Research Square

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Evaluation of Total Phenolic, Flavonoid Contents and Antioxidant Activities of Seed Extracted from Coriander (Coriandrum Sativum L) and Black cumin (Nigella Sativa) Spices

Dessie Tibebe

dessie.1977@gmail.com

University of Gondar

Abebaw Belete

University of Gondar

Yezbie Kassa

University of Gondar

Marye Mulugeta

University of Gondar

Zerubabel Moges

University of Gondar

Dereje Yenealem

University of Gondar

Tarekegn Fentie

University of Gondar

Agmas Amare

University of Gondar

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Abstract

*Black cumin (*Nigella sativa*), and Coriander (Coriandrum sativum L.) are common culinary spices that are used singly or combined in the diet of many populations of Ethiopia and there is a long-held belief of their health-enhancing properties. The present study was designed to investigate the antioxidant properties, phenolic and flavonoid contents* of aqueous *methanolic extracts from* Nigella sativa *and Coriandrum sativum L. seed samples. The contents of polyphenols and flavonoids were evaluated by colorimetric method and antioxidant activity was determined based on the ability to scavenge free radicals by using DPPH assay. An* aqueous *methanol extract of Coriandrum sativum L. resulted in the highest phenolic content from West Dembia (1.799 ± 0.0011 mg GAE/g), followed by* Nigella sativa *from East and West Dembia (1.737 ± 0.0014 & 1.734 ± 0.0013 mg gallic acid equivalent per gram (mg GAE/g) respectively) and Coriandrum sativum L. from West Dembia (1.678 ± 0.0018 mg GAE/g) measured by the Folin–Ciocalteu reagent method. The greatest flavonoid content was observed with extracts of Coriandrum sativum L. (1.114 ± 0.0005 mg quercetin equivalents per gram samples (mg QE/g)) from East Dembia district followed by* Nigella sativa *(1.002 ± 0.0007 (mg QE/g) as measured by an aluminum chloride colorimetric method, while the least was recorded for Coriandrum sativum L. (0.926 ± 0.0013 mg QE/g) and* Nigella sativa *(0.764 ± 0.0010 mg QE/g) from West Dembia district. Among the two selected spice extracts* Nigella sativa from East Dembia district *displayed the highest DPPH radical scavenging activity with an* IC_{50} *value 432 mg ascorbic acid equivalent per gram sample (AAE/g), while* Nigella sativa & *Coriandrum sativum L. from West Dembia and Coriandrum sativum L. (East Dembia) showed lower antioxidant activities having 372, 320 and 366 mg AAE/g of sample respectively. These results confirmed that* Nigella sativa *and Coriandrum sativum L. extracts possess appreciable natural antioxidant potentials, thereby providing good justification for their increased domestication and combined food consumption.*

1. Background

Aromatic plants like cumin, thyme, rosemary, sage, tarragon, coriander are excellent sources of secondary metabolites, in particularly phenolic compounds that are associated with antioxidative and antimicrobial action in all biological systems [1]. The use of spice in foods has been known since antiquity to improve the flavor or to possess antibacterial and antifungal properties. Spices represent natural sources of polyphenolic compounds [1].

Food provides not only essential nutrients needed for life but also other bioactive compounds for health promotion and disease prevention. Thus, consumers demand for natural foods with antioxidant property, which enhances health and food preservation. There is increased demand for natural dietary products which produce antioxidants in the body. Antioxidants prevent oxidative damage caused by free radicals; they interfere with the oxidation process by reacting with free radicals, chelating, and catalytic metals and also by acting as oxygen scavengers [2].

Spices are various parts (bud, fruit, seed, bark, rhizome, leaf and bulb) of plants used as a flavoring or seasoning, although many can also be used as herbal medicine. The term ‘spice’ originated from the Latin word ‘species’, meaning of specific kind. Spices have many functions in food. Primarily they are used for flavoring food products; in addition they are also used for preservation of food and for providing nutritional and health benefits [2]. Mix spices cooking paste is a type of ready-to cook food containing combination of various spices and condiments that were blended and thermally processed to enhance their shelf life. As several metabolic diseases are associated with oxidative processes in the human body, the antioxidant properties of spices have attracted considerable attention among researchers and consumers [3].

Plants (fruits, vegetables, medicinal herbs, etc.) and products made from plants may contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g., phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity [4]. Polyphenolic compounds are effective antioxidants due to their capability to scavenge free radicals of fatty acids and oxygen. They behave as antioxidants by a variety of way including trapping of reactive oxygen species, inhibition of enzymes responsible for superoxide anion production, chelation of transition metals involved in process forming radicals and prevention of the peroxidation process by reducing alcoxyl and peroxyl radicals [5]. Phenolic antioxidants, a specific group of secondary metabolites, play an important role in protecting organisms against harmful effects of oxygen radicals and other highly reactive oxygen species (ROS). Phenolics belong to the very important group of plant antioxidants. They are always substituted phenolic compounds with one to three hydroxyl groups on the aromatic ring in different positions. A carboxylic acid group may also be present as the main substituent or another ring may be linked to the aromatic ring. The antioxidant effect depends mainly on the number and position of hydroxyl groups and the identity of the main substituent’s, but also on many other factors [6, 7]. Natural antioxidants present in the diet increase the resistance to oxidative stress and they may have a substantial impact on human health. Recently, they have received much attention since many epidemiological studies suggested that consumption of polyphenol-rich foods and beverages is associated with a reduced risk of cardiovascular diseases, stroke and certain forms of cancer. Oxidative stress is the production of free radicals that cannot be neutralized due to insufficient number of antioxidants. Oxidative stress is controlled by a balance between pro-oxidants and antioxidants. Oxidative stress can lead to the development of cardiovascular diseases, cancer and can increase the rate of aging [8, 9]. Examples of antioxidant compounds found in spices may include eugenol, camphene, carvacrol, piperine, myristic acid, myristicin and ascorbic acid [10].

This research work aims, the determination of total polyphenol content, total flavonoid content and antioxidant activity of two commercially available spices (Coriander and Black cumin) extract in central Gondar zone, koladiba & Chuahit town.

2. Materials and Methods

2.1. Description of the Study Area

The spices samples were collected from the major planting areas and commercially available sites in East and West Dembia districts. Koladiba and chuahit are the main town of East and West Dembia districts respectively and located in Central Gondar Zone of Amhara Regional state which is situated at 12°15'0"N – 12°30'0"N latitude and 37°58'30"E – 37°37'0"E longitude (Fig. 1). The study area was selected based on the major planting of various types of spices by the local farmers with comfortable farming sites and the spices planted in the study area was used as traditional additives of foods.

2.2. Chemicals and reagents

The following chemicals, reagents, plant (Spices) seed samples were used for this research work:

Methanol (99%), ascorbic acid (99% from Blulux laboratory reagent, India), Gallic acid,

DPPHP (2,2-dephenyl-1-picryhydrazyl, 99% from Sigma-Aldrich Pty Ltd, Germany), folin-ciocalteu reagent, sodium carbonate (Blulux laboratory reagent, India), sodium hydroxide (from Loba Chemie Pvt. Ltd (Mumbai, India)), and *Coriandrum sativum* L. and *Nigella sativa* spices seed samples were purchased from local market in Koladiba and Chuahit town.

2.3. Apparatus and Instruments

The following equipment's and analytical instruments were used for this research work: Microprocessor UV-Vis double beam spectrophotometer (Abron, India), grinder, rotary shaker (Abron), Erlenmeyer flasks, Volumetric flasks, Beakers, Analytical balance, Magnetic stirrer, Spatula, Pipette with bulb, Micropipette, Mortar and pestle, Test tubes, Oven, Whatman No.1 filter paper, Filter funnel, and Refrigerator.

2.4. Collection of *Coriandrum sativum L.* and *Nigella sativa* spices seed samples

The plant of *Coriandrum sativum* L. and *Nigella sativa* spices seed samples were collected (purchased) from local market in Koladiba and Chuahit town, Central Gondar zone, Amhara regional state during January, 2022.

2.5. Preparation of the spices seed samples extract

The extraction was performed following the method [45] with slight modifications. The spices seed samples were washed thoroughly under running tap water to remove adhered dirt, dust, and other foreign debris. After washing, the seed samples were dried at laboratory room temperature for few days. The dried seed samples were ground further to get the fine powder by using a small laboratory grinder and passed through a sieve for further refining, and it was pack separately in air-tight plastic jars for further analysis. The above powdered spices samples were extracted with different v/v methanol: water solvent ratios (50, 60, 70, 80, 90 and 100%) following a 1:10 sample-to-solvent ratio. The extracts were

separated from solids by filtering through Whatman No. 1 filter paper. Afterward, the filtrate extracted samples were stored at 4°C until tested and analyzed [45].

2.6. Determination of Total Phenolic Content (TPC)

2.6.1. Preparation of Standard Gallic Acid for Calibration Curve

Total phenolic contents (TPC) in the spices seeds extracts were determined by Folin–Ciocalteu colorimetric method as described by Phuyal *et al*. [46] With some modifications. Standard Gallic acid solution was prepared by dissolving 0.1 gram of it in 100 mL of 70% aqueous methanol (1000 mg/L). Various concentrations of gallic acid solutions (10, 25, 50, 100, 150, 200 and 250 mg/L) was prepared from the stock solution. To each concentration, 2.5 mL of 10% Folin–Ciocalteu reagent (FCR) and 4 mL of 7.5% solution of Na_2CO_3 was added. Thus, the obtained blue colored mixture was shacked well and incubated for 30 minutes in a dark area. Then, absorbance of serious standard concentration was measure at 760 nm against blank. The FCR reagent oxidizes phenols in plant extracts and changes into the dark blue color, which is then measured by UV-visible spectrophotometer. All the experiments were carried out in triplicates, and the average absorbance values obtained at different concentrations of gallic acid were used to plot the calibration curve.

2.6.2. Preparation of Samples for Total Phenolic Content

Briefly, a 0.5 mL of each extract samples were mixed with 3.25 mL of distilled water and 0.25 mL of Folin-Ciocalteu reagent. After 5 min in the dark, 1 mL of 7.5% Na_2CO_3 was added and the solution was incubated for 90 min in the dark at room temperature. The absorbance was measured by using a double beam UV-Vis spectrophotometer at 760 nm. Total phenolic content of the spice extracts were expressed as mg Gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g). The total phenolic contents in all the samples were calculated by the using the formula:

$$C = c(V/m) \quad \text{..... (2)}$$

where C = total phenolic content mg GAE/g dry extract, c = concentration of samples obtained from Gallic acid calibration curve in mg/mL, V = volume of extract in mL, and m = mass of extract in gram [46].

2.6.3 Determination of Antioxidant activity

The radical scavenging activity (RSA) of the spices seed extracts was adopted to measure antioxidant activity using the DPPH method [16]. Briefly, 0.1, 0.2, 0.4, 0.8, 1.6 and 2.4mL portion of the extracts were mixed with 1.6 mL of DPPH (0.1 mM) solution and the final volume of each solution was adjusted to 4.0 mL with 70% aqueous methanol solution. The mixtures was keep aside in a dark area for 30 min and absorbance of the solution was measured at λmax 517 nm against an equal amount of DPPH and methanol as a control. Ascorbic acid was used as the standard and the solution was prepared by dissolving 0.1 gram of it with 100 mL of 70% aqueous methanol (1000 mg/L) and various concentrations

of the standard solutions (5, 10, 15, 30, 60, 90 and 120 mg/L) was prepared from the stock solution and 1.6 mL of methanolic solution of DPPH was added. Absorbance of the standard solutions was recorded at λmax 517 by UV-Visible spectrophotometer and the percentage of DPPH• scavenging activity (RSA %) was estimated using the equation:

% Scavenging of DPPH• = $[(A_0 - A_1)/A_0] \times 100$ ..... (3)

Where A_0 = absorbance of the control and A_1 = absorbance of the sample extracts.

2.6.4. Determination of Total Flavonoid Content

Total flavonoid contents in the extracts sample was determined by aluminum chloride colorimetric assay following previous standard method [47]. A volume of 500 µL of aliquot extract was diluted in 2.5 mL of distilled water followed by the addition of 150 µL of 5% (w/v) sodium nitrite ($NaNO_2$) to the solution. After 5 min, 300 µL of 10% (w/v) aluminum chloride ($AlCl_3$) was mixed and the solution was allowed to stand for 5 min before the addition of 1 mL of 1 M NaOH solution. Then the mixture was diluted with 550 µL of distilled water and it was shaking vigorously. The solution absorbance was measured at 510 nm using UV-Vis spectrophotometer. Quercetin was used as the standard and total flavonoid content was determined as quercetin equivalent. A calibration curve for quercetin was drawn for this purpose. The stock solution (4 mg/mL) of quercetin was prepared by dissolving 50 mg of quercetin standard in 50 mL of 70% aqueous methanol (1000 mg/L). The standard solution was diluted serially to make various concentrations of 5, 25, 50, 100, 150, 200 and 300 mg/L. 500 µl of each of the quercetin dilution was mixed with 2.5 mL of distilled water and then with 150 µL of 5% Sodium nitrite and allowed to stand for 6 minutes. Then 300 µL of 10% Aluminum chloride solution was added and allowed to stand for 5 minutes after which 1 mL solution of 1M Sodium hydroxide was added sequentially. Then the mixture was diluted with 550 µL of distilled water and has been shaking vigorously. The absorbance of this reaction mixture was recorded at 510 nm on UV-vis spectrophotometer. The results were reported as mg quercetin equivalent per gram (mg QE/g) of extract [47].

3. Results And Discussion

3.1. Determination of total phenolic contents of spice extracts

3.1.1. Linearity of standard solutions

Gallic acid standard curve was constructed (10–250 mg/L) and the calibration curve gave a strong relationship (R^2 = 0.999) between absorbance and concentration (Fig. 2).

3.1.2. Optimization of Extraction Parameters

3.1.2.1. Effect of Solvent Proportion on Extraction of Total Phenolic Content

The extractions of phenolic compounds from the spices were optimized in terms of methanol concentration to get the optimal total phenolic content yield. Effect of solvent proportion on extraction of polyphenols was carried out at a time of 6 h shaking on rotary shaker including 24 h maceration time by using methanol: water at different proportion (50, 60, 70, 80, 90, and 100%). Methanol has been generally found to be more efficient in extraction of polyphenols [48, 49]. Table 3 presents the effect of % of aqueous methanol and methanol alone on the TPC extracted from spices in East Dembia and West Dembia.

Table 1
Effect of methanol concentration on total phenol content of spices in mg GAE/g sample

	East Dembia		**West Dembia**	
[M]	Spices		Spices	
	Black cumin	Coriander	Black cumin	Coriander
50%	1.171 ± 0.0007	1.146 ± 0.0012	0.882 ± 0.0008	0.882 ± 0.0008
60%	1.441 ± 0.0010	1.337 ± 0.0007	1.199 ± 0.0010	1.063 ± 0.0009
70%	1.737 ± 0.0014	1.489 ± 0.0016	1.734 ± 0.0013	1.799 ± 0.0011
80%	1.713 ± 0.0015	1.678 ± 0.0018	1.662 ± 0.0019	1.661 ± 0.0014
90%	0.842 ± 0.0020	1.085 ± 0.0016	0.971 ± 0.0017	1.011 ± 0.0006
100%	0.664 ± 0.0023	0.906 ± 0.0010	0.789 ± 0.0022	0.984 ± 0.0010
Note: [M] - % of methanol: water proportion (v/v).				

As clearly indicated in Table 3, when the concentration of methanol increased from 50–70% the TPC values increased significantly from 1.171 ± 0.0007 to 1.737 ± 0.0014 (Black cumin), 1.146 ± 0.0012 to 1.489 ± 0.0016 (Coriander) and from 0.882 ± 0.0008 to 1.734 ± 0.0013 (Black cumin), 0.882 ± 0.0008 to 1.799 ± 0.0011 (Coriander) mg GAE/g sample for East Dembia (Koladiba) and West Dembia (Chuahit) respectively. While a decreased trend in the extraction efficiency of TPC was observed when the concentration of methanol was higher than 70%.

It is in line with previous reports suggesting that mixture of methanol and water is more efficient to extract phenolic constituents as compared to mono-solvent system and addition of small quantity of water to organic solvent usually creates a more polar medium which facilitate the extraction of polyphenols [50]. The results are also in agreement with Majeed et al. [51] who reported that highest

levels of total phenolics, flavonoids and antioxidant activity are extracted from the Leaves of the *Oreganum vulgare* in aqueous: methanol (30:70).

Table 2
Optimum total phenol content (mg GAE/g sample) of spices in two districts

Spices	Sample site	
	East Dembia	West Dembia
Black cumin	1.737 ± 0.0014	1.734 ± 0.0013
Coriander	1.678 ± 0.0018	1.799 ± 0.0011

The highest concentration in phenolic content was determined for Coriander from West Dembia (1.799 ± 0.0011 mg GAE/g), followed by Black cumin from East and West Dembia (1.737 ± 0.0014 & 1.734 ± 0.0013 mg GAE/g respectively) and Coriander from West Dembia (1.678 ± 0.0018 mg GAE/g) (Table 2 & Fig. 3). It can be said that total phenolic content of Black cumin and Coriander, with extraction of the same solvent composition, was relatively the same from both sampling sites. The results are in agreement with those reported by Słowianek and Leszczyńska (2016), and that the *Nigella sativa* and *Elettaria cardamomum* seed extracts obtained a high content of total phenolics (2.7 ± 0.059 and 2.2 ± 0.010 mg GAE/g sample) respectively [10]. The results are also comparable to those obtained by Aminah and Wan Aida (2011), recorded high value of TPC from Extracts of *Polygonum minus* (165.3 ± 1.0 mg GAE/100g DW) and *Zingiber officinale* (101.6 ± 0.6 mg GAE/100g DW) [52].

According to Nagy et al. (2015), the highest concentration in total phenolic contents was also found in Romania for *Piper nigrum L.* (338 mg GAE/100g), followed by *Allium sativum* (97.5 mg GAE/100g) and *Capsicum Annum L.* (92.5 mg GAE/100g) and almost comparable for the present study [8]. However, there are findings reported by Alam et al. (2020), on total phenolic contents for *Cili Burung* (1.00 mg GAE/g) followed by *Cili Putih* (0.76 mg GAE/g) and *Cili Ungu* (0.49 mg GAE/g) which is lower than our results [53]. These differences could be explained by the diversity of genotypes and their landraces, maturity stage, variations in sample preparation, extraction methods, and the use of fresh or dehydrated samples.

3.2. Analysis of Total Flavonoid Content

Quercetin standard curve was constructed (5-300 mg/L) and the flavonoid content of samples extract were determined & expressed as mg QE/g sample (Fig. 4).

The calibration curve of the standard gave a strong relationship (R^2 = 0.999) between absorbance and concentration.

Total flavonoid content was determined for optimum TPC yield extracts and all samples showed in a range of 0.764–1.114 mg QE/g sample as shown in Table 3below.

Table 3
Total flavonoid content (mg QE/g ± SD) of spices in the respective sampling sites with the optimum TPC yield

East Dembia		West Dembia	
Spices		Spices	
Black cumin	Coriander	Black cumin	Coriander
1.002 ± 0.0007	1.114 ± 0.0005	0.764 ± 0.0010	0.926 ± 0.0013

The optimum yield result indicates that Coriander from East Dembia (Koladiba) records high contents of total flavonoid with 1.114 ± 0.0005 mg QE/g extract followed by Black cumin (1.002 ± 0.0007) while the lowest value was Coriander with 0.926 ± 0.0013 mg QE/g extract sample and Black cumin (0.764 ± 0.0010 mg QE/g) for samples from West Dembia (Chuahit).

According to Nurcholis *et al*. (2022), the highest total flavonoid yield (3.202 mg of quercetin equivalent (QE) per g of dry weight (DW)) amomum compactum soland. Ex maton fruit was detected when the continuous shaking extraction (CSE) method was applied with 360 min of exposure [54] and this is higher than the present study. Pourmorad *et al*. (2006) also reported that higher contents of total flavonoid of the plants *Mellilotus officinalis*, *Adiantum capillus-veneris*, *Plant ago major*, *Equisetum maximum* and *Urtica dioica* with TFC values of 57 ± 5.4, 78.3 ± 4.5, 25.15 ± 0.18, 36.2 ± 1.2 and 43.3 ± 0.37 mg QE/g extracts respectively [55].

3.3. Determination of Antioxidant Activities of optimized samples

The antioxidant activity of spices is related to their chemical composition; primarily to the presence of polyphenolic and other biologically active compounds. The antioxidant activity of phenolic compounds is mainly due to their redox properties such as adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. The most effective antioxidants act via interrupting the free radical chain reactions. These compounds donate H radicals to the free radicals formed during oxidation becoming radicals themselves that are stabilized by the resonance delocalization of the electron within the aromatic ring [32, 56]. In the present study the antioxidant activities of *Coriandrum sativum* L. and *Nigella sativa* were determined by the method of DPPH radical scavenging assay. Results were compared with ascorbic acid which was used as a reference antioxidant compound & standard was constructed (1–500 mg/L) and control absorbance was 0.684 (Fig. 5). The values are expressed as milligram of ascorbic acid per gram of sample (mg AAE/g).

From the graph illustrated above (Fig. 5), it can be seen that 10 mg/L of standard ascorbic acid solution was required to inhibit 50% inhibition concentration (IC_{50}), which was used as a basement to calculate the concentration of both spices extracts. The IC_{50} value is the concentration that can scavenge 50% of DPPH free radicals [57]. Our results confirmed that all extracts possess significant antioxidant activity

with IC_{50} values of 432 mg AAE/g extract of black cumin from East Dembia & 372 mg AAE/g of West Dembia (Figs. 6 & 7), while 320 mg AAE/g extract of Coriander from East Dembia & 366 mg AAE/g of West Dembia (Figs. 8 & 9) respectively.

As shown in Fig. 10, the black cumin samples had high antioxidant activities with IC_{50} values of 432 and 372 mg AAE/g sample. On the other hand, the Coriander samples showed lower antioxidant activities having 320 and 366 mg AAE/g of sample; and DPPH scavenging activities of all samples were evaluated at 50% inhibition concentration (IC_{50}).

According to the estimated IC_{50} values in mg AAE/g samples of the present study, the antioxidant activity distribution fashion was East Dembia (Black cumin) > East Dembia (Coriander) and West Dembia (Black cumin) > West Dembia (Coriander) (Fig. 10).

4. Conclusion and recommendations

Phenolic, flavonoid, and antioxidant activity were evaluated from locally planted spices samples of *Coriandrum sativum L.* and *Nigella sativa.* It is noticed that the highest concentration of phenolic compounds in the extracts were obtained using solvents of high polarity; 70% and 80% aqueous methanolic extract manifested greater power of extraction for phenolic compounds from the two selected spices seed samples. From the results both of *Coriandrum sativum L.* and *Nigella sativa* is a potential source of TPC, TFC and antioxidant activities and could be used as a natural antioxidant and preservative in food systems. Further research will require on the effect of varying maceration time and effect of temperature on the extraction efficiency of total phenolic contents of the spices samples. The spice seed extracts shall be studied its antimicrobial activities.

Declarations

Ethics approval and consent to participate:-“Not Applicable (NA)”

Consent for publication: - “Not Applicable (NA)”

Availability of data and materials:-The datasets used and/or analyzed and included in the article

Competing interests:- The authors declare that they have no competing interests.

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Authors’ contributions:-Abebaw Belete, Tarekegn Fentie and Agmas Amare collected the samples and conducted the analysis. Dessie Tibebe designed the research and finalized the manuscript. Marye Mulugeta, Zerubabel Moges[1], and Dereje Yenealem edited the manuscript. All authors read and approved the final manuscript.

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Figures

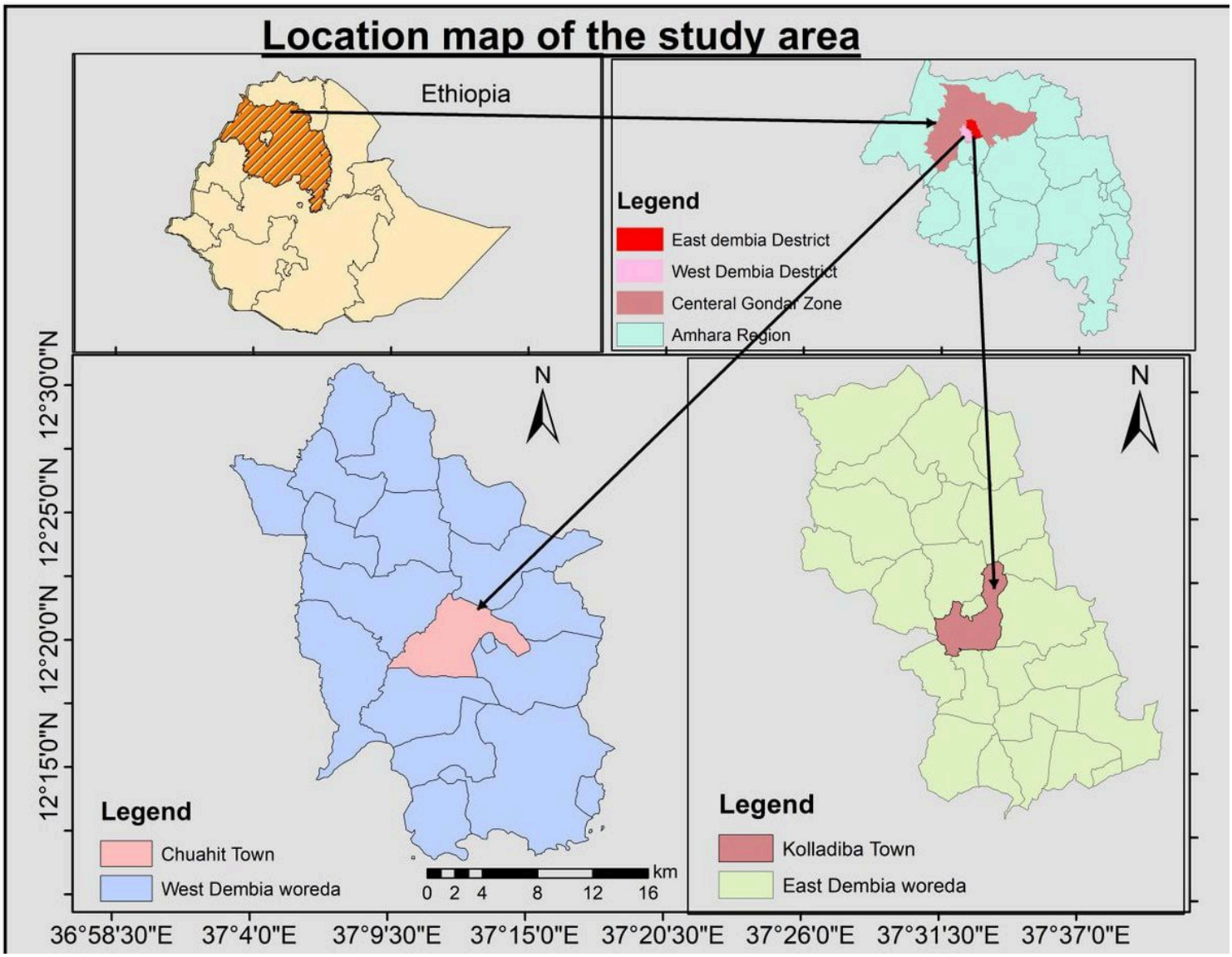


Figure 1

Map of the study area

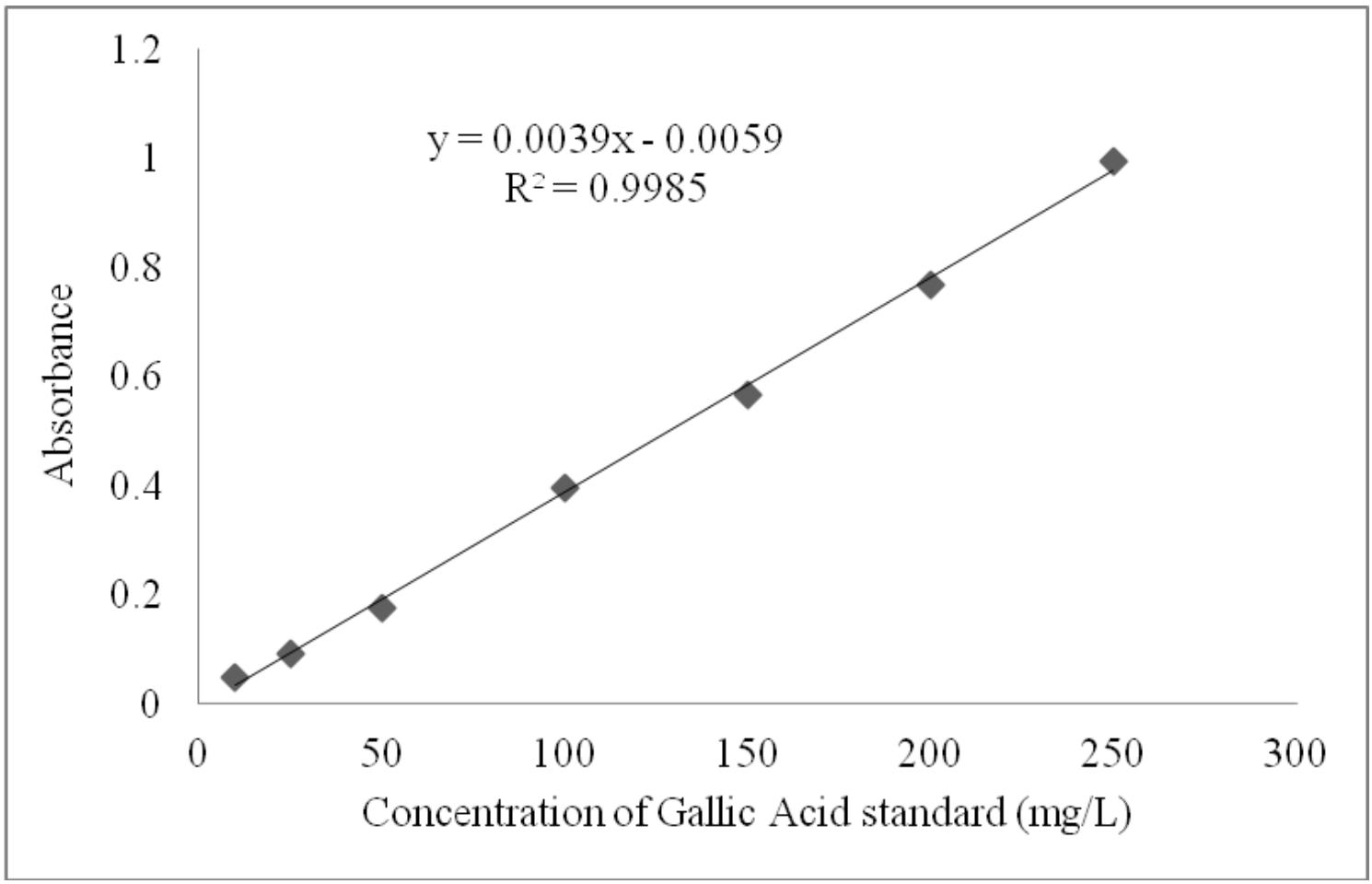


Figure 2

Calibration graph of Gallic acid standard solution

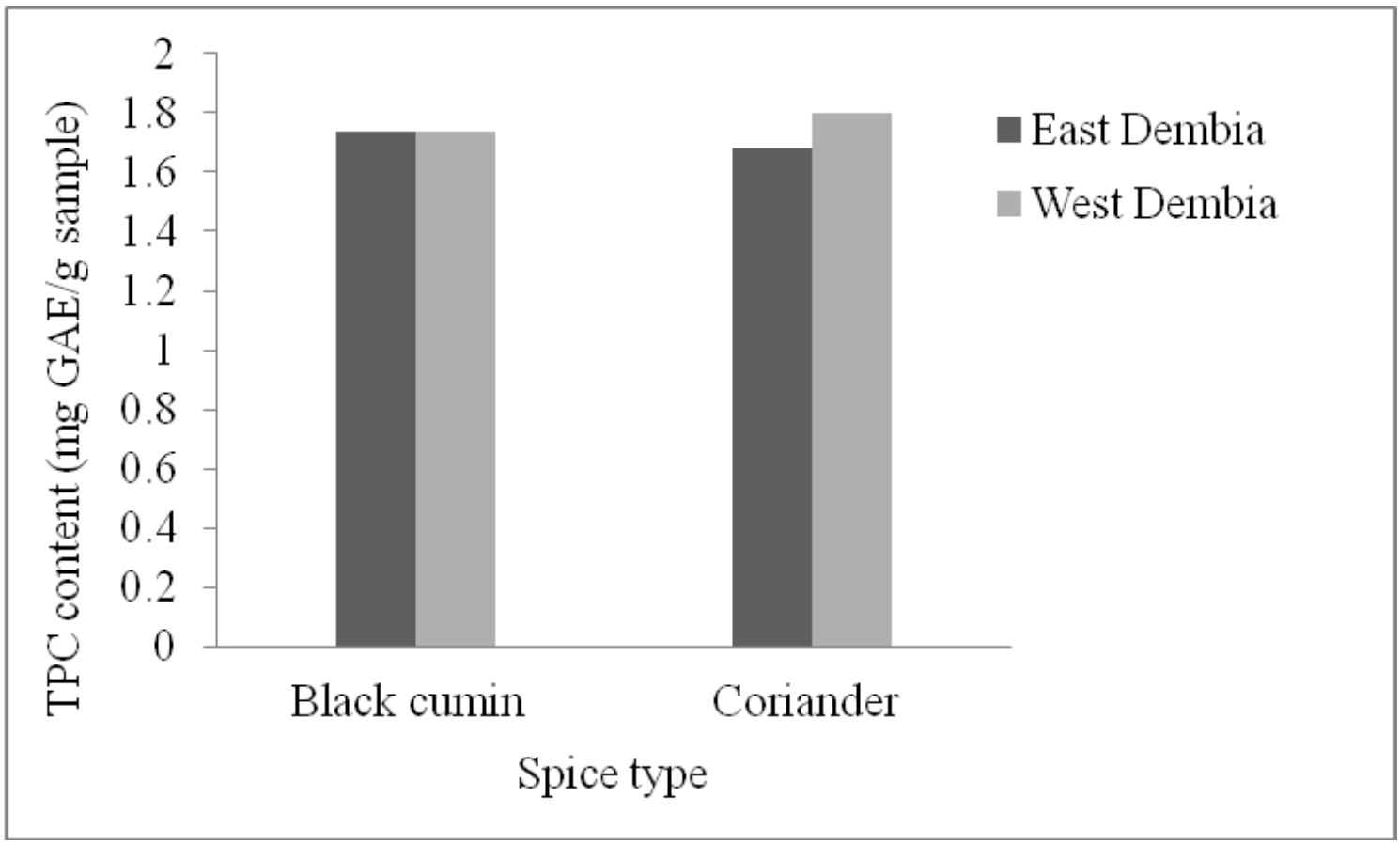


Figure 3

Comparison of optimized total phenol content yield of two spices in two districts

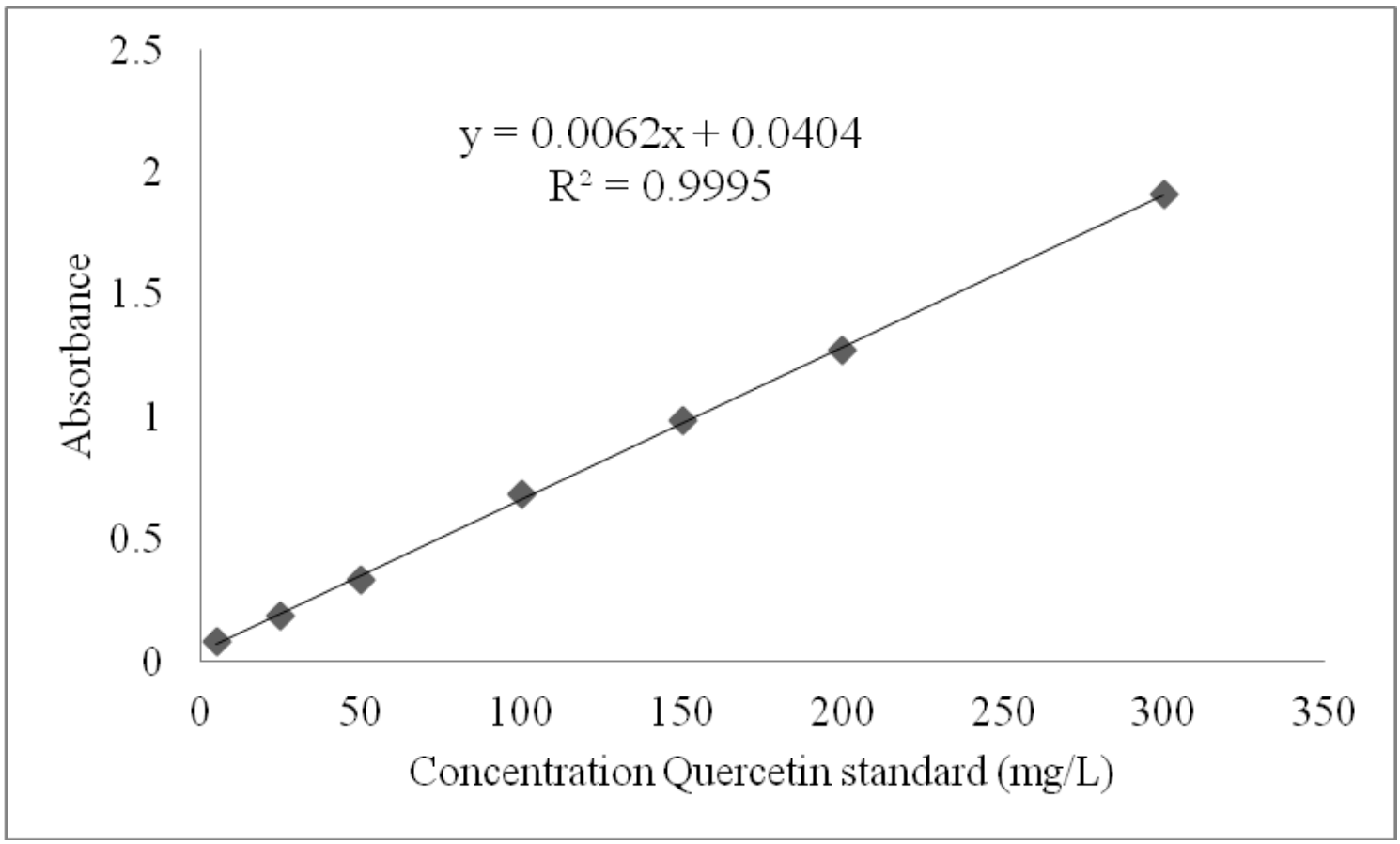


Figure 4

Calibration graph of Quercetin standard solution

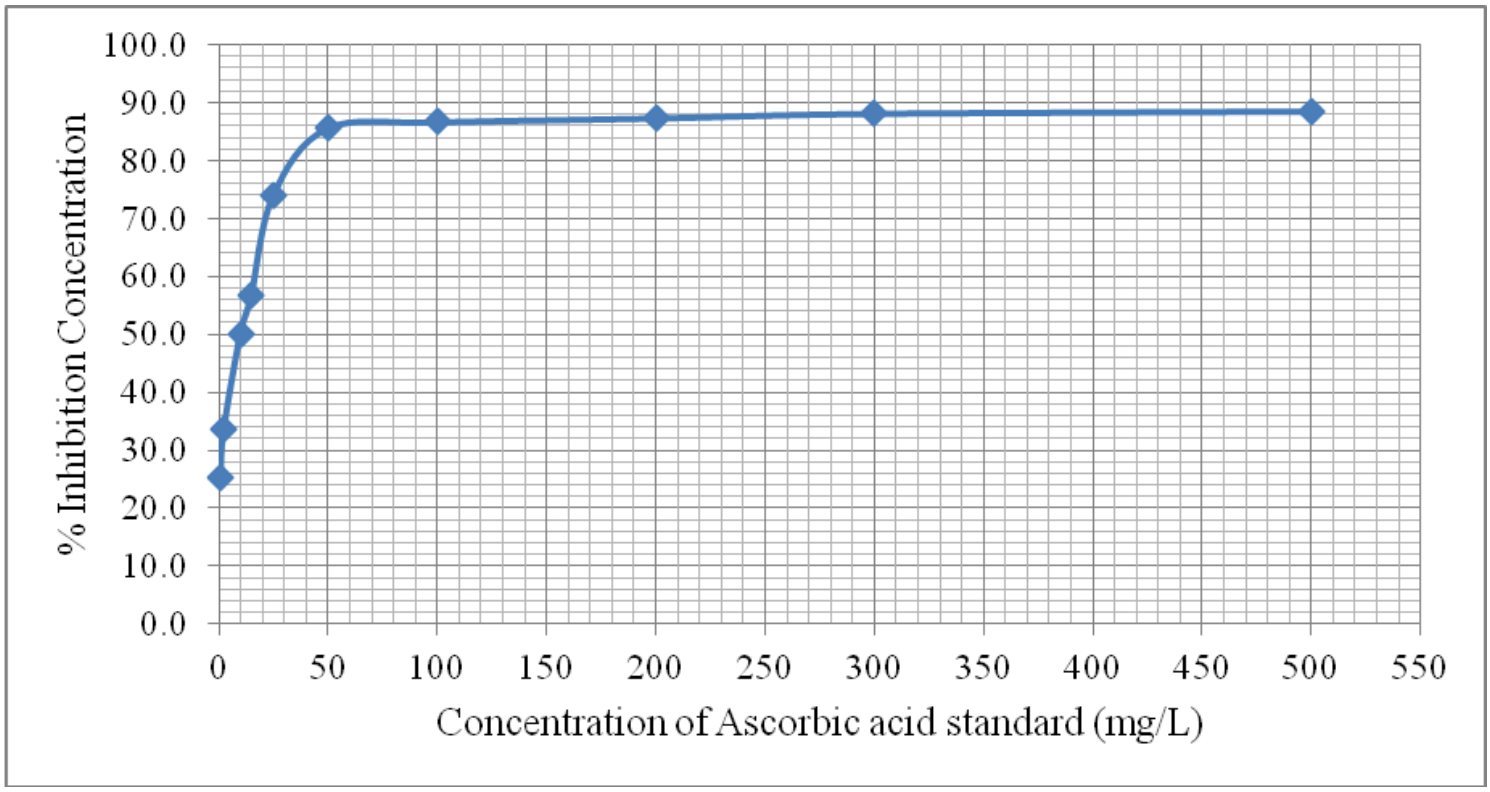


Figure 5

Standard curve of ascorbic acid

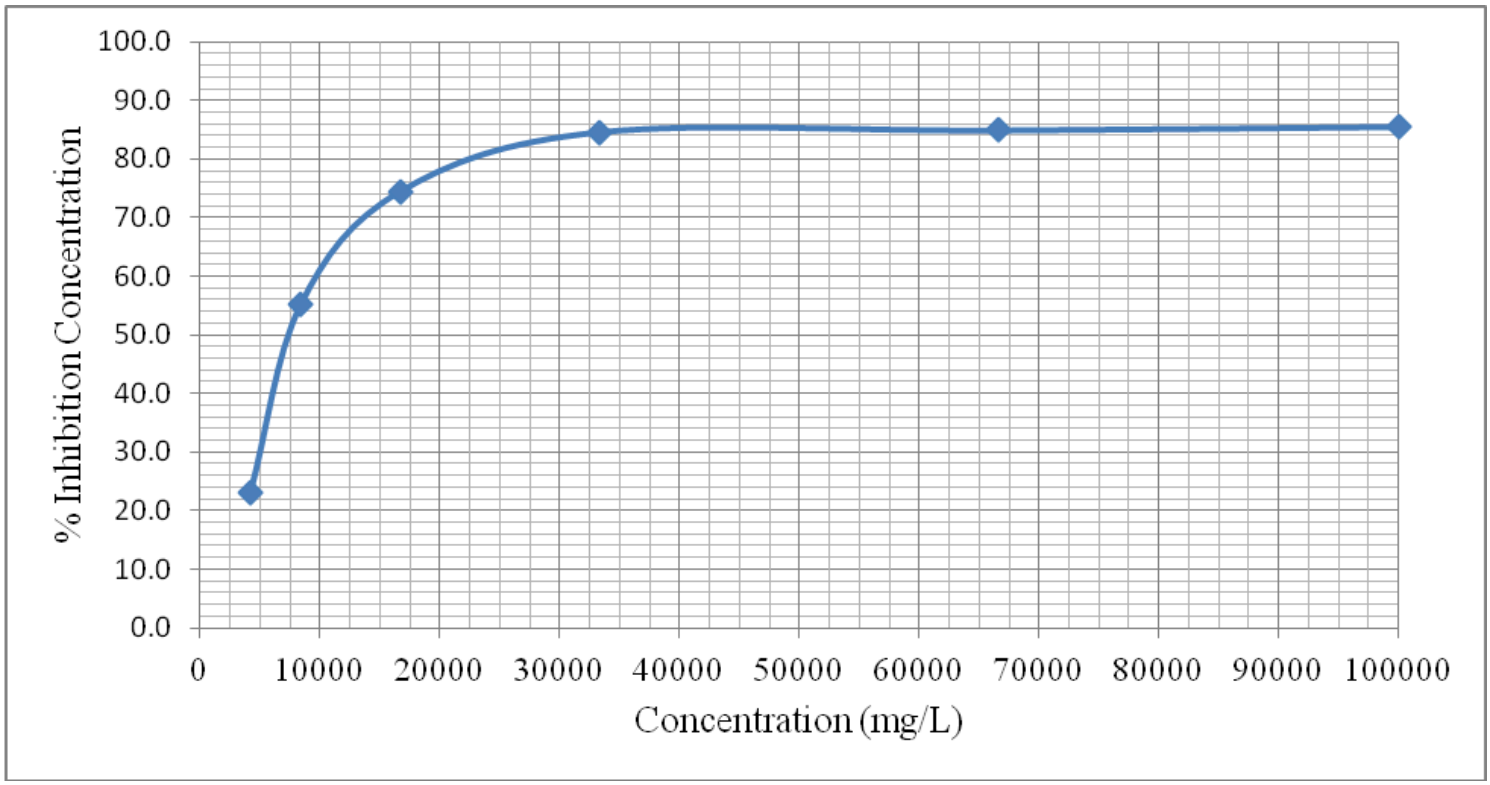


Figure 6

DPPH Free radical scavenging activity (% Inhibition) of Black cumin from East Dembia

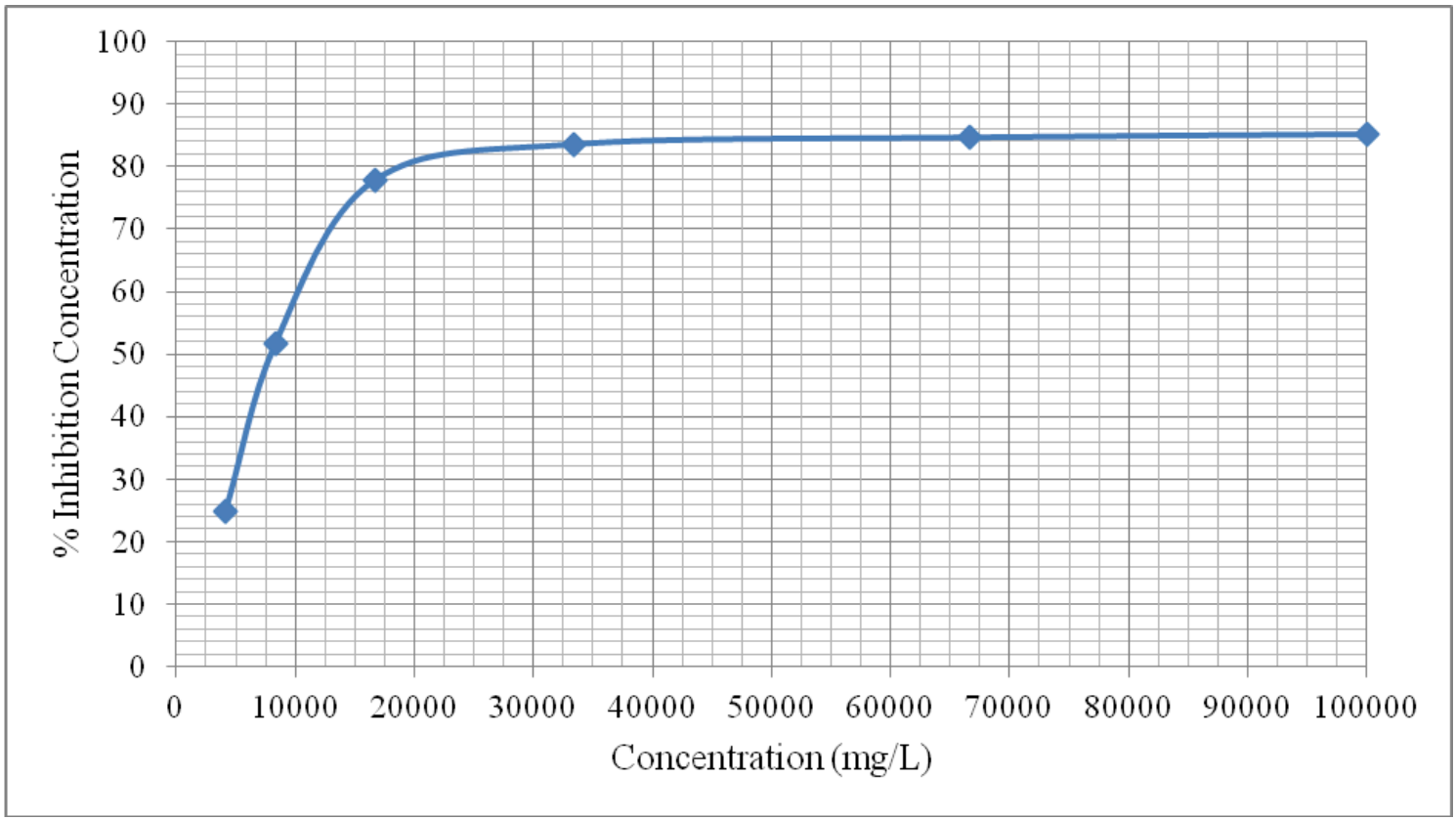


Figure 7

DPPH Free radical scavenging activity (% Inhibition) of Black cumin from West Dembia

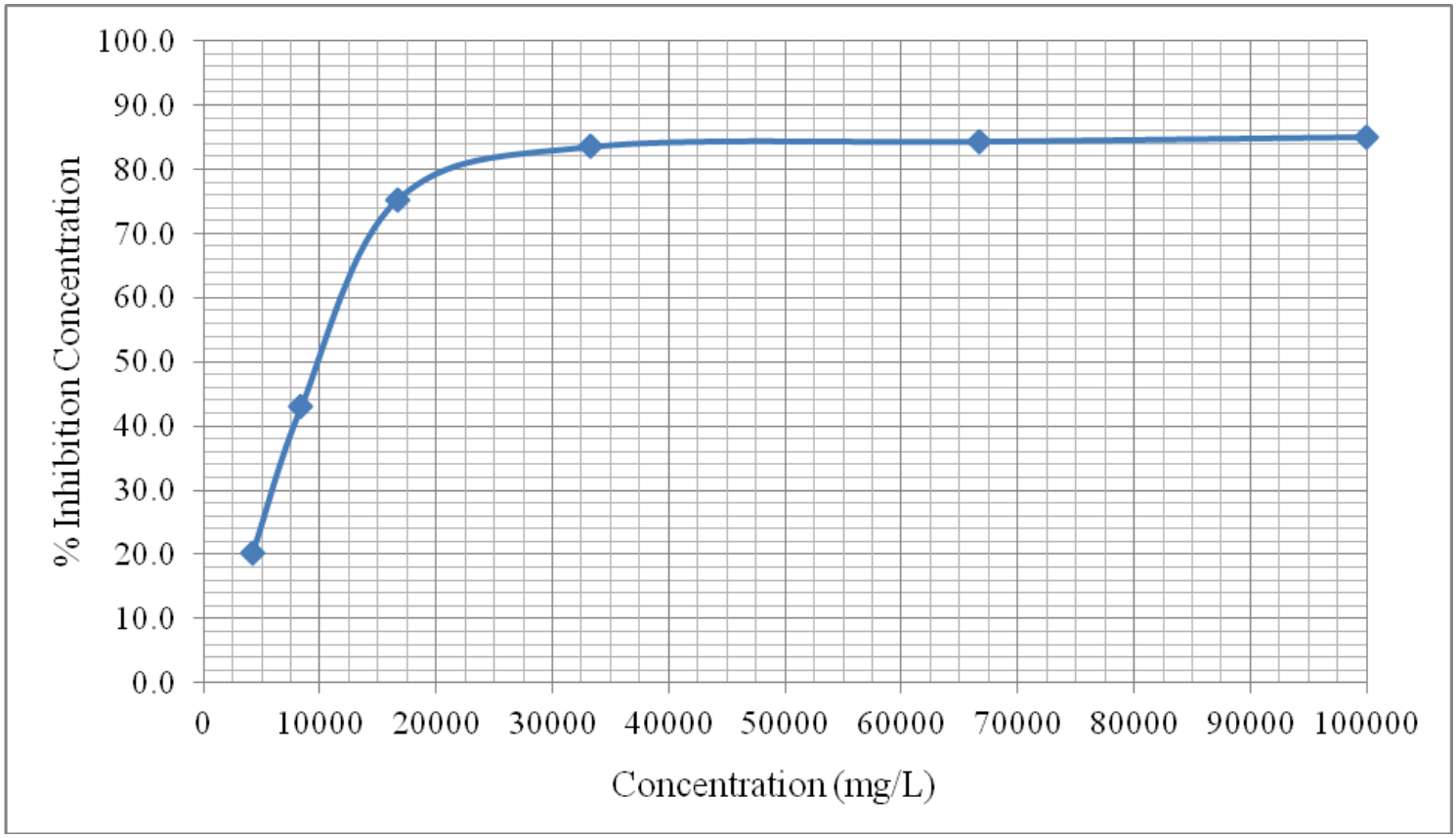


Figure 8

DPPH Free radical scavenging activity (% Inhibition) of Coriander from East Dembia

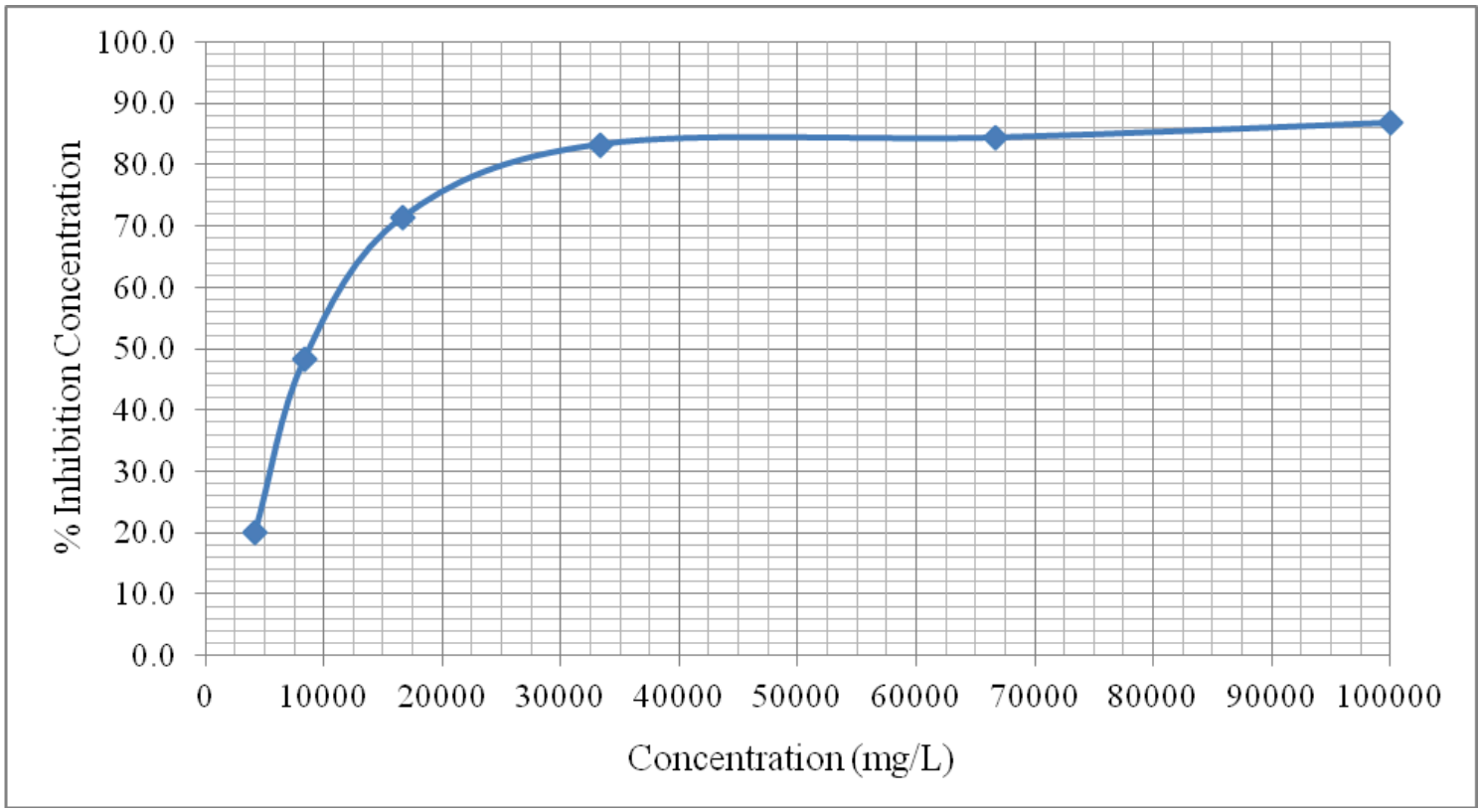


Figure 9

DPPH Free radical scavenging activity (% Inhibition) of Coriander from West Dembia

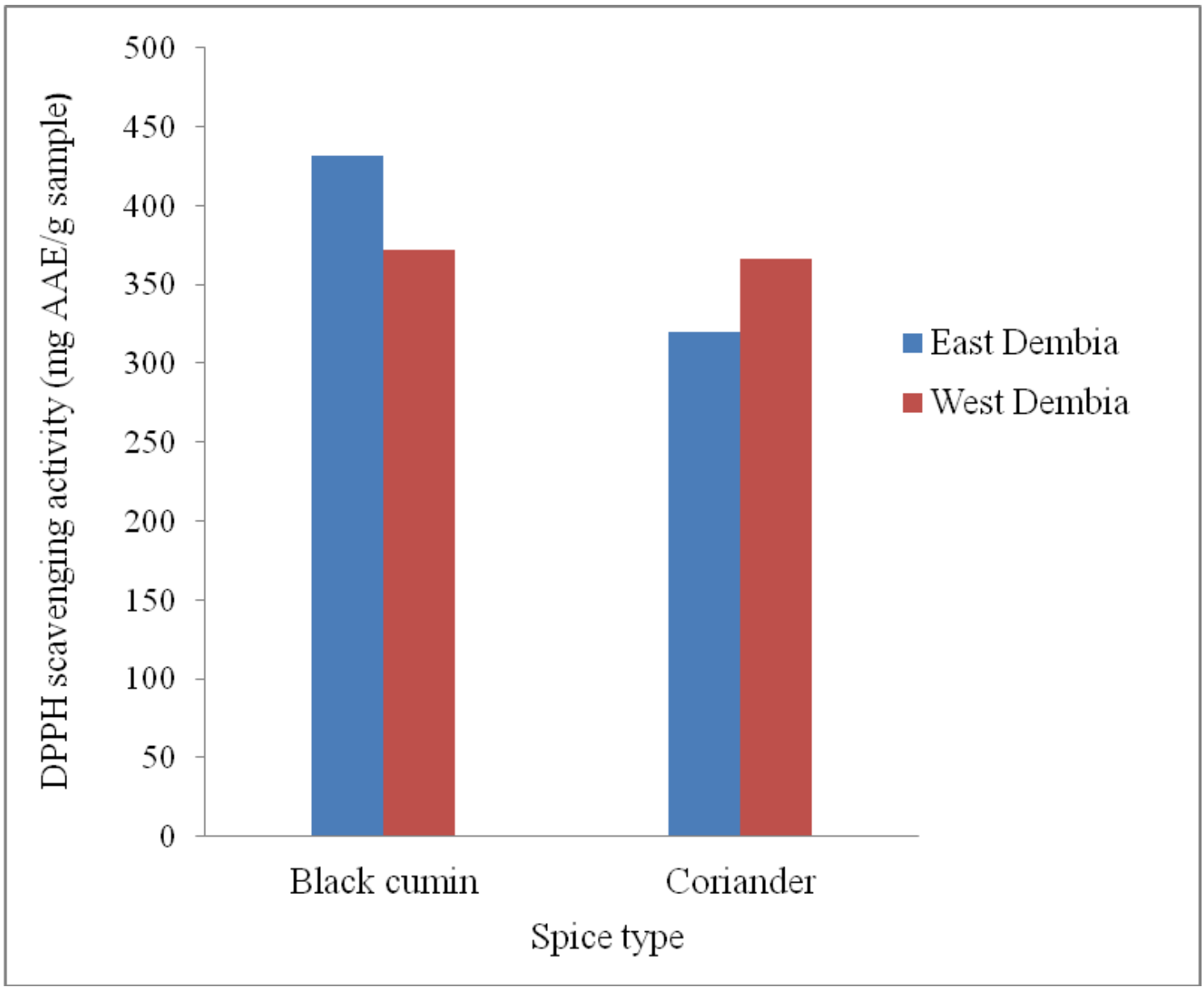


Figure 10

Comparison of antioxidant activities between Black cumin and Coriander in two different sampling sites