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By

Siyum Shewakena

A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirement for the Degree of Master of Science in Analytical Chemistry.

Advisor: Prof. B.S. Chandravanshi

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Title

Studies on the levels of total polyphenol, flavonoid, tannin and antioxidant capacity of selected Ethiopian fermented traditional beverages

College of Natural Sciences
Department of Chemistry

As thesis research advisor(s), we (I) hereby certify that we (I) have read and evaluated this thesis prepared, under our (my) guidance, by Siyum Shewakena entitled 'Studies on the levels of total polyphenol, flavonoid, tannin and antioxidant activity of selected fermented traditional beverages. We (I) recommend that it be submitted as fulfilling the thesis requirement.

1. Prof. B.S. Chandravanshi	RPC	25/06/15
Research advisor	**Signature**	**Date**

As member of the Board of Examiner(s) of the M.Sc. Thesis Open Defense Examination, we (I) certify that we (I) have read, evaluated the Thesis prepared by Siyum Shewakena and examined the candidate. We (I) recommended that the Thesis be accepted as fulfilling the Thesis requirement for the Degree of Master of Science in Chemistry (Analytical Chemistry).

2. Dr. Nigussie Negash		26/06/2015
Examiner	**Signature**	**Date**
3. Dr. Solomon Mehretie	Solo-eh	25 June 2015
Examiner	**Signature**	**Date**

ABSTRACT. The purposes of this study were to determine total phenolic content (TPC), total flavonoids content (TFC), total tannin content (TTC), and total antioxidant activity (TAA) and to compare these parameters of Ethiopian traditional alcoholic beverages from different origins of Ethiopia within the same sample type and between different sample types. For these purposes, methanolic extracts (70%) were prepared for semi-solid samples. Total phenolic content (TPC) was determined by Folin-Ciocalteu's method, total flavonoids content (TFC) was determined by aluminium chloride ($AlCl_3$) method, total tannin content (TTC) was determined by indirect method precipitating with gelatin, protein while DPPH radical scavenging activity method was used to determine the total antioxidant activity (TAA). The total phenolic content (TPC) obtained in gallic acid equivalent, GAE mg L^{-1} was: *korefe* (285-326), *tej* (369–390) and *tella* (435–459); the total flavonoids content (TFC) obtained in catechin equivalent, CE mg L^{-1} was: *korefe* (190-199), *tej* (183–190) and *tella* (211–216); the total tannin content (TTC) obtained in tannic acid equivalent, TAE mg L^{-1} was: *korefe* (19.9–25.9), *tej* (17.9–19.4) and *tella* (28.8–30.8) and the total antioxidant activity (TAA) obtained in ascorbic acid equivalent, AAE mgL^{-1} was: *korefe* (479–498), *tej* (465–479) and *tella* (541–561). *Tella* exhibited a highest scavenging effect and the reducing capability on DPPH solution absorbance compared to others. Similarly, total phenolic content (TPC), total flavonoids content (TFC) and total tannin content (TTC) also showed that *tella* had the highest content than the rest beverages. Statistically, Pearson correlation showed there were positive correlations between total phenolic content (TPC), total flavonoids content (TFC) and total tannin content (TTC) with antioxidant capacity assayed by DPPH radical scavenging assay ($r = 0.969$, $r = 0.931$ and $r = 0.944$, respectively). In brief, all phenolic parameters measured and antioxidants were highly remarkable in the sequence of *tella* > *korefe* > *tej*. At 95% confidence level one way ANOVA displayed that the levels of total polyphenol, flavonoid, tannin and antioxidant activity are significantly different between unlike sample types and the magnitude of these bioactive chemicals are in comparable amount with the few beverages reported in the literature.

KEY WORDS: Polyphenol level, Flavonoid level, Tannin level, Antioxidant capacity, Ethiopian traditional alcoholic beverages

Dedication

This Thesis is

Dedicated to my families and friends who have remarkable offer throughout my educations.

Declaration

First, I affirm that this thesis is my bonafide work and that all sources of materials used for this thesis have been accordingly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for the Degree of Master of Science in Chemistry (Analytical Chemistry) at Addis Ababa University. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate

Name: Siyum Shewakena

Signature: _____

Date: June / 2015

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Above all things, I have to first thank God for making it possible for me to finish this Degree of Master of Science.

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I also wish to convey my cordial thanks to everybody who has a hand in my accomplishment without mentioning each.

My most sincere appreciation goes to my parents who have disciplined me and nurtured me throughout life to give me strength and tools to achieve all the goals I set for myself.

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ABBREVIATIONS

AAE	Ascorbic acid equivalent
CE	Catechin equivalent
Df	Degree of freedom
DNA	Deoxyribose nucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
FCR	Folin-Ciocalteu reagent
GAE	Gallic acid equivalent
I	Inhibition
MS	Mean square
ROS	Reactive oxygen species
RI	Refractive index
RSD	Relative standard deviation
SD	Standard deviation
SG	Specific gravity
SS	Sum of squares
TAE	Tannic acid equivalent
TPC	Total polyphenol concentration
UV-Vis	Ultra Violet-Visible
V/V	Volume to volume ratio

1. INTRODUCTION

In all over the world, some types of alcoholic beverage native to their region are prepared and consumed [1]. Indigenous fermented alcoholic beverages from different parts of the world are described. Among these, information on the microbiology and biochemical properties of varieties of the indigenous African fermented alcoholic beverages is available. These include Egyptian *bouza*, Tanzanian *wanzuki*, *gongo*, *tembo-mnazi* and *gara*, Nigerian *palm-wine*, Kenyan *muratna* and *uragela*, and South African *kaffir* beer [2]. Similarly in Ethiopia, some of indigenous fermented beverages include *borde*, *korefe*, *shamita*, *tej* and *tella* which are very popular traditional drinks. *Borde* and *shamita* are mainly prepared in central and southern Ethiopia [3]. Whereas considering popularity, traditional alcoholic beverages namely, '*katikala' (areki*), *korefe* and *tella* are very common in northern part of Ethiopia. Fermented beverages vary considerably in type. Fermented beverages produced from cereals usually referred to as beers while those produced from fruits are classified as wines [4].

Among fermented foods, alcoholic beverages have been widely consumed by people around the world. Fermented products can play an important role, contributing to the livelihoods of rural and perturbing dwellers [5]. In developing countries, traditional fermentation serves many purposes. It can improve the taste of food, enhance the digestibility of food, preserve food from degradation by noxious organisms, and increase nutritional values. Further, it is used for medical reasons, recreational purposes, in marriages, in religious and non-religious ceremonies, at festivals and social gatherings, at burial ceremonies and as food substitutes [6].

One of the effective and most economical method of processing foods and beverages acceptable to man is fermentation. The method is inexpensive, easily acceptable, and adaptable at household level in traditional communities [4]. Fermentation processes enhance the nutritional quality of raw ingredient by improving the digestibility of nutrients and inactivating anti-nutritional factors [7]. It also improves acceptability of the food by destroying undesirable flavors of the raw ingredients [8].

The Ethiopian alcoholic beverages alcoholic levels [9-11], physico-chemical properties [9, 11, 12], preparation procedure [9-11, 13-15] and there types [9, 10] were studied. However, there is no any report on the total polyphenol, total flavonoid, total tannin and total antioxidant capacity of Ethiopian alcoholic beverages. So the aim of this work was to establish the chemical profile of *korefe*, *tej* and *tella* from selected area of Ethiopia belonging to the traditional beverage family. This study was designed because little scientific interests up to now have aroused about the biochemical activities of highly and widely consumed beverages namely *korefe*, *tej* and *tella* (Figure 1).

Korefe *Tej* *Tella*

Figure1. Pictures of *korefe*, *tej* and *tella*.

1.1. Statement of the problem

Despite the importance of information on chemical composition for the efficient control and improvement of *korefe*, *tej* and *tella* beverage quality, the chemical composition of these beverages currently used in the main production area of Ethiopia, like Shoa, Gojam and Gondar has not been extensively assessed and documented for any of the chemical compounds that are decisive for the production of high quality, disease resistant cultivars. Additionally, the level of beneficial chemical constituents has not been broadly assessed for the consumer's health. The objective of this study was to establish the chemical profile of *korefe*, *tej* and *tella* of selected area of Ethiopia. This study was designed because little scientific interests up to now have aroused about the bioactive chemicals of highly and widely consumed beverages namely *korefe*, *tej* and *tella.*

1.2. Purpose of the study

Current research findings will reveal that beverages have properties that improve running endurance, and decrease body fat composition while increasing one's energy expenditure, reduce mortality caused by degenerative diseases [8]. These findings point to various polyphenols, flavonoids, tannins and total antioxidant in many beverages as a causative factor for these beneficial results. However, there is no any research on Ethiopian traditional beverage to support this assumption. Therefore, this research is designed to determine total polyphenol, flavonoids, tannins and total antioxidant levels in selected Ethiopian traditional beverage. The findings will give credence to the hypothesis that described beverages have beneficial qualities. Consumers will then be able to brew their beverage with successful efforts in obtaining the greatest concentrations of health beneficial.

1.3. Objectives of the study

1.3.1. General objective

The main objective of this research work was to determine the levels of bioactive chemicals in Ethiopian traditional beverages based on UV-Vis spectroscopy techniques.

1.3.2. Specific objectives

The specific objectives of this research works are:

1. To optimize methods for the determination of total polyphenol, flavonoids, tannins and total antioxidant capacity in *korefe*, *tej* and *tella* based on UV-Vis spectroscopy methods.
2. To determine the levels of total polyphenol, flavonoids, tannins and total antioxidant capacity in *korefe*, *tej* and *tella* based on UV-Vis spectroscopy methods.
3. To investigate the variations of total polyphenol, flavonoids, tannins and antioxidant capacity between selected beverage of Ethiopia due to geographical variations.
4. To compare the levels of mentioned bioactive chemicals of these traditional beverages with other beverages from literatures.
5. To provide baseline information and make recommendation for the future on these beverages.

2. LITERATURE REVIEW

2.1. History of beverages

The history of alcohol in the ancient world dates back to before recorded time. Although no one knows when beverage alcohol was first used, it was presumably the result of accident that occurred at least ten thousands of years ago. Alcoholic beverages have been widely consumed since prehistoric times by people around the world, seeing use as a component of the standard diet, for hygienic or medical reasons, or recreational purposes, and for other reasons [16]. According to the report of Oladeinde [17], however, alcoholic beverages have been used since the landing of pilgrims.

The oldest alcoholic drinks were fermented beverages of relatively low alcoholic contents [18]. Before the advent of the distillation technique as introduced into Europe by Arabs, the oldest alcoholic drinks were fermented beverages, such as beers and wines. The development of distilled alcoholic beverage was associated with the advent and improvement of distillation techniques [19].

Fermentation is a widely practiced ancient technology and fermented foods are an essential part of diets in all regions of the world. Traditional fermented beverages are those that are indigenous to a particular area and have been developed by the local people using an age-old techniques and locally available raw materials [20]. Practically, every civilization has developed some type of fermented food and beverage. The early men probably used fermented beverage as a substitute for safe water (free from pathogens). Such beverages are usually non-intoxicating if consumed during the early stage of fermentation, as the alcohol level will still be low [18].

2.2. Ethiopian traditional fermented beverages

In Ethiopia, villagers prepare a wide range of traditional fermented beverages from different raw materials such as cereals, honey, hop, milk, etc. Some of the known Ethiopian traditional fermented beverages are *ergo, tella, tej, areki, borde, shamita, korefe, keribo* and *kineto*. These products, if properly exploited, could be of significant economic importance for the country. Most of the customs and rituals involving the Ethiopian traditional fermented beverages are still

prevailing today in urban areas, village communities and rural households [21]. From stated Ethiopian traditional beverages, this research focuses on the following beverages which were prepared from raw materials of local hop, honey, water and different cereals like barely, sorghum, maize, wheat, rice, malt.

2.2.1. Korefe

Korefe is the name of the local beer made in Begemder (Gondar) province among the Koumant ethnic group [22]. It is a beverage prepared for sales, holidays and different ceremonials from local hop, water, malt of barely and raw barely after processing.

Korefe **brewing**: First barely, malt of barely and leaf of hop are bought from market, then the barely under goes cleaning by hand peaking, cooking followed by sieving and drying of the barely on the sun; after cuisine on *mithad*, dehusked with *mukrcha*, subsequently that toasted and milled then after baked on *mithad* to prepare *derekot*. *Tinsis* is prepared by mixing of pounded malt of barely and leaf of local hop in the ratio of (2:1) with water and withstand for five days in a pot which is seasoned by smoking over smoldering local hop stems, *tinjut* and olive after cleaning by washing. *Kita* is prepared and mixed with the *tinsis* then tolerate for a day or over until foam is observed by properly covering. Here is the time to mix the fermented result with *derekot* which gives *difdif* in the big pot. Finally, after three days the *difdif* is mixed with water and ready for consumption.

2.2.2. Tej

Tej is a home-processed, but also commercially available honey wine. It is a beverage mainly used for great feasts, such as weddings and the breaking of fasting. It is a prestige beverage, and more expensive than the local beer. It is prepared from honey, water and local hop (*Rhamnus prenoides*). Sometimes, widely for commercial purposes, mixture of honey and sugar could be used for its preparation. In cases where sugar is used as part of the substrate, natural food coloring is added so that the beverage attains a yellow color similar to that made from honey [23]. Some people also add different concoctions such as barks or roots of some plants or secrete herbal ingredients to improve flavor or potency and to attract customers. Due to concoction, adulteration practices and possibly some other reasons, producers usually are not willing to tell

about additives used and their composition [24]. The total alcohol levels of *tej* were varied between the values of 8.94 to 13.16% (v/v) [11] and 2.7 to 21.7% (v/v)[25].

***Tej* brewing process**: The fermentation pot is seasoned by smoking over smoldering local hop stems and olive wood after cleaning by washing. Honey was mixed with water in 1:4-6 (v/v) proportions and is placed in the pot, covered with a cloth for 2 to 3 days to ferment after which wax and top scum is removed by sieving. Some portion of the filter is boiled with washed local hop and positioned back to the fermenting pot. The pot is covered and fermented continuously for farther 5 days, in warmer weathers, or for 10-12 days, in colder cases. The mixture is stirred daily and finally filtered through cloth to remove residue and *Rhamnus prenoides*. The local hop stems are heated on the *mitad* and added to the mixture, which is left to fermentation in a closed container for 5-6 days. Then after, it is ready for consumption.

2.2.3. Tella

Tella is one of the Ethiopian traditional beverages, which is prepared from different ingredients. It is, by far, the most commonly consumed alcoholic beverage in Ethiopia. It is assumed that over two million hectoliters of *tella* is brewed annually in households and drinking houses in Addis Ababa [26]. *Tella* is widely brewed and consumed in both rural and urban part of Ethiopia. It is well known as local beer [27] since it is malt based beverage like that of commercial beer [26]. Generally, *tella* is brewing from substrates such as barley, wheat, maize, millet, sorghum, *teff* or other cereals. Commercial beer is mostly made from malted barley and adjunct like corn, rice or wheat provide the carbohydrate substrates for ethanol production by *Saccharomyces carlsbergensis* [28]. With regard to the substrate, there is no as such basic difference between *tella* and beer. The alcoholic level of *tella* is between 3.5 to 6.48% (v/v) [11].

***Tella* brewing process:** *Tella* is made of barley, wheat, maize, sorghum, *teff* or other cereals and water. The brewing process differs as between the ethnic groups and depends on tradition and the economic situation. But according to the tradition of sample site selected in this study, malt of barley or wheat; bought in the local market or prepared at home, by steeping of the grains in water for 3-4 days and the moisten grains are separate by sieving and placed between fresh

leaves of *gullo* in depth of 7-12 cm at the ground, left to germinate for 3 days and after that dried and milled. Local hop is available in the market. The hop is dried again in the sun; the leaves are separated from the stems, which need a longer time to dry, and after that ground separately. The clay container (pot) is washed with *grawa* and water several times and after that smoked with wood from *weyra* (olive), and/or *tinjute* for about 12 min., in order to get it as clean as possible. The ground *gesho* leaves are placed in a clay container with water and left to ferment for 3 days. Some of the grains intended for *tella* preparation are toasted and milled, and then mixed with water and baked on the *mitad*. This *kita*, broken into small pieces, part of the milled malt and the ground *gesho* stems are added to the water mixture and allowed to ferment for 2-3 days. Then the flour of *asharo* is sprinkled with water and toasted on *mitad* until dark brown. This mixture *enkuro*, the fermented (*tinsis*), flour of malt, some grounded *gesho* and water are added to the big pot container. The mixture is kept covered for three days, after which more water is added and the container is kept sealed for 5-7 days, then after, the beverage is ready for consumptions.

In this research, sampled traditional beverages have local hop as a common ingredient that has the characteristics of bittering (to balance the sweetness of the malt), flavoring and aroma imparting agent due to its essential oils, bacteriostatic activity to inhibit the growth of most microorganisms [26]. The chemical substances such as emodin, physcion, rhamnazin, prinoidin, and many other emodin-derived compounds were reported from *Rhamnus prinoides*. Among different chemical substances found in *Rhamnus prinoides*, naphthalenic glucoside, geshoidin is the basic bittering agent for beverages [29]. In general hop is a major source of *p*-coumaric, caffeic and ferulic acids which are better antioxidant [30].

2.3. Chemical components of beverages

As pointed out above, these traditional beverages are prepared by fermentation from different cereals, malt, honey, hops and water. Hops are crucial as a source of bitterness (from hop resins), aroma (from the essential oils) and antioxidant-rich materials; malt also contains a range of flavonols (monomeric, dimeric, and trimeric flavonols) and higher molecular weight flavonoid tannins [31]. As a result, many minerals, volatile and non-volatile organic compound can be a chemical component of these beverages. But this research was designed to investigate chemical composition with emphasis to phenolic compounds in the groups of the beverages because

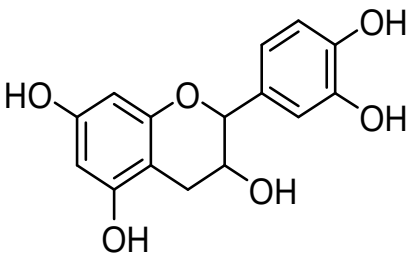
polyphenols have recently aroused considerable interest as a result of their potential beneficial biochemical and antioxidant effects on human health.

Different researches are conducted in different beverages to determine the levels of total polyphenol, flavonoid and antioxidants. Total phenolic compounds in liqueurs made from red fruits [8], wine [32-38], beer [39-41], traditional fermented sorghum beers “dolo” [33, 42], alcoholic beverages made from purple rice [43], ciders [44]; anthocyanin’s in liqueurs made from red fruits [8], dolo [42] and wine [33-35, 38]; total flavonoids in wines [20, 33, 34, 38]; total tannin [33, 34, 45] and antioxidants capacity in liqueurs made from red fruit [8], wine [33-38], beer [41]were determined.

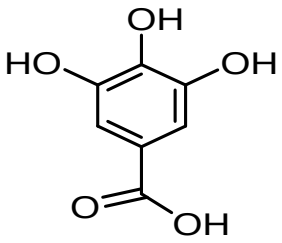
2.3.1. Polyphenol

Polyphenols 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 [12].Although polyphenols are chemically characterized as compounds with phenolic structural features, this group of natural products is highly diverse and contains several sub-groups of phenolic compounds. Fruits, vegetables, whole grains and other types of foods and beverages such as tea, chocolate and wine are rich sources of polyphenols. 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 and acylated sugars at different positions of the polyphenol skeletons [46].

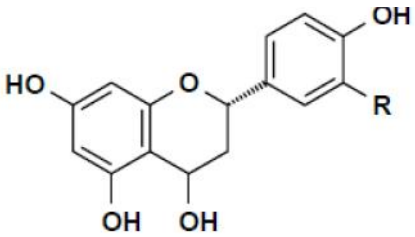
Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or violence by pathogens. In food, polyphenols may contribute to the bitterness, astringency, color, flavor, odor and oxidative stability. Polyphenols are the subject of increasing scientific interest because of their possible beneficial effects on human health [46]. Examples of polyphenol compounds are shown in Figure 2.



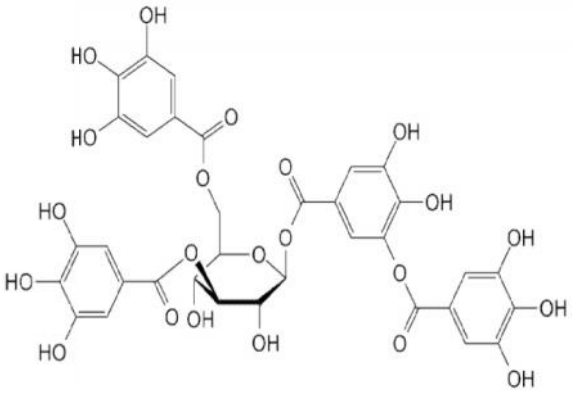
Catechin



Gallic acid



Flavan-4-ol

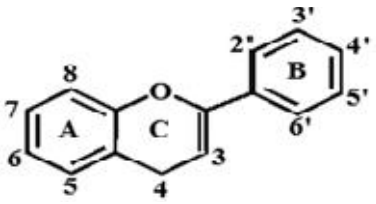


Tannic acid

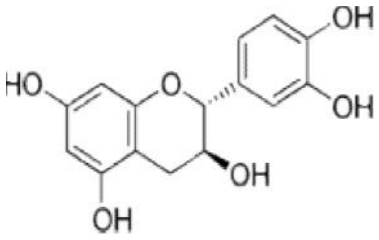
Figure 2. Examples of polyphenol compounds structure.

2.3.2. Flavonoids

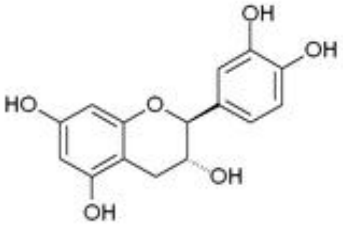
Flavonoids comprise the most studied group of polyphenols. Flavonoids have the C6–C3–C6 general structural backbone in which the two C6 units (ring A and ring B) are of phenolic nature (Figure 3). Due to the hydroxylation pattern and variations in the chromane ring (ring C), flavonoids can be further divided into different sub-groups such as flavan-3-ols, flavones, flavanones and flavonols. While the vast majority of the flavonoids have their ring B attached to the C2 position of ring C, some flavonoids such as isoflavones and neoflavonoids, whose ring B is connected at the C3 and C4 position of ring C, respectively, are also found in plants. These basic structures of flavonoids are aglycones; however, in plants, most of these compounds exist as glycosides. Biological activities of these compounds, including antioxidant activity, depend on both the structural difference and the glycosylation patterns. Quercetin, myricetin, Catechins, etc. are some most common flavonoids [47].



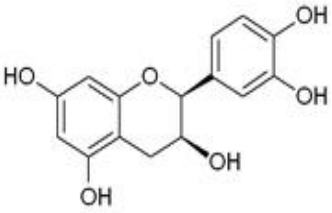
Basic flavonoid structure



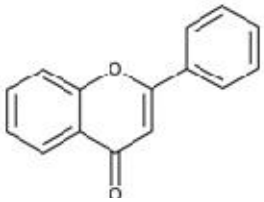
(+)-Catechin (2R, 3S)



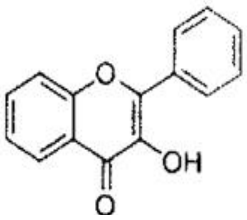
(-)-catechin (2*S*, 3R)



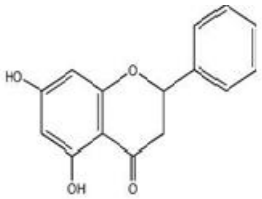
(+)-epicatechin (2*S*, 3S)



Flavones



Flavonols



Flavanones

Figure 3.Basic structure and examples of flavonoids.

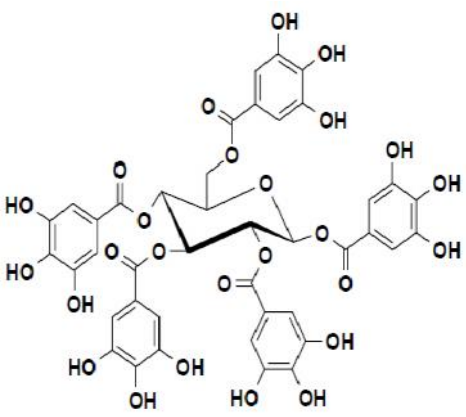
2.3.3. Tannin

Tannins are phenolic compounds of molecular weight from intermediate to high (500-3000 Dalton) and can be classified into two major groups: hydrolysable tannins and non-hydrolysable or condensed tannins. The hydrolysable tannins have a center of glucose or a polyhydric alcohol partially or completely esterified with gallic acid or ellagic acid, forming gallotannin and ellagitannins, respectively. These metabolites are readily hydrolyzed with acids, bases or enzymes. The condensed tannins are polymers of catechin and/or leucoanthocyanidin, not readily hydrolyzed by acid treatment, and constitute the main phenolic fraction responsible for the characteristics of astringency of the vegetables [28].

Tannins are astringent, bitter polyphenols that either bind and precipitate or shrink proteins. The astringency from the tannins is that which causes the dry and pucker feeling in the mouth

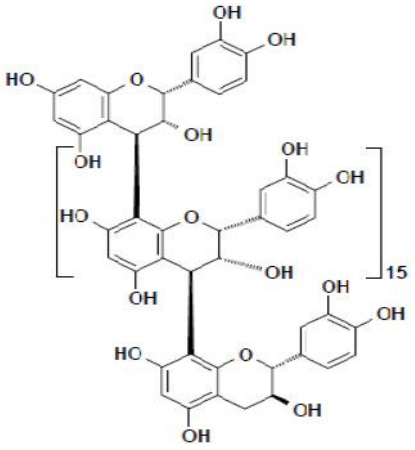
following the consumption of red wine, strong tea, or an un-ripened fruit. High amounts of tannins are in the hops of some beers. The amount of tannins in beer depends on quality of yeast along with other factors. The more bitter the taste, the more tannin, less bitter the taste, the less tannin [48].

Chemical structure of tannins consists of multiple adjacent polyhydroxyphenyl groups. This structure of tannins gives opportunity to bond with macromolecular compounds such as proteins, metal ions and polysaccharides [49]. Examples of tannins are shown in Figure 4.



A

Pentagalloyl glucose (gallotannins)



B

Procyanidin (polymer of catechins)

Figure 4. Examples of hydrolysable tannins (A) and condensed tannin (B).

2.3.4. Antioxidant

Antioxidants may be defined as compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. Antioxidants can also protect the human body from free radicals and reactive oxygen species (ROS) effects. They retard the progress of many chronic diseases as well as lipid peroxidation. Also, antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods. Food antioxidants such as ascorbic acid, amino acids, proteins, flavonoids and other phenolic compounds might also play a significant role as physiological and dietary antioxidants, there by supplementing the body's natural resistance to oxidative damage [21].

The following diagram shows that antioxidant species like polyphenol, ascorbic acid, amino acids, etc. are giving an electron for free radicals from meals, by product of meals which can react /complexes with essential nutrients such as menials and others.

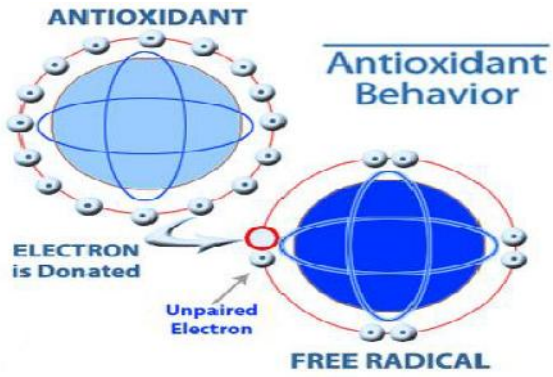


Figure 5. Free radical molecule [50].

2.4. Bioavailability of polyphenols

Bioavailability is the proportion of the nutrient that is digested, absorbed and metabolized through normal pathways. Bioavailability of each and every polyphenol differs however there is no relation between the quantity of polyphenols in food and their bioavailability in human body. Generally, aglycones can be absorbed from the small intestine. Importantly it is the chemical structure of polyphenols and not its concentration that determines the rate and extent of absorption and the nature of the metabolites circulating in the plasma. The most common polyphenols in our diet are not necessarily those showing highest concentration of active metabolites in target tissues; consequently the biological properties of polyphenols greatly differ from one polyphenol to another. Evidence, although indirect, of their absorption through the gut barrier is given by the increase in the antioxidant capacity of the plasma after the consumption of polyphenols rich foods [15].

Polyphenols also differs in their site of absorption in humans. Some of the polyphenols are well absorbed in the gastro-intestinal tract while others in intestine or other part of the digestive tract. In foods, all flavonoids except flavanols exist in glycosylated forms. Most of the glycosides probably resist acid hydrolysis in the stomach and thus arrive intact in the intestine where only aglycones and few glucosides can be absorbed. The absorption at gastric level is possible for

some flavonoids, such as quercetin, but not for their glycosides. Moreover, anthocyanins are absorbed from the stomach [15].

Condensed tannins differ from most of other plant polyphenols because of their polymeric nature and high molecular weight. This particular feature should limit their absorption through the gut barrier, and oligomers larger than trimers are unlikely to be absorbed in the small intestine in their native forms [46].

Though most of the polyphenols get absorbed in gastrointestinal tract and intestine but there are some polyphenols which are not absorbed in these locations. These polyphenols reach the colon, where microflora hydrolyze glycosides into aglycones and extensively metabolize these aglycones into various aromatic acids [15].

2.5. Polyphenols and human diseases

Epidemiological studies have repeatedly shown an inverse association between the risk of chronic human diseases and the consumption of polyphenolic rich diet [51]. The phenolic groups in polyphenols can accept an electron to form relatively stable phenoxyl radicals, thereby disrupting chain oxidation reactions in cellular components [52]. It is well established that polyphenol rich foods and beverages may increase plasma antioxidant capacity. This increase in the antioxidative capacity of plasma following the consumption of polyphenol rich food may be explained either by the presence of reducing polyphenols and their metabolites in plasma, by their effects upon concentrations of other reducing agents (sparing effects of polyphenols on other endogenous antioxidants), or by their effect on the absorption of pro-oxidative food components, such as iron [51]. Consumption of antioxidants has been associated with reduced levels of oxidative damage to lymphocytic DNA. Similar observations have been made with polyphenol rich food and beverages indicating the protective effects of polyphenols. There are increasing evidences that as antioxidants, polyphenols may protect cell constituents against oxidative damage and, therefore, limit the risk of various degenerative diseases associated with oxidative stress [15].

From different effects of polyphenolic compound families, cardio-protective effect (limits the incidence of coronary heart diseases), anti-cancer effect (most often protective and induce a reduction of the number of tumors or of their growth), anti-diabetic effect (affect glycemia through different mechanisms, including the inhibition of glucose absorption in the gut or of its uptake by peripheral tissues), exert preventive effects in treatment of asthma. Supplementation of diet with genistein, daidzein or their glycosides for several weeks prevents the loss of bone mineral density and trabecular volume and polyphenols also protect skin damages induced from sunlight are studied [15]. However, few investigators have examined their possible toxicity, but no acute toxicity was observed after oral administration of a grape seed proanthocyanidin extract at a dose of 0.5 or 2 gkg^{-1} body weights to rats or mice or after administration of an ellagitannin present in pomegranate juice at a dose of 60 gkg^{-1}diets to rats [53].

So as mentioned functions of polyphenol and variations on the absorption site of them in our body based on their structure and size triggered us to determine the levels of total polyphenol, total flavonoids, total tannins and total antioxidant activities in our vending fermented beverages which were very prehistoric.

2.6. Analytical methodologies for the analysis of the analytes

2.6.1. Determination of total polyphenols

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 lab [54]. 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 [55] which react with Folin-Ciocalteu phenol reagent (an oxidizing agent comprised of heteropolyphosphotungstate-molybdate) under basic conditions to form chromogens that can be detected spectrophotometrically at 760 nm. The reaction forms a blue chromophore constituted by a phosphotungsticphosphomolybdenum complex, Folin and Ciocalteu included lithium salts in the reagent, which prevented the turbidity due to excess Folin-ciocalteu reagent [56]. 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, spectrophotom- etrically measured [57].

2.6.2. Determinations of total flavonoids

The flavonoid content was determined according to mostly applied spectrophotometric methods based on the formation of aluminium-flavonoid complexes; complexation reaction is carried out in the presence of $NaNO_2$ in alkaline medium [58]. Total flavonoid content was determined by aluminium chloride method, using catechin as a standard, 5% sodium nitrite, 10% aluminium chloride, 1 M sodium hydroxide were used as a reagent. Then after appropriate dilution was made with distilled water, the reaction mixture was measured at 415 nm against a blank spectrophotometrically [59]. Here the method is based on the nitration of any aromatic ring bearing a catechol group with its three or four positions unsubstituted or not sterically blocked. After addition of Al(III), a yellow solution of complex was formed, which then turned immediately to red after addition of NaOH, 5% $NaNO_2$was added because the complexation of Al(III)with flavonoid, in the presence of $NaNO_2$in alkaline medium was very selective.1M sodium hydroxide was used for neutralization of the mixture [58].

2.6.3. Determination of tannins

Tannin was determined by indirect method, precipitating with gelatin at the right temperature, pH and ionic strength. The tannin-protein complexes readily precipitate under the right conditions of pH, ionic strength, solvent strength, and temperature [60]. The tannin was determined from the mean absorbance difference of total polyphenol and non-tannin polyphenol with the line equation of tannic acid standard curve. Total polyphenol and tannin free polyphenol were determined by Folin-Ciocalteau methods.

The sample blank was obtained as follows. The sample solution was pipetted into a 100 mL beaker containing of gelatin solution. To the mixture were added acidic sodium chloride solution followed by kaolin and the whole was shaken for several minutes. The precipitate was allowed to settle and the mixture was filtered. Then, the filtrate, distilled water, gelatin solution and acidic sodium chloride solution were pipetted into a 100 mL beaker followed by the addition of kaolin.

After shaking for several minutes, the mixture was filtered and the filtrate was treated as described under calibration graph [45].

The procedure for determining the gelatin blank was the same as that for the sample blank except that distilled water was used instead of the sample solution. The difference in absorbance between the sample blank and the gelatin blank gave the net sample blank. The difference in absorbance between the sample and net sample blank was due to tannins in the sample and their concentration was deduced from the calibration graph [45].

2.6.4. Determination of total antioxidant activity

Radical scavenging activities are very important due to the deleterious role of free radicals in foods and in biological systems. Diverse methods are currently used to assess the antioxidant activity of plant phenolic compounds. But DPPH radical scavenging methods are common spectrophotometric procedures for determining the antioxidant capacities of components because of DPPH radical is the stable chromogen compounds to be measured with UV-Vis spectroscopy [34], the violet DPPH radical is easy to use, have a high sensitivity, and allow for rapid analysis of the antioxidant activity of a large number of samples, inexpensive and provides firsthand information on the overall antioxidant capacity of the test system [21].

In the DPPH assay, the antioxidants were able to reduce the stable radical DPPH to the yellow colored diphenyl-picrylhydrazine. The method is based on the reduction of alcoholic DPPH radical solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH–H by the reaction. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [61].

With this method it was possible to determine the antiradical power of an antioxidant by measuring of a decrease in the absorbance of $DPPH^{\cdot}$ at 517 nm. The resulting color change from purple to yellow, the absorbance decreased when the $DPPH^{\cdot}$ was scavenged by an antioxidant through donation of hydrogen to form a stable DPPH-H molecule. In the radical form, this molecule had an absorbance at 517 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule [21].

Spectrophotometry was used and the level of the mixture of antioxidants was determined with ascorbic acid equivalent. The DPPH radical scavenging activity in terms of percentage was calculated according to the following equation [59].

$$\text{Percent inhibition} = [(A_{DPPH} - A_{sample})/A_{DPPH}] \times 100\%$$

Where: A_{DPPH} = Absorbance of DPPH, A_{sample} = Absorbance of sample (sample/ascorbic acid).

3. EXPERIMENTAL

3.1. Samples, sample collection and preparation

The sample types were selected based on universality in consumption from adolescent to agers, widely available on the market and household level than the rest of traditional beverages. Similarly sample areas were selected due to considerations of widely fermentations and consumptions areas of sampled beverages. Though, *korefe* is mainly familiar in the area of North Gondar, accordingly representative samples were purchased from three different localities namely Chilga, Gondar and Woreta. From each sample area, five representative samples were collected from different vending houses (0.5 L from each) which were selected randomly to prepare a bulk sample by mixing because instead of analyzing separately, analyzing of the mixture reduces the variance and resource consumption and triplicate analysis was performed for each. For *tej* and *tella* samples, three sample areas (Debre-Birhan, Debre-Markos and Gondar city) were selected; from these places five representative samples were purchased for each sample type to have a bulk sample for each type of beverages. From each vending rooms oral information's were gathered about their preparations, raw materials and purpose of the beverage. All the samples were collected using glass amber bottles, then the bulk solutions were kept in fridge at 4 ^{o}C until optimization of procedure was conducted and the sample to be analyzed was prepared. The study was planned to determine bioactive chemicals and to explore chemical variations in the *korefe*, *tej* and *tella* caused by differences in the geographical variations of fermented beverages regions in Ethiopia. Geographical locations of the sample areas are given in Table 1.

Table 1. Geographical location of the sample areas.

Sample area	Longitude	Latitude	Altitude in m	Distance from Addis Ababa in km
Chilga	37^{o}4'1"E	12^{o}33'0"N	2,146	784
Debre-Birhan	39^{o}31'59"E	9^{o}40'59"N	2,840	130
Debre-Markos	37^{o}43'47"E	10^{o}20'1"N	2,446	306
Gondar	37^{o}37'28"E	12^{o}12'39"N	2,133	740
Woreta	37^{o}42'0"E	11^{o}55'1"N	1,810	625

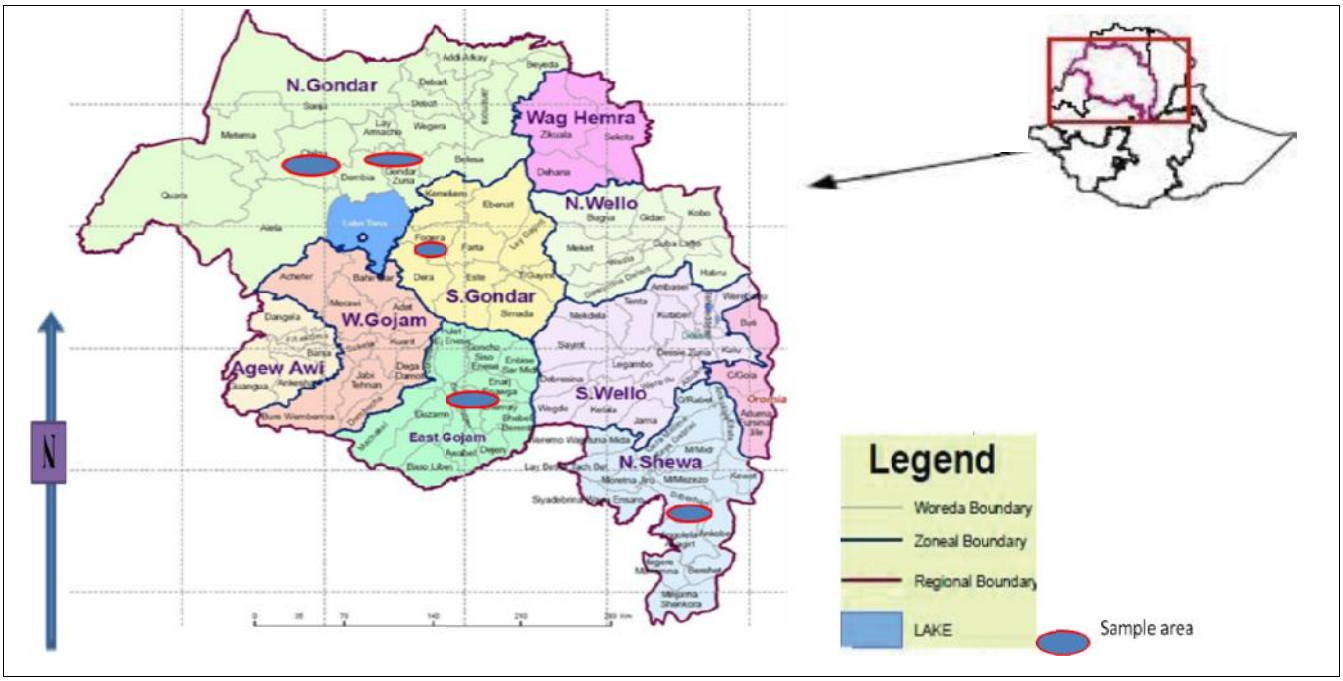


Figure 6. Map of Ethiopia showing the study area. (Source http://www.ochaeth.org/Maps/ Downloadable/Amhara.pdf accessed on 30 January 2015).

3.2. Materials, chemicals and methods of analysis

3.2.1. Materials

Volumetric flask, test tube, measuring cylinder, beaker, micropipette, funnel, weighing machine, brown glass amber bottles, Whatmann filter paper, safety goggles, safety dust mask, glove, cuvette, magnetic stirrer, refrigerator(Hitachi, Tokyo, Japan), oven (Griffin and George Ltd, Britain),centrifuge Plc series (K Gemmy industrial Corp., Taiwan), ion meter (Barloworld scientific Ltd, United Kingdom), conductor meter, Abbee Refractometer (ATAGO, USA)and UV-Visible spectroscopy(Lambda 950, Perkin Elmer, and UK) were used during experimentations.

3.2.2. Chemicals

All the reagents used were of analytical grade reagent and the water was distilled. Sodium molybdate dehydrate and sodium tungstate (BDH Laboratory Supplies, Poole, England); lithium sulfate, ascorbic acid and gelatin (BDH Chemicals Ltd, Poole, England); phosphoric acid, 2,2-

diphenyl-1-picrylhydrazyl (DPPH),gallic acid, catechin, aluminium chloride, sodium nitrite, tannic acid, pH standards, sulfuric acid andsodium chloride (Sigma Aldrich, Steinheim, Germany);sodium carbonate, kaolin and sodium sulfate anhydrous (Research–Lab Fine Chem. Industries, Mumbai, India); sodium hydroxide and methanol (Schartau Chemie S.A. Europian Union, Spain);concentrated hydrochloric acid (Fisher Scientific UK Limited, Bishop Meadow Road, UK), bromine (Guandong Guanghua Chemical Factory Co. Ltd, China)were used as received.

3.2.3. Methods of analysis

3.2.3.1. Analyte extraction

The extraction of bioactive compounds from solid or semi-solid materials is the first step in the utilization of phytochemicals in the preparation of dietary supplements, food ingredients, pharmaceutical, and cosmetic products. Among the samples, *korefe* is semi-solid. So *Korefe* was homogenized with aqueous:methanol solution (30:70%) [62].The extractions were performed in triplicates. The homogenate was stirred with magnetic stirrer at 900 rpm at room temperature for 90 min. Then it was put in centrifuge at 3000 rpm for 20 min. The supernatant was taken by decantation. Finally the supernatant was stored in the fridge until analysis time at 4 ^{o}C.

3.2.3.2. Determination of total polyphenol level

Reagents: Folin-Ciocalteu's phenol reagent preparation: 10 g sodium tungstate and 2.5 g sodium molybdate were dissolved in 70 mL water. 5 mL 85% phosphoric acid and 10 mL concentrated hydrochloric acid were added to the solution, refluxed for 10 h. Then the following were added 15 g lithium sulfate, 5 mL water and 1 drop bromine. Refluxed for 15 min. Cooled to room temperature and brought to 100 mL with water. Hexavalent phosphomolybdic/phosphotungstic acid complexes with the following formula were formed in solution [63].

$$3H_2O{\bullet}P_2O_5{\bullet}13WO_3{\bullet}5MoO_3{\bullet}10H_2O$$
$$3H_2O{\bullet}P_2O_5{\bullet}14WO_3{\bullet}4MoO_3{\bullet}10H_2O$$

Product description: Appearance: Clear bright yellow solution, acidic, Folin-Ciocalteu's phenol reagent was stored tightly capped at room temperature and the reagent was diluted with distilled water as required.
For 10% Na_2CO_3: 10 g sodium carbonate was dissolved in 100 mL distilled water.

Determinations: Total phenolic level was determined by Folin-Ciocalteu's method with some modifications [57]. 0.4 mL of sample/standard gallic acid (100, 250, 400, 550, 700 mgL^{-1}) was positioned into 25.0 mL volumetric flask and 4.0 mL of Folin-Ciocalteu's reagent (1:9) diluted with distilled water was mixed and shaken with hand shake. After 5 min, 4.0 mL of 10% sodium carbonate was added to the mixture for color development and volume made up to 25 mL with distilled water. The analysis of samples was performed in triplicates.

It was allowed to incubate for 90 min at room temperature. Intense blue color was developed. After incubation, absorbance was measured at 760 nm spectrophotometrically against a blank sample that contains all the reagents except the sample that was replaced by distilled water using UV-Visible (Lambda 950, Perkin Elmer, UK) instrument. The spectrum was scanned against blank using spectrophotometer from 1390–400 nm to avoid decomposition of ultraviolet sensitive compounds. The absorbance measurement was performed in triplicate and the average absorption was noted for each sample in triplicate. The calibration curve was plotted using standard gallic acid. Quantitative analysis was done based on the equation of regression line: $y = 0.00121x - 0.0578$ (where y = absorbance (Abs. at $_{max}$ – Abs. at $_{base}$) and x = concentration in mg L^{-1}). The data for total polyphenol contents of beverage were expressed as mg of gallic acid equivalent weight (GAE)/L of beverage.

3.2.3.3. Determination of total flavonoid level

Total flavonoid level was measured with the aluminium chloride colorimetric assay [58]. 1 mL of sample/standard catechin solutions (50,100, 200, 400, 600 mgL^{-1})were positioned into 20 mL volumetric flask, 4 mL of distilled water and 0.3 mL of 5% sodium nitrite solution were added into each flask. After 5 min, 0.3 mL of 10% aluminum chloride was added. At 6^{th} min, 2 mL of 1 M sodium hydroxide was added. Finally, volume was made up to 20 mL with distilled water and mix well. The samples were performed in triplicates.

It was allowed to incubate for 10 min. Orange yellowish color was developed. The absorbance was measured at 415 nm spectrophotometerically using UV-Visible (Lambda 950, Perkin Elmer, UK) instrument against the blank sample that contain distilled water instead of sample and all reagents. The spectrum was scanned from 750–350 nm. The absorbance measurement was performed in triplicate and the average absorption was noted for each sample in triplicate.

Catechin was used as standard. The calibration curve was plotted using standard catechin. Quantitative determination was done based on the equation of regression line: y = 0.00159x+0.049 (where y = absorbance (Abs. at $_{max}$ – Abs. at $_{base}$) and x = concentration in mg L^{-1}). The total flavonoids content of the samples were expressed as mg of catechin equivalent weight (CE)/L of beverage.

3.2.3.4. Determination of total tannin level

Reagents**:** Gelatin solution, 0.3% m/v prepared by soaking 1.5 g of gelatin in 10% sodium chloride solution for 1 h, then warmed to dissolve the gelatin and finally diluted to 0.5 L with 10% (m/v) sodium chloride solution in distilled water after cooling. Acidic sodium chloride solution was prepared by adding 25 mL of concentrated sulfuric acid to 375 mL of 10% sodium chloride solution and 10% NaCl was prepared by adding of 10 g NaCl in 100 mL distilled water [45].

Determination: Calibration graph: 0.4 mL standard tannic acid solutions (12.5, 25, 50, 100 and 200 mgL^{-1}) were pipetted separately into a series of 25 mL calibrated flasks. To each of these flasks 4.0 mL of Folin-Ciocalteu's reagent (1:9) diluted with distilled water was mixed and shaken. Then after 5 min, 4.0 mL of 10% sodium carbonate was added to the mixture for color development and volume was made up to 25 mL with distilled water.

It was allowed to incubate for 90 min at room temperature. Intense blue color was developed. After incubation, absorbance was measured at 760 nm spectrophotometrically against a blank sample that contains all the reagents except the sample that was replaced by distilled water using UV-Visible spectrophotometer. The absorbance measurement was performed in triplicate and the average absorption was noted for each sample in triplicate.

Sample and sample blank determinations: 0.4 mL of the sample solutions were treated as described above. The determinations were performed in triplicates. The sample blank was obtained as follows. A 10 mL volume of the sample solution was pipetted into a 100 mL beaker containing 5.0 mL of gelatin solution. To the mixture were added 10.0 mL of acidic sodium chloride solution followed by 2.0 g of kaolin and the whole mixture was shaken for 15 min. The precipitate was allowed to settle and the mixture was filtered. Then, 10.0 mL of the filtrate, 6.0 mL of distilled water, and 3.0 mL of gelatin solution and 6.0 mL of acidic sodium chloride solution were pipetted into a 100 mL beaker followed by the addition of 2.0 g of kaolin. After shaking for 20 min, the mixture was filtered and 0.4 mL of the filtrate was treated as described under calibration graphs [45]. The difference in absorbance between the sample and sample blank was due to tannins in the sample and their concentration was deduced from the calibration graph.

3.2.3.5. Determination of total antioxidant activity

Spectrophotometric measurements were performed using a UV-Visible spectrophotometer. Antioxidant activity of beverage was determined using the DPPH method [59]. 1 mL sample (1:9) diluted with distilled water was mixed in test tubes with 2 mL of DPPH solution (160 mg L^{-1}DPPH in methanol) and made up to final volume of 6 mL with methanol. The mixture was allowed to react at room temperature in the dark for one hour. The positive control assays were prepared with 2 mL of DPPH solution and 4 mL of methanol only. Methanol was used as a blank. The absorbance was measured by scanning from 850–450 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. Quantification of antioxidant capacity was made by calibration curves obtained from methanolic solutions of ascorbic acid (1:3) diluted; the antioxidant capacity of compounds was expressed as milligram of ascorbic acid equivalent (AAE) using the equation of regression line: $y = -0.0032x + 1.3786$ (where y = absorbance (Abs at $_{max}$ – Abs at $_{base}$) and x = concentration, AAE in mg L^{-1}). The degree of decolorization of DPPH from purple to yellow indicated the scavenging efficiency of the sample. The percentage inhibition of DPPH free radical scavenging activity was calculated using the following equation [64]:

$$\text{Percent inhibition} = [(A_{DPPH} - A_{sample})/A_{DPPH}] \times 100\%$$

Where: A_{DPPH} = Absorbance of DPPH, A_{sample} = Absorbance of sample (sample/ascorbic acid).

3.3. Instruments

Spectrophotometric measurements were performed on a UV-Vis spectrophotometer (Lambda 950, Perkin Elmer, and UK) equipped with 1 cm path length quartz cells.

3.4. Statistical analysis

Analysis of variance (ANOVA) is a statistical method used to test differences between two or more means. In this study, a one way ANOVA analysis was used at 95% confidence level using version20 SPSS software to know the variation of mean of total polyphenol, flavonoid, tannin and antioxidant activity are significantly different within and between groups or not and Pearson correlation coefficient was used to measure the degree of linear relationship and direction of relations between total polyphenol, flavonoid and tannin with total antioxidant activity of the samples.

4. RESULTS AND DISCUSSIONS

4.1. Phyisco-chemical properties

The results of phyisco-chemical properties of the samples from different areas have been summarized in Table2. Based on the results discussions were made accordingly for each physical parameter. For each samples the measurements were done in triplicate and the results were noted as mean±SD.

Table 2. Physico-chemical properties of the samples (n = 3, triplicates analysis).

Sample type	Sample area	Conductivity mS cm^{-1}	pH	Refractive index at 20 ^{o}C	Specific gravity
Korefe	Chilga	1.06±0.07	4.89±0.05	1.34_{19}±0.01	1.02±0.04
	Gondar	1.09±0.08	4.76±0.01	1.34_{25}±0.02	1.04±0.02
	Woreta	1.02±0.05	4.92±0.03	1.34_{11}±0.02	1.05±0.01
Tej	Debre-Birhan	0.38±0.02	3.98±0.02	1.34_{38}±0.01	1.03±0.03
	Debre-Markos	0.37±0.06	3.91±0.04	1.34_{12}±0.02	1.05±0.06
	Gondar	0.40±0.01	3.87±0.02	1.35_{15}±0.07	1.02±0.03
Tella	Debre-Birhan	0.76±0.02	4.53±0.05	1.33_{48}±0.06	1.02±0.02
	Debre-Markos	0.66±0.01	4.41±0.03	1.33_{55}±0.06	1.02±0.01
	Gondar	0.72±0.03	4.62±0.01	1.33_{43}±0.06	1.01±0.05

From the determined physico-chemical properties of these traditional beverages: *korefe* has highest ionic strength than the rest of the samples. Whereas the pH value of *tej* is lower than the other beverages which tells us it is acidic. But the refractive index and specific gravity of these beverages are in comparable amount. So the ionic strength and pH value have been taken under consideration, during adjusting of them for gelatin-tannin complexations.These properties can help us for demonstrating the substance and thus understand how this substance will behave under various conditions.

4.2. Total polyphenols

The calibration curve was constructed with standard gallic acid in the concentration range of 100-700 mgL^{-1}(Table 3) and the calibration curve is shown in Figure 7. Then the levels of total polyphenols in the samples were determined with the regression line equation and the results of total polyphenols of the samples from different areas have been summarized in Table 4. For each result discussion were made. The results were compared with different research results of beverage family and with each others of samples from different areas.

Table 3. Data for calibration curve construction using standard gallic acidfor the determination of polyphenols in the sample(n = 3, triplicates analysis).

Standard gallic acid in mgL^{-1}	Standard gallic acid in mL	Distilled H_2O in mL	Folin-Ciocalteu in mL	Na_2CO_3 in mL	Mean absorbance at 760 nm
100	0.40	16.6	4.00	4.00	0.063
250	0.40	16.6	4.00	4.00	0.244
400	0.40	16.6	4.00	4.00	0.423
550	0.40	16.6	4.00	4.00	0.607
700	0.40	16.6	4.00	4.00	0.786

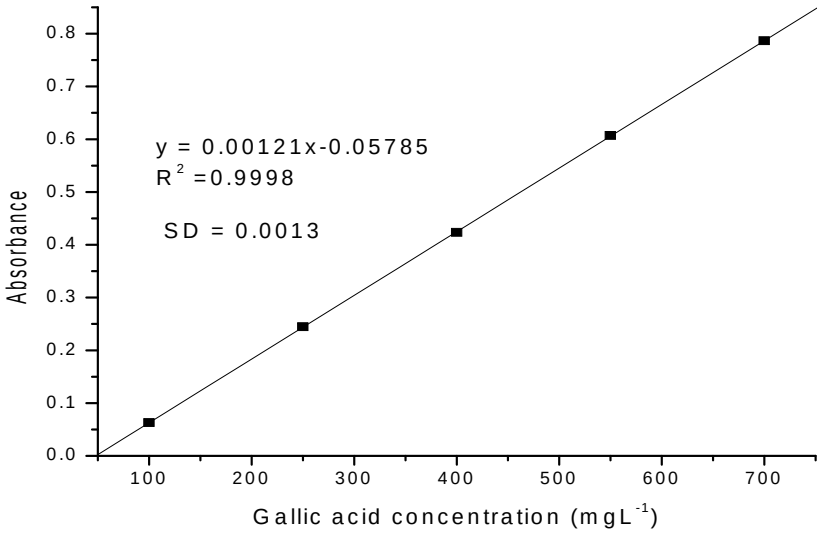


Figure 7. Calibration curve of gallic acid for total polyphenol determinations.

Table 4. Polyphenol determination (mgL^{-1}) results of the samples (n = 3, triplicates analysis).

Sample name	Sample area	Average absorbance	Concentration in mgL^{-1}	%RSD
Korefe	Chilga	0.337	326±0.5	0.153
	Gondar	0.333	323±0.5	0.155
	Woreta	0.299	285±0.9	0.316
Tej	Debre-Birhan	0.414	290±0.8	0.276
	Debre-Markos	0.389	269±0.9	0.335
	Gondar	0.414	290±0.4	0.138
Tella	Debre-Birhan	0.497	459±1.0	0.218
	Debre-Markos	0.487	450±0.2	0.044
	Gondar	0.469	435±0.8	0.184

The levels of total polyphenol were expressed in terms of gallic acid equivalent (GAE), determined by the method of Folin-Ciocalteu [56] and quantified from the equation of regression line: $y = 0.0012x + 0.05785$with $r^2 = 0.9998$ (Figure 7) where y is mean absorbance, x is concentration in mgL^{-1}. On the levels of total polyphenol, the beverages are in the order: *tella* >

korefe > *tej* (Table 4). The highest concentration of total polyphenol was found in Debre-Birhan *tella* with the value of 459±0.7 mgL^{-1} of sample volume and the lowest concentration of total polyphenol was found in Debre-Markos *tej* with the value of 269±1.0 mgL^{-1} of the sample volume. The levels of total polyphenol in the same families of beverage, i.e. *korefe*, *tej*, *tella* families were Chilga > Gondar > Woreta; Debre-Birhan = Gondar > Debre-Markos; Debre-Birhan > Debre-Markos > Gondar, respectively, which is supported by spectra given in the appendix (Figures 11b, c and d, correspondingly).

The levels of total polyphenols in beverages were reported by different authors. The reported total phenolic contents in GAE mg L^{-1} of sample volume are: in beer, 270–600 [65], 206–374 [24] and 400–700 [66]; in wine, 178–284 [24], 189–3130 [32] and 1648–4495 [11]; similarly in dolo, 506[42] and437–578[33]. The levels of total polyphenols in sampled beverage of this research are in comparable amount with several beverages except few wines that have much higher values. So the consumers of these traditional beverages relatively can get total polyphenol in the level that found in beer and dolo, this might be due to having of common raw materials used in their preparations.

ANOVA at 95% confidence level shows that the mean concentrations of total polyphenolswithin the same families of beverages were significantly different (Table 11a-c). Similarly,the mean concentrations of total polyphenol between different beverage families were significantly different; the result was supported by Table 11d; the magnitudes of variances were determined and expressed in Table 11a-d. This difference may arise because of different factors. The main difference is topographical location; the samples were collected from five different geographically located areas, which have different soil composition, annual rain fall, cultivation of the raw materials, and the variation of brewing process between groups of samples, the ingredient amount and types. All these lead to the difference in total polyphenol content of the samples.

4.3. Total flavonoids

The calibration curve was constructed with standard catechinin the concentration range of 50-600 mgL^{-1}(Table 5) and the calibration curve of standard catechin is shown in Figure 8. The levels of total flavonoids were determined with the regression line equations and the results of total flavonoids of the samples from different areas have been summarized in Table 6. The result of this parameter has been discussed and comparison was made with different research results of beverage family and with each others of samples from different areas.

Table5. Data for the calibration curve construction with standard catechin for total flavonoids determinations in sample (n = 3, triplicates analysis).

Standard catechin inmgL^{-1}	Standard catechin in mL	Distilled H_2O in mL	5% $NaNO_2$ in mL	10% $AlCl_3$ in mL	1 M NaOH in mL	Mean absorbance at 415nm
50	1.00	16.4	0.30	0.30	2.00	0.089
100	1.00	16.4	0.30	0.30	2.00	0.125
200	1.00	16.4	0.30	0.30	2.00	0.219
400	1.00	16.4	0.30	0.30	2.00	0.378
600	1.00	16.4	0.30	0.30	2.00	0.542

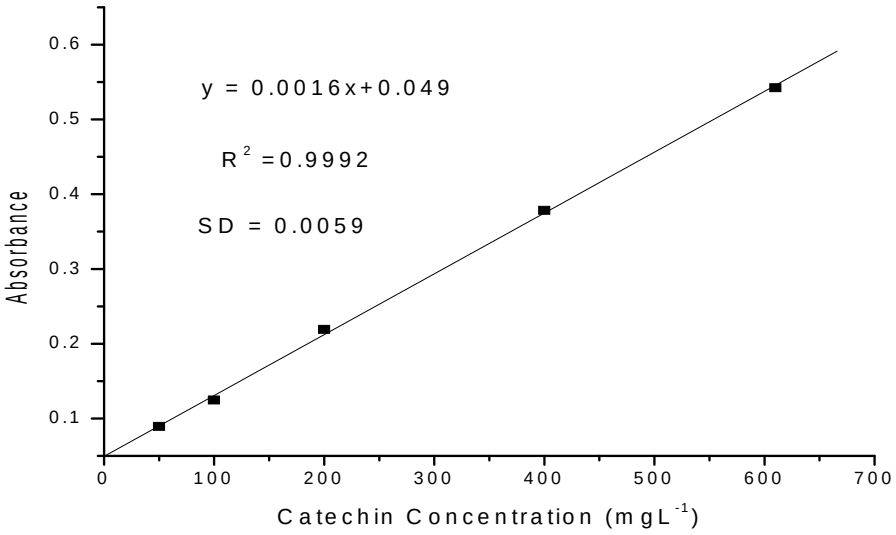


Figure 8. Calibration curve of standard catechin for total flavonoid determinations.

Table 6. Total flavonoid determinations (mgL^{-1}) result of the samples (n = 3, triplicates analysis).

Sample name	Samplearea	Mean absorbance	Concentration in mgL^{-1}	%RSD
Korefe	Chilga	0.337	199±1.3	0.653
	Gondar	0.333	196±1.1	0.561
	Woreta	0.299	190±2.4	1.263
Tej	Debre-Birhan	0.414	188±1.5	0.798
	Debre-Markos	0.389	183±1.6	0.874
	Gondar	0.414	190±0.8	0.421
Tella	Debre-Birhan	0.497	216±1.3	0.60
	Debre-Markos	0.487	211±0.9	0.437
	Gondar	0.469	212±1.6	0.755

The levels of total flavonoids were expressed in terms of catechin equivalent (CE), determined by aluminium chloride assay [57] and quantified from the equation of regression line: $y= 0.0016x + 0.049$ and $r^2 = 0.9992$ where y is mean absorbance; x is concentration in mgL^{-1}. From the samples the highest concentration of total flavonoids was found in *tella* whereas the lowest amount of total flavonoid was found in *tej* (Table 6). The levels of total flavonoid in the same

families of beverage, i.e. *korefe*, *tej*, *tella* families were Chilga > Gondar > Woreta; Gondar > Debre-Birhan > Debre-Markos; Debre-Birhan > Debre-Markos > Gondar, respectively, supported by spectra given in the appendix (Figures 11f, g and h, respectively).

The amounts of total flavonoids in beverages were reported by different authors. The reported total flavonoid contents in CE $mgL^{-1}$of sample volume are: in white wine ranges from 31.0–283[34], 0.3–233[35]; red wine 461–1596 [34], 240–680 [35]; non-alcoholic wine 2076–3832 [38] and alcoholic wine 1960–3827 [38]. The level of total flavonoid in this research samples are approximately similar with few white wine. So we can consider them as a good source of flavonoids like few white wines which have lower quantity of flavonoids.

ANOVA at 95% confidence level shows that the level of total flavonoids with in the same families of beverage which were from different areas has no significant differences (Table 11a-c). But the mean concentrations of total flavonoids between different families of beverages were significantly different. This is supported by the data in Table 11d; the magnitudes of variances were determined and expressed in Table 11d. The sources of variations were might be the points that declared at total polyphenol discussion point.

4.4. Total tannins

The calibration curve was constructed with standard tannic acid in the concentration range of 12.5-200 mgL^{-1}(Table 7) and the calibration curve of standard tannic acid are shown in Figure 9. The levels of total tannin were determined with the regression line equations and the results of total tannin of the samples from different areas have been summarized in Table 8. The result of this parameter has been discussed and comparison was made with different research results of beverage family and with each others of samples from different areas.

Table 7. Data for calibration curve construction using standard tannic acid for the determination of total tannin in the samples(n = 3, triplicates analysis).

Tannic acid in mgL^{-1}	Tannic acid in mL	Distilled H_2O in mL	Folin-Ciocalteu reagent in mL	Na_2CO_3 in mL	Mean absorbance at 760 nm
12.5	0.40	16.6	4.00	4.00	0.045
25	0.40	16.6	4.00	4.00	0.065
50	0.40	16.6	4.00	4.00	0.116
100	0.40	16.6	4.00	4.00	0.203
200	0.40	16.6	4.00	4.00	0.360

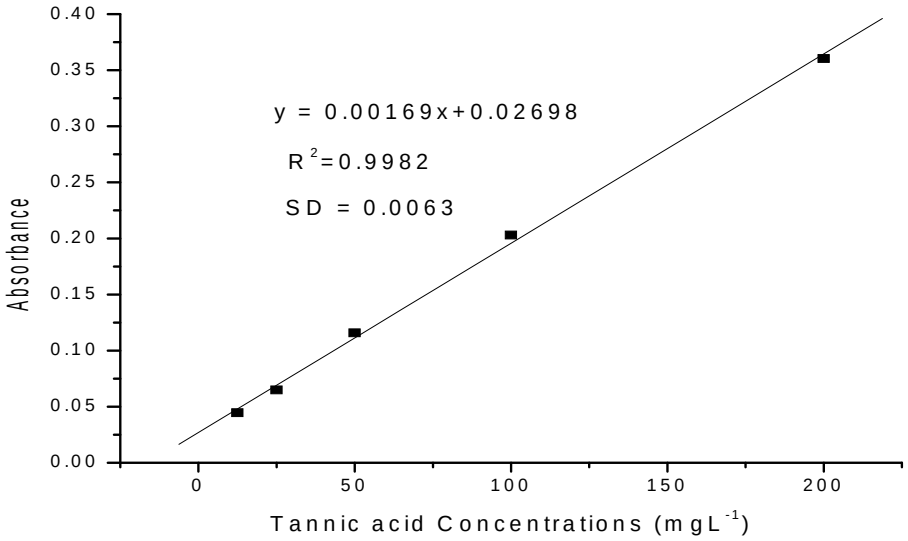


Figure 9. Calibration curve of standard tannic acid for total tannin determinations.

Table 8. Total tannin determinations (mgL^{-1}) result of the samples (n = 3, triplicates analysis).

Sample name	Sample area	Net mean absorbance	Concentration in mgL^{-1}	%RSD
Korefe	Chilga	0.071	25.9±0.6	2.32
	Gondar	0.066	23.2±0.4	1.72
	Woreta	0.061	19.9±0.9	4.52
Tej	Debre-Birhan	0.058	18.2±0.5	2.75
	Debre-Markos	0.056	17.1±0.6	3.51
	Gondar	0.060	19.4±0.6	3.09
Tella	Debre-Birhan	0.079	30.8±0.9	2.92
	Debre-Markos	0.076	28.8±0.6	2.08
	Gondar	0.078	30.6±0.6	2.32

The level of total tannin was expressed in terms of tannic acid equivalent (TAE),determined by precipitating with protein followed by Folin-Ciocalteu method [45, 56]and quantified from the equation of regression line: $y = 0.00169x + 0.02698$ with $r^2 = 0.9982$ where y is mean absorbance, x is concentration in mgL^{-1}. From the samples, the highest concentration of total tannin was found in *tella* whereas lowest concentration of total tannin was found in *tej* which are given in Table 8. The levels of total tannin between the same families of beverage i.e. *korefe*, *tej*, *tella* families were Chilga > Gondar >Woreta; Gondar > Debre-Birhan > Debre-Markos; Debre-Birhan > Gondar >Debre-Markos, respectively, which is supported by the spectra given in the appendix (Figures 11j, k and l, respectively).

The amount of total tannin in beverages was reported by different authors. The reported total tannin contents in TAE mgL^{-1} of sample volume are: in dolo ranges from 34-45 [33], in wine 997-1197 [33], in beer 73.8–101.5 [57], in alcoholic and non-alcoholic wine 421-1576 and 452-3383, respectively [38]. The level of total tannin in the samples of this research (Table 8) is much lower than the beverage which is cited here except that total tannin level in *tella* is near to the total tannin level in dolo. The reason for such variation might be monomers of tannin (mainly catechin) in the samples of this research are lower than the cited beverages which have higher level of total tannin.

ANOVA at 95% confidence level shows that the mean concentrations of total tannins within the same family of beverages were not significantly different (Table 11a-c).Between different sample types, the mean concentration of total tannins shows the presence of significant differences. This variation is supported by Table 11d; the magnitude of variances was determined and expressed in Table 11d. This is might be due to the variation of manufacturers, the content and type of ingredients, soil difference.

4.5. Total antioxidant activities

The calibration curve was constructed with ascorbic acid standard in the concentration range of 25-250 mgL^{-1}(Table 9) and the calibration curve of standard ascorbic acid is shown in Figure 10. The levels of total antioxidant were determined with the regression line equations and the results of total antioxidant of the samples from different areas have been summarized in Table 10. The result of this parameter has been discussed and comparison was made with different research results of beverage family and with each others of samples from different areas.

Table 9. Data for constructing of calibration curve using standard ascorbic acid solutions for determination of antioxidant activity of the samples (n = 3, triplicate analysis).

Ascorbic acid in mgL^{-1}	Ascorbic acid in mL	Methanol in mL	Amount of DPPH in mL	Absorbance at 517 nm	% of inhibition
25	1.00	3.00	2.00	1.269	4.29
50	1.00	3.00	2.00	1.212	12.4
100	1.00	3.00	2.00	1.048	28.7
150	1.00	3.00	2.00	0.884	44.9
250	1.00	3.00	2.00	0.571	77.4

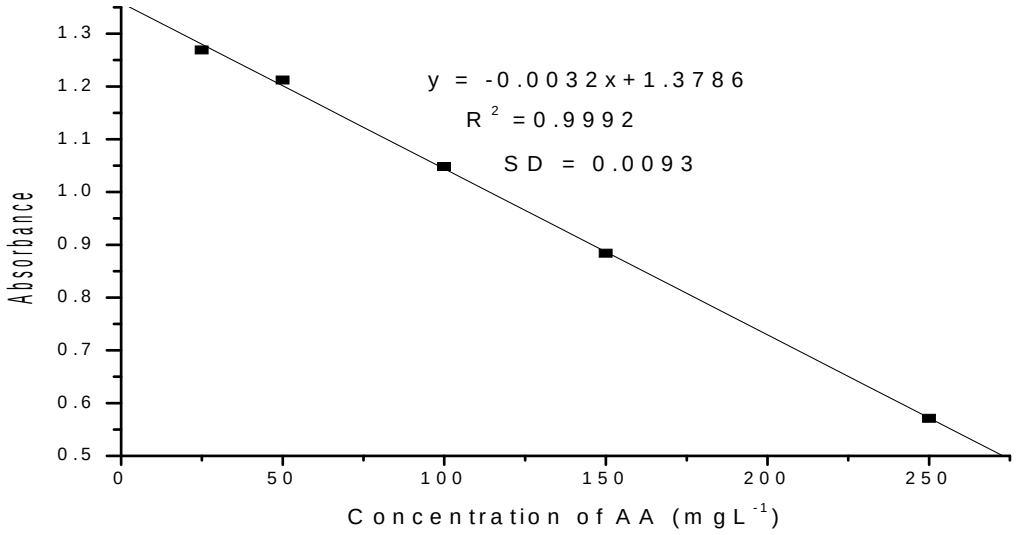


Figure 10. Calibration curve of AA to determine total antioxidant activity of the samples.

Table 10. Total antioxidant determination results of the samples (n = 3, triplicates analysis). (Mean absorbance of sample control DPPH: 1.340).

Sample name	Sample area	Mean absorbance	% of inhibition mean±SD	TAC(mg AAE/L) mean±SD	%RSD
Korefe	Chilga	0.729	45.6±0.4	508±4.9	0.805
	Gondar	0.740	44.8±0.5	498±3.1	0.518
	Woreta	0.758	43.4±0.4	484±3.5	0.602
Tej	Debre-Birhan	0.765	42.9±0.2	479±1.2	0.209
	Debre-Markos	0.775	41.9±0.4	470±2.3	0.407
	Gondar	0.782	41.6±0.4	465±1.5	0.268
Tella	Debre-Birhan	0.659	50.8±0.1	561±0.7	0.104
	Debre-Markos	0.666	50.3±0.7	556±3.1	0.464
	Gondar	0.685	48.9±0.3	541±2.1	0.323

The total antioxidant capacity was expressed in terms of ascorbic acid equivalent (AAE),determined by DPPH radical assay [58] and quantified from equation of regression line:

$y = -0.0032x + 1.3786$ with $r^2 = 0.9992$ where y is mean absorbance, x is concentration in mgL^{-1}. In all of the investigated parameters, *tella* have higher values than the rest. This might be due to the amount of ingredients such as malt, hop and their compositions. The levels of total antioxidant capacity in the same families of beverage, i.e. *korefe*, *tej*, *tella* families were Chilga> Gondar > Woreta; Debre-Birhan > Debre-Markos > Gondar; Debre-Birhan >Debre-Markos > Gondar, respectively, which is supported by spectra given in the appendix (Figures11n, o and p, respectively).

The amounts of total antioxidant activity in beverages were reported by authors. The reported total antioxidants in trolox equivalent mgL^{-1} of samples are: in dolo14.3-87.3 [33], in wine 822-1362 [34] and in non-alcoholic beverage (wine) 132-933 [38]. The level of total antioxidant activity in the samples of this research is comparable with dolo and wines which have lower total antioxidant activities of the beverages cited at this point.

ANOVA at 95% confidence level shows that the mean concentration of total antioxidant capacity within the same sample type shows the absence of significant difference (Table 11a-c). Within different groups of the samples, there were significant differences in the mean concentrations of total antioxidant capacity which were supported with Table 11d; the magnitude of variances were determined and expressed in Table 11d. This might be due to the variation in the types and amount of ingredients used, and inconsistency in the preparation process. Moreover, the variation is dependent on the type of phenolic compounds present.

4.6. Analysis of variance

All determinations were carried out in three replicates of each sample. For all analyses which were conducted, the data were subjected to an analysis of variance (ANOVA). The results obtained for total polyphenol, total tannin, total flavonoid and total antioxidant activities variations are summarized in Table 11.

Table 11. General comparison of samples (ANOVA at 95% confidence level).

Table 11a. ANOVA for *korefe* samples from different areas at 95% confidence level.

		Sum of squares	Df	Mean square	$F_{calculated}$	$F_{critical}$	Remark, at p = 0.05 level
Polyphenol	Between groups	1045	2	523	1188	5.14	The means are significantly different.
	Within groups	2.62	6	0.44			
Flavonoid	Between groups	40	2	20	4.55	5.14	The means are not significantly different.
	Within groups	26.4	6	4.4			
Tannin	Between groups	18.1	2	9.05	4.31	5.14	The means are not significantly different
	Within groups	12.6	6	2.1			
Antioxidant	Between groups	2.51	2	1.26	5.04	5.14	The means are not significantly different.
	Within groups	1.49	6	0.25			

Table11b. ANOVA between *tej* samples from different areas at 95% confidence level.

		Sum of squares	Df	Mean square	$F_{calculated}$	$F_{critical}$	Remark , at p = 0.05 level
Polyphenol	Between groups	294	2	147	272	5.14	The means are significantly different.
	Within groups	3.22	6	0.54			
Flavonoid	Between groups	24	2	12	4.76	5.14	The means are not significantly different.
	Within groups	14.9	6	2.52			
Tannin	Between groups	1.49	2	0.75	2.34	5.14	The means are not significantly different.
	Within groups	1.94	6	0.32			
Antioxidant	Between groups	0.88	2	0.44	3.67	5.14	The means are not significantly different.
	Within groups	0.72	6	0.12			

Table11c. ANOVA between *tella* samples from different areas at 95% confidence level.

		Sum of squares	Df	Mean square	$F_{calcuted}$	$F_{critical}$	Remark , at p = 0.05 level
polyphenol	Between groups	294	2	147	263	5.14	The means are significantly different.
	Within groups	3.36	6	0.56			
Flavonoid	Between groups	11	2	5.5	3.29	5.14	The means are not significantly different.
	Within groups	10.1	6	1.67			
Tannin	Between groups	2.34	2	1.17	2.29	5.14	The means are not significantly different.
	Within groups	3.06	6	0.51			
Antioxidant	Between groups	1.94	2	0.97	4.85	5.14	The means are not significantly different.
	Within groups	1.18	6	0.2			

Table11d. ANOVA between*korefe*, *tej* and *tella* samples at 95% confidence level.

		Sum of squares	Df	Mean square	$F_{calculated}$	$F_{critical}$	Remark , at p = 0.05 level
Polyphenol	Between groups	6042	8	755	845	2.51	The means are significantly different.
	Within groups	16.1	18	0.894			
Flavonoid	Between groups	762	8	95.3	35.4	2.51	The means are significantly different.
	Within groups	48.5	18	2.69			
Tannin	Between groups	234	8	29.3	76.3	2.51	The means are significantly different.
	Within groups	6.91	18	0.384			
Antioxidant	Between groups	86.5	8	10.8	71.6	2.51	The means are significantly different.
	Within groups	2.72	18	0.151			

The analysis of variance (ANOVA) revealed that at 95% (p = 0.05), there are significant differences on the level of total polyphenol between the same sample types (Table 11a-c), whereas between different sample types all parameters are significantly different (Table11 d) since $F_{calculated} > F_{critical}$. The source of variations might be due to the difference in preparation of

the beverages, the amount and kind of ingredients used, and the types and amounts of phenolic compounds present in the raw materials, the variation of environment, soil texture, etc.

4.7. Pearson correlation

The relationship between total phenolic families and antioxidant activities were investigated using Pearson correlation. The correlations of the samples are compared in Table 12.

Table12. Pearson correlation between determined parameters of the samples.

	Total polyphenol	Antioxidant	Tannin	Flavonoids
Total polyphenol	1			
Antioxidant	0.969	1		
Tannin	0.951	0.944	1	
Flavonoids	0.883	0.931	0.895	1

Total polyphenol, total flavonoids and total tannins were found to have correlation coefficients with their antioxidant activity assayed: ($r = 0.969$ and $r = 0.931$, $r = 0.944$, respectively). The results indicate that the presence of positive and very good correlation between these variables. The positive correlation indicates that when the concentrations of total polyphenolic compounds, total flavonoids and total tannins increase, total antioxidant activity also increases. This illustrates that phenolic compounds are important contributors to antioxidant activity of the beverages.

CONCLUSION

In the samples of this research, the presence of phenolic compounds usually called polyphenols were proved by employing chemical analytical methods followed by UV-Vis spectroscopy techniques. By this study the level of bioactive compounds: total phenolic, total flavonoids, total tannin and antioxidant activities of the beverages were investigated. The results obtained indicated that at 95% ($p = 0.05$) confidence level, there were significant difference on the level of total polyphenol, flavonoids and tannins between different sample types and within the same sample type, there was significant difference on the level of total polyphenol. Similarly total antioxidant activity of these fermented alcoholic beverages was significantly different between different sample types. Among the fermented beverages, *tella* has showed higher total phenolic and antioxidant capacity than the rest. The results of this study also indicated that the concentration of total tannin is much lower than the concentration of total flavonoid. The investigation indicated that the total phenolic components of alcoholic beverages depend on the raw materials and the brewing processes. According to the data obtained from this study, the content of total polyphenol, total flavonoids and total antioxidant activity also present remarkably while total tannin was very low. These research findings conclude that *korefe*, *tej* and *tella*, Ethiopian traditional fermented alcoholic beverages are as a promising source of biologically active polyphenol and flavonoid compounds and contributing effective protection from free radicals, cancer, and aging problems in comparable manner of beer, dolo and few wines which were cited.

RECOMMENDATIONS

1. Even if, all phenolic compounds have similar functions like antioxidant, anticancer, antiaging, etc.; they differ on absorption site of body and rate of mentioned functions based on structures and size. So these beverages need further individualizations studies of phenolic compounds.
2. For variations of the total quantity of determined parameters within and between groups of the samples, different conditions were estimated as factors. So further investigations are required to disproof the suspected conditions.
3. The one who consumes these beverages can get health benefits rather than traditional purposes. So it is advised to drink these beverages to get such benefits.
4. This research was done on mentioned beverages by collecting them on the matured date /ready to drink. So anyone who has interest can do investigations, the effect of under maturations and long shelf life on these bioactive chemicals.
5. In several studies, total polyphenols were reported as mg gallic acid equivalents per liter which is the commonly used standard. It will be important to measure the polyphenols using catechin as the standard and compare if the same results can be obtained. Catechin is the second most used standard after gallic acid and can be advantageous access of standard varieties.

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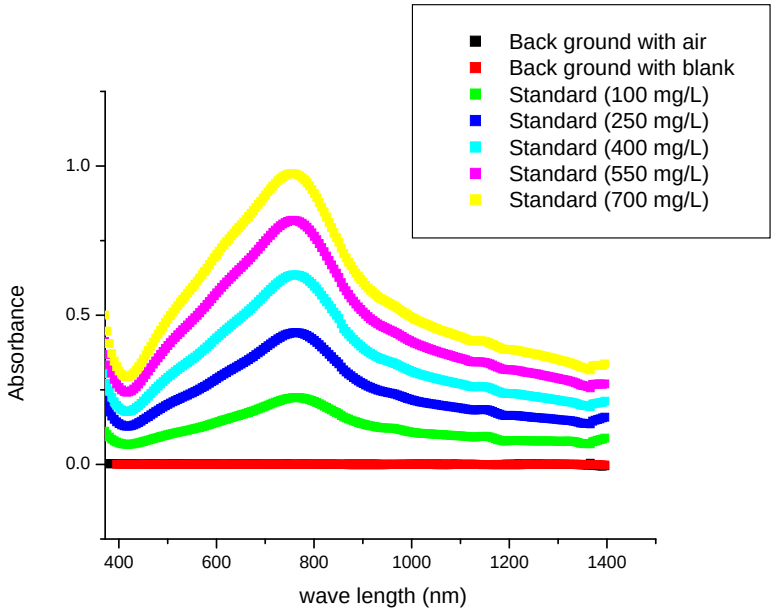
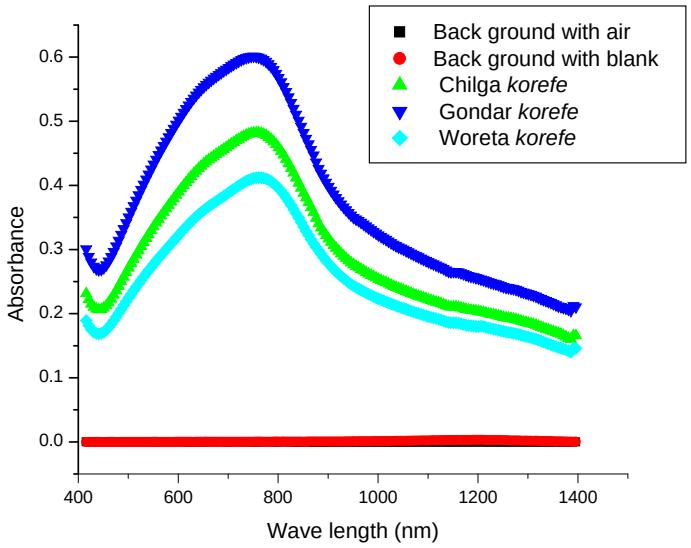
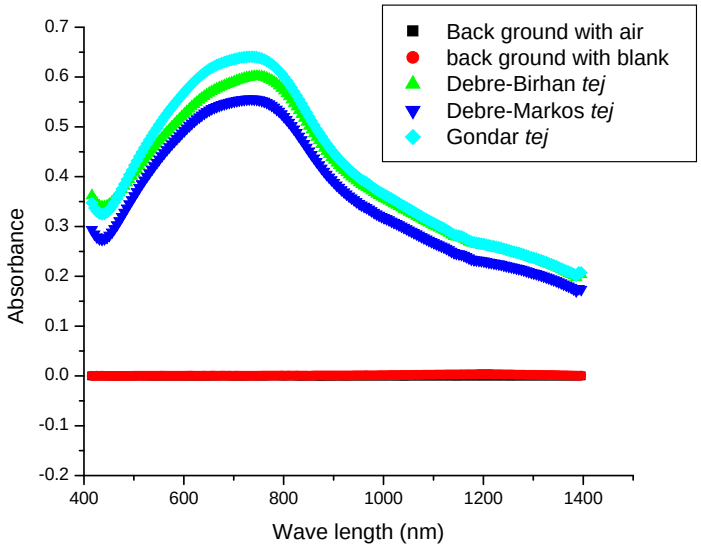
APPENDICES



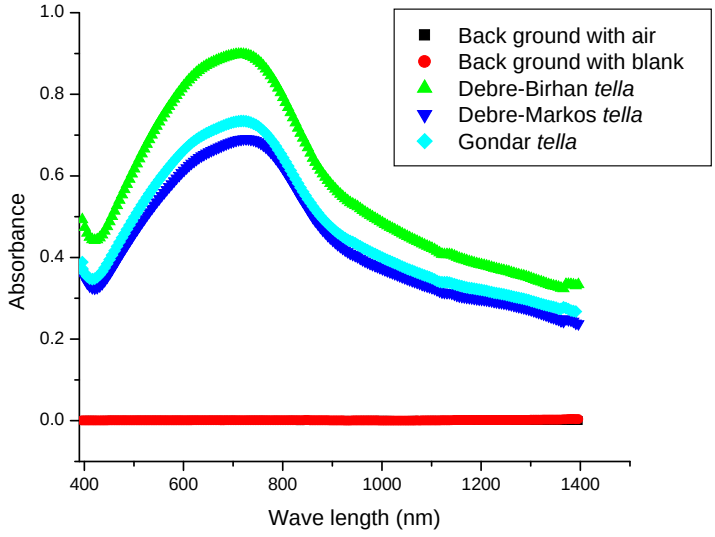
(a). Gallic acid standard spectra for total polyphenol determinations.



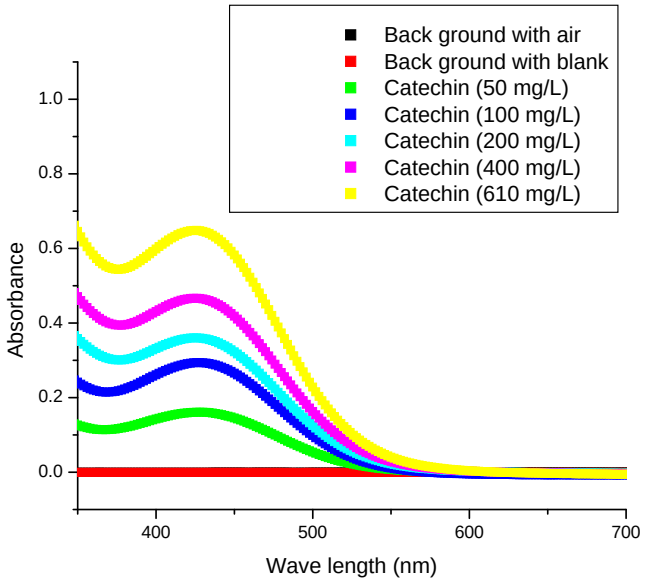
(b).The spectra of total polyphenol for *korefe* samples.



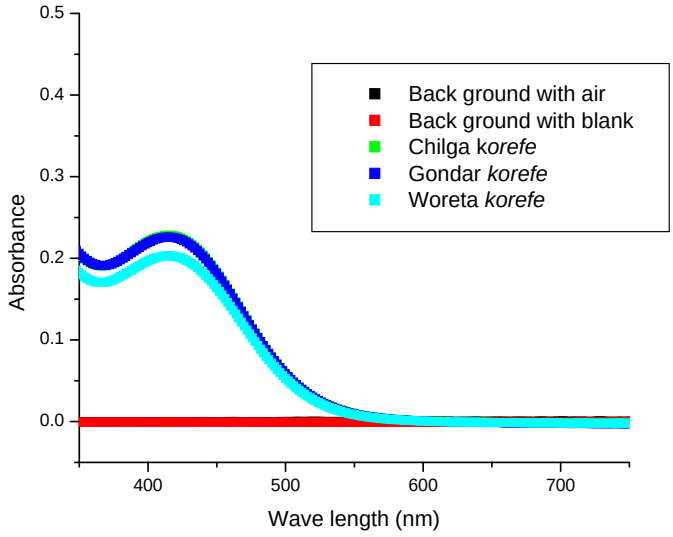
(c).The spectra of total polyphenol for *tej* samples.



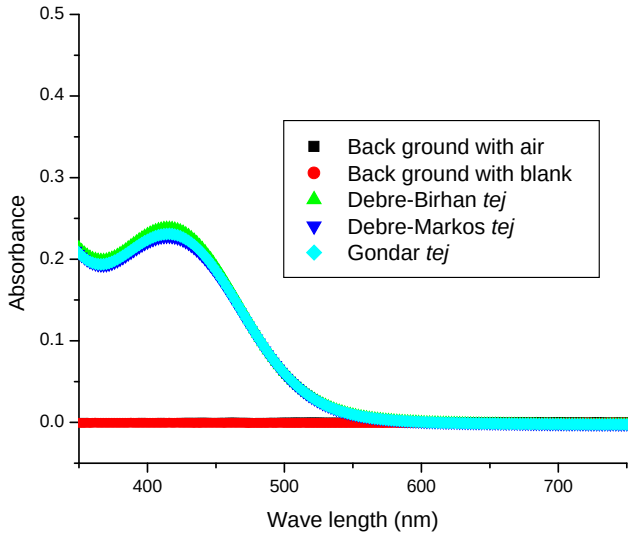
(d).The spectra of total polyphenol for *tella* samples.



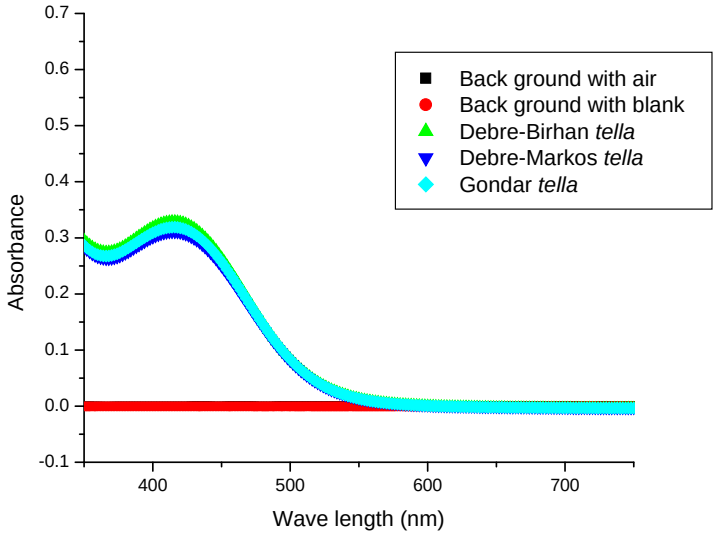
(e).Spectra of standard catechin for total flavonoid determinations.



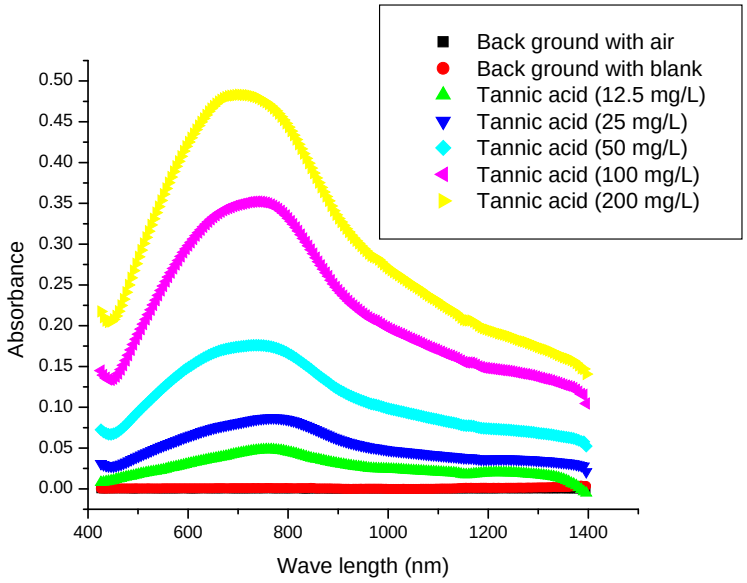
(f).The spectra of total flavonoid for *korefe* samples.



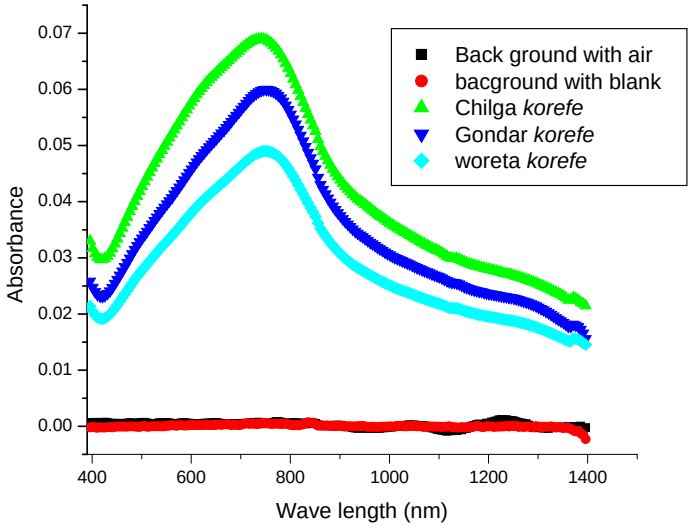
(g).The spectra of total flavonoid for *tej* samples.



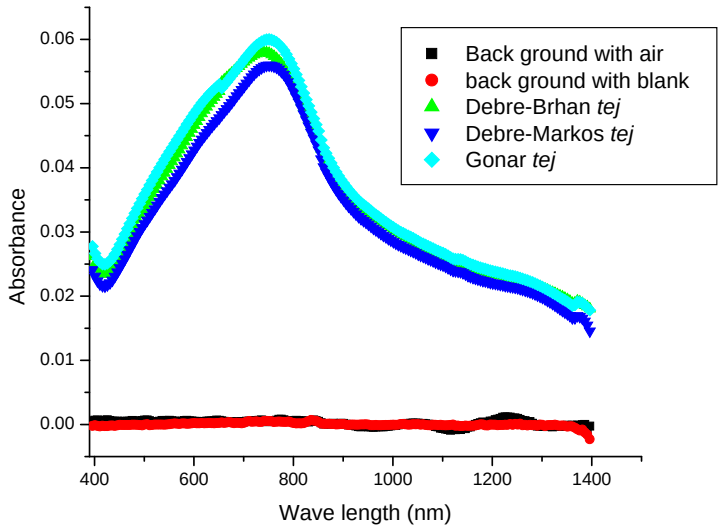
(h).The spectra of total flavonoid for *tella* samples.



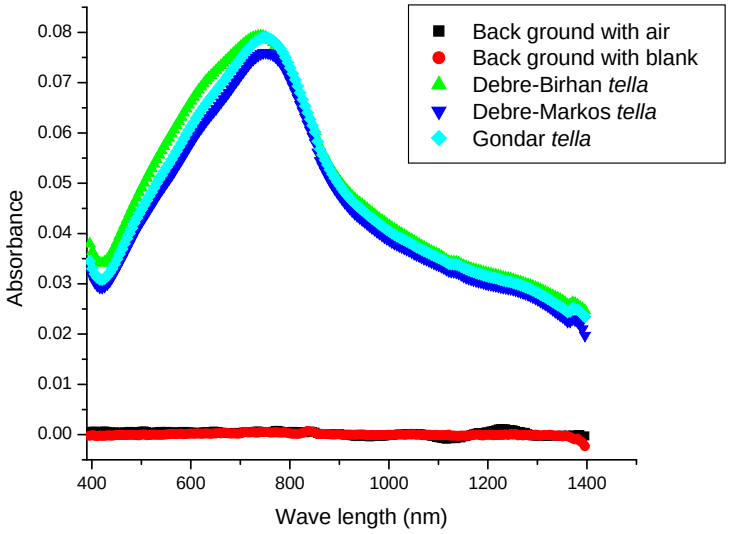
(i). Spectra of tannic acid for total tannin determinations.



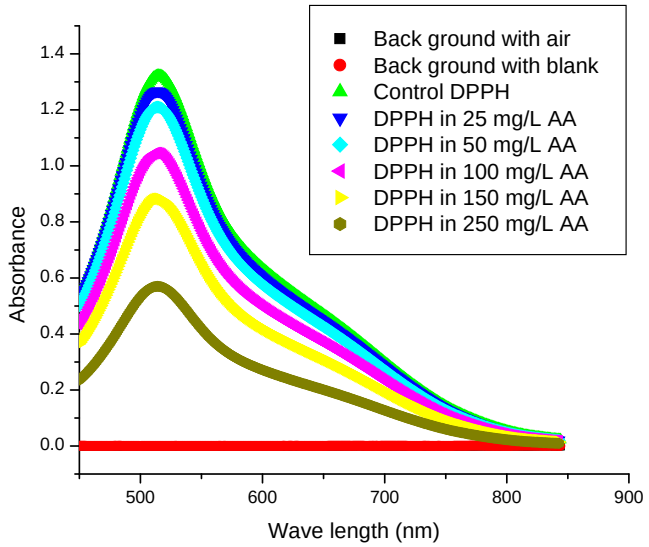
(j).The spectra of total tannin for *korefe* samples.



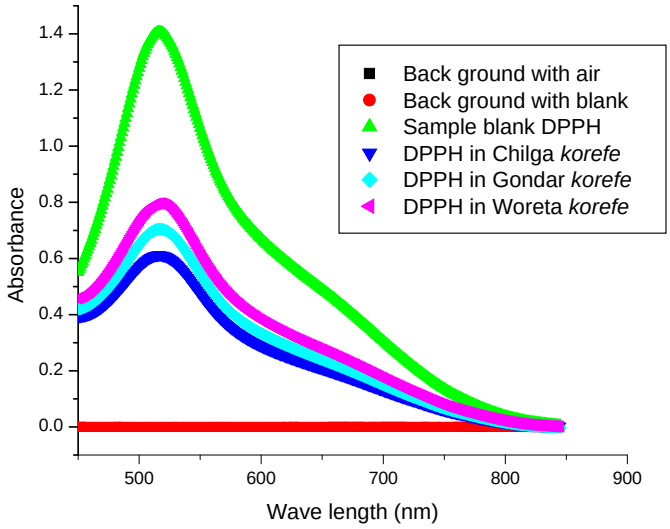
(k).The spectra of total tannin for *tej* samples.



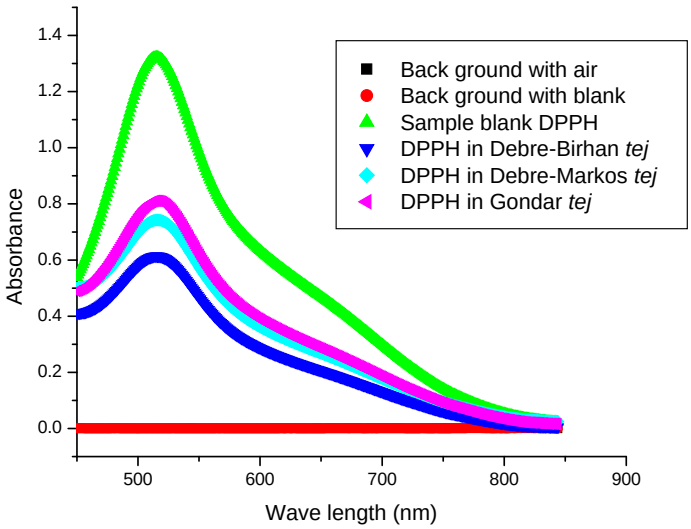
(l).The spectra of total tannin for *tella* samples.



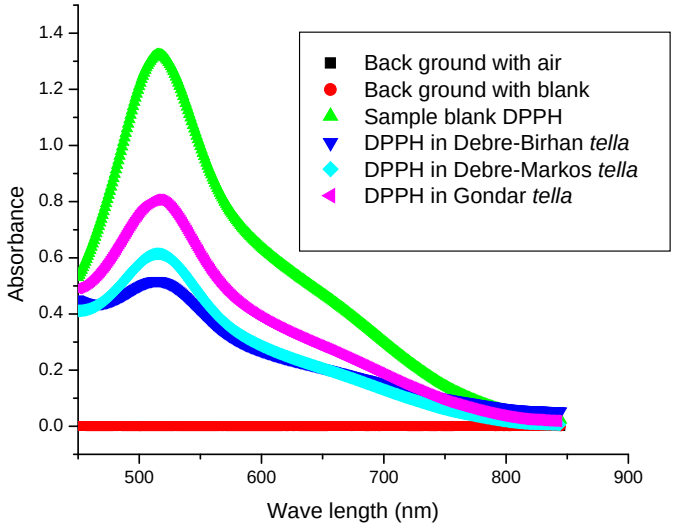
(m). Spectra of DPPH with ascorbic acid standards.



(n). Spectra of DPPH with sample blank and *korefe* samples.

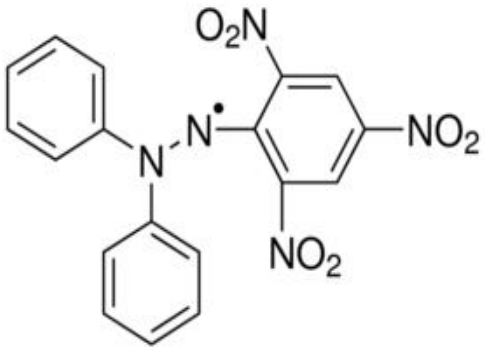


(o). Spectra of DPPH with sample blank and *tej* samples.

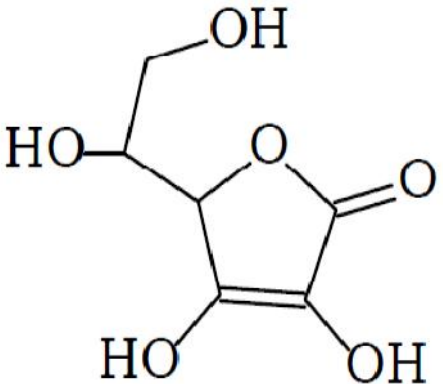


(p). Spectra of DPPH with sample blank and *tella* samples.

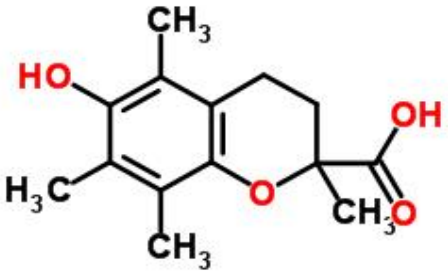
Figure 11.Spectra of standards and samples.



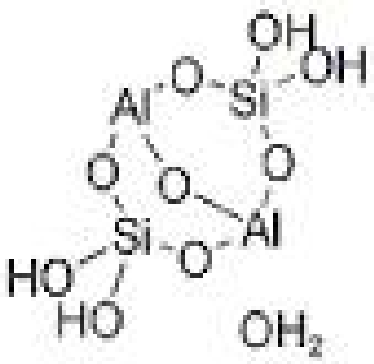
2, 2-Diphenyl-1-picrylhydrazyl.



Ascorbic acid.



Trolox(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).



Kaolin ($Al_2Si_2O_5(OH)_4$).

Figures 12. Structures of few reagent and standards which are not included in the text (main body).