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DETERMINATION OF TOTAL PHENOLIC CONTENT FROM COMMERCIALLY AVAILABLE ETHIOPIAN BLACK TEAS

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Jimma, Ethiopia

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DECLARATION

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

Tefera Teklu

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ACRONYMS /ABBREVIATION

C.SINESIS	Camellia Sinensis
FCR	Folin-ciocalteu Reagent
GAE	Gallic acid Equivalent
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
ISO	International Standard Organization
LLE	Liquid–Liquid Extraction
LOD	Limit of detection
LOQ	Limit of quantification
MAE	Microwave- Assisted Extraction
PLE	Pressurized Liquid Extraction
TLC	Thin Later Chromatography
TPC	Total Phenol Content
UAE	Ultrasound Assisted extraction
UV/VIS	Ultraviolet- Visible Spectroscopy

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ABSTRACT

Tea is one of the most popular beverages in the world, is an infusion of the leaves of the tea plant, Camellia sinensis which is under the Theaceae family and the Camellia species (Camellia sinensis). The main types of tea are green tea, oolong tea, white tea and black tea. Among the four types, black tea is the most commonly consumed in the world. Therefore, the aim of this study was to determine total phenol content from some commercially available Ethiopian black tea. To find the best extraction condition, heat extractions were conducted at different solvent, temperature and time. Finally, the tea samples were analyzed for phenol content with Uv/Vis Spectroscopy at 760 nm. Under optimum conditions of 80% ethanol solvent ratio, 80°C temperature and extraction time of 15 minutes, the TPC found in Gumaro, Wush-wush and Back lion tea were 28.5 ± 0.96 mg GAE/g, 26.5 ± 0.82 mg GAE/g and 23 ± 0.24 mg GAE/g, respectively. From the three Ethiopian black teas Gumaro black tea have the highest total phenol content and black lion have the lowest phenolic content. The detection limit (LOD) and quantification limit (LOQ) were 1.3 and 3.8 mg GAE/g, receptively. Even if the samples were belong to the same black tea type, the level of TPC in the three tea type were significantly different ($p<0.05$) at 95% confidence interval. The possible reason for this difference total phenolic content could be different ways of tea cultivation, period of harvesting, different processing procedure, fermentation time and other process.

Keyword: *Tea, Phenol, extraction, spectrophotometer, Folin*-Ciocalteu's reagent

1. INTRODUCTION

Tea is one of the most popular beverages in the world, is an infusion of the leaves of the tea plant, *Camellia sinensis* which is under the *Theaceae* family and the *Camellia* species (*Camellia sinensis*). *C.sinensis* consists mainly of two varieties, *C.sinensi*s variety sinensis (The China tea plant) and *C.sinensis* variety assamica (The Assam tea plant) [1]. There are four major categories of tea which are distinguished by different processing methods and, consequently, different concentrations of the chemical components in tea. The main types of tea are green tea, oolong tea, white tea and black tea. Black tea is the most commonly, consumed tea in the world and the one processed in the largest amount among the four types. Black teas usually undergo full oxidation leading to the development of the characteristic dark brown and black colors of the leaves [1].

Tea beverage is consumed for centuries [2] and it is the second most consumed beverage in the world with an estimated 18-20 billion cups consumed daily. The economic importance of tea backs to a very long years and it is a source of income for many countries including Ethiopia. For instance, it is the leading export item in Kenya, India and Sri Lanka [3].

A recent awareness of health benefits has increased consumers' interest in this beverage especially green tea. Green tea is derived from drying and steaming the fresh tea leaves and thus no oxidation occurs, resulting in high levels of catechins. Most commercially prepared tea is obtained from the leaf of the plant *C.sinensis*. *C.sinensis* variety sinensis (China tea) is grown extensively and used in China and Japan, while C. sinensis variety assamica (Assam tea) predominates in South and South-East Asia [4].

Traditionally, green tea was used to improve blood flow, eliminate alcohol and toxins, improve resistance to disease, and relieve joint pain and to clear urine and improve its flow [5]. Tea contains large amounts of polyphenolic compounds with antioxidant properties, and these may prevent oxidative damage of DNA [6]. Tea is also rich in flavonoids and other polyphenol compounds which have different beneficial activity such as anti carcinogenic or prevent tumor cell growth, cholesterol lowering, antiviral, and antibacterial, reduce cardiovascular disease, and induce body weight loss [7, 8].

Chemical composition of tea varies with climate, season, variety, horticultural practices and the age of the leaf [9] which suggests that various levels of the bioactive compounds could be expected from tea that is grown in different parts of the world. The most important compounds present in tea, which are of considerable pharmacological significance, are the polyphenols and caffeine. Ethiopia has a unique climatic conditions and soil chemistry affecting the composition of plant secondary metabolites composition as it has been reflected in many studies on samples from Ethiopia [10].

Nowadays, the commercial tea in Ethiopia, which is black and green type, is processed from the C. assamica variety or the same type of Camellia sinesis grown in Wush-wush, Gumero and Chewaka tea plantations located in Southwestern part of the country and more than ten different types of tea are commercially available for local and export market under different brand names originated from these plantations. In contrast to the considerable amount of research on tea leaves, there are relatively few studies concerned with total phenol content of tea leaves.

Recently the total phenolic content of tea has been studied intensively in the world. However, to the best of our knowledge there is no literature report that shows the determination of total phenolic content of Ethiopian Black tea using Folin-Ciocalteu reagent. Therefore, the objective of this study is to determine the total phenolic content of three commercially available Ethiopian tea samples using Folin-Ciocalteu reagent.

1.1. Statement of the problem

Teas have been consumed in different parts of Ethiopia for different reason since the early time like enjoyment, during lamentation, and cold time. Epidemiological studies strongly suggest that regular consumption of plant polyphenols, such as those found in tea and tisanes, can protect against the development of disorders caused by free radical damage, including cardiovascular disease, cancer, diabetes, osteoporosis, and neurodegenerative diseases However ,the users may not understand why they drink it and what benefit does the drink have.

Several reports have shown that the composition of total polyphenols in tea sample one of the parameters for quality of tea regarding its biological properties [11], therefore this study should be applied for the quality control of manufactured teas. Thus, the determination of total poly phenol content of tea is very important in assessing the standard and quality of tea as well as any potential implications to health. Since tea is beverage which is a part of our daily dietary intake and frequency consumed, assessment of the nutrient composition the aforementioned one in tea plant grown in Ethiopia is of great importance from quality and standards, nutrition and health perspectives.

Based on this fact this study was conducted to answer the following questions.

- Do black tea have significant amount of Total phenolic content?
- Do extraction parameters influence on the amount of total Phenolic content yield?
- Are the three black teas differing in their total phenol content?

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1.2 OBJECTIVES

1.2.1 General objective

- The general objective of this study is to extract and know the total phenolic contents from commercially available Ethiopian black tea samples.

1.2.2 Specific objectives

- To optimize the extraction condition of solvent, time, and temperature for the extraction of total phenolic content from black tea.
- To determine total phenolic content of wush-wush, Gumaro and Black lion tea sample.
- To compare the total phenolic content of three different Ethiopian tea sample.

1.3 Significant of the study

In Ethiopia like the other countries tea is consumed largely in our daily life, therefore determination of total phenolic content from black tea is very vital. The finding of this study could provide information concerning the potential of black teas as a source of high value products like phenolic compounds. This thesis is intended to analyze the total phenol contents in commercially available Ethiopian black tea so that the total phenol content of the black tea and amount of the phenol content obtained from different brand were compared. Then, the results obtained might initiate further studies on nutritional, medicinal and toxicological effects of the commercially available Ethiopian tea**.**

2 LITERATURE REVIEW

2.1 History and Origin of Tea

Tea (Camellia sinensis) is the species of plant whose leaves and leaf buds are used to produce Chinese tea. It is of the genus Camellia, a genus of flowering plants in the family Theaceae. The scientific classification of tea is kingdom-plante, Order-Ericales, Family-Theaceae, Genus-camellia, Species-C.Sinensis and Binomial name camellia senensis [12].

Tea (Camellia sinensis) is native to the southern regions of China and parts of India, Laos, Thailand, Vietnam, and Myanmar [12]. Tea is said to have first been discovered as a drink and medicine in China around 2737 BC. It was then introduced to Japan during the early 13th century and to Europe in the 16th century, then to America, Africa and other regions of the world [12, 13]. Tea is presently cultivated in over 30 countries around the world and the tea beverage is second only to water in terms of worldwide consumption. All kinds of tea originate from Camellia sinensis (earlier called The a sinensis) which has the two sub species: variety sinensis (China tea) is grown extensively and used in China and Japan and the other subspecies is variety assamica (Assam tea) which predominates in South and South-East Asia [14].

2.2 Tea plantation and related aspects

Like other plants need certain requirements to grow, develop and give desired products, tea plantation also requires favorable conditions to give the expected products, such as soil, rainfall, water, nutrients and others. According to Mauskar et al [15] the following are key factors for tae plantation.

Soil: Tea is grown in a wide range of soil types found in tropical, subtropical and temperate climate conditions. These soil types have developed from diverse parent rock material and under different climate conditions. In China, Indonesia, Sri Lanka, South India, Turkey and Georgia (USSR) tea is mostly grown on sedimentary soils derive from genesis or granite.

In north-east India, except in Darjeeling, tea is grown on flat alluvial lands which occupy the vast area of the Brahmaputra Valley in Assam. Peat soils that have been drained are successfully used in Catcher to grow tea. But in Kyoto and Konya, the main areas of Japanese tea, the crop is grown on soil types derived from volcanic ash and in Taiwan tea occupies a tract of tertiary

rocks derived from a residual formation. However, all these soils have one common characteristic i.e. they grows best in acid soils. Despite the diversity of soil types on which tea is grown, all the soils exist in high rainfall conditions, as this is the most important climate factor for successful tea growing. These soils get formed under special type of weather (permanent moist conditions) combined with intensive leaching of the products of weathering. The degree of leaching and hence the character of the resulting soil depends not only on the rainfall but also on the temperature [15]. Because of differences in temperature, soils formed in tropical climates are likely to have certain characters different from those formed in the sub-tropical or semi-tropical conditions. The tea areas in tropical climates experience minimal temperature variations as compared with those in sub-tropical climates.

Rainfall: The relationship between tea production and rainfall is well known. Tea grows well in areas having a precipitation of about 1,150 to 8,000 mm. The effect of rainfall is perhaps more manifested by its influence on moisture status of the soil and in inducing vegetative growth. Therefore, distribution of rainfall is as important as the total annual rainfall. It is difficult to say what exactly is water requirement of tea in different phases of its growth and development. It is natural to expect that water requirement of tea would vary according to the prevailing environmental conditions, but it is reasonably assumed that tea on an average may transpire 900mm per annum. Ideally, the crop water requirement should be such that it does not cause any stress in the plant system.

Humidity: Humidity is of importance in tea physiology primarily because of its influence in determining the loss of moisture by evaporate-transpiration. High humidity reduces water loss, but low humidity increases it, and also increases water stress during rainless drought period. In relation to temperature, low humidity may be advantageous because by increasing transpiration rate it also reduces leaf temperature, though the effect will be more pronounced in un shaded conditions. In north-east India the humidity level generally remains high during the harvesting period and this is generally considered to be conducive to growth.

Air Temperature: Tea is grown in tropical to temperate conditions, under a regime of air temperature that varies between –8°C and 35°C the suitable temperature for growth being the one common to the habitat of the plant, that is, where it grows. But photosynthetic rate of tea is

at maximum between 30°C and 35°C, falls rapidly at 37°C, and between 39°C and 42°C there is virtually no net photosynthesis. There is also no uptake of carbon dioxide at about 42°C; respiration may continue up to about 48°C but the leaf is irrevocably damaged. Therefore, there has to be an optimum range of temperature for growth and productivity of tea.

Fertilizer: Fertilization is an important part of the normal intensive production of tea. Tea is normally grown as a long-term monoculture. Without applied fertilizer the supply of nutrients available in the soil will become exhausted leading to mineral deficiencies in the plants, severe reduction in yield and ultimately, to the death of plants and a degraded plantation. Nutrients are removed from a field of tea in a number of ways. There is an irreversible loss in the crop. The net loss of nutrients in old leaves and pruning depend on the extent to which these items are retained in the field. Soil erosion, drainage of excess water containing nutrients in solution and decomposition to gases create further losses. Uncontrolled weeds also absorb substantial quantities of nutrients. In addition to oxygen, hydrogen and carbon which are obtainable in air & water, tea plant requires fourteen (14) mineral nutrients for its ideal growth. These are Nitrogen (N), Phosphorus(P), Potassium (K), Calcium (Ca), Magnesium (Mg), Sulphur (S), Manganese (Mn), Zinc (Zn),Copper (Cu), Aluminum (Al), Iron (Fe), Boron (B), Molybdenum (Mo) and Chlorine (Cl). Of these essential nutrients mentioned above, the first nine are considered principal nutrients as they are required in relatively larger amounts [15].

2.3 Botanical Classification of Tea Plant

The tea shrub is a perennial evergreen plant. It is under the Theaceae family and the Camellia species (Camellia sinensis). Camellia sinensis consists mainly of two varieties, Camellia sinensis variety sinensis (The China Tea Plant) and Camellia sinensis variety assamica (The Assam tea plant). In fact there are other varieties in addition to the previous two types of varieties. These varieties differ in the height of the tea bush, the number of stems and characteristics of their leaves [16]. In nature, tea trees can have a height of 20-30 m but usually the plant is kept as an evergreen shrub by pruning, pruned when they are around 1.5-2.0 meters. Old tea plants with age of 1500 years old are still thriving in their original forests of Yunnan Province in the Southwestern China [17].

2.4 Tea Plant in Ethiopia

The production of tea-leaves in Ethiopia has got a history of more than half a century. Nevertheless, it is only three decades since the consolidated and organized development of tea production began. The production of tea-leaves was begun in 1984 with the support of the state and up to 1996 not more than 2,000 hectares were produced.

In the 2005/2006 fiscal year, tea plantation on seven household farms was executed and a contract with tea plantation development organizations was made. Data obtained from the plantation of Wush-wush, Gumero, and Chewaka varieties show that the land covered by the tea plantations covered in 2004/2005 exceeds that of the 2002/2003 by 190 hectares, and the yield correspondingly increased by 390 tons. Compared to that of 2003/2004, land covered by plantation surpassed by 75 hectares and production likewise exceeded by 302 tons. Tea is one of the cash crops in Ethiopia [18].

In Ethiopia, tea is mostly grown in the highland dense forest regions where the land is fertile and thus the use of fertilizer is very minimal. Moreover, the availability of abundant and cheap labor in the country has made the use of manual weeding, instead of chemical weeding, possible. Because of this mostly organic cultivation, Ethiopian tea is increasingly sought for its aroma and natural flavors. Ethiopia exports teas regularly to different countries [19].The following table shows certain areas of Ethiopia where tea plantation is found, Wush-Wush and Gumaro tea plantations.

Table 1. Regions of tea plantations in Ethiopia

Brand name	Location	Distance from Addis Ababa (km)	Altitude (m)	Annual Rainfall (mm)	Temp (°C)	Soil property
Wush-Wush Tea	Kaffa (SNNP)	460 SW of Addis Ababa	1900	1820	10-30	fertile & rich in org.matter
Gumaro Tea	Illubabor (Oro. Reg.)	637 SW of Addis Ababa	1718	2089	10-30	fertile & rich in org.matter

2.5 Economic Importance of Black Tea

The economic importance of tea backs to a very long year. A Chinese document published in 347 A.D. states that people in Southwest China used teas for paying tribute to the Chinese emperors as early as 1066 B.C. In the essay "Tong Yue", written by a country landlord Wang Bao and published in 59 B.C., there is mention of the making and sale of tea. It showed that tea was commercially available in the local country market, suggesting that tea processing and marketing as early as 59 B.C. in Southwest China [17].

The principal teas produced and consumed in the world are black and green teas, with small amount of other types [17]. Black tea represents approximately 78% of total consumed tea in the world, where as green tea accounts for approximately 20% [21]. The major producers of tea are India, China, Sri Lanka, and Kenya, while the major consumers are India, China, Turkey, and Japan. During the 1990s, the world production and consumption of tea has increased steadily with occasional fluctuation in some years. Thus, Camellia sinensis has become a very important agricultural and commercial product, with unique horticultural and processing methods [17]. It is easy to see how tea is commercially important. For instance, tea is the leading export crop in Kenya, which places Kenya to be the third largest producer of black tea after India and Srilanka and large amount of money is earned [22].

2.6 Types of Tea

There are several major categories of tea, which are distinguished by different processing methods and, consequently, different concentrations of the chemical components in tea. The main types of tea are green tea, Oolong tea and black tea. The fermentation process involves an enzymatic oxidation of polyphenols, leading to the formation of chemical compounds that generate both the aroma and color of black tea [20].

The Green tea is a yellow to green color which is preferably manufactured from the Camellia sinensis var. Sinensis. Its chemical constituents are not altered by fermentation and the enzyme is inhibited by tea.

The Oolong tea is a semi-fermented tea which is manufactured by letting the tea leaf to undergo incomplete fermentation. The Oolong tea, with a large twisted leaf, brownish in color with white

tips, produces a light green, slightly coppery infusion. Its chemical composition is in between the green tea and the black tea [23]. Oolong tea contains monomeric catechins, theaflavins, and thearubigins. Some characteristic components, such as epigallocatechin esters, theasinensins, dimeric catechins, and dimeric proanthocyanidins, are also found in Oolong tea [24].

The Black tea is a completely fermented tea leaves. Fresh tea leaves are rich in polyphenolic compounds known as catechins. When tea leaves are intentionally broken or rolled during processing catechins become oxidized through the action of polyphenol oxidase enzymes present in the tea leaves. The oxidation of catechins, known as fermentation in the tea industry, causes them to polymerize and to form larger, more complex polyphenols known as theaflavins and thearubigins. This brings a difference in the color, aroma and chemical composition of the tea leaf as compared to green tea and oolong tea [24].

In fact there are other two types of tea, the white and yellow teas that have been regarded as two subclasses of green tea. These two types of tea are different from green tea due to differences in variety, processing, geographical and traditional distributions [17].

2.7 Manufacturing of Black Tea

The green, glossy leaves and young new shoots are plucked and immediately processed for optimal freshness [25]. The first step in the manufacturing of black tea is withering, which removes a large proportion of the water from the fresh leaf by evaporation. The leaves become limp and suitable for rolling and undergoing fermentation.

Rolling consists of twisting or breaking up the leaves so that preparing them for fermentation and transforming them into particles corresponding to the type of commercial tea required.

Fermentation is the most important stage in the manufacture of black tea. It involves the enzymatic oxidation (fermentation) of the polyphenols which are converted into theaflavines and thearubigines, with the leaves turning from green to coppery brown color. In this process, the monomeric flavan-3-ols undergo polyphenol oxidase-dependent oxidative polymerization leading to the formation of bisflavanols, theaflavines, and thearubigines. These chemical compounds possess benzotropolone rings with dihydroxy or trihydroxy substitution systems, which give the characteristic color and taste of black tea [24].

Firing will follow the fermentation step which stops the fermentation and reduce the water content of the tea which makes handling and transportation easy. The firing leads to the destruction of the polyphenol-oxidizes. Then the tea will be packed after sorting, which consists of extracting the fibers with the aid of winnowing machines and grading the tea by size and volumetric weight [24].

2.8 Chemical Composition of Black Tea

The chemical composition of a black tea may vary depending on different parameters such as the variety of leaf, growing environment, application of fertilizers, manufacturing, particle size of ground tea leaves and infusion preparation. In Table 2 the average values for the different constituents present in black tea are given [21].

Table 2. Principal components of black tea

Constituent	**Weight of extract solid (%)**
Catechins	3-10
Theaflavins	3-6
Thearubigens	12-18
Flavonols	6-8
Phenolic acids	10-12
Amino acid	13-15
Methylxanthines	8-11
Carbohydrates	15
Protein	1
Mineral matter	10
Volitailes	<0.1

2.9 Health Benefits of Black Tea

The chemical components in black tea have a health benefit to human. Although the oxidization process modifies the type of flavonoids present, the total level and their overall antioxidant activity, is similar in both green tea and black tea. Research is now suggesting that antioxidants, such as those found in both green and black tea may have a protective role to different diseases.

Different health benefit of black tea is reported [26]. For instance, black tea polyphenols are antioxidants in cancer chemo preventive [27]. The tea polyphenols may reduce oxidative stress through one of several mechanisms that relate to their structural chemistry. For example, the tea flavanoids directly scavenge free radical species through hydrogen/electron donation [26]. Black tea also has antigen toxic effect [28]. It also represents a promising tool for the prevention and treatment of cardiovascular disorders [29]. It has also been reported that the aqueous extract of black tea prevents chronic ethanol toxicity [30].

The amount of intake of black tea infusion gives health benefit. For instance, one to six cups per day for significant increases in plasma antioxidant capacity and less than eight cups of tea per day for the avoidance of adverse effects on hydration and iron status [31].

2.9.1 Medicinal use of tea plant

The tea plant is commonly used in Chinese herbalist, where it is considered to be one of the 50 fundamental herbs. Modern research has shown that there are many health benefits to drinking tea, including its ability to protect the drinker from certain heart diseases.

It has also been shown that drinking tea can protect the teeth from decay, because of the fluoride naturally occurring in the tea. However, the tea also contains some tannin, which is suspected of being carcinogenic. The leaves are cardio tonic, diuretic, expectorant, stimulant and astringent. They exert a decided influence over the nervous system, giving a feeling of comfort and exhilaration, but also producing an unnatural wakefulness when taken in large doses. They are used internally in the treatment of diarrhea, dysentery, hepatitis and gastro-enteritis.

Tea is reportedly effective in clinical treatment of amoebic dysentery, bacterial dysentery, gastro-enteritis, and hepatitis. It has also been reported to have ant atherosclerotic effects and vitamin B activity. Excessive use, however, can lead to dizziness, constipation, indigestion, palpitations and

insomnia. Externally, they are used as a poultice or wash to treat cuts, burns, bruises, insect bites, ophthalmic, swellings etc. Only the very young leaves and leaf buds are used, these can be harvested throughout the growing season from plants over three years old and are dried for later use [32- 36].

2.9.2 Other uses of tea plant

Besides its medicinal use the tea plant has also other uses, an essential oil is distilled from the fermented and dried leaves. It is used in perfumery and in commercial food flavoring. Non-drying oil is obtained from the seeds. Refined tea seed oil, made by removing the free fatty acids with caustic soda, then bleaching the oil with Fuller's earth and a sprinkling of bone black, makes an oil suitable for use in manufacture of sanctuary or signal oil for burning purposes, and in all respects is considered a favorable substitute for rapeseed, olive, or lard oils. The oil is different from cottonseed, corn, or sesame oils in that it is a non-drying oil and is not subject to oxidation changes, thus making it very suitable for use in the textile industry; it remains liquid below -18 0C. A grey dye is also obtained from the pink or red petals. The leaves contain 13 - 18% tannin. The leaves also contain quercetin, a dyestuff that, when found in other plants, is much used as a dye [34].

2.10 Polyphenol

Polyphenols (Figure 1) are naturally occurring organic compounds found largely in the fruits, vegetables, cereals and beverages. More than 8000 phenolic structures are currently known, and among them over 4000 flavonoids have been identified [37]. Although polyphenoles are chemically characterized as compounds with phenolic structural features (Figure 1), these groups of natural products is highly diverse and contain several sub-groups of phenolic compounds. The diversity and wide distribution of polyphenols in plants have led to different ways of categorizing these naturally occurring compounds. Polyphenols have been classified by their source of origin, biological function and chemical structure. Also, the majority of polyphenols in plants exist as glycosides with different sugar units such as Tannic acid and acylated sugars at different positions of the polyphenol skeletons (Figure 1) [36].

Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or violence by pathogens. In food, polyphones may contribute to the bitterness, astringency, color, flavor, odor and oxidative stability. Polyphenols are the subject of increasing scientific interest because of their possible beneficial effects on human health [38].

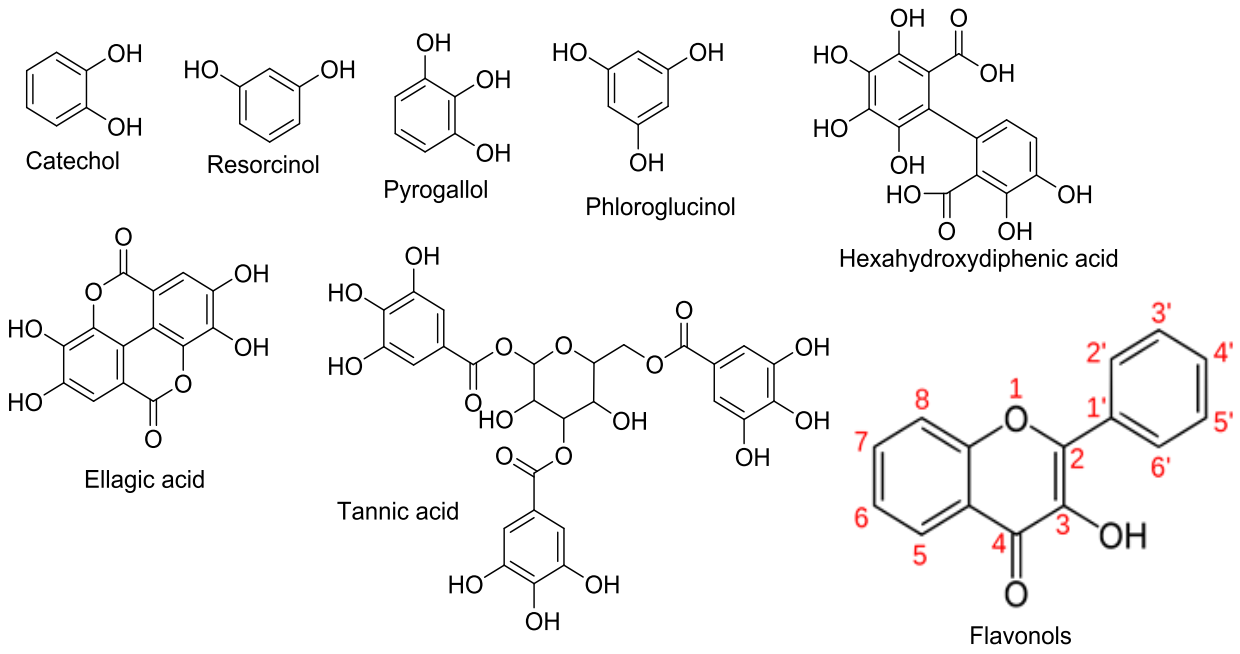

Figure 1. Chemical structure of Polyphenol representatives

2.11 Analytical techniques for determination of TPC

2.11.1 Extraction

Complete extraction of phenolic compounds is the next critical step after sample preparation. The most common techniques to extract phenolics employ solvents, either organic or inorganic. Several parameters may influence the yield of phenolics, including extraction time, temperature, solvent-to-sample ratio, the number of repeat extractions of the sample, as well as solvent type. Furthermore, the optimum recovery of phenolics is different from one sample to the other and relies on the type of plant and its active compounds.

The choice of extraction solvents such as water, acetone, ethyl acetate, alcohols (methanol, ethanol and propanol) and their mixtures will influence the yields of phenolics extracted [39].

As mentioned above, extraction is generally influenced by the sample nature, particle size, solvent type as well as extraction techniques employed. Extraction is separation or the isolation of the target compounds from their original matrix the choice of the suitable technique depends on: the desired class of compounds to be extracted; the structural characteristics of the botanic matrix (fruits, stems, seeds, leaves, root, flowers, etc.). Soxhlet, heated reflux extraction and maceration are conventional procedures frequently used to recover phenolics from solid samples. The Soxhlet and heated reflux methods are normally performed at 90 °C for several hours while maceration is performed over days at ambient temperature. These methods are simple, require relatively cheap apparatus and result in adequately high phenolic extraction rates [40].

Due to problems associated with conventional extraction procedures, a demand for alternative techniques for extraction of phenolic compounds has arisen. The use of ultrasound-assisted extraction (UAE) [41], microwave-assisted extraction (MAE) [42], ultrasound-microwave-assisted extraction (UMAE) [43] and supercritical fluid extraction (SFE) [38, 39,] is increasing. These methods shorten extraction times, decrease the release of toxic pollutants through reducing organic solvent consumption, and are relatively simple to perform.

Water bath is an indirect heating method used for decades. The plant material is slowly heated so as to enable maximum extraction. As the temperature rises, the plant tissue starts releasing its inner content into the medium extraction. Longer time of extraction gives better results. Conventional extraction and concentration of polyphenols using a water bath is typically conducted at temperature ranging from 20 to 80 °C, temperature. Higher temperature can increase the efficiency of the extraction since heat render the cell walls permeable, increasing solubility and diffusion coefficient of the compounds to be extracted and decrease the viscosity of the solvent thus facilitating its passage through the solid substrate mass. However, the use of temperature higher than 80 °C decrease the total polyphenol and proanthocyanidins yield, which is probably due to their degradation [46].

2.11.2 Quantification Methods of Phenolics

Despite a very large number of published investigations, quantification of various phenolic structural groups remains difficult [47]. Thus there is great scope for developing quantification methods based on the type of phenolic group [48]. High performance liquid chromatography (HPLC)[49] and Gas chromatography (GC)[50], or their combinations, with mass spectrometry are the two most commonly applied methods to quantify phenolic compounds. Other relevant technique includes spectrophotmetric [51].

2.11.3 Spectrophotmetric

Spectrophotometry is one of the relatively simple techniques for quantification of plant phenolics. The Folin-Denis and Folin-Ciocalteu methods were the two widely used specrophotometric assays to measure total phenolics in plant materials for many years [49]. Both methods are based on a chemical reduction involving reagents containing tungsten and molybdenum. The products of this reduction in the presence of phenolic compounds have a blue color with a broad light absorption spectrum around 765 nm.

The Folin-Ciocalteu method is a widely used method for the determination of total phenolic content. The Folin-Ciocalteu was chosen for this purpose due to its wide applicability for biological materials and its simplicity to use in the laboratory [50]. The method also provides reasonably good and reliable estimates of concentration of total reducing phenolic groups. The method is based on the reducing power of the phenolic hydroxyl groups [51] which react with Folin-Ciocalteu phenol reagent (an oxidizing agent comprised of hetero polyphospho tungstate molybdate) under basic conditions to form chromo spheres that can be detected spectrophotometrically at 765 nm. The reaction forms a blue chromospheres constituted by a phosphor tungstic phosphor molybdenum complex, Folin and Ciocalteu included lithium salts in the reagent, which prevented the turbidity due to excess Folin-ciocalteu reagent [52]. Sodium carbonate, which yields an appreciable concentration of the phenolate ions, the phenolates reduces the yellow Folin-Ciocalteu reagent; the reaction changing it into a blue pigment, spectrophotometrically measured [53].

3 MATERIALS AND METHODS

3.1 Apparatus and instruments

UV/Vis spectrometer (JENWAY 6705, England), centrifuge (Gemmy Industrial corp. Taiwan), analytical digital balance (Balance instrument Co.Ltd, China) what man No1filter paper, different size volumetric flasks, were used. Thermometer (Gallen kamp Griffin, England) water bath, plastic containers, crusher and cuvate were also used.

3.2 Chemicals and reagents

All chemicals and reagents used in this study were analytical grade. Sodium carbonate (Blux laboratory reagent, India) 90% ethanol (Laboratory reagent, India) Folin-Ciocalteau's reagent (Sisco research laboratory, India), and Gallic acid (Indian industry, India). Distilled water was used for cleaning and dilution purpose.

3.3 Sample Collection

3.3.1 Sample collection strategies

Three Ethiopian black tea brands (Wush-Wush, Gumaro, and Black lion) were purposely selected for this study since (i) they are widely consumed and most available in the local market of Ethiopia and (ii) the three tea brands represent the three different tea plantations found at three different locations in Ethiopia, The Wush-wush and Gumero black teas are processed from the Wush-wush and Gumero tea plantation, respectively. The Black Lion black tea is packed after blending black tea from the Chewaka plantation. Therefore the three tea sample was collected from Jimma local market for analysis.

3.3.2 Sampling and Sample Preparation

Three different types of commercially available Ethiopian black teas namely Wush-Wush, Gumero, and Black Lion tea, were collected from Jimma local market with the pack size of 100g. For each of the black tea brands three packages of 100 g were used. The three packs were mixed thoroughly and grinded to fineness using an electronic blender. The grinded 0.5µm black tea powder was used for the extraction of total phenolic content.

3.4 Methods

3.4.1 Procedure for solution preparation

3.4.2 Folin-Ciocalteu's reagents

Ten fold diluted folin-ciocalteu's reagent was prepared by taking 10 mL of FCR into 100 mL beaker and dilute to 1:10 by adding 90 mL distill water**.**

3.4.3 Preparation of 7.5% W/V Na_2CO_3 solution

Na_2CO_3 solution was prepared by taken 7.5 g of Na_2CO_3 into 100 mL volumetric flask and distilled water was added and shaken until dissolved then the flask was filled up to the mark with distilled water.

3.4.4 Preparation of Blank Solution

Blank solution was prepared by the same procedure with the sample by adding 5 mL of 10% FCR reagent in distilled water instead of sample and 4 mL of 7.5% of Na_2CO_3 solution.

3.4.5 Optimization strategy for extraction of Total Phenolic Content

The three black tea samples were extracted by using water bath. One factor at time experiment was carried out to determine the optimal extraction condition of total phenol contents from black tea, which means finding the best condition of one variable by fixing the other constant. 0.2 g of each black tea sample were extracted with 10 mL of ethanol/water (v/v) at different ratio 100:0, 50:50, 70:30, 80:20, 90:10 and 100:0 with different time of 5, 10, 15, 20, 25, and 30 minutes at different temperature of 65, 70, 75, 80, and 85 ^{o}C.

3.4.6 Optimization of extraction solvent

The optimizations of extraction solvent (ethanol/water) ratio were done by keeping the time and temperature constant at 10 min and 70 °C, respectively. 0.2 g of tea sample was extracted by varying the ratio of ethanol: water at 0:100, 50:50, 70:30, 80:20, 90:10 and 100:0. The best Extraction solvent ratio that gave highest total phenolic content (TPC) (mg Gallic acid equivalent, GAE/g) was selected.

3.4.7 Optimization of extraction time

The effects of extraction time were studied by fixing solvent ratio and temperature constant. 0.2 g samples were extracted with optimal ethanol water ratio and Temperature at 70 ^{o}C constant and by varying extraction time at 5, 10, 15, 20, 25, and 30 minutes. The best extraction time which gave highest values of total phenolic content (TPC) (mg Gallic acid equivalent, GAE/g) were selected.

3.4.8 Optimization of extraction temperature

The effect of extraction temperature was performed at different temperatures using optimal solvent ratio of aqueous ethanol solution and time. Sample of 0.2 g was extracted at different temperature, which was 60, 70, 75, 80, and 85 ^{o}C. The best extraction temperature which related to the values of total phenolic content (TPC) (mg Gallic acid equivalent, GAE/g) was selected.

3.5 Preparation of Standard Gallic acid solution

1 g of gallic acid standard was weighed and dissolved into 1000 mL volumetric flask and 10 mL of ethanol was added and filled with distilled water up to the mark. Then series dilution was performed in order to prepare different concentrated working solution. 10, 20, 30, 40, 50 and 60 mg/L by taking 1, 2, 3, 4, 5 and 6 mL from stock solution, 5 mL of folin-ciocalteau reagent and 4 mL of Na_2CO_3 (75 %) was added into each 100 mL volumetric flask and fill up to mark by distilled water the mixture was shaken and the flasks were covered with aluminum foil and allowed to stand for one hour in dark place. And absorbance of each was measured using UV-VIS spectrophotometer at 760 nm against a blank sample. All tests were performed in triplicate.

3.6 Determination of total phenolic content

The total phenolic content of the samples was determined by the Folin-Ciocalteu's reagent method ISO [52], with slight modification. An aliquot of 1.0 mL of the diluted sample extract was transferred into 100 mL of volumetric flask containing 5.0 mL of a 1/10 dilution of folin-Ciocalteu's reagent in water. Then, 4.0 mL of a sodium carbonate solution (7.5% w/v) was added after three minute and the beaker was filled up to the mark by distilled water. During the oxidation of phenolic compounds, phosphomolybdic and phosphotungstic acid, contained in the

Folin-Ciocalteau's reagent, are reduced to blue-colored molybdenum and tungsten oxides. After one hour, the absorbance of blue coloration was measured using UV-VIS spectrophotometer at 760 nm against a blank sample. In this work, Gallic acid was used as the standard; therefore the results were expressed as mg Gallic acid equivalent (GAE)/g extract. All tests were performing in triplicate.

3.7 Method validation

In order to validate the spectrophotmetric method for total phenol determination in black tea the following performance parameters were verified: linearity, detection and quantification limits, accuracy and precision.

3.7.1 Linearity

Linearity of response for standards was tested by assaying in triplicate using six levels of concentrations, ranging from 10- 60 mg/L Gallic acid covering in this way all the expected values and the regression coefficient (R^2)was calculated from plot of concentration and absorbance.

3.7.2 Limit Detection (LOD)

Limit of detection is the minimum concentration that can be detected by the analytical method with a given certainty. It is also the smallest concentration or amount of an analyte that can be detected or measured under defined condition. The LOD was calculated by three times standard deviation of the blank divided by the slope.

$$LOD=3\times SD/b$$

3.7.3 Limit of quantification (LOQ)

The limit of quantification (LOQ) of individual analytical procedure as the lowest amount of analyte in a sample which can be quantitative determined with suitable precision and accuracy. LOQ were calculated ten times standard deviation of blank divided by slope.

$$LOQ=10\times SD/b$$

3.7.4 Recovery test

As there is no any certified reference material used to compare the results with, the efficiency of the method used was assessed by spiking experiments. The precision of the method was determined as relative standard deviation and the accuracy of the method was determined as percent recovery, which was carried out by spiking known concentration of standard Gallic acid solution in black teas Then, the absorbance of solution before spiked and after spiked was measured by UV-Vis spectrometer. The percent of standard Total phenol recovered from the solution were calculated by using formula:

$$\%\text{Recovery} = \left(\frac{\text{C spiked sample} - \text{C unspiked sample}}{\text{C added (Spiked)}}\right) \text{x}100$$

3.8 Statistical analysis

Statistical tools such as one way ANOVA and person correlation was used to compare total phenol content of the black tea sample. Single factor Analysis of variance (ANOVA) was used to test the level of significant difference at $\alpha = 0.05$ [60].Variation in the levels of TPC between the samples was tested to decide it was due to random error.

4 RESULTS AND DISCUSSION

4.1 Calibration curve for Gallic acid.

The calibration curve was constructed with standard Gallic acid in the concentration range of 10-60 mg/L and the calibration curve is shown in Figure 2 with R^2 value of 0.997. Then the levels of total phenols in the samples were determined with the regression line equation and the results of total polyphenols of the samples were reported as average concentration and standard deviations of triplicate measurement.

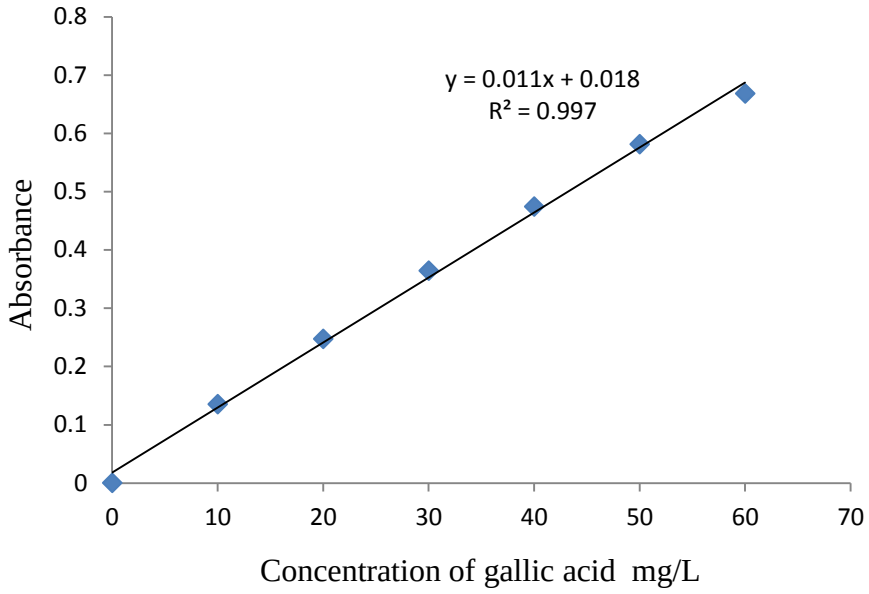

Figure 2. Calibration curve of gallic acid for total polyphenol determination

4.2 Optimization of parameters for Extraction of Tea Polyphenols

To obtain bioactive compounds from plant materials it is very important to choose the optimal extraction conditions. Processes which are more environmentally friendly and shorter extraction time and less organic solvent are of great interest. Effects of extraction time, extraction temperature, and extraction solvent were investigated and results are shown in Figures 3-5.

4.2.1. Effect of solvent on extraction of total polyphenol

To study the extraction efficiency of solvents, 0.2 g of each ground samples at fixed temperature 70 ^{0}C, time at 10 min and various ratios of water and ethanol solvents were done. For this different ethanol /water ratio of 0:100, 50:50 ,70:30, 80:20, 90:10 and 100:0 were studied as shown in Figure 3. The highest yield was obtained at 80:20 (v/v) of ethanol/water ratio. The effect of ethanol concentration on extraction of TPC shown that instead of mono-component solvent, the ethanol-water mixture was more efficient for higher TPC yield. Similarly in literature, it was reported that it is necessary to add a certain amount of water in the extraction solvent in order to improve the extraction of phenolic compounds [54]. Therefore, 80% aqueous ethanol is found to be the optimum extraction solvent mixture.

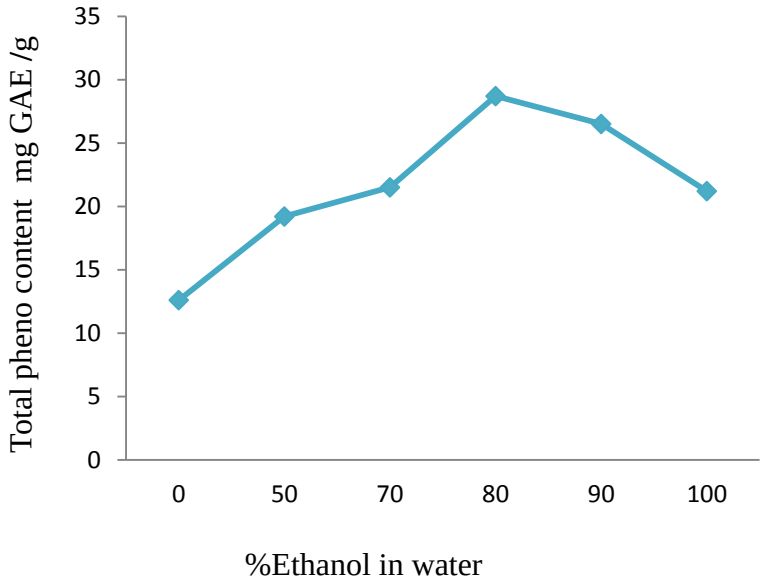

Figure 3. Effect of ethanol concentration on total phenolic content (at constant time and temperature)

4.2.2. Effect of time on extraction of total polyphenol

Extraction time is one of the main factors which influence the extraction for TPC. For this study to find the extraction efficiency of time, 0.2 g of grounded tea samples at fixed temperature of at 70 ^{0}C, solvent at 80:20 Ethanol/Water and various time at 5, 10, 15, 20, 25, and 30 minutes were

studied as it was shown in Figure 4 the effect of extraction time on the yield of Total Phenol at constant temperature and solvent for tea samples were highest at extraction time of 15 min. Researchers reported that, the total phenolic content on green tea did not significantly change, in between 10-30 minutes of extraction time [55]. So, for this study 15 min was selected as best extraction time.

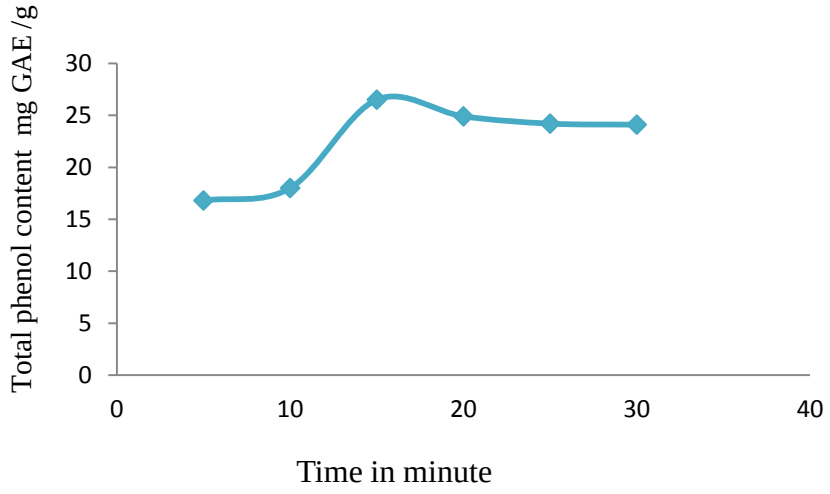

Figure 4.Effect of time on extraction of total polyphenol content (at constant solvent and temperature)

4.2.3 Effect of temperature on extraction of total polyphenol

For the study of temperature on the extraction efficiency, 0.2 g of grounded tea samples at fixed time of at 15 min solvent at 80:20 E/W and various temperatures of 65, 70, 75, 80, and 85^{o}C. The result depicted that high yield of TPC was obtained at a temperature of 80 ^{o}C Figure 5. Higher temperature can increase diffusion of extracted molecules, reduce its viscosity and improve mass transfer. However, excessive temperatures during extraction the stability of some phenol compounds due to reactions involving chemical and enzymatic degradation, or a thermal decomposition of some compounds [57].

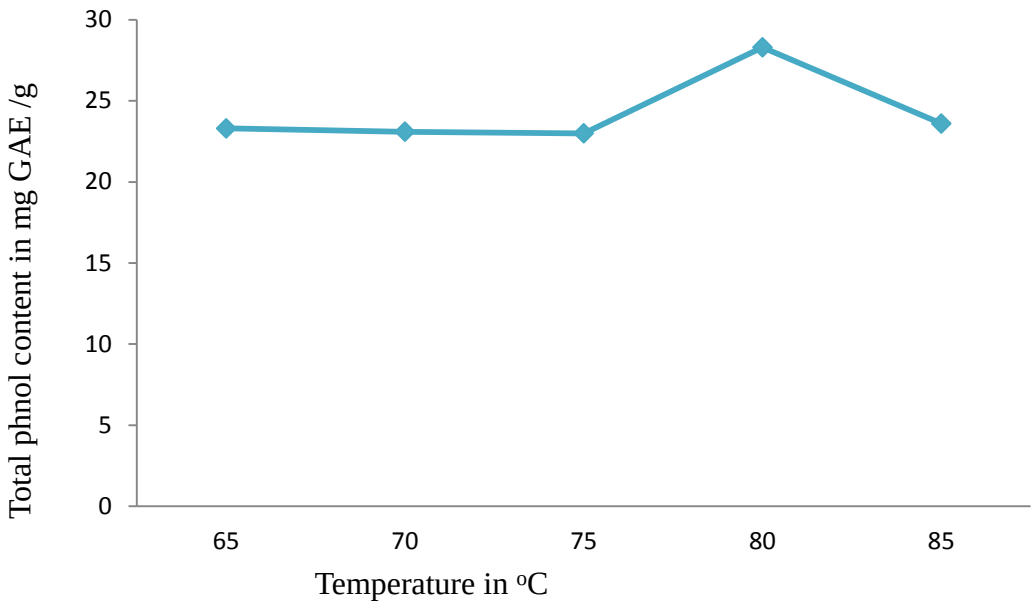

Figure 5. Effect of temperature on total phenolic content (at constant time and solvent)

4.3 Analysis of TPC in black tea sample

The total phenolic contents of three black tea samples were estimated using the Folin–Ciocalteu method, which relies on the transfer of electrons from phenolic compounds to the Folin–Ciocalteu reagent in alkaline medium, and is a simple and rapid method [52]. As shown in Table 3, the total phenol contents with optimized extraction conditions of 80 ^{o}C, 80:20 and 15 min varied from 23.0 ± 0.24 to 28.50 ± 0.96 GAE mg/g. The decreasing order of total polyphenols contents of tea crude extracts follow; Gumaro >Wush-wush >Black lion. The variation in polyphenol content in the studied commercial tea brands might be due to several factors such as age of the harvested tea plant, climate, agricultural practices, post harvest processing, and packaging.

Table 3. Total polyphenol content of three Ethiopian black tea samples. The result expressed mg GAE /g (n=3 mean ± SD)

Tea brand	**TPC mg GAE/g**
Black lion	23.0 ± 0.24
Wush-wush	26.5 ± 0.82
Gumaro	28.5 ± 0.96

Among the black tea brands, gumero brand had the highest (28.5 ± 0.96 mg GAE/g) while black lion had the lowest (23.0 ± 0.24 mg GAE/g) TPC as compared to wush-wush. Similar result was observed in the other study on black tea from Ethiopia [11]. The difference in TPC within the black tea brands could be attributed to variation of factors like the agronomic conditions, harvested leaf age, and storage during and after transport, as well as the degree of fermentation.. According to report on Korean tea, the total phenol content of green tea was found to be 7.45 mg GAE/g) [58]. On the other study of the chemical profile of black tea in Pakistan with the highest value of 11.5 ± 0.15 mg GAE/g) was recorded in ethanol followed by methanol (7.22 ± 0.12 mg GAE/g) while the lowest (3.54 ± 0.05 mg GAE/g) in water extract [24]. Moreover, according to reports from Malaysia [58] and Turkey [59] on the variation of the content of TPC of tea samples were varies with maturity level of the leaves and seasonal variation respectively. Thus, from the aforementioned reports, it is possible to conclude that variation in geographical origin have significant influence on the accumulation of phenolic compounds in tea in addition to other factors mentioned above.

4.4. Result for method of validation

4.4.1. Accuracy

The accuracy or validity of the optimized procedure was assessed by spiking experiments. For this purpose 40 mg/L. Gallic acids was prepared from 1000 mg/L. Then 40mg/L was added on each concentration of sample (wush-wush, Black lion and Gumaro) which optimized the result for accuracy showed that a percentage recovery ranges from 93.5-105% .This value are in the literature range 80-120%.This indicates the optimized/proposed method is accurate/valid for determination of TPC of black tea by heat extraction method.

Table 4. Results of recoveries test for optimized procedure of three black tea (n=3)

Gallic acid added (mg/L)	**Recovery (%) in wush-wush**	**Recovery (%) in Black lion**	**Recovery (%) in Gumaro**
40	99.4	105.0	99.9
40	98.0	93.5	99.6
40	103.3	95.4	98.7

4.4.2 Limit of detection and Limit of quantification

Limit of detection is the lowest concentration of analyte that can be detected and calculated from three times standard deviation of blank divided by slope. Limit of quantification is the lowest concentration level at which the measurement quantitatively detected and calculated from ten times the standard deviation of the blank and divided by slope and the result shows 1.13 and 3.8 mg GAE/g respectively

Table 5. Result of linearity, Limit of detection (LOD), and limit of quantification (LOQ) from calibration curve.

Parameter	Gallic acid (standard)
Concentration	10-60 mg/L
Slope	0.011
Y-intercept	0.018
R^2	0.997
LOD in mg/g	1.3
LOQ in mg/g	3.8

4.5. Comparison of the present study with literature values

The present study in the black lion and Gumaro tea brand has higher phenolic content than the previous study but wush-wush tea has a little less TPC value than the previous study as shown in table 6 this is may be due to extraction technique and reagent used. For the present study water bath heat extraction technique was used but the other study used maceration techniques. Also for the present study folin-ciocalteu's reagent is used but the previous study used folin-Dennis reagent. The other factors which affect the yield may be extraction time, temperature and solvent ratio [39].

Table 6. Comparison of the present study with literature values from Ethiopia

Tea brand	Present study TPC in mg GAE/g mean ± SD	Other study TPC in mg GAE/g mean ± SD	Reference
Wush-wush	23.0±0.24	23.6±0.0.72	[11]
Black lion	26.5±0.82	22.2±0.39	[11]
Gumaro	28.5±0.96	25.0±0.37	[11]

4.6. Statistical evaluation

Statistical analyses of the results were made to verify whether there were significant differences in concentrations of the TPC in the black tea samples. For the present study, the significance of variation between samples has been studied using a Single Factor ANOVA [60]. The three samples were analyzed in triplicate and submitted to ANOVA. At 95% confidence level, significant difference was observed between black lion with wush-wush and Gumaro with ($p<0.05$). But there is no significant difference between wush-wush and Gumaro at the levels of TPC between samples.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The present study was carried out to determine the total polyphenol content in Ethiopian black teas. The three most common and representative teas of the country have been carried out to meet the objectives of the study. The optimization experiments showed that, the best condition for the extraction of phenolic compound where ethanol ratio of 80; 20, extraction temperature 80 ^{o}C and extraction time 15 min. The highest TPC was detected in Gumaro black tea 28.5 ± 0.96 mg GAE/g followed wush-wush black tea 26.5 ± 0.82 mg GAE/g and lastly black lion black tea 23 ± 0.24 mg GAE/g. Even if the samples were belong to the same black tea type, the level of TPC in Black lion sample is significantly different than other black tea sample ($p<0.05$) at 95% confidence interval. The possible reason for this difference could be different ways of tea cultivation, period of harvesting, different processing procedure, fermentation time and other process.The amounts of TPC obtained were found to be higher than the values obtained from literature from Ethiopia except for wush-wush, which was more or less similar to the values found in literature. In general, the optimized extraction condition was appropriate for the determination of TPC with respect to the application of environmentally friendly solvent.

5.2 Recommendation

- ✓ Even if, all phenolic compounds have similar functions like antioxidant, anticancer, anti-aging, etc. they differ on absorption site of body and rate of mentioned functions based on structures and size. So these black teas need further individualizations studies of phenolic compounds.
- ✓ More research should be under taken to determine the proper condition for process optimization of the determination of total phenol content from black tea

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Appendix

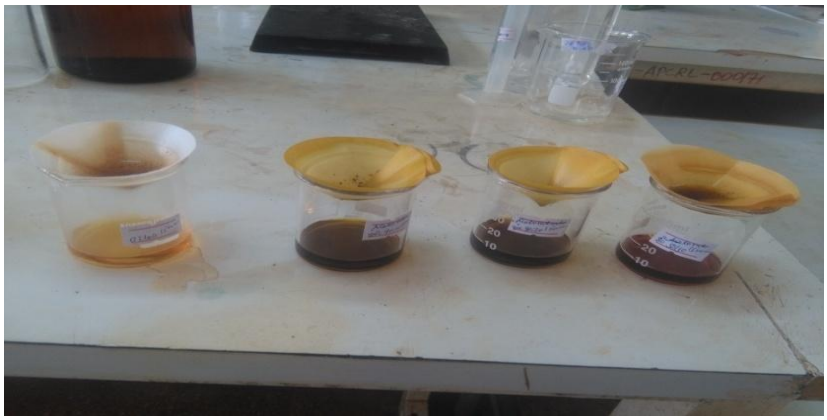
Photos during experimental session

Part I Tea sample

Part II. During heat extraction

Part III. Extracted sample

Part. IV During filtration of extracted sample



Part V.Diluted extracted sample

PartVI. During standard solution preparation

Part VII. Solution prepared from extracted sample