deviation = $0.35 \div 20$

= 0.035

10 = number of samples selected.

To get the mean deviation, you divide it by X_2

i.e. $0.035 \div 2.28 = 0.015$

Also, % mean deviation was gotten by multiplying mean deviation by 100

i.e. $0.015 \times 100 = 1.5\ \%$

Appendix 3; Sample calculation of moisture content (M.C) studies (G-2 in %).

M.C (%) = Weight of air-dried sample – Weight of oven dried sample × 100

Weight of dried sample

In order to minimize error it was performed in duplicate as follows:-

B_1	B_2
46.2871— W_1	46.2871— W_1
47.2959— W_2	47.2872— W_2
47.1759— W_3	47.1700— W_3
0.12	0.1172

M.C = $\frac{0.12\ g}{1\ g} \times 100$

M.C= 12 %

M.C = $\frac{0.1172\ g}{1\ g} \times 100$

M.C= 11.72 %

X= 11.86, ± SEM = 0.90

W_1= weight of crucible.

W_2= weight of crucible + sample before heating in the oven.

W_3= weight of crucible + sample after heating and cooling.

1g= weight of sample measured.

X= mean.

SEM= standard error of the mean.

Appendix 4; Sample calculation of Total ash studies (G-2 in %).

This was performed four times in other to minimized error as follows:-

B_1	B_{11}	B_2	B_{22}
45.15 —W_1	45.15 —W_1	48.64 —W_1	48.64 —W_1
47.15 —W_2	47.15 —W_2	50.64 —W_2	50.64 —W_2
45.25 —W_3	45.26 —W_3	48.72 —W_3	48.72 —W_3

Ash value = $\frac{W_3 - W_1}{2g} \times 100$

B_1	B_{11}	B_2	B_{22}
= $\frac{0.1g}{2g} \times 100$	= $0.11g \times \frac{100}{2g}$	= $0.08g \times \frac{100}{2g}$	= $0.11g \times \frac{100}{2g}$
= 5 %	= 5.5 %	= 4 %	= 5.5 %

X = 5, ± SEM = 0.35

W_1= weight of crucible.

W_2= weight of crucible + sample before heating in the oven.

W_3= weight of crucible + sample after heating and cooling.

2g= weight of sample measured.

X= mean.

SEM= standard error of the mean.

Appendix 5; Sample calculation of Water soluble ash studies (G-2 in %).

Two samples (B_1& B_{11}) among the four samples (in total ash) were used for this study as follows:-

$$\text{Water soluble ash} = \frac{W_2 - W_1}{2g} \times 100$$

B_1	B_{11}
W_1= 1.02g	W_1= 1.03g
W_2= 2.29, 1.55, 1.36, 1.14, 1.10, 1.05g	W_2= 2.22, 1.86, 1.32, 1.19, 1.09, 1.07g
$= \frac{1.05g - 1.02g}{2g} \times 100$	$= \frac{1.07g - 1.03g}{2g} \times 100$
= 1.5 %	= 2 %

X= 1.75, ± SEM= 0.35

W_1= weight of filter paper.

W_2= weight of filter paper + sample at constant weight.

2g= weight of sample measured.

X= mean.

SEM= standard error of the mean.

Appendix 6; Sample calculation of Acid insoluble studies (G-2 in %).

Two samples (B_2& B_{22}) among the four samples (in total ash) were used for this study as follows:-

$$\text{Acid insoluble ash} = \frac{W_2 - W_1}{2g} \times 100$$

B_2	B_{22}
W_1= 1.04g	W_1= 1.09g
W_2= 3.66, 2.99, 1.75, 1.14, 1.32, 1.19, 1.12, 1.08, 1.06g	W_2= 4.71, 3.72, 2.62, 1.49, 1.12,1.12g
$= \frac{1.06g - 1.04g}{2g} \times 100$	$= \frac{1.12g - 1.09g}{2g} \times 100$
= 1 %	= 1.5 %

X= 1.25, ± SM= 0.25

W_1= weight of filter paper.

W_2= weight of filter paper + sample at constant weight.

2g= weight of sample measured.

X= mean.

SEM= standard error of the mean.

Appendix 7; Sample calculation for conversion of AAS analysis, (Fe in G-2)

Concentration of metals in sample = $\frac{amount\ \ in\ ppm\ x\ Df\ x\ Volume\ \ of\ water\ \ added}{mass\ \ of\ \ samlpe\ \ measured}$

Df = Dilution factor

Amount in ppm = 0.6937

Df = Nil

Volume of water added = 100

Mass of sample measured = 1g

Using the above relation:-

Conc. of metal in sample = $\frac{0.6937\ x\ 100}{1}$

= 69.37 mg/g

Appendix 8; Sample calculation for concentration of EGCG in tea samples (G-2)

y = 0.1349x – 0.0279 (regession equation)

$X = \frac{y + 0.0279}{0.1349}$

y = 1.057, 1.049, 1.051 (y is the absorbances)

$X_1 = \frac{1.057 + 0.0279}{0.1349}$

ss = 804µg/ml

$X_2 = \frac{1.049 + 0.0279}{0.1349}$

= 798 µg/ml

$X_3 = \frac{1.051 + 0.0279}{0.1349}$

= 800 µg/ml

Conversion from µg/ml to mg/g

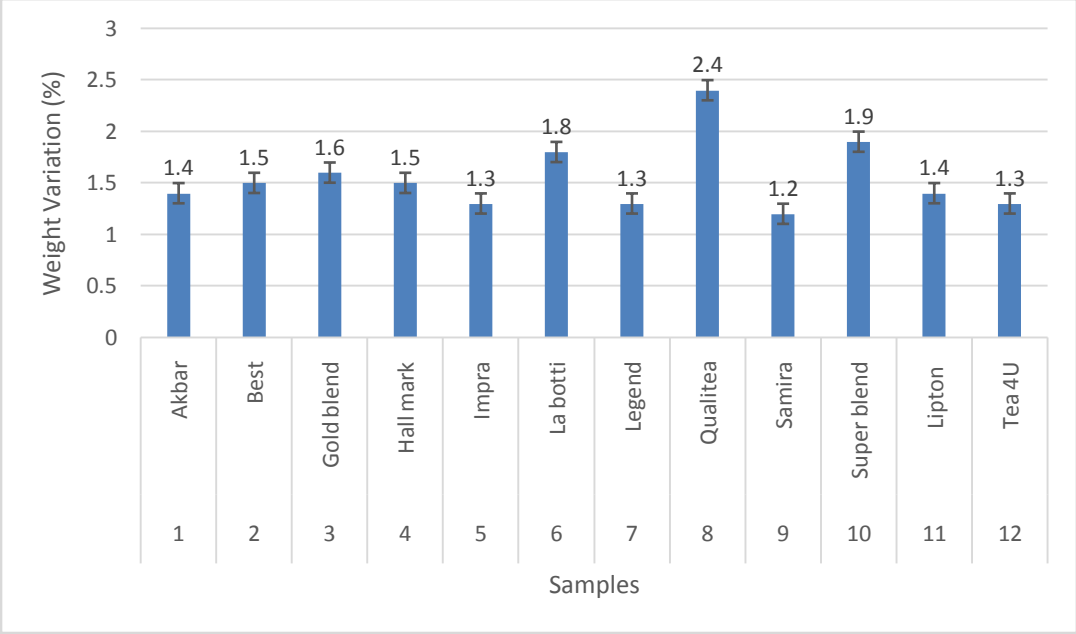
1000 µg ⟶ 1 mg

804 µg/ml ⟶ Xmg/g (Recall; 1g ≡ 1ml from density of water)

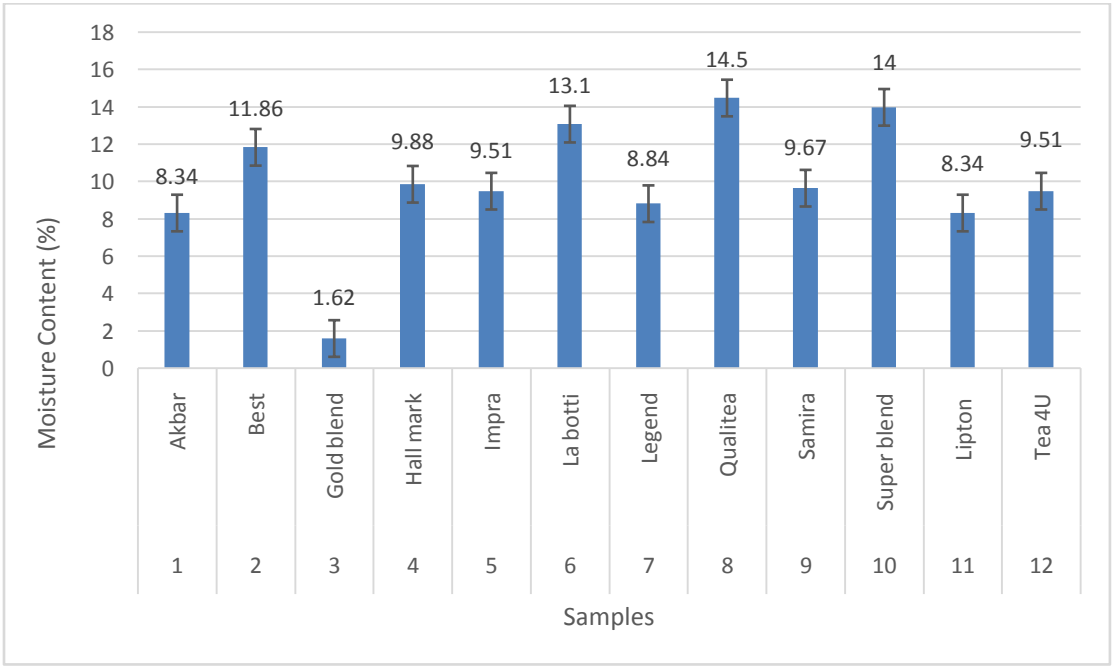
Therefore, $\frac{804 \text{ µg/ml} + 1\ mg}{1000 \text{ µg}}$

= 0.84 mg/g

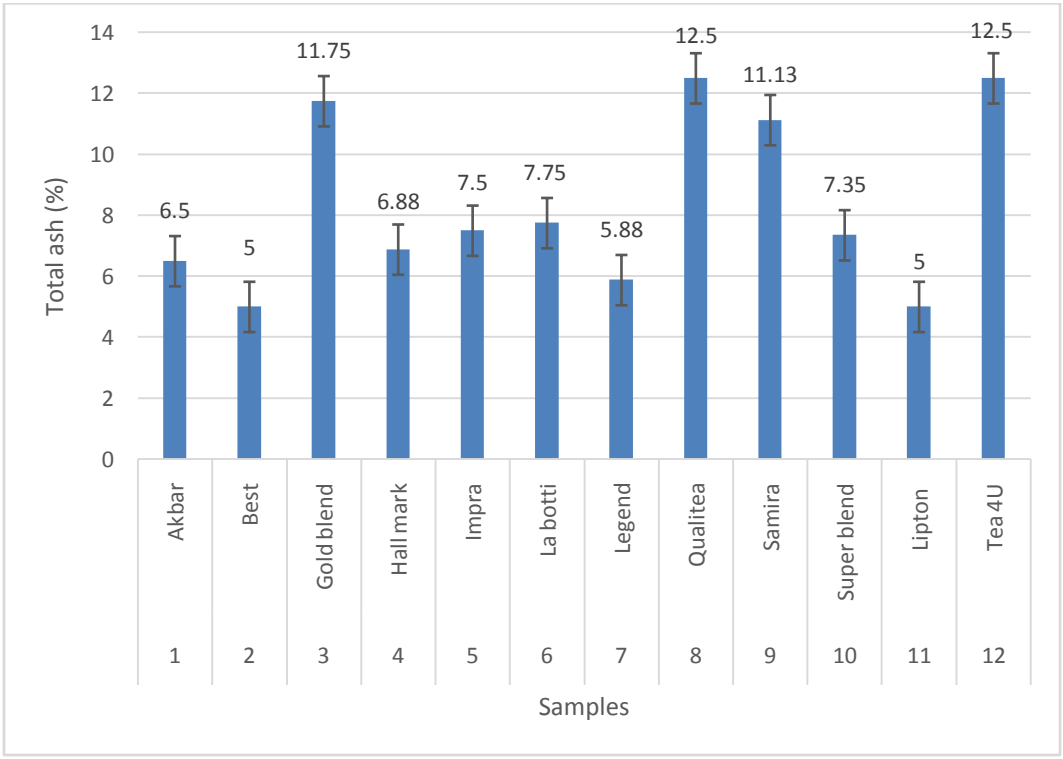
Appendix 9; Bar charts showing results of quality control of the various green tea brands.



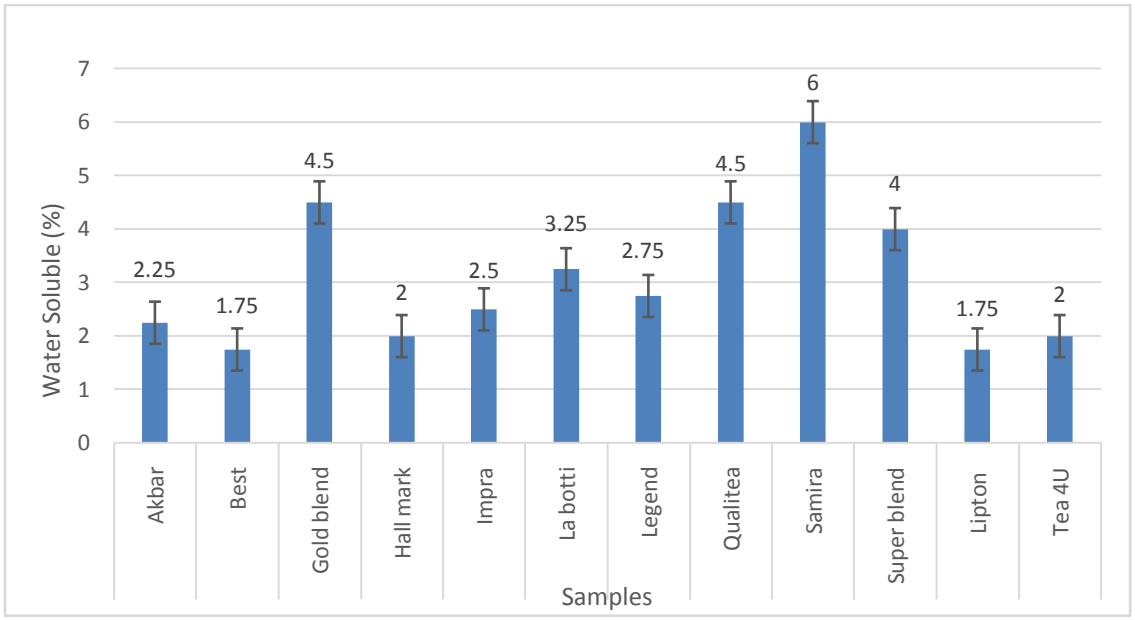
Appendix 9a: Uniformity of weight studies of various green tea brands.



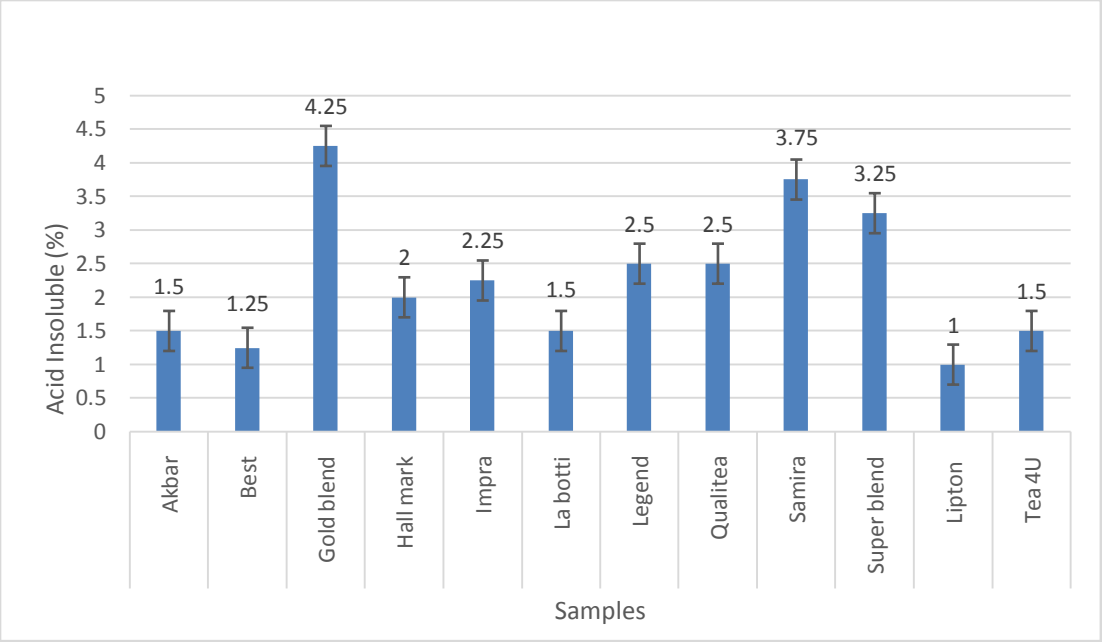
Appendix 9b: Moisture content of various green tea brands.



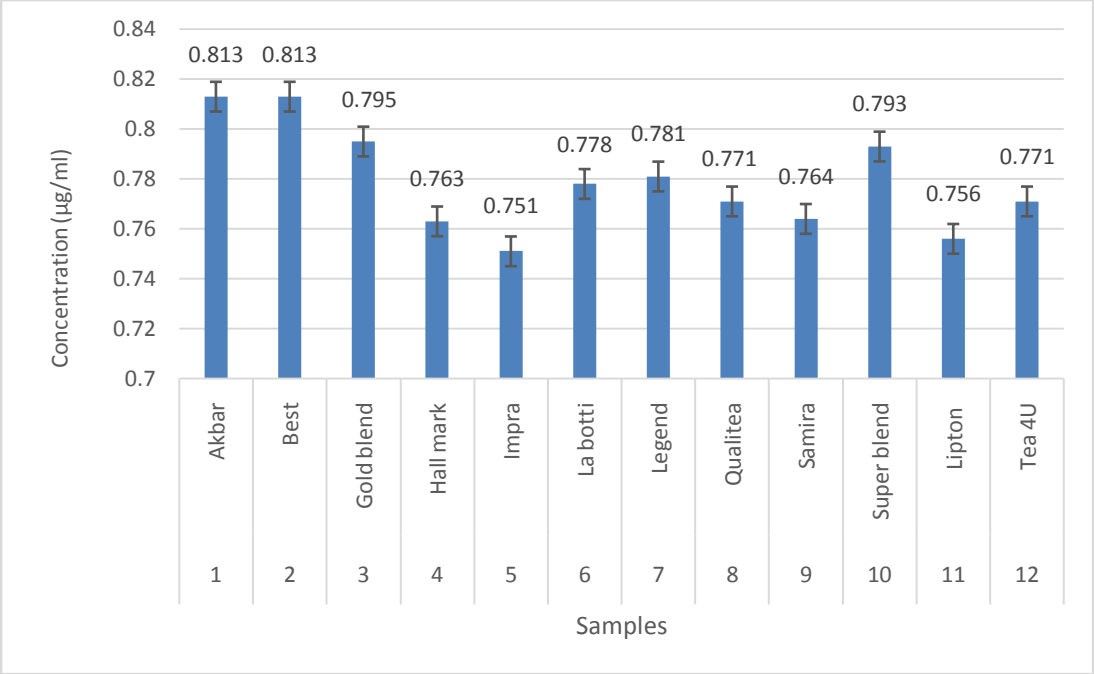
Appendix 9c: Total ash of various green tea brands



Appendix 9d: Water soluble of various green tea brands.



Appendix 9e: Acid insoluble of various green tea brands.



Appendix 9f: concentrations EGCG in the various green tea brands.