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CENTER FOR FOOD SCIENCE AND NUTRITION

STUDIES ON PHENOLIC CONTENTS AND BIOFUNCTIONAL ACTIVITIES OF SELECTED ETHIOPIAN SPICES AND HERBS

By

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Dedication

This dissertation is dedicated for those eighty four civilians, including my Dad, who were beheaded and shot down by Somali invaders in a place called Biyo Keraba, Doba Woreda, West Harrarghe (Eastern Ethiopia), on June 30, 1977 G.C.

May your souls continue to rest in peace?

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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ACRONYMS

3-Hydrb	3- Hydroxybenzoic acid
AA	Ascorbic acid
ABTS	2,2[1]-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)
AC	*Aframomum corrorima*
AGE	Advanced Glycation End product
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytolene
Caf	Caffeic acid
Cat	Catechin
Chl	Chlorogenic acid
Cinam	Hydroxycinamic acid
CSF	*Coriandrum sativum*, fruit
CSL	*Corinadrum sativum*, leaf
CSS	*Corinadrum sativum*, seed
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
ECG	Epicatechingallate
EGC	Epigallocatechin
EGCG	Epigallocatechingallate
Epicat	Epicatechin

Fer	Ferulic acid
FR	Ferric reducing power
FRAP	Ferric reducing Ability Plasma
FTC	Ferric thiocyanate
Fum	Fumaric acid
GC-MS	Gas Chromatography- Mass Spectroscopy
HPLC	High Performance Liquid Chromatography
HT-29	Cultured colon cancer cell lines
KB cells	Keratin-forming tumor cell line
IC_{50}	Concentration of antioxidant needed to reduce the original amount of radical by 50%
Isofer	Isoferulic acid
LAA	*Lippia adoensis* var. adoensis
LAK	*Lippia adoensis* var. koseret
LC-MS	Liquid Chromatography-Mass Spectroscopy
LDL	Low Density Lipoprotein
MCF-7	Human breast adenocarcinoma cell line
MDA	Malondialdehyde
ORAC	Oxygen Radical Absorbance Capacity
Phdz	Phloridzin
Phlor	Phloritin
PUFA	Polyunsaturated fatty acid
Q3-arglu	Quercetin-3-O- arabinoglucoside
Q3-gal	Quercetin-3-O- galactoside
Q3-glu	Quercetin-3-O- glucoside

Q3-rha	Quercetin-3-O- rhaminoside
Q3-rut	Quercetin-3-O- rutinoside
Qr	Quercetin
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
Suc	Succininc aid
Syr	Syringic acid
TA	Total antioxidant
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substance
TBHQ	*tert*- butylhydroquinone
TS	*Thymus schimperi*
TV	*Thymus vulgaris*

ABSTRACT

Spices and herbs have been added to foods since ancient times, not only as flavoring agents, but also as folk medicine and food preservatives. They show potential health benefits as they possess antioxidant and biologically active phytochemicals. In Ethiopia, people use dietary spices and herbs to flavor different cultural foods and also as folk medicine. *Thymus schimperi* (TS), *Lippia adoensis* var koseret (LAK), *Lippia adoensis* var. adoensis (LAA), *Aframomum corrorima* (AC), *and Coriandrum sativum* leaf (CSL), *Coriandrum sativum* fruit (CSF), and *Coriandrum sativum* seed (CSS) have been used as an important food flavoring agents in Ethiopia, and are also claimed to have various health benefits. TS, LAK, and LAA are endemic herbs to Ethiopia. In this study, the phenolic contents, the *in vitro* antioxidant activities and the effects of heat treatments on total phenolic content (TPC) and antioxidant potentials from the leaves extract of TS, LAK, LAA, CSL, fruit extract of CSF and seed extracts of CSS and AC were reported.The *in vitro* antidiabetic activity of TS and *T. vulgaris* (TV) and antiproliferative activity of AC on liver cancer HepG2 cell lines were evaluated. LAK, LAA, AC, CSL, CSF, and CSS were collected from Sidama Zone South Ethiopia. The TS was collected from North Shoa, Ethiopia. The leaf of TV was collected from campus of Dalhousie Agricultural College, Canada. Each dried sample was extracted with five solvents (petroleum ether, water, acetone, methanol, and aqueous: methanol, 20: 80, v/v). The antioxidant activity was determined using 2, 2-dipheny-l-2-picryl-hydrazyl (DPPH) assay method, ferric ion reducing, phosphmolybdenum, ferric ion chelating, and lipid peroxidation assays. The effects of thermal treatments (heated at 100 ^{o}C, 150 ^{o}C, and 180 ^{o}C for 1 and 2 h) on TPC and antioxidant capacity were investigated. The *in vitro* antiproliferation activity of petroleum ether and aqueous: methanol (20:80, v/v) extracts of AC on liver cancer HepG2 cell lines was conducted using [3-(4, 5-dimethylthiazol-2-yl)-5-(3-

carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] assay. Based on α–amylase and α–glucosidase inhibitions, the *in vitro* anti-diabetic activity of hot water and aqueous: methanol (20:80, v/v) extracts of TS and TV was evaluated.

The TPC ranged from 6.39 ± 0.62 mg GAE/g dried extract up to 122.04 ± 11.59 mg GAE/g of dried extract, while the TFC varied between 0.02 ± 0.01 and 45.11 ± 5.09 mg QE/g of dried extract. Five flavan-3-ols, seven phenolic acids, six flavonols, two dihydrochalcones, and two aliphatic organic acids were identified and quantified. Highest concentrations of total flavonols and phenolic acids were found in TS. Highest flavan-3-ols were found in CSS and highest aliphatic carboxylic acid levels were found in CSL. Q3-Rut was the most abundant flavonol and hydroxycinnamic acid was the most abundant phenolic acid in TS. Catechin (Cat) was the most abundant flavan-3-ols in CSS.

The antioxidant activity was concentration dependent. The aqueous: methanol (20:80, v/v) extract of LAA showed highest DPPH radical scavenging (IC_{50} = 7.96 ± 2.11 μg/mL), reducing power (IC_{50} = 79.55 ± 6.32 mg AAE/g of dried extract), and total antioxidant activity (1.98 ± 0.14 mg BHTE/g dried extract). Water extract of CSS showed the strongest iron chelating activity (IC_{50} = 53.95 ± 1.22 μg/mL), the leaf methanol extract of CSL had the highest percentage of linoleic peroxidation (78.30 ± 2.50%) and CSS showed the highest (82.64 ± 2.47%) inhibition of secondary lipid decomposition products. There were positive relationships (R^2 = 0.55–0.95) between TPC and DPPH scavenging activity (%) of the tested plant extracts but negatively correlated with ferrous chelating activity (%).

The TPC and DPPH scavenging activity (%) of most of these dietary spices and herbs were increased with the increasing heating temperature and prolong heating time. But LAK and LAA

showed reduction in TPC and antioxidant activities when heated at high temperatures and others showed different variation in all the activities. The DPPH scavenging activity (%) was strongly correlated (R^2 = 0.86, R^2 = 0.80) with TPC whereas the total antioxidant activity was moderately correlated (R^2 = 0.59, R^2 = 0.45) for all spices and herbs heated at different heating temperatures for 1 and 2 h, respectively.

Both aqueous: methanol (20:80, v/v) and boiling water extracts from TS and TV exhibited inhibitory activities against α–amylase and α-glucosidase. Aqueous: methanol (20:80, v/v) extract of TS showed the strongest α-amylase inhibition activity (IC_{50} = 0.33 ± 0.05 mg/mL), whereas, the hot water extract exhibited the strongest α-glucosidase inhibition (IC_{50} = 0.05 ± 0.01 mg/mL) activities. The petroleum ether extract of AC showed stronger inhibition of proliferation of HepG2 cells (IC_{50} = 105.36 ± 6.92 μg/mL) than that of aqueous: methanol (20:80, v/v) extract (IC_{50} = 282.01 ± 43.40 μg/mL) treated for 24 h.

The findings suggested that the methanol, acetone, and aqueous: methanol (20:80, v/v) herbal extracts (CSL, LAK, LAA, and TS) exhibited stronger antioxidant activities than that of spice (CSS, CSF, and AC) extracts. Except for LAK and LAA, thermal treatment increased the TPC and DPPH scavenging activities of the plant extracts. There is high potential for the polar solvent extracts to be utilized as sources of natural antioxidants in preventing various oxidative stresses, in the control of blood glucose for diabetes and as food preservatives in the functional food industry. However, on the contrary the nonpolar solvent extracts from the seeds of AC showed stronger inhibition of proliferation of HepG2 cell lines than polar solvent extracts suggesting that the nonpolar compounds are also important for their anticancer activity.

Key words: *Afamomum corrorima; antioxidant;* α–amylase; *Coriandrum;* α–glucosidase; HepG2; LC-MS*; Lipia adoensis;* phytochemical; Spices and herbs; thermal treatment*; Thymus,*

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CHAPTER 1: GENERAL BACKGROUND

1.1 General Introduction

Oxidation is essential to many living organisms for the production of energy to fuel biological processes. In healthy humans, free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are constantly formed in the human body by normal metabolic action. However, the uncontrolled production of free radicals is involved in the onset of many chronic diseases. Exogenous chemical and endogenous metabolic processes in the human body or in the food system might produce highly reactive free radicals, especially oxygen derived radicals, which are capable of oxidizing biomolecules, resulting in cell death and tissue damage (Volko *et al.*, 2007). Their action is opposed by a balanced system of antioxidant defenses including antioxidant compounds and enzymes. Upsetting this balance causes oxidative stress and if these excess free radicals are not eliminated by antioxidants, they may damage crucial extracellular or cellular components which can lead to the pathogenesis of certain chronic human diseases, including cancer, aging, diabetes and atherosclerosis (Moskovitz, *et al*., 2002; Christine and John, 2008). These chronic diseases have been linked to "oxidation and damage" of "cellular molecules" such as proteins, lipids and DNA. Therefore, there has been an increased interest in finding natural antioxidants because they protect the human body from free radical attacks and retard the progress of chronic diseases as well as retard lipid rancidity in foods (Edwin, 1996).

Antioxidants are a group of substances which, can inhibit, delay or reduce oxidative processes, while often being oxidized themselves. Antioxidants can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. In foods, they are capable of

delaying, retarding or preventing the development of food rancidity or other flavor deterioration due to oxidation (Jayathilakan *et al*, 2007). They delay the development of off-flavors by extending the induction period. Antioxidants have also been used in the health-related area because of their ability to protect the body against damage caused by free radicals. Although there are several enzyme systems within the body that scavenge free radicals, some bioactive phytochemicals, present in food, have been reported to exhibit antioxidant activity because of their free radical inhibition.

The appearance of foods is one of the major determinants of its appeal to the consumers and consequently influences its sales. Lipid oxidation and bacterial contamination are the main factors that determine food quality loss and shelf-life reduction. Therefore, delaying lipid oxidation and preventing food borne microorganisms are highly relevant to the nutritional quality, color, flavor, texture and safety of foods (Sema *et al.*, 2007; Karin and Daren, 2010). At present there are increasing interest both in the industry and in scientific research community for dietary phytochemials because of their strong antioxidant, antimicrobial properties and natural origin (Sevil *et al*., 2010), which exceed many currently used synthetic antioxidants.

Many food products undergo irreversible chemical changes when exposed to oxygen and light. The process of oxidation deteriorates color and flavor of food and beverage products containing susceptible fats, and can eventually create odors that affect the quality of a product. During production, processing, distribution and storage, food can undergo deterioration from chemical processes (Lucija *et al*., 2007). In addition, lipid oxidation is a cause for the problems associated with food losses and human diseases due to generation of free radicals. Fats, oils and lipid-based foods deteriorate through several degradation reactions both on heating and on long term storage. The main deterioration processes are oxidation reactions and the decomposition of oxidation

products which result in decreased nutritional value and sensory quality. The retardation of these oxidation processes is important for the food producer and, indeed, for all persons involved in the entire food chain from the factory to the consumer. Oxidation may be inhibited by various methods including prevention of oxygen access, use of lower temperature, inactivation of enzymes catalyzing oxidation, reduction of oxygen pressure, and the use of suitable natural antioxidants (Nicolalde *et al*., 2006).

Chemically synthesized preservatives such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly used as antioxidants in foods to prevent or retard lipid oxidation. However, restrictions on the use of these compounds are being imposed because of their carcinogenicity and some side effects (Velioglu, *et al*., 1998). Consumers are becoming more conscious of the nutritional value and safety of their foods and ingredients. The preference for natural foods and food ingredients that are believed to be safer, healthier and less subject to hazards is increasing compared to their synthetic counterparts (Farag *et al*., 1986).

The consumption of foods contaminated with some microorganisms represents a serious health risk to humans. The growth of microorganisms in foods may lead to spoilage, formation of toxins and quality deterioration of food products (Celiktas *et al*., 2007). In recent years, the essential oils and the herbal extracts from various species of edible and medicinal plants have attracted a great deal of scientific interest due to their potential as a source of natural agents to increase the safety and shelf life of foods and of natural biologically active compounds (Ste´phanie *et al*., 2006).

Dietary herbs and spices have been added to foods since ancient times as flavoring and preservatives. They are natural agents possessing a multiple purposes such as antioxidant (Iris *et*

al., 2006; Hui-Yin *et al.*, 2007), antimicrobial properties (Bin *et al*., 2011; Gian *et al*., 2011) and enriching the food with nutrients, vitamins, minerals and more over medicines against cancer agents (Pinent *et al*., 2008; Miloˇs *et al*., 2014), high blood pressure and cholesterol lowering (Dhanapakiam *et al*., 2008), and antidiabetic (Adeneye *et al*., 2008) in folk medicine. More over, these natural agents have an advantage of being readily accepted by consumers, as they come from naturally consumed edible sources (Jekyll, 1999). Therefore, the use of spices and herbs as antioxidants and food preservative (Mario *et al*., 2007; Gebrehana and Shimelis, 2013) activities in processed foods is becoming of increasing importance in the food industry as an alternative to synthetic antioxidants (Nuala *et al*., 2006).

Ethiopia is among the largest consumer of dietary spices and herbs in Africa (Nigist & Sebsebe, 2009). People use dietary spices and herbs to flavor different cultural foods and also as folk medicine. The spiced red pepper locally known as Berbere, widely used in daily meal of the people, is processed from different spices and herbs. However, the ratio and types of spices and herbs used may differ from home to home or region to region. Similarly, people use different combination of the mixture of the spice and herb plant materials to prepare datta (paste consisted of chill pepper, spice and herb ingredients) and also to flavor and preserve butter, milk, bread, meat, soups, and different vegetables (Nigist & Sebsebe, 2009).

1.2 Literature Review

1.2.1 Dietary spices and herbs

Spices and herbs are strongly flavored aromatic parts of plants, used in small quantities in food as a preservative, or flavoring in cooking and as traditional medicines (Belewu *et al*., 2005). Herbs are herbaceous plant materials (which are green, leafy parts of plants) with a pleasant

taste. Spices are dried material produced from seed, bark, root, fruit, or flower of shrubs and trees. Generally, the leaf of a plant used in cooking (e.g. thyme, mint and basil) may be referred to as a culinary herb, and any other part of the plant, often dried are named as spices. Spices can be the buds (cloves), bark (cinnamon), roots (ginger), berries (peppercorns), and aromatic seeds (cumin) (Nakatami, 1994). As demonstrated by coriander, some plants can even produce herbs (fresh leaves) and spices (fruits and dried seeds).

1.2.2 Dietary spices and herbs in Ethiopia

Spices and herbs are the most ancient and traditional products known from Ethiopia. According to tradition, the legendary Ethiopian Queen of Sheba has taken large quantities of different spices among other precious gifts when she had visited King Solomon of Israel about 992 BC (Caroline 2007). Today, spices and herbs still widely found and used in Ethiopia and they are important for flavoring food, as sources of color, for medicinal use, as sources of cash income and employment, and as a means of export-oriented diversification or to improve local livelihood.

Some species found in Ethiopia are indigenous, others are introduced. The main indigenous spices are *Aframomum corrorima* (korarima), Piper nigrum (Long pepper), and *Brassica spp* (Mustard), whereas *Lippia adoensis* var. koseret (koseret*), Lippia adoensis* var. adoensis (kesse), *Thymus shimperi* (Tosign) and *Thymus serrulatus* (Tessene) are endemic dietary herbs to the country. Other spices have been introduced in the country since the 15th century, like *Zingiber officinale* (ginger), *Bunium persicum* (cumin), *Pimpinella anisum* (anis), *Coriandrum sativum* (coriander), *Trigonella foenum-graecum* (fenugreek), and *Ocimum basillicum* (ocimum). Others have been introduced more recently since the 1960s and are still under development like

Zeylanicum (cinnamon), *Piper nigrum* L. (black pepper) and cardamom (*Elettaria cardamomum*) (Caroline, 2007).

In Ethiopia, production of spices is an important area to cater to the domestic and the export market. Spice-bearing plants are cultivated in the Southern and South-Western parts of the country. The country exports significant quantities of raw dried spice such as ginger, cardamoms, red pepper and coriander to Sudan, Yemen, Saudi Arabia, UAE, Djibouti and Egypt. Spices are high value and export oriented commodity crops, which play an important role in agricultural economy of the country. In addition, the Ethiopian spice extraction factory produces the oleoresin crude extract of paprika annum, ginger, and turmeric and the factory exports these products to different countries (http://www.ethiopianspiceextraction.com/profile.htm).

1.2.3 General description of selected dietary spice and herb plants used for the study

1.2.3.1 *Aframomum corrorima*

Aframomum corrorima, locally known as korarima (Amharic, Oromifa, Tigrigna) or the Ethiopian cardamom is a renowned spice and medicinal crop of the family Zingiberaceae, native to Ethiopia. The fruit (Figure 1.1 A) is widely cultivated in the south and south western part of Ethiopia. Ethiopian cardamom is known only from Ethiopia where it grows naturally. The fruit is dried (Figure 1.1 B) and the seeds are collected from in side of the pod. Korarima seeds (Figure1.1 C) are used in Ethiopia as spice in the preparation of hot red pepper and to flavor stew coffee, tea, paste of chili pepper (datta), and butter. They have also medicinal properties being used as tonic, carminative and pungative (Caroline, 2007) and to treat sore throat (Eyob *et al*, 2008) in traditional medicine. In spite of its high relative price compared with other local spices, korarima is widely consumed which shows the interest and importance that people attached to it.

The ground seed is often mixed with other spices to perfume traditional sauce stew dishes like "wat or waxxi". It can also be used to spice coffee, tea, bread, and butter.

(A) (B) (C)

Figure 1.1 *Aframomum corrorima*: fruit (A), dried fruit (B), and seed (C).

1.2.3.2 *Coriandrum sativum*

Coriander (*Coriandrum sativum* L.) is an annual herb (Figure 1.2 A) used as spice that belongs to the family *Apiaceae* (*Umbelliferae*), mainly cultivated from its seed throughout the year (Hedburg and Hedburg, 2003). The herb is cultivated commercially in Europe, Asia and Africa. It is used as a spice in culinary, medicine (Delaquis *et al*., 2002), in perfumery, food, beverage, and pharmaceuticals industries (Jansen, 1981). The essential oil and fatty oil of the fruits are used as raw materials for industrial use and for further processing. The green herb is used as spice and vegetable (Dyulgerov and Dyulgerova, 2013).

The ripe fruits (Figure 1.2 C) of coriander have a pleasant flavour owing to the particular composition of the essential oil. The fruits are used in the preparation of fish and meat, but also for baking. In India, the fruits are also extensively employed as a condiment in the preparation of

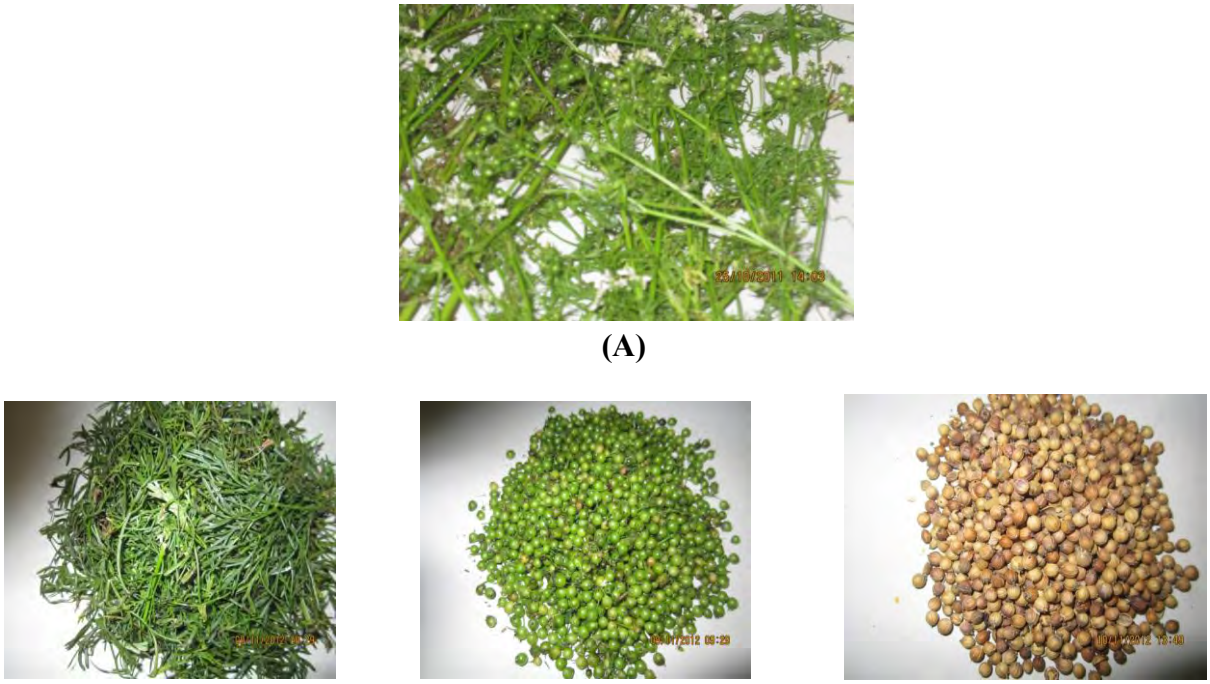
pickling spices, sausages and seasonings, and for flavoring pastry, cookies, buns and cakes, and tobacco products. The entire young plant is used to prepare chutneys and sauces. Coriander is used also to flavour several alcoholic beverages, e.g. gin (Jansen, 1981).

In Ethiopia, coriander is widely used along with other spices to add flavor to 'berbere' which is a spiced, hot red-pepper powder used for numerous meat, bread and vegetarian dishes. The leaves (Figure 1.2 B) and fruits are powdered to prepare a paste known locally as datta made up of chilli pepper and different spice and herbs which when consumed is known to improve appetite (Nigist and Sebsebe, 2009).

The seeds (Figure 1.2 D) are mainly responsible for the medical use of coriander and have been used as a drug for indigestion, against worms, rheumatism and pain in the joints (Chandan *et al.*, 2011). The seeds are boiled in water and drunk on an empty stomach to treat stomachache (Jansen, 1981). It is also traditionally used for treatment of ascariasis and hepatitis (Giday *et al*., 2007). Studies have also demonstrated hypoglycaemic action and effects on carbohydrate metabolism (Kamran, *et al.*, 2012). Volatile components in essential oil, from both seeds and leaves, have been reported to inhibit growth of a range of micro-organisms (Suganya, *et al.,* 2012; Darughe *et al.*, 2012), cholesterol lowering (Dhanapakiam, *et al.*, 2008), and inhibition of lipid peroxidation (Anita *et al,*, 2014).

Different studies on leaf, seed and root of this herb demonstrated anticancer activity. According to the report by Tang *et al* (2013), the ethyl acetate extract of *Coriandrum sativum* roots showed the highest antiproliferative activity on MCF-7 cells (IC_{50} = 200.0 ± 2.6 μg/mL), inhibited DNA damage and prevented MCF-7 cell migration induced by H_2O_2, suggesting its potential in cancer

prevention and inhibition of metastasis. Also study conducted on HT-29 cell showed that ethanol extract of the leaf possesses significant anticancer activity (Nithya, *et al.*, 2014).



(A) **(B)** **(C)** **(D)**

Figure1.2 *Coriandrum sativum*: the whole plant (A), leaf (B), fruit (C), and seed (D)

1.2.3.3 *Lippia adoensis*

The genus Lippia (*Verbenaceae*) is widely distributed in tropical and subtropical regions of the Americas and Africa, and it consists of approximately 200 species of herbs, shrubs, and small trees (Terblanche & Kornelius, 1996). Most of these species are traditionally used in the indigenous systems of medicine for the treatment of a variety of human aliments. The majority of them have been used for the treatment of stomach ailments, cardiovascular troubles, coughs, colds and asthma, tranquillizing remedy, prevention of gastritis, headache etc. (Raul *et al.*, 2011; Mamun-Or-Rashid *et al*., 2013). Some of the species exhibited strong antioxidant (Naznin &

Hasan, 2009), antidiabetic (Rangachari and Savarimuthu, 2011), insecticidal (Okonkwo & Ohaeri 2013) and antimicrobial (Sandra *et al*., 2012**)** activities.

Lippia adoensis is one of the five *Lippia* species in Ethiopia where it occurs as an erect woody shrub up to 1-3 m tall (Hedberg *et al*., 2006). It is endemic herb to the afromontane region of Ethiopia. The leaves of *L. adoensis* are used in Ethiopian traditional medicine for the treatment of various skin diseases including eczema and superficial fungal infections (Hailu *et al.*, 2004), also for food flavoring agent and preservative (Riot *et al.*, 2005). Two varieties are recognized in Ethiopia, the wild variety (var. adoensis) and the cultivated variety (var. koseret sebsebe).

Lippia adoensis var. koseret Sebsebe (Figure 1.3 A), locally known as koseret, is widely grown in the central and southern highlands of the Ethiopia. Traditionally, the dried leaves are used as one of the ingredients in the preparation of spiced butter. The special taste and flavor of the Gurage kitfo (minced meat with spiced butter) is attributed to essential oil imparted by the leaves (Nigist & Sebsebe, 2009). The dried leaves powdered together with barley are consumed to get relief from stomach complaints (Megersa *et al*, 2013). The recent study conducted against human pathogenic bacteria and fungi, indicated that the alcohol based extracts (methanol and ethanol) showed stronger antimicrobial activity than water extract of leaf of *Lippia adoensis* var. koseret (Gemechu *et al*., 2015).

Lippia adoensis var. adoensis (Figure 1.3 B), the wild variety, locally known as kesse, has lemon-like odour. In different parts of the country people use the leaf to flavor milk, butter and also as one of the ingredients to prepare spiced red pepper, locally known as berebere (Nigist & Sebsebe, 2009). In some parts of the country, the dried leaf is ground with roasted barley and mixed with butter to prepare a paste known as “Chuko”, the traditional food prepared from

roasted flour of barely and butter (Personal communication). In addition the fresh leaves of this wild herb are used for washing wooden and ceramic kitchen utensils to impart fresh and spicy fragenance.

(A)

(B)

Figure 1.3 *Lippia adoensis* var. koseret (A) *Lippia adoensis* var. adoensis (B)

1.2.3.4 *Thymus schimperi* Ronniger

The genus *Thymus* (Lamiaceae) includes about 350 species worldwide and is widely distributed in temperate zones (Sebsebe, 1993). The essential oil known as thyme oil is used in the food flavoring and preservatives (Ehivet *et al*., 2011), perfumery and pharmaceutical industries (Ballester-Costa *et al*., 2013). *Thymus schimperi* Ronniger (Figure 1.4), locally known as 'Tosign' in Ethiopia, is a wild endemic aromatic herb to Ethiopia, occurring in open grassland between bare rocks on slops and tops of mountains, sometimes growing near ditches. The dried leaves are used to flavor tea, coffee, food and also boiled as a tea substitute and are believed to be good for diabetic patients (Nigist & Sebsebe, 2009). A tea made by the herb in water is also recommended as a local medicinal remedy for respiratory problems (cough, bronchitis, sore

throat), gastrointestinal disorders, (colic, dyspepsia gastritis, flatulence, and diarrhea) and liver disease (Abebe & Ayehu, 1993). Essential oil obtained from steam distillation of the freshly collected leaf also exhibited an antihelmentic effects (Jemal *et al*., 2011).

Figure1.4 *Thymus schimperi*

1.2.4 Introduction to phenolic phytochemicals

Phytochemicals are bioactive, non-nutritive, naturally occurring plant products found in fruits, vegetables, spices, herbs, and whole grains (cereals, legumes and oilseeds) and even in some animal tissues. There are large numbers of known phytochemicals the majorities of phenolic compounds and the most important groups are the flavonoids and phenolic acids, have protective or disease preventive properties. They represent a class of substances that vary widely in chemical structure, and have diverse mechanisms of action. It is well known that plants produce these chemicals to protect themselves. But recent researches demonstrate that they can protect humans against diseases because of their strong antioxidant and antimicrobial properties (Eric *et al*., 2012).They are naturally occurring components of foods, can be obtained through the consumption of a diet from a normal food supply, which includes fruits, vegetables, cereals grains, legumes, dietary herbs, spices and oil seeds except for some refined foods such as sugar or alcohol. Some of them may be found as rich deposits into specific foods, such as soybeans, wheat germ, green tea, flaxseed, colored vegetables, and fruits (Hai, *et al*., 2006).

1.2.4.1 Phenolic compounds

Phenolics are a large and heterogeneous group of phytochemicals of plant-based foods. The structural diversity of phenolics extends from simple one-phenol hydroxybenzoic and hydroxycinnamic acids to large polymeric macromolecules like proanthocyanidins and tannins. Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups. They are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants (Mumper, 2010).

Despite their wide distribution, the health effects of dietary phenolics have come to the attention of nutritionists only in recent years. Researchers and food manufacturers have become more interested in phenolic compounds due to their potent antioxidant properties, their abundance in the diet, and their credible effects in the prevention of various oxidative stress associated diseases (Manach *et al*., 2004). The preventive effects of these secondary plant metabolites in terms of cardiovascular, neurodegenerative diseases and cancers are reduced from epidemiologic data as well as *in vitro* and *in vivo* studies (Rasmussen *et al*., 2005). Furthermore, phenolics were found to modulate the activity of a wide range of enzyme and cell receptors. In this way, in addition to having antioxidant properties, phenolics have several other specific biological actions such as antimicrobial (Celiktas *et al*., 2007) and antidiabetic activities (Shobana, *et al*., 2009).

Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions (Velioglu *et al*., 1998). The antioxidative effect is mainly due to phenolic components, such as flavonoids, phenolic acids, and phenolic diterpenes (Zhao *et al*., 2003). The antioxidant activity of phenolics is mainly due to their redox properties which make them act as reducing agents, hydrogen donors, singlet

oxygen quenchers, decomposers of peroxides, and metal chelating agents (Izabela *et al*., 2010; Peng *et al*., 2011). Many of these phytochemicals possess significant antioxidant capacities that may be associated with lower incidence and lower mortality rates of cancer in several human populations (Velioglu *et al*., 1998).

Phenolic compounds show their antioxidant action by scavenging free radical *via* hydroxyl group of phenols and their reactivity attributed to aromatic phenolic moiety (Heim *et al*., 2005). They generally donate their hydrogen atom to reduce reactive species (Demiray *et al*., 2009) and themselves are converted into phenoxy radical ($ArO^{\cdot}$), which get resonance stability due to delocalization of unpaired electron over aromatic ring (Figure 1.5) furthermore which change into quinines as shown (Figure 1.6).

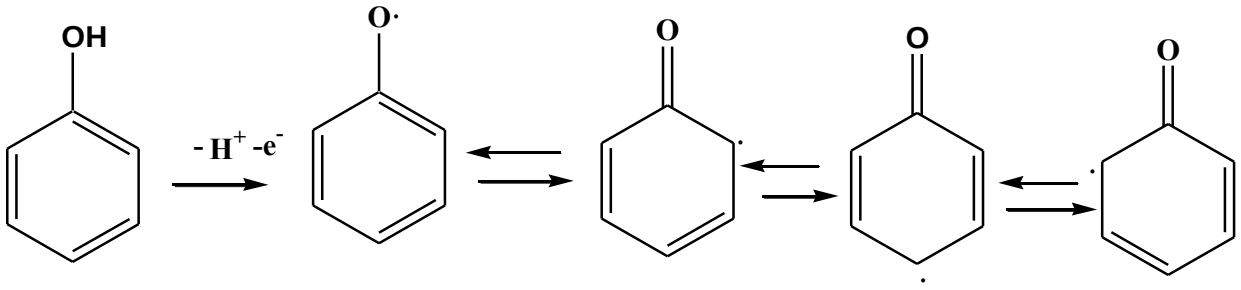
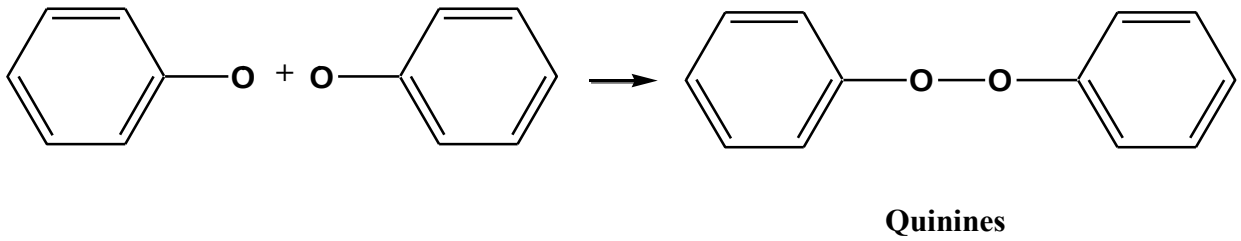


Figure1.5 Stabilization of phenol by delocalization of electron



Quinines

Figure1.6 Termination reaction of phenoxy radical

These numerous phenolic phytochemicals are classified depending on their ring structure and the number of carbon atoms that substitute the ring. Phenolic structure of these compounds could chemically differ from being simple molecules (e.g phenolic acids with a unique ring structure), presenting 2-3 phenolic rings (biphenyls and flavonoids), or being polymers of 12-16 phenolic groups, such as proanthocyanidins. The main groups of phenolics are: flavonoids, phenolic acids, antocyanins, stilbenes, chalcones, and lignans (Figure 1.7).

Flavonoids

Flavonoids are the largest group of naturally occurring, low molecular weight phenolic compounds in human diets that are widely distributed in the plant kingdom. They are a class of secondary plant metabolites that are thought to exert beneficial health effects through their antioxidant and chelating properties being the major contributor to the antioxidant capacity (Williams *et al*., 2004). In addition to their antioxidant function, flavonoids have biological effects, such as modulate cell signalling pathways, modulation of enzymatic activity, and inhibition of cellular proliferation, antimicrobial, and antidiabetic activities (Choudhary *et al*., 2010; Tajkarimi and Ibrahim, 2010). They show also antibiotic activities and could be used as antiallergic, antidiarrheal, antiulcer, and antiinflammatory agents (Cherng *et al*., 2007). The possible beneficial effects of flavonoids have resulted in their use as food supplements.

Natural flavonoids, especially their glycosides, are the most abundant phenolics in food and over 15,000 flavonoids have been separated and identified from plants and the list is constantly growing (Wahajuddin *et al*., 2013). They possess a unique C_6-C_3-C_6 structure (diphenylpropane structure) with phenolic OH groups (Figure 1.8). The general structure is two benzene groups connected by a three-carbon (propane) bridge. With the exception of chalcones, all flavonoids

found in foods have a pyran ring (oxygen-containing heterocyclic ring), which is formed by the addition of oxygen to position 2 of chalcones and subsequent cyclization of the three-carbon chain with the "A" ring. Their structure consists of two moieties: benzopyran (A and C rings) and phenyl (B ring) groups. Depending on the C ring type and to the linkage between the benzopyran and phenyl groups, six groups of flavonoids have been catergorized: flavones, flavonols, flavanones, isoflavones, flavanols (or flavan-3-ols), and anthocyanidins (Perla *et al*, 2012) (Figure 1.9). Their structural variation in each subgroup is partly due to the degree and pattern of hydroxylation, methoxylation, or glycosylation. The various subclasses of flavonoids are derived from this basic structure by changing the oxidation state and substitution (primarily hydroxylation) of the propane portion of the molecule.

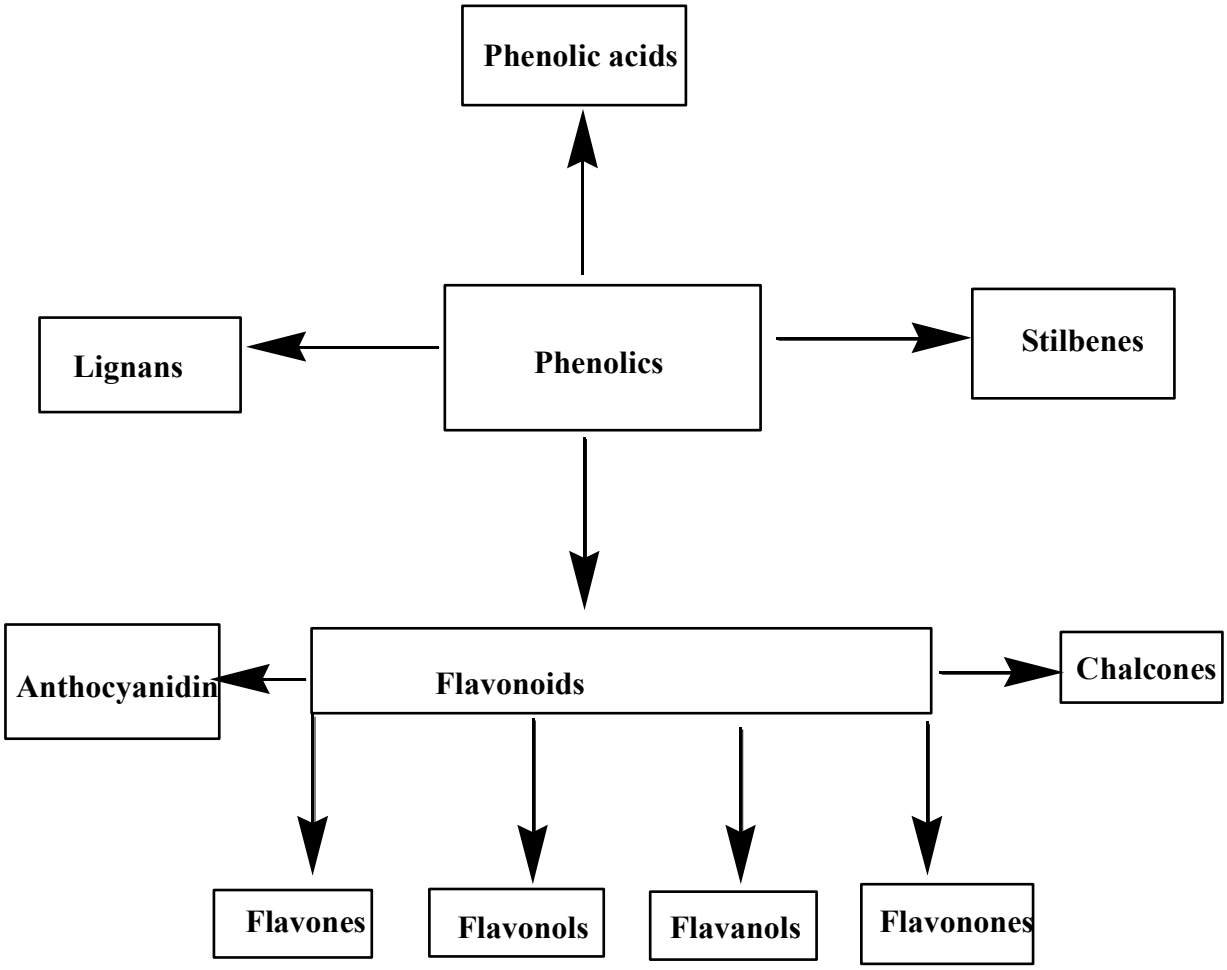


Figure1.7 Classification of phenolics

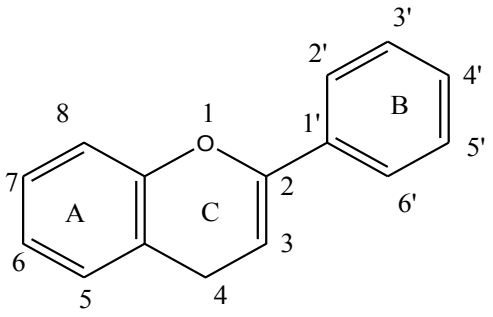
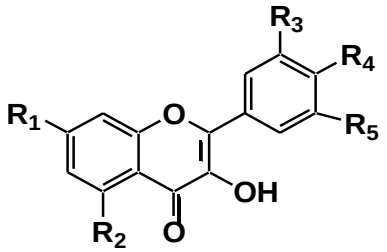


Figure1.8 Basic structure of flavanoids

The flavonols are the most widely distributed flavonoids, and the most common are quercetin, kaempferol and myricetin. Quercetin is a principal flavonoid in many plants; particularly high levels are found in onion, apples, kale, broccoli, and tea (Aneta *et al*., 2007).

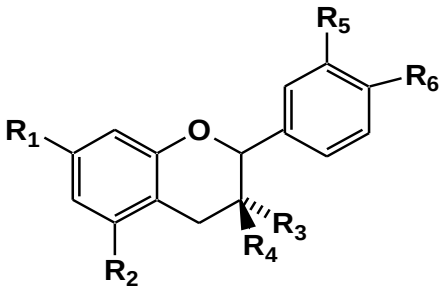
The prominent flavonoids in tea are the flavanols catechin, epicatechin, epicatechin gallate, epigallocatechin (EGC), and epigallocatechin gallate (EGCG) and their fermentation products. Propolis (a resinous hive product collected by honey bees from different parts of plants) is a rich source of flavanols (Soraia *et al.*, 2014). The flavanols, catechin and epicatechin are also found in red wine, condensed tannin polymers in fruits (eg, pomegranate and cranberry), some legumes and grains such as in sorghum grain varieties.

Flavonol



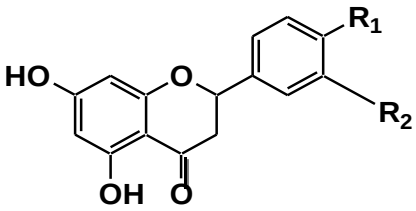
Quercetin: $R_1 = R_2 = R_3 = R_4 = OH, R_5 = H$
Myricetin; :$R_1 = R_2 = R_3 = R_4 = R_5 = OH$

Flavanol



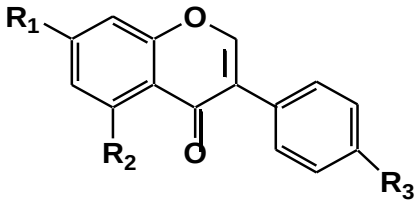
Catechin: $R_1 = R_2 = R_4 = R_5 = R_6 = OH, R_3 = H$
Epicatechin: $R_1 = R_2 = R_3 = R_5 = R_6 = OH, R_4 = H$

Flavanone



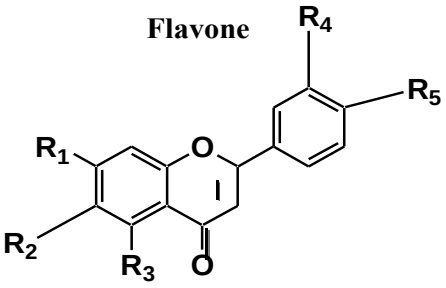
Hesperetin: $R_1 = OCH_3, R_2 = OH$
Naringenin: $R_1 = OH, R_2 = H$

Isoflavone



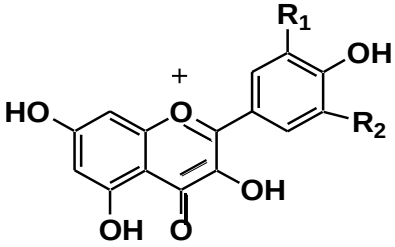
Genistein: $R_1 = R_2 = R_3 = OH$
Daidzein: $R_1 = R_3 = OH, R_2 = H$

Flavone



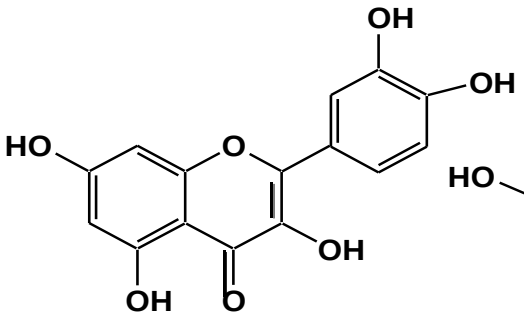
Apigenin: $R_1 = R_3 = R_5 = OH, R_2 = R_4 = H$
Luteolin: $R_1 = R_3 = R_4 = R_5 = OH, R_2 = H$

Anthocyanidin

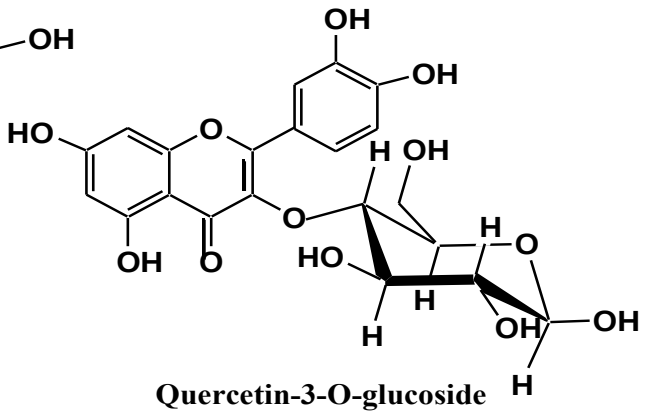


Cyanidin: $R_1 = OH, R_2 = H$
Delphinidin; $R_1 = R_2 = OH$

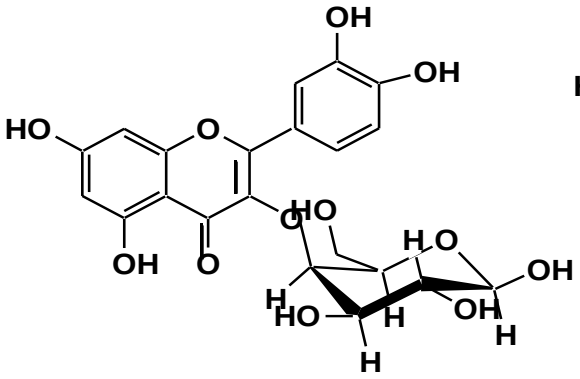
Figure1.9 Chemical structures of the six main classes of flavonoids; flavones, flavonols, flavanones, flavanols, isoflavones, and anthocyanidins.



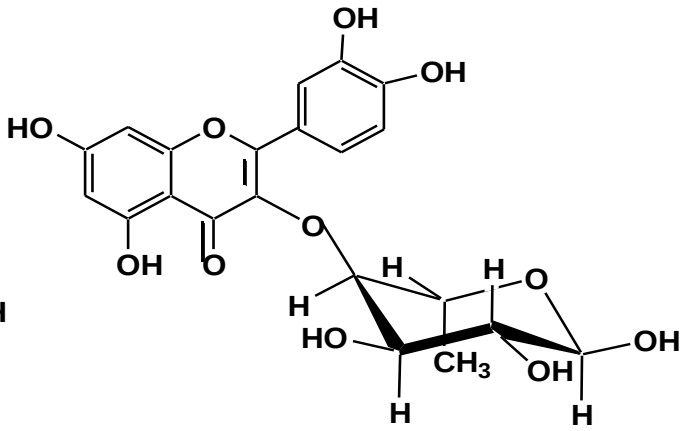
Quercetin aglycone



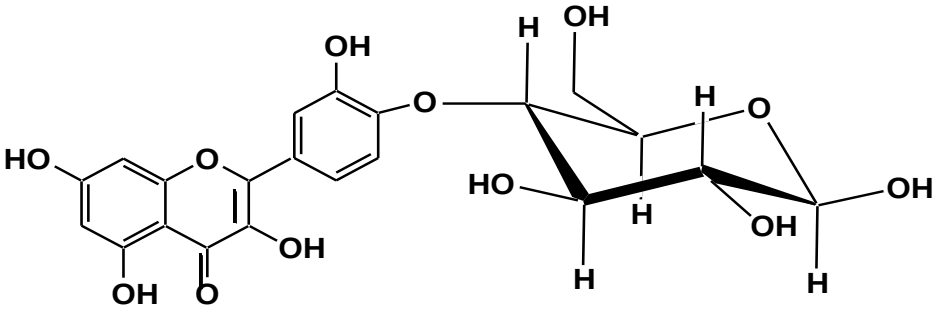
Quercetin-3-O-glucoside



Quercetin-3-O-galactoside



Quercetin-3-O- rhamonoside



Quercetin-4^{1}-O-glucoside

Figure 1.10 Typical quercetin glycosides found in foods

Most flavonoids are present in nature as glycosides and other conjugates, which contribute to their complexity and the large number of individual molecules that have been identified (Figure 1.10) (Harborne and Williams, 2002). In plants and most plant-derived foods, flavonoids are largely present as conjugates with the flavonoid aglycone linked to a variable sugar moiety by a β-glycosidic bond (Christina *et al*., 2012). Aglycones (the forms lacking sugar moieties) occur less frequently. At least 8 different monosaccharides or combinations of these (di- or trisaccharides) can bind to the different hydroxyl groups of the flavonoid aglycone (Jianbo *et al*., 2014). The most common sugar moieties include D-glucose and L-rhamnose. The glycosides are usually O-glycosides, with the sugar moiety bound to the hydroxyl group at the C-3 or C-4 position (Hasim *et al*., 2013).

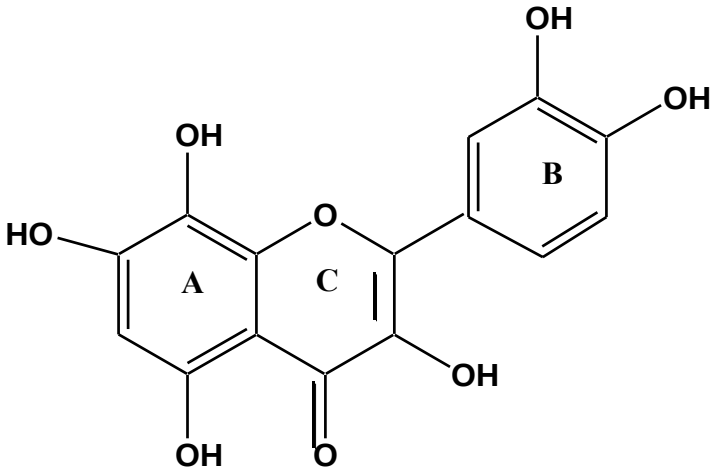


Figure 1.11 Structural features of flavonoids with high free radical scavenging activity

The antioxidant activity of flavonoid is due to their hydrogen donation ability, and their structural requirement considered being essential for effective radical scavenging, it has been reported that this activity may result from:

a) The presence of a 3', 4'-dihydroxy, i.e., o-dihydroxy group (catechol structure) in the B ring, possessing electron donating properties and being a radical target.

b) The 3-OH moiety of the C ring is also beneficial for the antioxidant activity of flavonoids.

c) The C2-C3 double bond conjugated with a 4-keto group, which is responsible for electron delocalization from the B ring, enhances further the radical-scavenging capacity.

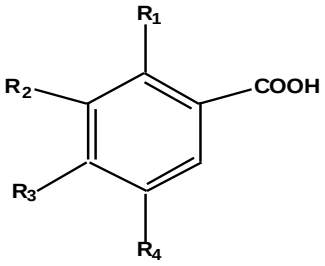
d) The presence of both 3-OH and 5-OH groups in combination with a 4-carbonyl function and C2-C3 double bond.

e) The presence of hydroxyl substituents in a catechol structure on the A-ring, which are able to compensate the absence of the o-dihydroxy structure in the B-ring, and becomes a larger determinate of flavonoid antiradical activity (Amić *et al*., 2003) (Figure 1.11).

Phenolic acids

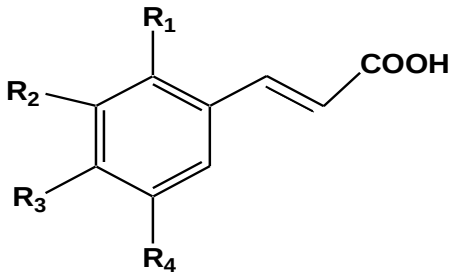
Phenolic acids are among the most important non-vitamin antioxidant phytochemicals naturally present in almost all vegetables fruits and whole grains. They are non-flavonoid phenolic compounds which can be divided into two main types (Figure 1.12), benzoic acid and cinnamic acid derivatives based on C1–C6 and C3–C6 backbones respectively (Tsao, 2010). Caffeic acid is the most abundant phenolic acid in many fruits and vegetables, most often esterified with quinic acid as in chlorogenic acid, which is the major phenolic compound in coffee (Aneta *et al*., 2007). Another common phenolic acid is ferulic acid, which is present in whole grains and is esterified with hemicelluloses in the cell wall (D'Archivio, *et al*., 2006). The most common hydroxybenzoic acid derivatives are *p*-hydroxybenzoic, vanillic, gallic and protocatechuic acids which are mainly present in the form of glucosides in plant diets (Zheng *et al*., 2001).

Hydroxybenzoic acid



Gallic acid; $R_2 = R_3 = R_4 = OH$, $R_1 = H$
Vanillic acid; $R_1 = R_3 = H$, $R_2 = OH$, $R_4 = OCH_3$
Procatechuic acid; $R_1 = R_2 = H$, $R_3 = R_4 = OH$

Hydroxycinnamic acid



Caffeic acid; $R_1 = R_2 = H$, $R_3 = R_4 = OH$
Ferulic acid; $R_1 = R_4 = H$, $R_3 = OH$, $R_2 = OCH_3$
Cinnamic acid; $R_1 = R_2 = R_3 = R_4 = H$
p-Coumaric acid; $R_1 = R_2 = R_3 = H$, $R_4 = OH$

Figure1.12 Chemical structures of some phenolic acids.

Anthocyanins

Anthocyanins are generally accepted as the largest and most important group of water soluble pigments in nature (Harborne, 1998). They are, one of the six subgroups of a large group of plant phenolic which are responsible for the orange, red, blue and purple colors of many fruits and vegetables such as apples, berries, beets, apples and onions (Mazza, 2007).

They are distinguished from other flavonoids due to their capacity to form flavylium cations (Figure 1.9) (Mazza, 2007). They occur principally as glycosides of their respective aglycone anthocyanidin chromophores with the sugar moiety generally attached at the 3-position on the C-ring or the 5- position on the A-ring. There are about 17 anthocyanidins found in nature, but only six (cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin, with cyanidin being the most common) are ubiquitously spread and of great importance in human diets (Jaganath and Crozier, 2010).

Tannins

Tannins are generally defined as naturally occurring phenolic compounds of high enough molecular weight present in many plant foods (Scalbert and Williamson, 2000).The name tannins is given to polymeric phenolic substances which are capable of tanning leather or precipitate proteins. They form complexes with proteins, carbohydrates, gelatin and alkaloids. They are divided into two groups based on their structures; hydrolysable tannins and condensed tannins (Fecka, 2009).

Hydrolyzable tannins are compounds containing a central core of glucose or another polyol esterified with gallic acid, also called gallotannins, or with hexahydroxydiphenic acid, also called ellagitannins (Figure 1.13). Hydrolyzable tannins, upon hydrolysis, produce gallic acid and ellagic acid and depending on the type of acid produced. Condensed tannins are all oligomeric and polymeric proanthocyanidins formed by linkage of C-4 of one catechin with C-8 or C-6 of the next monomeric catechin. Condensed tannins are oligomeric and polymeric proanthocyanidins consisting of coupled flavan-3-ol (catechin) units. Biosynthetically the condensed tannins are formed by the successive condensation of the single building blocks, with a degree of polymerization between two and greater than fifty blocks being reached (Tatjana *et al*., 2009).

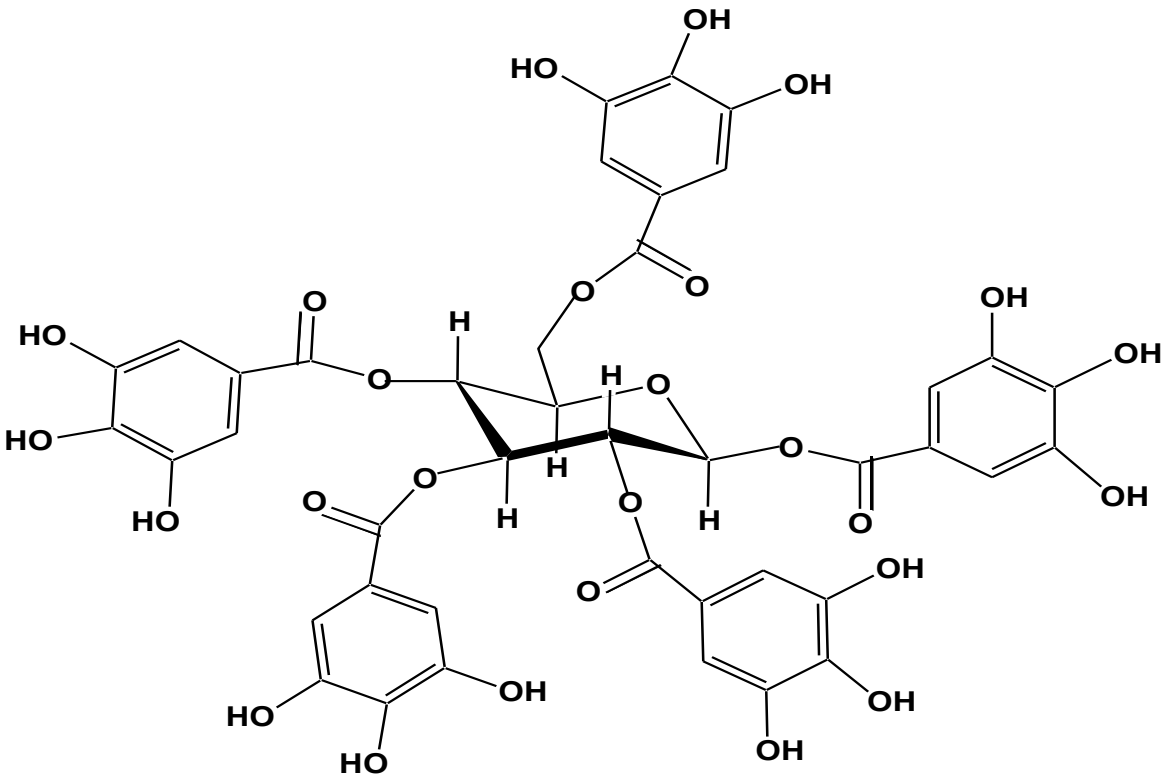
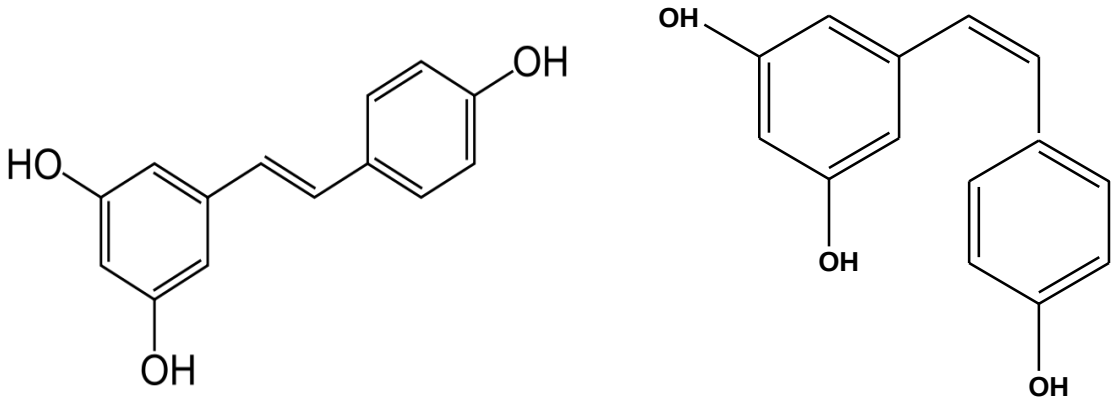


Figure 1.13 Gallotannin

Stilbenes

Stilbenes are non-flavonoid phenolics with diphenyl ethene derivatives, found in numerous plants distributed in several botanical families (Rivière *et al*., 2012). They are constituents of vine and wine and may be involved in health benefits brought by moderate wine consumption (Renaud and Lorgeril, 1992). Stilbenes are very interesting for their biological properties, such as potential antioxidative activity on human low density proteins (Jang *et al*., 1997), antimicrobial activity (Albert *et al*., 2011), and as cancer-chemopreventive natural products (Pace-Asciak *et al*., 1995). Resveratrol (3, 5, 4'-trihydroxystilbene) is present in relatively large amounts in grapes and red wine. It exists in both *cis* and *trans* isomeric forms (Figure 1.14), mostly in

glycosylated forms. In smaller quantities, resveratrol is also present in many plant species, where it has been found to act as an anti-fungicide and disease resistance in the plant kingdom (Rivière *et al*., 2012). In addition, resveratrol has been proven to possess antioxidative, anticarcinogenic, and antitumor properties (Fauconneau *et al*., 1997; Takao *et al*., 2002).



Trans- Resveratrol *Cis*- Resveratrol

Figure 1.14 Structures of *trans* and *cis*- Resveratrol

1.3 Statement of the Problem

Dietary herbs and spices have been used to preserve and flavor food for thousands of years before modern refrigeration was developed. In Ethiopia, people use different dietary spices and herbs or mixtures to flavour cultural foods and also as folk medicines

Little is known about antioxidant and biological activities of spices and herbs grown in Ethiopia. The studies conducted so far focused on essential oil of *Lippia adoensis, Ocimum basillicum, Zingiber officinale and Aframomum corrorima* for their antioxidant and antimicrobial properties (Hailu *et al*., 2004; Riot *et al*., 2005). So far no study has been conducted on the phenolic content

and antioxidant activities of the various solvent extracts and the effects of heat treatment on the phenolic content and antioxidant capacity of the selected dietary spices and herbs from Ethiopia. Also no study has been conducted on *in vitro* antidiabetic activity of *Thymus schimperi* and antiproliferitive effect of seed extracts of *Aframomum corrorima*.

This study focused on the leaves of *Lipia adoensis* var. adoensis (LAA), *Lippia adoensis* (LAK) var. koseret and *Thymus schimperi* (TS), fruits of *Coriandrum sativum* (CSF) and seeds of *Aframomum corrorima* (AC) and *Coriandrum sativum* (CSS). Among these plants, *Thymus schimperi*, *Lippia adoensis* var. koseret, and *Lippia adoensis* var. adoensis are endemic dietary herbs to the Ethiopia. Therefore, this study was carried out to evaluate the phenolic composition, *in vitro* antioxidant activities of various solvent extracts of these spices and herbs. In the addition, effect of thermal treatment on antioxidant activity and total phenolic content was also assessed before heating and the changes of phenolic content and antioxidant activity after heating at different temperatures (100 ^{o}C, 150 ^{o}C and 180 ^{o}C) for 1 and 2 h. Furthermore, the *in vitro* antidiabetic activity of *Thymus schimperi* and *Thymus vulgaris* and antiproliferative activity of *Aframomum corrorima* on liver cancer cells were also evaluated.

The relation of phenolic content and antioxidant activities of several species of spices and herbs which are commonly available in Ethiopia is still unknown. Hence, the phenolic content of different extracts was also conducted so that to investigate the correlation between total phenolic content (TPC) and total flavonoid contents (TFC) and antioxidant capacity of these selected spices and herbs.

The work of this PhD dissertation was specifically directed to address the following research questions:

a) do the selected spice and herb extracts exhibit antioxidant activities as compared to synthetic antioxidants?

b) do heating time and heating temperatures affect the total phenolic content and antioxidant capacity of the extracts?

c) which one of the two *Thymus* species (*Thymus schimperi* or *Thymus vulgaris*) showed stronger *in vitro* antidiabetic activity?

d) do seed extracts of *Aframomum corrorima* exhibit antiproliferative activity on liver cancer cell lines?

e) is there any correlation between phenolic contents and antioxidant activities?

1.4. Objectives

1.4.1. General objective

The overall objective of the study was to identify and quantify the phenolic composition and to determine the antioxidant activity of leaves of *Lipia adoensis* var. adoensis (LAA), *Lippia adoensis* (LAK) var. koseret and *Thymus schimperi* (TS), *Coriandrum sativum* (CSL), and *Coriandrum sativum* fruits (CSF) and seeds of *Aframomum corrorima* (AC) and *Coriandrum sativum* (CSS), evaluate the *in vitro* antidiabetic activites of TS and *Thymus vulgaris* (TV) also *in vitro* anticancer activity of AC. The selection of plant materials was based on the known use of the plants as food or herbal remedies.

1.4.2 Specific objectives

The specific objectives of the present study are to:

- determine the phenolic composition of the selected spices and herbs extracts.
- investigate the antioxidant capacity of various solvent extracts of the selected spices and herbs.
- assess the effect of thermal treatment on total phenolic contents and antioxidant capacity of the selected spices and herbs extracts.
- determine the *in vitro* antidiabetic potential of leaf extracts of *Thymus schimperi* and *Thymus vulgaris*.
- evaluate the effect of *Aframomum corrorima* seed extract on human liver cancer cell lines by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay.

CHAPTER 2: EVALUATION OF PHENOLIC COMPOSITION OF *Aframomum corrorima, Thymus schimperi, Lippia adoensis,* AND *Coriandrum sativum*

Abstract

Thymus schimperi (TS), *Lippia adoensis* var koseret (LAK), *Lippia adoensis* var. adoensis (LAA), *Aframomum corrorima* (AC), *Coriandrum sativum,* leaf (CSL), *Coriandrum sativum,* fruit (CSF), and *Coriandrum sativum,* seed (CSS) have been used as important food flavoring agents in Ethiopia since a time in memorial, and are also claimed to have various health benefits. The aim of this work was to carry out a chemical analysis focusing on secondary metabolites, particularly phenolic compounds, which have several roles in the plant physiological processes and have demonstrated significant capacity in the prevention of human health diseases. The total phenolic (TPC) and flavonoid (TFC) contents were evaluated using the Folin-Ciocalteu and aluminum chloride colorimetric method, respectively. Phenolic acids, flavonols, flavan-3-ols, dihydrochalcones, and aliphatic organic acids were characterized and quantified using ultra high-performance liquid chromatography coupled with diode array and electrospray ionization mass spectrometric detection (LC-MS). After extraction with (aqueous: methanol, 20:80, v/v), 22 compounds were characterized based on retention time, molecular ions, and the comparison with reference compounds. The TPC has ranged from 6.39 ± 0.62 to 122.04 ± 11.59 milligram of gallic acid equivalent per gram of dried weight (mg GAE/g dw). The TFC has varied between 0.02 ± 0.01 and 45.11 ± 5.09 milligram of quercetin equivalent gram of dried weight (mg QE/g dw). Five flavan-3-ols, seven phenolic acids, six flavonols, two dihydrochalcones, and two aliphatic organic acids were identified and quantified in the extracts. The highest concentrations

of total flavonols (133.93 ± 12.62 μg/g dw) and phenolic acids (827.81 ± 62.24 μg/g dw) were found in TS. The highest flavan-3-ols were found in CSS and highest aliphatic carboxylic acid levels were found in CSL, with values of 11.33 ± 1.10 and 2532.69 ± 60.70 μg/g dw, respectively. Quercetin-3-O-rutinoside (Q3-Rut) was the most abundant flavonol (81.50 ± 7.60 μg/g dw) and hydroxycinnamic acid was the most abundant phenolic acid (548.81 ± 71.59 μg/g dw) in TS. Catechin (Cat) was the most abundant flavan-3-ols in CSS (5.81 ± 2.29 μg/g dw). This is the first study on the phenolic composition of selected Ethiopian dietary spices and herbs, highlighting the importance of these natural products as a source of natural antioxidants.

Keywords: flavoniod; Folin-Ciocalteu; galic acid; herb; hydroxycinnamic acid; LC-MS; phenolic compounds; quercetin-3-O-rutinoside; spice

2.1. Introduction

Phenolic compounds or polyphenols are chemically complex and large family of phytochemicals. They are, by far, the main secondary metabolites in plants, and therefore are present in all foods of plant origin. The common structural feature of all phenolic compounds is the presence of phenolic hydroxyl group (s). Simple monomeric phenolics, such as benzoic acid derivatives and hydroxycinnamic acid derivatives, usually co-exist in plants with oligomeric and polymeric derivatives (tannins and lignans) (Fecka, 2009).

Aromatic plants such as herbs and spices are rich in the phenolic compounds, and have been widely used to extend the shelf life of foods (Jayathilakan *et al*., 2007) and in traditional medicine as treatment for many diseases (Botsoglou *et al*., 2002). Beside their antioxidant properties, they exhibit a wide range of biological activities, such as antiallergenic, antiartherogenic, antiinflammatory, antimicrobial, antidiabetic, and anticarcinogenic effects

(Jeffery and Keck 2008; Ranilla *et al*., 2010). The favorable effects derived from phenolic compounds consumed with foods have been most often attributed to their antioxidant activity (Scalbert *et al*., 2005). The positive effects of phenolics in relation to cardiovascular diseases are probably associated with their ability to increase the antioxidative capacity of the blood plasma and prevention of low density lipoprotein (LDL) oxidation and platelet aggregation. Overall, these substances have the capacity for acting as potent radical scavengers, inhibitors of enzymes, and have also an antihemorrhagic activity by tightening blood vessels (Cherng *et al*., 2007).

Analysis of the essential oils from different *Thymus* species indicated the presence of different components mainly the high phenolic monoterpenes such as carvacrol, thymol and α-terpineol (Ehivet *et al*., 2011; Zouari *et al*., 2011) and solvent extracts contain many phenolic acids such as rosmarinic, ferulic, caffeic, chlorogenic and p-coumaric acids and also different flavonoids (Iness *et al*., 2012; Zeghad and Merghem, 2013).

The essential oil from TS is a pale yellow liquid with a rich aromatic, warming, herbaceous odor. The main constituents of the essential oils are p-cymene, γ-terpinene, thymol, and carvacrol (Nigist *et al*., 2000). The chemical compositions of LAK and LAA investigated so far are essential oils. The oils from both forms were found to differ in their physical characteristics and chemical composition. Linalool, which was absent in the oil of the wild form, is the major component in the oil of the cultivated variety. Also limonene, perillaldehyde and piperitenone were reported in oils from the wild plants but not in oils from the cultivated plants. The uncommon monoterpene ketone, 2-methyl-6-methylene-2, 7-octadien-4-one (ipsdienone), was found in the oils of the cultivated and the wild plants **(**Berhanu *et al*, 2001**)**.

The seeds of AC contain different types of essential oil components with a pleasant odor (Jansen, 1981; Abegaz *et al*., 1994). According to Baser and Kürkcüoglu (2001) and Hymete *et al* (2006), the major components of the essential oil from dried seeds were 1,8-cineole and the sesquiterpene (E)-nerolidol which was most abundant in the dried pods collected from local markets. According to (Eyob *et al*., 2007) study conducted on fresh materials, the major constituents of the oil were found to be γ-terpinene in pods and 1,8-cineole in seeds, while sesquiterpenes were the main constituents of the husks, with (*E*)-nerolidol, β-caryophyllene and caryophyllene oxide as the main representative structures of the essential oils. Later on Eyob *et al*, 2008, reported the major component of the oil of the leaf was β-caryophyllene and rhizome oil was dominated by γ-terpinene and β-pinene.

The chemical compositions of coriander vary considerably with region and age of the product. The seeds cultivated from different countries contain an essential oil and the monoterpenoid linalool, as the main component (Nigist & Berhanu, 1998). The phenolic compounds, apigenin, catechin, p-coumaric acid, and aliphatic alkenals and alkanals were reported in *C. sativum* aerial parts (Rajeshwari and Andallu, 2012). According to various studies, coriander leaves and fruits are rich in phenolic compounds, mainly phenolic acids and flavonoids (Barros *et al*., 2012; Andrea, *et al*., 2013).

So far different studies indicated the chemical composition of essential oils of LAK, AC, LAA, and TS mainly monoterpenes and sesquiterpenes. To our knowledge no study has been reported on phenolic profile of these plants. Also no phenolic compound was reported form leaf, fruit, and seed extracts of *Coriandrum sativum* grown in Ethiopia. Therefore, the aim of the present study was to determine the TPC and TFC and also to characterize and quantify the major phenolic

compounds by using high-performance liquid chromatography coupled with mass spectrometry (LC–MS).

2.2. Materials and Methods

2.2.1. Standards and chemicals

Caffeic acid (Caf), ferulic acid (Fer), isoferulic acid (Isofer), quercetin-3-O-rutinoside (Q3-rut), epigallocatechin (EGC), catechin (Cat), epicatechingallate (ECG), epigallocatechingallate (EGCG), and epicatechin (Epicat) purchased from Chroma Dex (Santa Ana, CA, USA). Quercetin-3-O-galactoside (Q3-gal) and quercetin-3-O-rhamnoside (Q3-rha) were purchased from Indofine Chemical Co. (Hillsborough, NJ, USA). Quercetin-3-O-glucoside (Q3-glu), quercetin-3-O-arabinoglucoside, (Q3-arglu), chlorogenic acid (Chl), 3-hydroxybenzoic acid (3-Hydrob), hydroxycinnamic acid (Cinam), fumaric acid (Fum), succinic acid (suc), (syringic acid (syr), quercetin (Qu), quercetin-3-O-rutinoside (Q3-rut), phloritin (phlor), phloridzin (phdz), sodium carbonate, and Folin-Ciocalteu reagent, were purchased from Sigma-Aldrich (St. Louis, MO, USA). The other chemicals and solvents used in this experiment were of analytical grade reagent.

2.2.2. Plant materials

The leaves of TS were collected from 50 km north of Addis Ababa on the roadside to Fiche town, North shoa, Ethiopia. The leaves of LAK, and LAA were collected form 5 km south east of Chuko town. CSS, CSF, and CSL were collected from 10 Km east of Hawassa town from the farm near the Tula town, and the fruits of AC were collected from South west of Yirgalem town, Sidama Zone, Southern Ethiopia. All samples were collected in October, 2011.

2.2.3 Preparation of plant extracts for the determination of total phenolic and flavonoid contents

Fresh leaves of LAA, LAK, CSL and TS were air dried for ten days and CSF (fruit), CSS (seed), AC (seed) were air dried for 20 days at room temperature. Then the dried samples were ground to fine powder using electric grinder (FM100 model, China).The extracts were prepared by dissolving 1g of each fine powder separately in 10 mL of each solvent (petroleum ether, water, acetone, methanol, and aqueous:methanol, 20:80, v/v). The contents were kept in orbital shaker for 6h at room temperature. Thereafter, each extract was filtered using Whatman no.1 filter paper and evaporated to dryness under vacuum at 40 ^{o}C by using a rotary evaporator (Buchi, 3000 series; Switzerland). The extraction procedure was done in triplicate for each extracts and the resulting extract was dissolved in methanol and stored in a sealed plastic container at -20 ^{o}C until further investigation.

2.2.4. Sample extraction for LC-MS analysis

Each extract was prepared by dissolving 1 g of the fine powder sample separately in 10 mL of aqueous: methanol (20:80, v/v). The contents were kept in orbital shaker for 6 h at room temperature. Thereafter, each extract was filtered using Whatman no.1 filter paper and evaporated to dryness under vacuum at 40 ^{o}C by using a rotary evaporator (Buchi, 3000 series, Switzerland). The extraction was done in triplicate and the resulting extract was dissolved in 2 mL methanol and stored in a sealed plastic container at -20 ^{o}C until further investigation. Following extraction samples were sonicated (model 750D, VWR Intl. Ltd., Montreal, QC, Canada) for 1 min and then centrifuged (model Durafuge 300, Precision Scientific, Richmond, VA, USA) at 5000 rpm for 15 min. Then the supernatant was filtered through 0.2 cm syringe filters prior to the chromatography analysis.

2.2.5. Determination of total phenolic content (TPC)

The TPC was determined by Folin-Ciocalteu method as described in Shan *et al* (2005) with slight modification using gallic acid as standard. To 0.1 mL of the extract (1 mg/mL), 1 mL Folin-Ciocalteu reagent (diluted ten times) was added and the mixture was left for 5 min and then 1 mL (75 g/L) of sodium carbonate was added. The absorbance of the resulting blue color was measured at 765 nm with a UV- visible spectrophotometer (JENWAY, 96500, UK) after incubation for 90 min at room temperature. The TPC was estimated from gallic acid (1-100 µg/mL) and results were expressed as milligram gallic acid equivalent/gram of dried extract (mg GAE/g) using calibration curve $y = 0.02x + 0.09$, $R^2 = 0.99$ (Figure 2.1 A).

2.2.6. Determination of total flavonoid content (TFC)

The TFC was determined as described in Ayoola *et al* (2008) with minor modifications. The analysis was based on the formation of yellow color of flavonoid-aluminum complex. Aluminum chloride (2 mL, 2%) was mixed with the same volume of the leaf extract (1 mg/mL). Individual blanks were prepared consisting of 2 mL of sample solution and 2 mL of methanol without aluminum chloride. Then absorbance readings at 415 nm were taken after 1 h of incubation at room temperature against a blank sample. The TFC was determined using a standard curve (Figure 2.1 B) of quercetin at (1- 40 µg/mL) and values were calculated as milligram quercetin equivalents/gram of dried extract (mg QE/g) ($y = 0.024x + 0.112$, $R^2 = 0.98$).

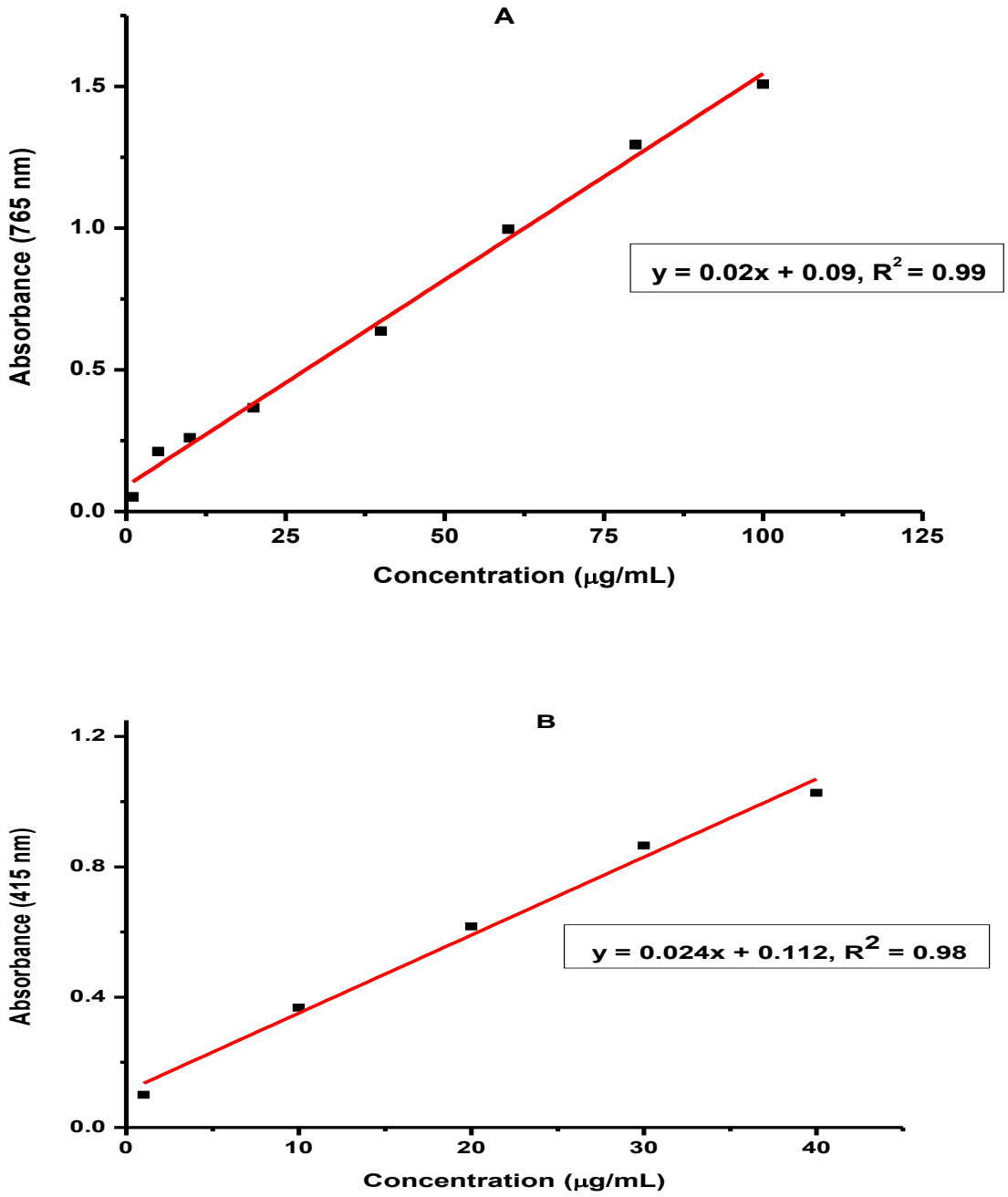


Figure 2.1 Gallic acid calibration curve for the determination of total phenolic (A) and quercetin calibration curve for determination of total flavonoid (B)

2.2.7 Identification of phenolic compounds

Separation and identification of phenolic compounds were carried out by using HPLC coupled to electrospray ionization and triple quadrupole mass spectrometry (LC-MS) as described by Rupasinghe *et al* (2010). The analyses of phenolic compounds in the extracts were performed using a Waters Alliance 2695 separations module (Waters, Milford, MA, USA) coupled with a Micromass Quattro micro API MS/MS system and controlled with Mass Lynx V4.0 data analysis system (Micromass, Cary, NC, USA). Electrospray ionization in negative ion mode was used in the multiple reaction mode of mass spectrometric analysis. The column used was an Allure biphenyl (100 mm x 2.1 mm) (Restek Chromatography Products, Bellefonte, PA, USA). For the separation of the flavonol, flavan-3-ol, phenolic acid and dihydrochalcone, and aliphatic organic acids, the mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetinitrile (solvent B) at a flow rate of 0.35 mL/min. A linear gradient profile was used with the following proportions of solvent A applied at time t (min); (t, A %): (0, 94%), (2, 83.5%), 2.61, 83%), (2.17, 82.5%), 3.63, 82.5%), (4.08, 81.5%), 4.76, 80%), 6.75, 20%), (8.75, 94%), 12, 94%). The retention time of each compound was compared with the retention time of the standards in different mobile phases. The peaks, showing the same retention time as that of the standards, were preliminary identified and were further analyzed by MS. The MS was used to get the molecular weights of the compounds by scanning from 50 to 1500 m/z.

2.2.8 Quantification of phenolic compounds

For the quantitative analysis of phenolic compounds, a calibration curve was obtained by injection of known concentrations (Table 2.1) of different standards compounds. The results were expressed in µg per g of dry weight (dw), as mean ± SD of three independent analyses.

Table 2.1 Regression equations of standard phenolic compounds for quantification of compounds in the extracts

No	Standard chemical used (range)	Regression equation	R^2
1	Cat (0.6–30 μg/mL)	y = 7135.87x + 4805.90	0.996
2	Epicat (0.44–22 μg/mL)	y = 10586.10x + 15241	0.972
3	EGCG (0.52–26 μg/mL)	y = 2523.35x + 2589.52	0.982
4	EGC (0.52–26 μg/mL)	y = 1751.87x- 290.08	0.999
5	ECG (0.48–24 μg/mL)	y = 4867.07x + 6811.45	0.972
6	Caf (0.466–23.3μg/mL)	y = 12161.281x + 15193.95	0.999
7	Chl (0.533–26.7 μg/mL)	y = 2426.04x + 2982.54	0.998
8	Fer (0.4–20 μg/mL)	y = 2590.53x + 4051.16	0.995
9	Isofer (0.4–20 μg/mL)	y = 461.42x + 39.11	0.999
10	Cinam (0.4–20 μg/mL)	y = 1534.72x + 330.12	0.956
11	Fum (0.4–20 μg/mL)	y = 180.777x	0.978
12	Suc (0.4–0.20 μg/mL)	Y = 1266.91x + 151.77	0.950
13	3-hydrob (0.4–20 μg/mL)	y = 847.02x + 161.43	0.989
14	Syr (0.4–20 μg/mL)	y = 1259.55x + 222.59	0.998
15	Q3-glu (0.458–22.9 μg/mL)	y = 9649.17x + 4449.30	0.999
16	Q3-gal (0,428.4–21.4 μg/mL)	y = 2853.93x + 8285.48	0.999
17	Q3-rut (0.428–21.4 μg/mL)	y = 7947.03x + 2168.42	0.999
18	Qu (0.486–24.3 μg/mL)	y = 19925.94x + 33058.53	0.986
19	Q3-rha (0.4–20 μg/mL)	y = 10784.83x + 8917.24	0.995
20	Q3-arglu (0.372–18.6 μg/mL)	y = 6688.89x + 1751.29	0.999
21	phlor (0.432–21.6 μg/mL)	y = 23269.70x + 40179.48	0.977
22	phdz (0.466–23.3 μg/mL)	y = 5664.83x + 611.43	0.977

2.3 Result

2.3.1Total phenolic content

Results showed that TPC content varied considerably in different plant materials and extracting solvents (Table 2.2). Among the methanol extracts, TS had the highest TPC (92.35 ± 2.68 mg GAE/g), followed by the LAA (66.23 ± 12.33 mg GAE/g), LAK (52.20 ± 5.37 mg GAE/g), CSL extracts (38.12 ±1.91 mg GAE/g) extracts. AC, CSS, and CSF contained the lowest TPC with the values of (23.79 ± 1.03 mg GAE/g), (21.33 ± 2.28 mg GAE/g), and (12.99 ± 2.52 mg GAE/g), respectively. The TPC were significantly different ($p < 0.05$) among LAA, LAK, and CSL. But no significant difference ($p > 0.05$) was observed between CSF and CSS in their TPC. The lowest TPC was detected in methanolic extract of AC ($p < 0.05$).

Similarly, in the aqueous: methanol (20:80, v/v) extracts, the TS had the highest TPC (95.92 ± 4.49 mg GAE/g) followed by LAA (72.47 ± 5.69 mg GAE/g), LAK (67. 61 ± 9.89 mg GAE/g), CSL (41.91 ± 3.11 mg GAE/g), and CSF (23.23 ± 1.83 mg GAE/g). AC (19.68 ± 1.34 mg GAE/g) and CSS (16.89 ± 0.59 mg GAE/g) had the lowest TPC. The TPC of TS was significantly higher ($p < 0.05$) than the rest of the extracts. No significant TPC variation was observed ($p > 0.05$) among AC, CSS, and CSF and also between LAA and LAK ($p > 0.05$). In acetone extract, among all the tested plants, TS leaves contained the highest amount of TPC (mg GAE/g) (122.04 ± 11.59 mg GAE/g) followed by LAA (50.53 ± 7.41 mg GAE/g), LAK (26.65 ± 1.27 mg GAE/g), AC (25.91 ± 3.36 mg GAE/g), CSL (25.13 ± 1.72 mg GAE/g). Both CSS (18.58 ± 0.98 mg GAE/g) and CSF (16.75 ± 2.41 mg GAE/g) contained the lowest TPC ($p < 0.05$).

In water extract, the highest TPC (mg GAE/g) was observed in LAA (45.71 ± 4.49 mg GAE/g) followed by TS (30.43 ± 5.65 mg GAE/g), LAK (33.93 ± 1.58 mg GAE/g), CSS (31.43 ± 1.03 mg GAE/g), CSL (21.08 ± 1.02 mg GAE/g), and CSF (17.85 ±1.55 mg GAE/g). The lowest amount of TPC ($p < 0.05$) was recorded in the AC (6.39 ± 0.62 mg GAE/g). No significant difference ($p > 0.05$) in the TPC was observed between CSF and CSL also among CSS, TS and LAK. But these values were significantly lower ($p < 0.05$) than the TPC of LAA. The lowest TPC was observed in petroleum ether extracts. Among the petroleum ether extracts, the CSS contained the lowest amount of TPC ($p < 0.05$).

Variation of TPC was also observed in various solvent extracts of the given plant material. The TPC of AC found the following order: acetone > aqueous: methanol (20:80, v/v) > methanol > petroleum ether > water. The CSS water extract contained the highest amount of TPC followed by methanol extract. The CSF methanol extract showed the highest TPC followed by aqueous: methanol (20:80, v/v), water, acetone, and petroleum ether extracts. In CSL extracts, aqueous: methanol (20:80, v/v) extract showed the highest TPC followed by methanol, acetone, water, and petroleum ether extracts. There was no significant difference ($p < 0.05$) in TPC between methanol and aqueous: methanol (20:80, v/v) extracts of CSF but these values were significantly higher ($p > 0.05$) than the TPC of the rest of extracts.

The TPC in various solvent extracts vary widely for leaf of TS, ranged from 11.25 ± 1.00 to 122.04 ± 11.59 mg GAE/g. The TPC followed the order: acetone > aqueous: methanol (20:80, v/v) > methanol > water > petroleum ether extracts. There was no significant difference ($p > 0.05$) in TPC between aqueous: methanol (20:80, v/v) and methanol extracts but these values were significantly different ($p < 0.05$) from petroleum ether, water, and acetone extracts. Acetone extract had the highest TPC ($p < 0.05$).

Table 2.2 Total polyphenols (mgGAE/ g of dried extract)* contents of *Aframomum corrorima* (AC), *Coriandrum sativum* seed (CSS), *Coriandrum sativum* fruit (CSF), *Coriandrum sativum* leaf (CSL), *Lippia adoensis* var koseret (LAK), *Lippia adoensis* var. adoensis (LAA) and *Thymus schimperi* (TS) obtained after extraction with different solvents (petroleum ether, water, acetone, methanol and aqueous: methanol)

	Extract				
Plant sample	Petroleum. ether	Water	Acetone	Methanol	Aqueous: methanol (20:80, v/v)
AC	14.23 ± 1.89^{bB}	6.39 ± 0.62^{aA}	25.91 ± 3.36^{bD}	12.99 ± 2.52^{aB}	19.68 ± 1.34^{aC}
CSS	6.43 ± 0.56^{aA}	31.43 ± 1.03^{dD}	18.58 ± 0.98^{aB}	21.33 ± 2.28^{bC}	16.89 ± 0.59^{aB}
CSF	10.70 ± 1.49^{bA}	17.85 ± 1.55^{bB}	16.75 ± 2.41^{aB}	23.79 ± 1.03^{bC}	23.23 ± 1.83^{aC}
CSL	10.70 ± 1.49^{bA}	21.08 ± 1.02^{cB}	25.13 ± 1.72^{bC}	38.12 ± 1.91^{cD}	41.91 ± 3.11^{bD}
LAK	10.20 ± 2.22^{bA}	33.93 ± 1.58^{dC}	26.65 ± 1.27^{bB}	52.20 ± 5.37^{dD}	67.61 ± 9.89^{cE}
LAA	11.57 ± 0.49^{bA}	45.71 ± 4.49^{eB}	50.53 ± 7.41^{cC}	66.23 ± 12.33^{eD}	72.47 ± 5.69^{cE}
TS	11.25 ± 1.00^{bA}	30.43 ± 5.65^{dB}	122.04 ± 11.59^{dD}	92.35 ± 2.68^{fC}	95.92 ± 4.49^{dC}

*Results are expressed as milligram of gallic acid equivalents per gram of dried extract (mg GAE/g). Values are expressed as mean ± SD (n = 3). Different letters after the means indicate significant differences among solvents (upper case) and plant sources (lower case) ($p < 0.05$).

The TPC from the leaf of LAK varied widely, ranging from 10.20 ± 2.22 to 67.61 ± 9.89 mg GAE/g. The value followed the order: aqueous: methanol (20:80, v/v) > methanol > water > acetone > petroleum ether extracts. There was no significant difference ($p > 0.05$) in TPC between acetone and water extracts but these values were significantly different ($p < 0.05$) from

petroleum ether, methanol, and aqueous: methanol (20:80, v/v) extracts. Also the TPC in various solvent extracts from the leaf of LAA varied widely, ranging from 11.57 ± 0.49 to 72.47 ± 5.69 mg GAE/g (Table 1). The TPC followed the order: aqueous: methanol (20:80, v/v) > methanol > acetone > water > petroleum ether extracts. There was no significant difference ($p > 0.05$) in TPC between acetone and water extracts but these values were significantly different ($p < 0.05$) from petroleum ether, methanol, and aqueous: methanol (20:80, v/v) extracts.

The variation among TPC may be assumed due to the presence of different types of phenolics and other constituents in different extracts, selectively extractable depending upon the extraction medium, *i.e.*, solvent employed. Having used several solvents, it was found that the best yields of phenolic compounds, for all the samples, were obtained in high polar medium, *i.e.*, acetone, methanol, and aqueous: methanol extracts. This is in agreement with a recent study on different plant extracts, where was found that the yield in TPC depends on the method and the choice of solvent, and that the highest amount was obtained in highly polar extracts (Alak *et al*., 2012; Duong *et al*., 2015). These Results showed that among all the solvent extracts; the aqueous methanol, methanol and acetone extracts had the highest TPC. This may be due to the fact that phenolics are often extracted in high amounts in more polar solvents such as aqueous methanol/ethanol as compared with absolute methanol/ethanol (Siddhuraju and Becker, 2003; Hismath *et al*., 2011).

2.3.2 Total flavonoid content (TPC)

TheTFC of various plant materials, extracted each with five different solvents, are given in Table 2.3. Using the $AlCl_3$ reagent, the TFC was reported as quercetin equivalents (QE) in reference to the standard curve $y = 0.24x + 0.11$, $R^2 = 0.98$. The TFC varied from 0.07 ± 0.251 to 45.11 ± 5.09 mg QE/g. In methanol extract, LAA had the highest TFC (45.11 ± 5.09 mg QE/g), followed

by CSL (29.45 ± 2.4 7 mg QE/g), TS (20.04 ± 2.30 mg QE/g), LAK (15.31 ± 1.48 mg QE/g), CSS (7.4 ± 0.34 mg QE/g), CSF (5.53 ± 0.4 mg QE/g) and AC (5.01 ± 0.56 mg QE/g). There was no significant difference ($p > 0.05$) in TFC between CSF and AC but these values were significantly lower ($p < 0.05$) than the TFC of the rest of the plants. The TFC of aqueous: methanol, v/v) extracts followed the order SCL (30.75 ± 2.04 mg QE/g) > TS (26.77 ± 1.91mg QE/g) > LAA (25.51 ± 3.86 mg GAE/g) > LAK (22.78 ± 0.29 mg QE/g) > AC (19.00 ± 0.13 mg QE/g) > CSF (17.98 ± 1.20 mg QE/g) > CSS (10.70 ± 0.61 mg QE/g). In acetone extracts, no significant difference ($p > 0.05$) of TFC was observed in LAK, LAA, and TS also among CSS, CSF, and CSL. AC contained the lowest TFC.

The TFC of various solvent extracts of TS varied from 0.12 ± 0.11 to 26.77 ± 2.91 mg QE/g and decreased in the order of aqueous : methanol (20:80, v/v) > acetone > methanol > water > petroleum ether extracts. The TFC in petroleum ether, water, methanol and aqueous: methanol (20:80, v/v) extracts were significantly different ($p < 0.05$), but in the acetone extract was not significantly different ($p > 0.05$) from aqueous: methanol (20:80, v/v) extracts.

For LAA, the TFC varied from 0.81 ± 0.32 to 45.11 ± 5.09 mg QE/g and decreased in the order of methanol > aqueous: methanol (20:80, v/v) > acetone > water extracts > petroleum ether (Table 1). The TFC in acetone and aqueous: methanol (20:80 v/v) extracts were not significantly different ($p > 0.05$), but these values were significantly different ($p < 0.05$) from water and methanol extracts. Methanol extract of LAA sample had the highest levels of TFC ($p < 0.05$). In LAK extracts, the TFC varied from 0.17 ± 0.10 to 25.24 ± 0.43 mg GAE/g and decreased in the order of acetone > aqueous: methanol (20:80, v/v) > methanol > water > petroleum ether extracts. The lowest TFC was detected in petroleum ether extract ($p < 0.05$). The TFC in water,

petroleum ether, and methanol extracts were significantly different ($p < 0.05$), but in the acetone extract was not significantly different ($p > 0.05$) from aqueous: methanol (20:80, v/v) extract.

Table 2.3 Total favonoid contents (mg QE/g of dtird extract)* of *Aframomum corrorima* (AC), *Coriandrum sativum* seed (CSS), *Coriandrum sativum* fruit (CSF), *Coriandrum sativum* leaf (CSL), *Lippia adoensis* var koseret (LAK), *Lippia adoensis* var. adoensis (LAA) and *Thymus schimperi* (TS) obtained after extraction with different solvents (petroleum ether, water, acetone, methanol and aqueous: methanol).

	Extract				
Plant sample	Pet. ether	Water	Acetone	Methanol	Aqueous: methanol (20:80, v/v)
AC	0.10 ± 0.01^{aA}	0.20 ± 0.05^{aA}	0.07 ± 0.01^{aA}	5.01 ± 0.56^{aB}	19.00 ± 0.13^{bC}
CSS	0.02 ± 0.01^{aA}	2.41 ± 0.12^{bB}	3.82 ± 0.21^{bB}	7.4 ± 0.34^{aC}	10.70 ± 0.61^{aD}
CSF	0.05 ± 0.01^{aA}	3.92 ± 0.40^{bB}	5.90 ± 0.45^{bB}	5.53 ± 0.40^{aB}	17.98 ± 1.20^{bC}
CSL	0.08 ± 0.02^{aA}	0.29 ± 0.21^{aB}	6.61 ± 0.71^{bB}	29.45 ± 2.47^{cC}	30.75 ± 2.04^{eC}
LAK	0.17 ± 0.10^{bA}	6.61 ± 0.19^{cB}	25.24 ± 0.43^{cD}	15.31 ± 1.48^{bC}	22.78 ± 0.29^{cD}
LAA	0.81 ± 0.32^{cA}	9.83 ± 0.34^{cB}	26.01 ± 0.63^{cC}	45.11 ± 5.09^{dD}	25.51 ± 3.86^{dC}
TS	0.12 ± 0.11^{bA}	10.9 ± 0.86^{cB}	21.40 ± 0.40^{cD}	20.04 ± 2.30^{cC}	26.77 ± 2.91^{dD}

* Results are expressed as milligram of quercetin equivalents per gram of dried extract (mg QE/g). Values are expressed as mean ± SD (n = 3). Different letters after the means indicate significant differences among solvents (upper case) and plant sources (lower case) ($p < 0.05$).

For CSL, the methanol and aqueous: methanol (20:80, v/v) extracts had significantly higher TFC ($p < 0.05$). Similarly, aqueous: methanol (20:80, v/v) extract of AC, CSS, and CSF contained higher TFC. Whereas, the acetone extract of AC had the lowest TFC. The petroleum ether

extracts of CSS and CSF showed the lowest TFC ($p < 0.05$). The result showed there was no significant difference ($p > 0.05$) among the TFC of the petroleum ether, water and acetone extracts from the seed extracts of AC. But the TFC of the aqueous; methanol (20:80, v/v) extract of AC was significantly higher ($p < 0.05$) than those of the methanol, acetone, water, and petroleum ether extracts.

2.3.3 Characterization of phenolic compounds

This study was carried out using LC–MS in the negative ion mode because of its higher sensitivity in the analysis of the different phenolic classes (Cuyckens and Claeys, 2004). Seven phenolic acids, six flavonols, five flavan-3-ols, two dihydrochalcones, and two aliphatic organic acids were identified by comparing the retention times (tR) and the molecular mass ion data of the peaks of the samples. Major chemical classes and representative constituents of phenolic compounds identified in this study were summarized in Table 2.4. Figure 2.2 displays chemical structures of major phenolics identified in the selected spices and herbs.

2.3.4 Quantification of the phenolic compounds

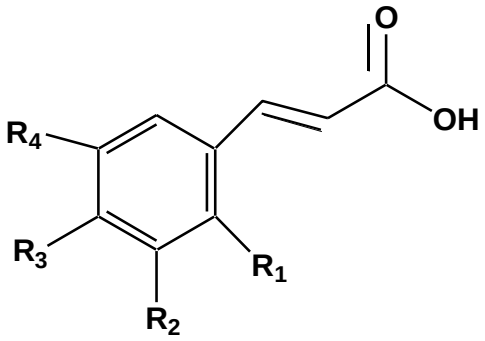
All calibration curves were linear over the concentration ranges tested with correlation coefficients > 0.995. Table 2.5 shows quantitative results of major individual phenolics identified in different spice and herb extracts. In general, the phenolic profiles of the selected spice and herb extracts were dominated by three categories; phenolic acids, flavonols, and aliphatic organic acid. The concentrations of aliphatic acids were extremely high when compared with the other two phenolic groups. Flavan-3-ols and dihydrochalcones contained the lowest amount.

Table 2.4 LC-MS Spectral information of identified Peaks of *Aframomum corrorima* (AC), *Coriandrum sativum* seed (CSS), *Coriandrum sativum* fruit (CSF), *Coriandrum sativum* leaf (CSL), *Lippia adoensis* var koseret (LAK), *Lippia adoensis* var. adoensis (LAA) and *Thymus schimperi* (TS)

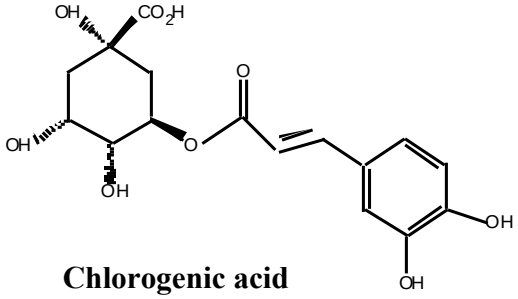
Compound	MW	Parent ion $[M-H]^-$ m/z	tR (min)
Phenolic acids			
Chl	354.31	353	3.33
3-hydrohb	138.12	137	4.03
Syr	198.17	197	3.87
Fer	194.15	193	5.59
Isofer	194.18	193	6.07
Caf	180	179	3.78
Cinam	164.18	163	7.27
Flavonols			
Qu	302.24	301	7.13
Q3-Glu	464.38	462.78	5.90
Q3-Gal	464.38	462.75	5.71
Q3-Rha	448.38	446.75	6.61
Q3-Rut	610.52	608.75	5.48
Q3-Arglu	596	594.75	4.86
Flavan-3-ols			
Cat	290.26	289	3.36
Epicat	290.26	289	3.96
EGC	306	305	3.02
ECG	442,37	441	5.80
EGCG	458.37	457	4.01
Others			
Fum	116.07	115	1.17
Suc	118.09	117	1.21
Phlor	274.25	272.75	7.30
Phldz	436.44	434.75	6.87

Chl: chlorogenic acid; 3-Hydroxyb: 3- Hydroxybenzoic acid; Syr: syringeic acid; fer: ferulic acid; Isofer: isoferulic acid; Caf: caffeic acid; Cinnam: cinnamic acid; Qu: quercetin; Q3-Glu: Quercetin-3-O-glucoside; Q3-Gal: quercetin-3-O-galactoside; Q3-Rham: quercetin-3-O-rhaminoside; Q3-Rut: Quercetin-3-O-rutinoside; Q3-arglu: Quercetin-3-O-arabinoglucoside; Cat: catechin; Epicat: Epicatechin; EGC: epigallocatechin; ECG Epicatechingallate EGCG: epigallocatechingallate; Fum: fumaric acid; Suc: succininc acid; Phlor: Phloretin; Phldz: phloridzin.

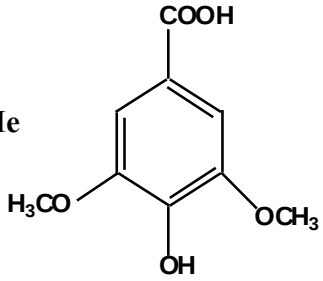
Phenolic acids



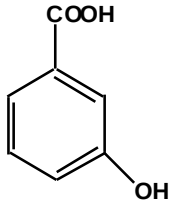
	R_1	R_2	R_3	R_4
Ferulic acid	H	H	OH	OMe
Caffeic acid	H	OH	H	OH
Isoferulic acid	H	OH	OMe	H
2-Hydroxycinnamic acid	OH	H	H	H



Chlorogenic acid

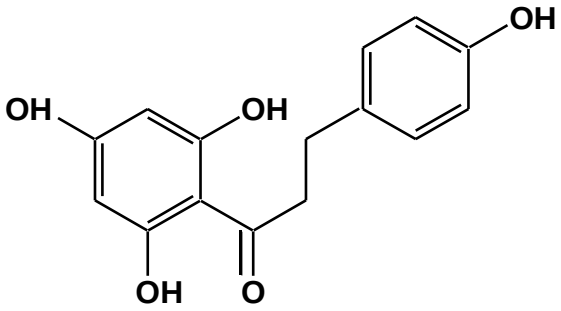


Syringic acid

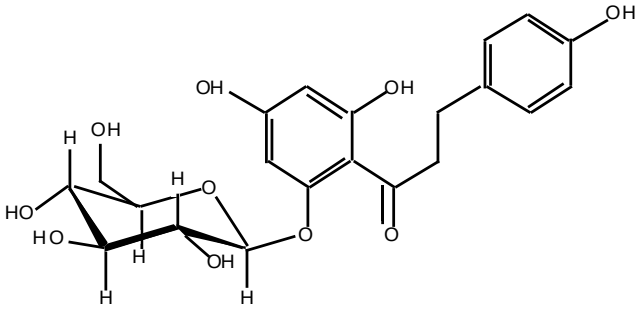


3-Hydroxybenxoic acid

Dihydrochalcones

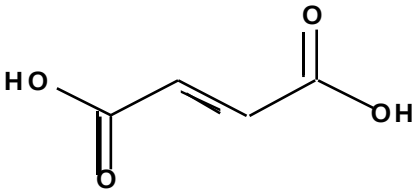


Phloretin

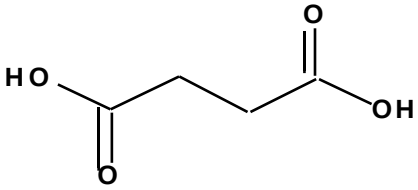


Phloridzin

Aliphatic organic acids

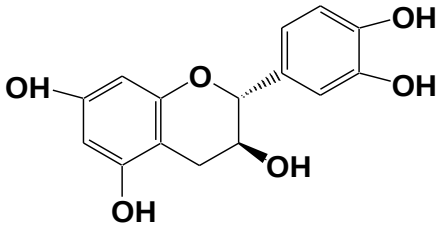


Fumaric acid

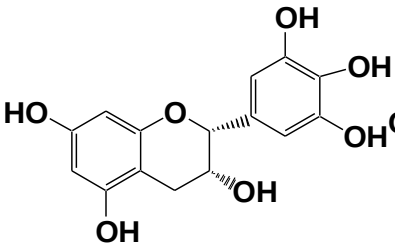


Succininc acid

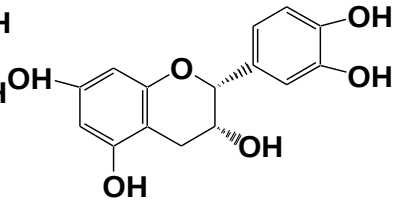
Flavanols



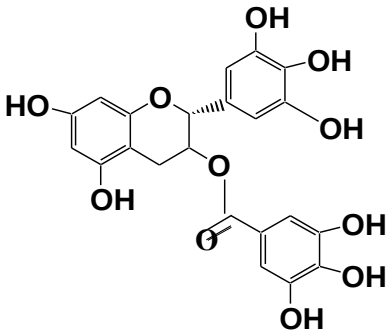
Catechin



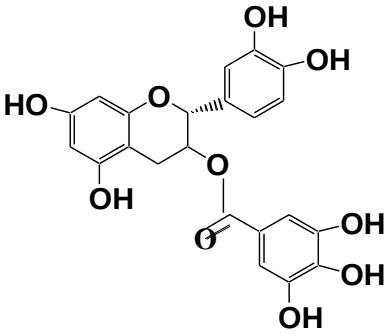
Epigallocatechin (EGC)



Epicatechin

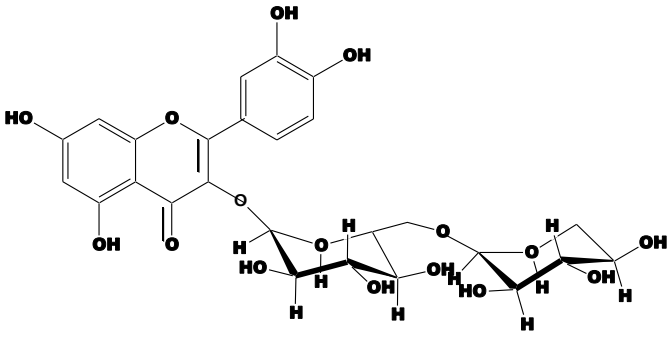


Epigallocatechingallate (EGCG)

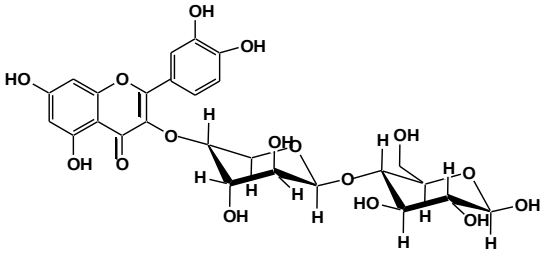


Epicatechingallate (EGC)

Flavonols



Quercetin-3-O-Rutinoside



Quercetin-3-O-arabinoglucoside

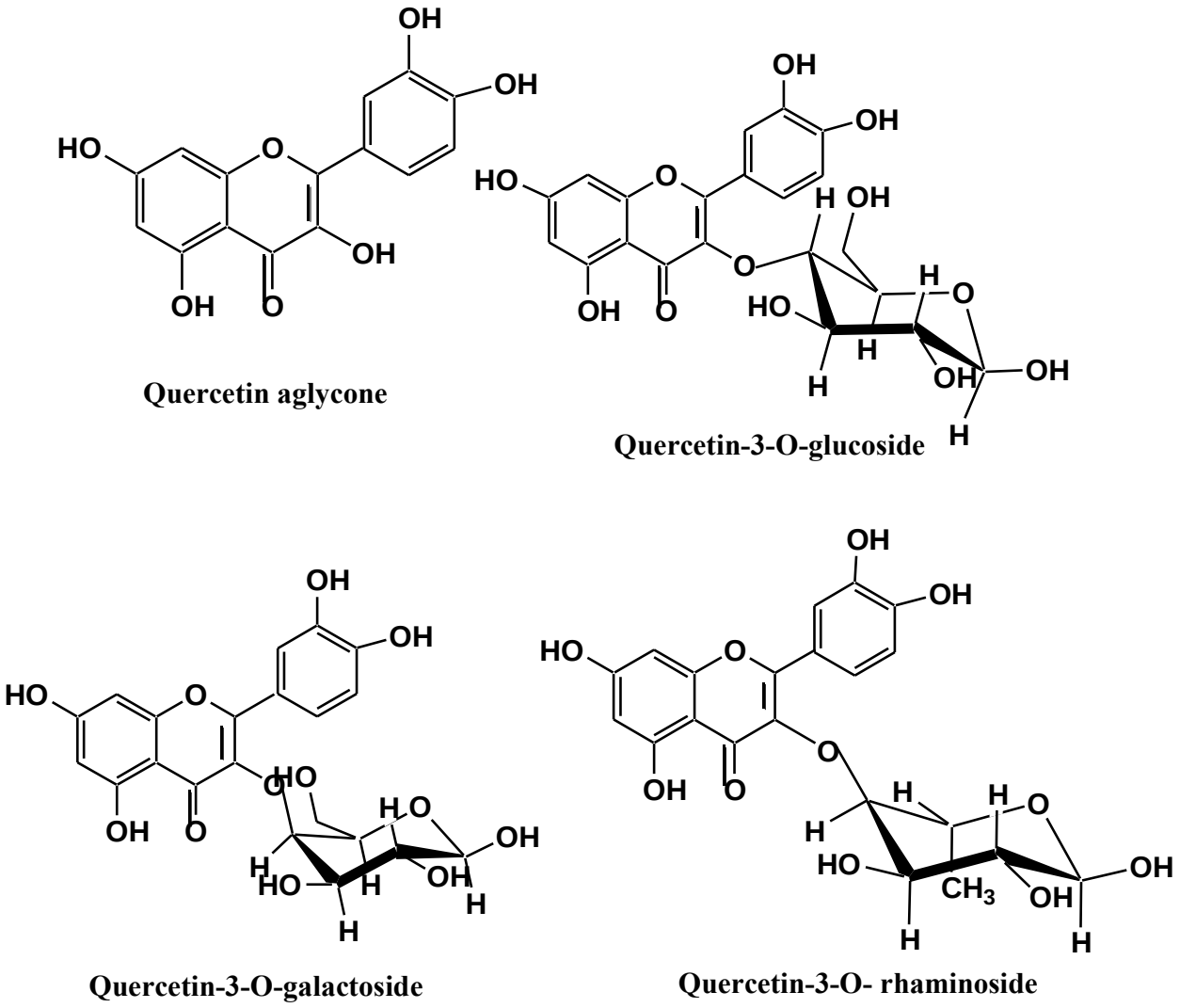


Figure 2.2 Chemical structures of the constituents in *Aframomum corrorima* (AC), *Coriandrum sativum* seed (CSS), *Coriandrum sativum* fruit (CSF), *Coriandrum sativum* leaf (CSL), *Lippia adoensis* var koseret (LAK), *Lippia adoensis* var. adoensis (LAA) and *Thymus schimperi* (TS)

2.3.4.1 Phenolic acids

As shown in Table 2.5, seven phenolic acids (syringic acid, chlorogenic acid, hydroxycinnamic acid, 3-hydroxybenzoic acid, cafeic acid, ferulic acids, and isoferulic acid) were identified and quantified in the studied spices and herbs. All phenolic acids were detected in all spices and herb extracts with total amounts ranged from 201.42 ± 72.03 to 827.81 ± 62.24 μg/g dw. The total phenolic acid contents were 827.81 ± 62.24, 552.79 ± 59.37, 450.89 ± 66.17, 441. 82 ± 3.04, 307.58 ± 4.53, 262.36 ± 25.02, and 201.42 ± 72.03 μg/g dw in TS, LAK, CSF, CSL, LAA, AC and CSS, respectively.

There was no significant difference ($p < 0.05$) in the total phenolic acid content between CSS and AC, and also between CSF and CSL, but there was significant difference ($p < 0.05$) between LAK and TS. With regard to the individual phenolic acids, it was noted that ferulic acid, cafeic acid, and hydroxycinnamic acid were the major phenolic acids in TS. Whereas, 3-hydroxybenzoic acid and syringic acid were the most abundant phenolic acids in LAK. The CSF exhibited the highest amount of chlorogenic acid (291.67 ± 78.26 μg/g dw) followed by CSL (215 39 ± 15.00 μg/g dw), CSS (76.46 ± 23.35 μg/g dw), TS (29.46 ± 9.46 μg/g dw). 3-hydroxybenzoic acid was highest in LAK (251.66 ± 76.51 μg/g dw), followed by LAA (110.32 ± 7.69 μg/g dw), and TS (49.26 ± 25.10 μg/g dw dw). The LAK had also the highest level of synergic acid (238.44 ± 26.62 μg/g dw), followed by AC (76.44 ± 13.05 μg/g dw), CSS (69.31 ± 25.16 μg/g dw), CSF (52.68 ±14.53 μg/g dw), LAA (51.44 ± 6.45 μg/g dw), TS (39.60 ± 13.05 μg/g dw) and CSL (31.13 ± 5.45 μg/g dw).

2.3.4.2 Flavonols

Within the class of flavonols, quercetin and its derivatives were found in all dietary spices and herbs. A total of six flavonols were identified and quantified in all plants considered in this study. The TS contained the highest total flavonol (133.93 ± 12.62 µg/g dw), followed by CSL (62.35 ± 1.07 µg/g dw). CSF (13.37 ± 1.76 µg/g dw), AC (11.54 ± 2.44 µg/g dw) LAK (11.16 ± 3.450 µg/g dw). LAA (8.51 ± 0.32 µg/g dw), and LAK (6.99 ± 2.06 µg/g dw). There were no significant differences ($p > 0.05$) in the total flavonol content among the AC, CSS, CSF, and LAK. But these values were significantly lower ($p < 0.05$) than the total flavonol content of CSL and TS. Of these, the highest levels of Q3-arglu (7.00 ± 0.34 µg/g dw), Q3-Gal (30.55 ± 3.46 µg/g dw), and Q3-Rut (81.50 ± 7.6 µg/g dw) were found in TS. The highest amount of quercetin was found in CSS (3.51 ± 0.78 µg/g dw).

Table 2.5 Quantification of phenolics in the Extracts by LC-MS.

		AC	CSS	CSF	CSL	LAK	LAA	TS
Phenolic acid	Caf	0.30 ± 0.08^{a}	9.53 ± 2.90^{a}	3.65 ± 0.77^{a}	5.62 ± 0.24^{a}	3.72 ± 1.06^{a}	2.86 ± 0.52^{a}	107.31 ± 13.28^{b}
	Fer	3.17 ± 1.97^{a}	10.39 ± 3.28^{b}	19.71 ± 3.94^{c}	20.60 ± 0.72^{c}	3.27 ± 1.35^{a}	1.89 ± 0.13^{a}	31.90 ± 3.65^{d}
	Isofer	80.32± 10.33^{b}	8.23 ± 2.41^{a}	69.61± 13.83^{b}	73.67 ± 4.02^{b}	8.79 ± 0.61^{a}	11.09 ± 7.58^{a}	21.44 ± 6.37^{a}
	Chl	0.37 ± 0.11^{a}	76.46 ± 23.35^{b}	291.67± 78.26^{d}	215.39 ± 7.00^{c}	4.04 ± 1.37^{a}	1.72 ± 0.37^{a}	29.46 ± 9.46^{ab}
	Hydroxyb	98.07 ± 14.85^{a}	11.70 ± 2.82^{a}	11.70 ± 4.86^{a}	50.67 ± 1.46^{a}	42.87 ± 10.07^{a}	128.26 ± 29.54^{a}	548.81 ± 71.59^{b}
	3- Hydr	3.69 ± 0.19^{a}	15.80 ± 2.3^{g}	1.87 ± 0.40^{a}	44.74 ± 9.05^{ab}	251.66 ± 76.51^{c}	110.32 ± 7.69^{b}	49.26± 25.10a^{b}
	Syr	76.44 ± 13.05^{c}	69.31 ± 25.16^{bc}	52.68 ± 14.53^{abc}	31.13 ± 5.45^{a}	238.44 ± 26.82^{d}	51.44 ± 6.45^{abc}	39.60± 13.05^{ab}
Total		262.36 ± 25.02^{a}	201.42 ± 72.03^{a}	450.89 ± 66.17^{b}	441. 82 ± 3.04^{b}	552.79 ± 59.37^{c}	307.58 ± 4.53^{ab}	827.81 ± 62.24^{d}
Flavonol	QR	1.44 ± 0.19^{a}	3.51 ± 0.78^{b}	3.00 ± 0.48^{b}	2.91 ± 0.07^{b}	1.08 ± 0.28^{a}	1.01 ± 0.04^{a}	2.60 ± 0.37^{b}
	Q3-Gal	0.60 ± 0.10^{a}	2.19 ± 0.69^{ab}	4.66 ± 0.92^{b}	15.79 ± 0.55^{c}	0.45 ± 0.20^{a}	0.22 ± 0.06^{a}	30.55 ± 3.46^{d}
	Q3-Glu	6.76 ± 0.76^{d}	0.40 ± 0.08^{a}	1.90 ± 0.29^{b}	11.61 ±0.52^{e}	3.80 ± 1.6^{c}	2.31 ± 0.02^{c}	7.06 ± 0.83^{d}
	Q3-Rha	1.06 ± 0.20^{a}	0.53 ± 0.05^{a}	1.00 ± 0.21^{a}	5.16 ± 0.3^{c}	2.91 ± 0.31^{b}	3.70 ± 024^{b}	5.22 ± 1,12^{c}
	Q3-Rut	0.34 ± 0.08^{a}	0.22±0.04^{a}	2.70 ±0.81^{a}	26.48 ± 0.57^{b}	0.32 ± 0.17a	0.16 ± 0.03a	81.50 ± 7.60c
	Q3-Arglu	1.34 ± 0.20^{c}	0.14 ± 0.04^{a}	0.11 ± 0.02^{a}	0.40 ± 0.05^{ab}	2.60 ± 1.18^{d}	1.11 ± 0.04^{bc}	7.00 ± 0.34^{e}
Total		11.54± 2.44^{a}	6.99 ± 2.06^{a}	13.37±1.76^{a}	62.35 ±1.07^{b}	11.16±3.45^{a}	8.51±0.32^{a}	133.93±12.62^{c}
Flavan-3-ols	Cat	0.32 ± 0.25^{a}	5.81 ± 2.29^{c}	4.58 ± 1.01^{c}	2.45 ± 0.09^{b}	0.38 ± 0.05a	0.19 ± 0.01^{a}	1.53 ± 0.4ab
	Epicat	0.16 ± 0.11^{a}	1.03 ± 0.37^{c}	0.72 ± 0.17^{b}	0.65 ± 0.03^{b}	0.18 ± .0..06^{a}	0.17 ± 0.06^{a}	0.15 ± 0.04^{a}
	ECG	1.7 ± 1.44^{c}	0.62 ± 0.18^{ab}	1.36 ± 0.29^{c}	0.69 ± 0.02^{ab}	0.19 ± 0.05^{a}	0.15 ± 0.03^{a}	0.17 ± 0.04^{a}
	EGC	1.93 ± 0.33^{abc}	3.13 ± 0.48^{cd}	2.45 ± 0.68^{bc}	4.34 ± 0.13^{d}	1.00 ± 0.18ab	2.34 ± 0.98^{bc}	0.51 ± 0.08^{a}
	EGCG	ND	0.74 ± 0.17^{a}	0.98 ± 0.21^{a}	0.71 ± 0.03^{a}	1.65± 0.03^{b}	1.54 ± 0.05^{b}	0.71 ± 0.06^{a}
Total		4.11 ± 1.34^{a}	11.33 ± 1.10^{b}	10.09 ± 1.02^{b}	8.84 ± 0.92^{b}	3.40 ± 0.54^{a}	4.39 ± 0.44^{a}	3.07 ± 0.23^{a}
Organic acids	Fum	298.88 ±46.04^{c}	1105.80 ±50.81^{e}	163.39 ± 4127^{b}	8.78.73 ± 28.90^{d}	120.52 ± 7.54ab	48.01 ± 8.01^{a}	312.33 ± 98.25c
	Suc	642.32 ± 55.03^{a}	1017.23 ± 102.5^{ab}	1420.09 ± 66.15^{bc}	1653.45 ± 35.7^{c}	860.82 ± 22.21a	696.34 ± 31.10^{a}	1036.14 ± 97.73^{ab}
Total		941.2 ± 92.83^{a}	2123.03 ± 150.61^{bc}	1583.48 ± 123.45^{b}	2532.18 ± 60.70^{bc}	981.34 ± 99.97a	744.35 ± 74.90^{a}	1348.47 ± 76.33^{ab}
Chalcone	Phlor	0.06 ± 0.01^{a}	0.05 ± 0.00^{a}	0.04 ± 0.02^{a}	0.04 ± 0.02^{a}	0.03 ± 0.00a	0.05 ± 0.00a	0.05 ± 0.00a
	Phlzd	0.94 ± 0.32	1.04 ± 0.28	0.82 ± 0.14	0.82 ± 0.14	1.10 ± 0.23	1.00 ± 0.06	0.34 ± 0.08
Total		1.00	1.09	0.86	0.86	1.13	1.05	0.39

Results are expressed as mean ± standard deviation. Different letters after the means within the rows (lower case) indicates significant difference at $p < 0.05$.

The concentration is given in µg/g of the dry plant material for triplicate injections.

2.3.4.3 Flavan-3-ols

Five flavan-3-ols (catechin, epicatechin, EGCG, ECG, and EGC) were detected and quantified in the samples. The highest ($p < 0.05$) total flavan-3-ol content was detected in CSS (11.33 ± 1.10 µg/g dw), CSF (10.09 ± 1.02 µg/g dw) and CSL (8.84 ± 0.92 µg/g dw). The TS contained the lowest total flavan-3-ols with the value of 3.07± 0.23 µg/g dw) significantly different ($p < 0.05$) from the total flavan-3-ols of CSS, CSL and CSF but similar ($p > 0.05$) with the contents found in LAK and LAA. Among the flavan-3-ols, the highest level of catechin was detected in extract of CSS (5.81 ± 2.29 dw) followed by the CSF (4.58 ± 1.01µg/g dw), CSL (2.45 ± 0.09 µg/g dw), TS (1.53 ± 0.4 µg/g dw), LAK (µg/g dw), AC (0.32 ± 0.25 µg/g dw), and LAA (0.19 ± 0.01 µg/g dw), respectively. The highest amount of EGC was found in the leaf extract of CSL (4.34 ± 0.13 µg/g dw). The HPLC analysis showed that no EGCG was present in AC extract.

2.3.4.4 Aliphatic organic acids and diydrochalcones

Fumaric and succinic acids were the two aliphatic organic acids detected in the samples. With respect to individual carboxylic acids, CSF had the highest amount of fumaric acid (1089.60 ± 81.61 µg/g dw), followed by CSL (847.88 ± 40.92 µg/g dw), CSS (496.72 ± 66.67 µg/g dw), TS (312.33 ± 98.25 µg/g dw), AC (232.53 ± 46.04 µg/g dw), LAK (120.52 ± 7.54 µg/g dw), and, LAA (48.01 ± 8.01 µg/g dw). There was no significant difference in fumaric acid content of AC, LAK, and LAA and between CSS and TS ($p > 0.05$). The CSL contained the highest amount of succcinic acid with the value of 1651.45 ± 35.71 µg/g dw followed by CSF (1420.76 ± 376.85 µg/g dw), TS (1036.14 ± 297.73 µg/g dw), CSS (1017.23 ± 102.60 µg/g dw), LAK (860.82 ± 222.21 µg/g dw), LAA

(696.34 ± 31.10 µg/g dw), and AC (642.32 ± 55.90 µg/g dw). Dihydrochalcones, phloridzin and phloritin were found in low amounts. The contents of phlorizin ranged from 0.82 ± 0.14 µg/g dw to 1.10 ± 0.23 .70 µg/g dw, whereas the phloritin content of all plant materials was less than one microgram per gram of dried weight of the samples.

Conclusions

The present study describes both qualitative and quantitative analyses of the major phenolic compounds in the leaves (*Thymus schimperi*, *Lippia adoensis* var koseret, *Lippia adoensis* var. adoensis, *Coriandrum sativum*), fruit (*coriandrum sativum*), and seeds (*Aframomum corrorima* and *Coriandrum sativum*) from Ethiopia. So far there is no information on the phenolic composition of these dietary spices and herbs from Ethiopia. Twenty phenolic compounds from four phenolic families (i.e. flavanols, phenolic acids, flavonols and dihydrochalcones) and two aliphatic organic acids have been characterized and quantified in the selected spices and herbs by using LC-MS. The TS was recognized as the richest source of TPC. The LAA was recognized as the richest source of TFC. The TS sample gave the highest levels of total flavonols and phenolic acids. Whereas, CSS represented the highest amount of total flavanols. The CSF, and CSL gave the highest amount of total aliphatic carboxylic acids. The additional peaks observed in the samples chromatograms indicate the presence of many soluble compounds extracted along with the phenolic compounds under study which warrants further investigation to identify the compounds in the aqueous and organic solvent extracts of these dietary spices and herbs. Further study is also needed in relation to the thermal stability of each bioactive phenolic compound during cooking of the spicy traditional foods used in Ethiopia.

CHAPTER 3: *IN VITRO* ANTIOXIDANT ACTIVITIES OF *Aframomum corrorima, Thymus schimperi, Lippia adoensis,* AND *Coriandrum sativum*

Abstract

Antioxidant compounds in food play important roles as health-protecting factors. They are also widely used as additives in fats and oils and in food processing to prevent or delay spoilage of foods. Dietary spices and herbs have received increased attention as sources of many effective antioxidants. The aim of the present study was to evaluate the antioxidant properties in model system studies of leaves of *Lipia adoensis* var. adoensis (LAA), *Lippia adoensis* var. koseret (LAK) and *Thymus schimperi* (TS), and *Coriandrum sativum* fruits (CSF) and seeds of *Aframomum corrorima* and *Coriandrum sativum,* and for their inhibitory action of linoleic acid and flaxseed oil oxidation in an aqueous emulsion systems. The antioxidant activity was concentration dependent. The aqueous: methanol (20:80, v/v) extract of LAA showed the highest DPPH radical scavenging activity (IC_{50} = 7.96 ± 2.11 μg/mL), reducing power (79.55 ± 6.32 mg of ascorbic acid equivalent/g of dried extract), and total antioxidant activity (1.98 ± 0.14 mg butylated hydroxytoluene equivalent/g dried extract). Water extract of CSS showed the strongest iron chelating activity (IC_{50} = 53.95 ± 1.22 μg/mL). The methanol extract of CSL had the highest percentage of linoleic peroxidation (78.30 ± 2.50%) and CSS showed the highest inhibition potential (82.64 ± 2.47%) of secondary lipid oxidation products. There were positive relationships (R^2 = 0.55–0.95) between TPC and DPPH scavenging activity (%) of the tested plant extracts but negatively correlated with ferrous chelating activity (%).

The result showed that, in addition to their traditional usage in food flavoring and folklore medicine, these plants represent a valuable source of natural antioxidants, and thus may be considered as great potential for the food industry, representing possible alternatives to the synthetic additives.

Key words: antioxidant; dietary spices and herbs; DPPH; reducing power; linoleic peroxidation

3.1. Introduction

Free radicals are highly reactive compounds with an odd (unpaired) number of electrons that are created in the body or introduced from the environment. These reactive oxygen species (ROS) and reactive nitrogen species (RNS) are formed regularly as a result of normal metabolic pathways of human organ functions, or as a result of excess oxidative stress. The reactive species superoxide ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), nitrogen oxide ($NO^{\bullet}$), and peroxynitrite ($ONOO^{\bullet}$), when present in excess they can exert a harmful compounds. To maintain a homeostasis balance, organs protect themselves from the toxicity of excess ROS/RNS in different ways, including the use of endogenous and exogenous antioxidants (Zheng and Wang, 2001).

Antioxidants are a group of substances which, significantly inhibit or delay oxidative processes, while often being oxidized themselves. In food, they are capable of delaying, retarding or preventing the development of food rancidity or other flavor deterioration due to oxidation (Bin and Clifford, 2007; Jayathilakan *et al*., 2007). They delay the development of off-flavors by extending the induction period. Antioxidants can safely interact with free radicals and terminate the chain reaction before vital molecules are

damaged. Although there are several enzyme systems within the body that scavenge free radicals, some bioactive phytochemicals, present in foods, have been reported to exhibit antioxidant activity due to the presence of radical inhibitors such as phenolics (Souad *et al*., 2004), carotenoids (Endakkadath *et al*., 2010), and tocopherols (Muller *et al*., 2010).

Antioxidants work in several ways: they may reduce the energy of the free radical, stop the free radical from forming in the first place, or interrupt an oxidizing chain reaction to minimize the damage caused by free radicals. They inhibit or retard oxidation in two ways: either by scavenging free radicals, in which case the compound is described as a primary antioxidant, or by a mechanism that does not involve direct scavenging of free radicals, in which case the compound is a secondary antioxidant. Primary antioxidants or chain breaking antioxidants are free radical scavengers that delay or inhibit the initiation step, or interrupt the propagation or autooxidation. Secondary antioxidants operate by a variety of mechanisms including binding of metal ions (Praveen *et al*., 2012), scavenging oxygen, and deactivating singlet oxygen (Eunok and David, 2009).

Many food products undergo irreversible chemical changes when exposed to oxygen and light. The process of oxidation deteriorates color and flavor of products containing susceptible fats, and can eventually create odors that affect the quality of a product. During production, processing, distribution and storage, foods can undergo deterioration from chemical and microbiological processes (Lucija *et al*., 2007). In addition, lipid oxidation is a cause for the problems associated with food losses and human diseases due to generation of free radicals. Fats, oils and lipid-based foods deteriorate through several degradation reactions both on heating and on long term storage. The main deterioration processes are oxidation reactions and the decomposition of oxidation products which

result in decreased nutritional value and sensory quality. Therefore, from the nutritional and technological points of view, it is highly desirable to control oxidation by addition of antioxidants, inhibitory substances providing suitable food quality (Jayathilakan *et al*., 2007).

Oxidative rancidity greatly affects the quality of processed food products, especially products with relatively high fat content like meat and butter. To combat oxidative rancidity, antioxidants are often added to these products to help improve the shelf life (Baohua *et al*., 2010). The most common antioxidants in use today are butylated hydroxy anisole (BHA), butylatedhydroxy toluene (BHT), and *tert*- butylhydroquinone (TBHQ). However, because of toxicological concerns of these synthetic antioxidants, nowadays the new trend in food industry includes an enhanced concern for the quality and safety of food products, increased preference for natural products over synthetic ones, and broadened regulations related to nutritional and toxicity levels of active ingredients. Consequently food market is demanding for natural food ingredients free of chemical additives orientated to promote the use of natural products.

Spices and herbs that since ages have been used as flavoring compounds in food products are nowadays widely used and accepted as natural food constituents. They are traditionally added to food for improving organoleptic properties and also as antioxidants and preservatives. Many spices and herbs or their extracts have been assessed for antioxidant activity in a variety of food products and lipid systems. According to the study conducted by Shan *et al* (2005), the antioxidant activities of 26 spices vary widely. Clove, cinnamon, oregano, thyme, rosemary and mint exhibited very strong antioxidant activity. Anise, nutmeng, and sweet basil showed lower activity. However coriander, parsley, Lemon

grass, green cardamom, chili, and black pepper showed quite low antioxidant. Research conducted on *Rosmarinus officinalis* L (Tavassoli and Emam, 2011) showed that the antioxidant activity of the leaf extracts was stronger than the antioxidant activity of BHA**.** Also many researchers have looked at the potential for spices and herbs as important natural antioxidants (Priyanjali *et al*., 2005; Iris *et al*., 2006; Stoilova *et al*., 2007; Politeo *et al*., 2007; Biljiana *et al*, 2008 ;Yara *et al*, 2009; Baohua *et al*., 2010). Their antioxidant activity has been attributed to the presence of polar phenolic compounds and essential oils (Aline *et al*., 2008; Demiray et *al.,* 2009).

Many spices and herbs have components that act as antioxidants and protect cells from free radicals. Some spices have more antioxidant properties than others depending on the type of compounds they contain. Combining spices with herbs or other spices or antioxidants such as tocopherols and ascorbic acid produces synergistic effects. (Dimitrios *et al.,* 2007). It has been suggested that the interactions between structurally different compounds with variable antioxidant activity provides additional protection against increased oxidative stress (Monica *et al.*, 2002). Such cooperative effects of are known as synergism.

The aim of the study was to determine the antioxidant capacity of selected dietary spices and herbs of Ethiopian origin commonly consumed by the Ethiopian population as an important constituent of their traditional food. So far limited information is known about the antioxidant potential of Ethiopian dietary aromatic plants. Studies have investigated the *in vitro* antioxidant activity of the essential oil of LAK (Riot *et al*., 2005; Workalemahu *et al.*, 2007), TS (Gebrehana and Shimelis**,** 2013), and AC **(**Eyob *et al***.,** 2008**).** So far, there is no report on *in vitro* antioxidant activity (using different assays) of

various solvent extracts from these dietary herbs and spices. Also no study has been reported on antioxidant activity of leaf, fruit, and seed extracts of *Coriandrum sativum* grown in Ethiopia. For this purpose the antioxidant activity of various extracts obtained from leaves of *Lipia adoensis* var. adoensis (LAA), *Lippia adoensis* (LAK) var. koseret and *Thymus schimperi* (TS), and *Coriandrum sativum* fruits *(CSF*) and seeds of *Aframomum corrorima* and *Coriandrum sativum* were studied. Therefore, the objectives of this study were (1) to evaluate and compare antioxidant activity of these selected dietary spice and herb extracts; and (2) to establish the relationship between antioxidant activity and phenolics (i.e., analyzed as TPC and TFC) of these selected spice and herb extracts to confirm that the phenolic constituents are responsible for their antioxidant activity.

3.2. Materials and Methods

3.2.1. Chemicals

Gallic acid, butylated hydroxytoluene (BHT), Folin–Ciocalteu reagent, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), quercetin, ferrozine, L-ascorbic acid, Tween-20, linoleic acid, flaxseed oil, thiobarbituric acid (TBA), and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma-Aldrich. The other chemicals and solvents used in this experiment were of analytical reagent grade.

3.2.2. Preparation and extraction of plant materials

Fresh leaves of TS, LAK, LAA, and CSL were air dried for ten days. The AC, CSF, and CSS were air dried for twenty days. Then each sample was ground to fine powder using electric grinder (FM 100 model, China). The petroleum ether, water, acetone, methanol, and aqueous: methanol (20:80, v/v) extracts of all were prepared by dissolving 10 g of the

sample separately in 100 mL each solvent. After carrying out several preliminary investigations on proportions of water and methanol, the aqueous: methanol (20:80, v/v) was selected for extracting solvent.The contents were kept in orbital shaker for 6 h at room temperature. Thereafter, each extract was filtered through Whatman no.1 filter paper and evaporated to dryness under vacuum at 40 ^{o}C by using a rotary evaporator (Buchi, 3000 series, Switzerland). The extraction was done in triplicate for each solvent and the resulting extracts were stored in a sealed plastic container at -20 ^{o}C until further investigation. Unless specifically mentioned all analysis were conducted on triplicate analysis.

3.2.3. Determination of antioxidant activity

3.2.3.1 DPPH method

This assay measures the free radical scavenging capacity of a compound or crude extract. DPPH is a molecule containing a stable free radical. In the presence of an antioxidant which can donate hydrogen to DPPH, the violet color which is typical to free DPPH radical, decays with change in absorbancy (Figure 3.1) which can be determined either spectrophotometrically (Jara *et al*., 2008) or by detecting changes in the concentration of the starting materials, using HPLC analysis (Angela *et al*., 2007).

In this work the DPPH radical scavenging activity of the extracts was determined as described by Katerere and Eloff (2005) with slight modification. Different concentrations (50-1000 µg/mL) of the extracts were taken in different test tubes. Freshly prepared DPPH solution (2 mL, 0.006%, w/v) in methanol was added in each of the test tubes containing 1 mL of the extract. The reaction mixture and the reference standards (ascorbic acid and

BHT) were vortex mixed and left to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was then taken at 520 nm. Methanol was used as blank. The ability to scavenge the DPPH radical was calculated using the equation:

DPPH scavenging (%) = $[(A_c - A_s)/A_c)]$ x100

Where A_c is the absorbance of the control and A_s is the absorbance in presence of the sample of the extracts. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration in µg/mL of extracts that scavenges the DPPH radical by 50%.

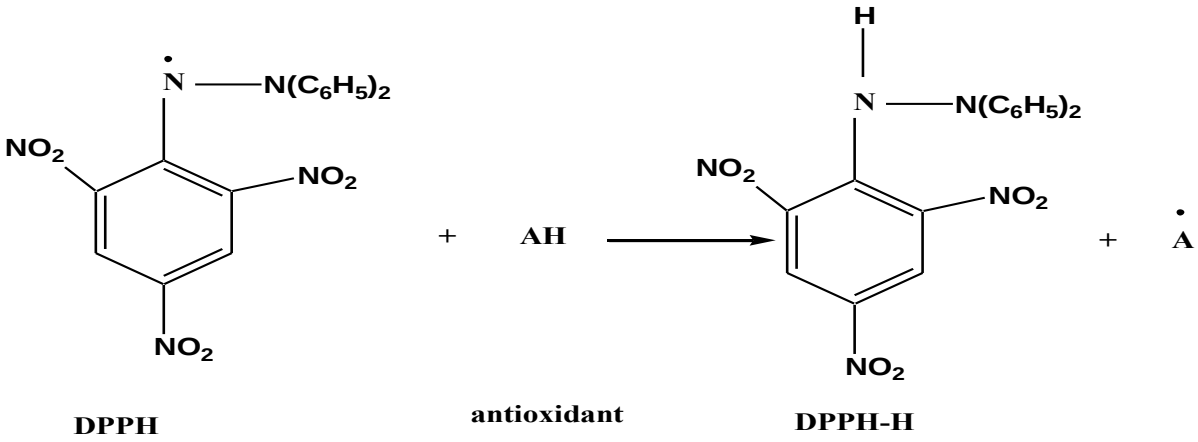


Figure 3.1 Reaction of DPPH with antioxidant

3.2.3.2 Ferric ion reducing power

The presence of antioxidants in the extract causes the reduction of the yellow ferric/ferricyanide complex to the ferrous form which can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Amarowicza *et al*., 2004). This assay was carried out as described previously by Oyaizu (1986). Plant extract (1 mL, 1 mg/mL) was

mixed with 2.5 mL sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. Then the mixture was incubated at 50 ^{o}C for 20 min. Trichloroacetic acid (2.5 mL, 10%) was added to the mixture, which was then centrifuged at 3000 rpm (Centurion, 1000 series, UK) for 5 min. Finally, 2.5 mL of the supernatant solution was mixed with 2.5 mL of distilled water and 0.5 mL $FeCl_3$ (0.1%) and absorbance was measured at 700 nm. The reducing power was expressed as milligram ascorbic acid equivalents/gram of dried extract (mg AAE/g) based on L-ascorbic acid calibration curve (Figure 3.2 A).

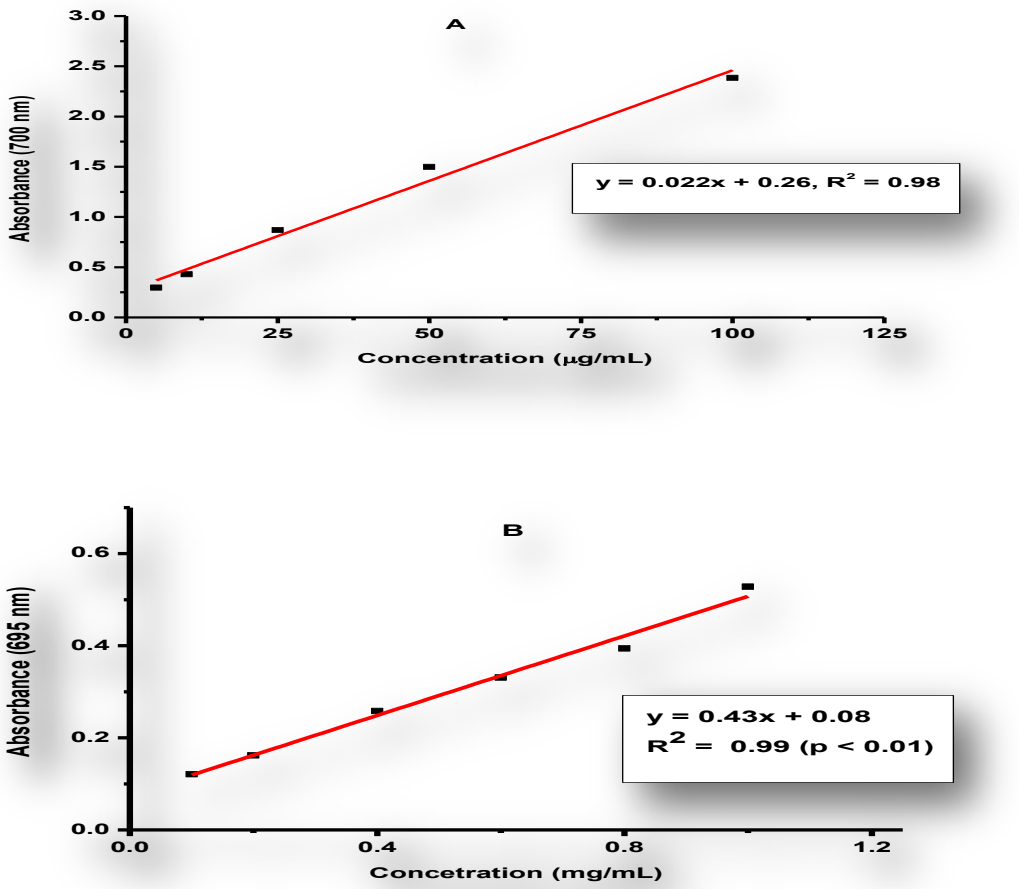


Figure 3.2 Calibration curve for the determination of ferric reducing power (ascorbic acid equivalent) (A) and total antioxidant using phosphomolybdenum method (butylated hydroxytoluene equivalent) (B).

3.2.3.3 Total antioxidant capacity using phosphomolybdenum assay

The total antioxidant activity of the crude extracts was evaluated by the phosphomolybdenum method (Prieto *et al*., 1999) with slight modification. The method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds or crude extract and subsequent formation of green Mo (V) complexes with a maximal absorption

at 695 nm in acidic medium (Mohamed *et al*., 2011). Plant extract (0.3 mL, 1 mg/mL) was mixed with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The samples were incubated at 95 ^{o}C for 90 min, cooled to room temperature and absorbance was measured at 695 nm and methanol (3 mL) was used as blank. The total antioxidant activity was expressed as mg butylated hydroxytoluene equivalents/g of dried extract (mg BHTE/g) based on the calibration curve (Figure 3.2 B).

3.2.3.4 Chelating effects on ferrous ions

The ferrous ion chelating activity was determined according to the method of Dinis *et al* (1994). Various concentrations (100-800 µg /mL) of the extracts (3 mL) in methanol were added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.1 mL). Then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was measured at 562 nm. The EDTA, L-ascorbic acid, quercetin, and BHT were used as positive controls. The inhibition percentage of ferrozine–Fe^{2+} complex formation was calculated by using the formula given below:

Ferrous chelating activity (%) = [(Ac -As / Ac)] × 100

Where Ac is control absorbance (the control contains $FeCl_2$ and ferrozine, complex formation molecules) and As is test sample absorbance.

3.2.3.5 Extraction of flaxseed oil

Figure 3.3 shows a schematic representation of solvent extraction of flaxseed. Flaxseed (100 g) purchased from the market and the seed was cleaned to remove stones or dirt and washed with distilled water. After air dried, the seed was then ground with mortar. The ground sample was soaked in 300 mL of n-hexane and the contents were kept in orbital shaker for 8 h at room temperature. Using separatory funnel, the mixture was partitioned by ethanol (99% v/v), then the hexane layer was separated from ethanol layer. Finally, the hexane was evaporated at 4 ^{o}C and the oil was kept under -20 ^{o}C.

3.2.3.6 Inhibitory activity toward lipid peroxidation (FTC assay)

The antioxidant activities of the methanol and water extracts were determined by the thiocyanate method (Yen & Hsieh, 1998) as described by Siddhuraju (2006). From the stock solutions (1 mg/mL), 0.5 mL of each solution was added to linoleic acid (2.5 mL, 40 mM, pH 7.0). The emulsion was prepared by mixing 0.280 g linoleic acid or flaxseed oil, 0.280 g Tween-20 as emulsifier in 50 mL 40 mM phosphate buffer and the mixture was then homogenized. After incubation at 37 ^{o}C, 0.1 mL aliquot of the reaction solution was mixed with 4.7 mL of ethanol (75%), 0.1 mL $FeCl_2$ (20 mM) and 0.1 mL ammonium thiocyanate (30%). The absorbance of this mixture was measured at 500 nm, after stirring it for 3 min, and it was measured again every 24 h until the time when the absorbance of the control reached the maximum value.

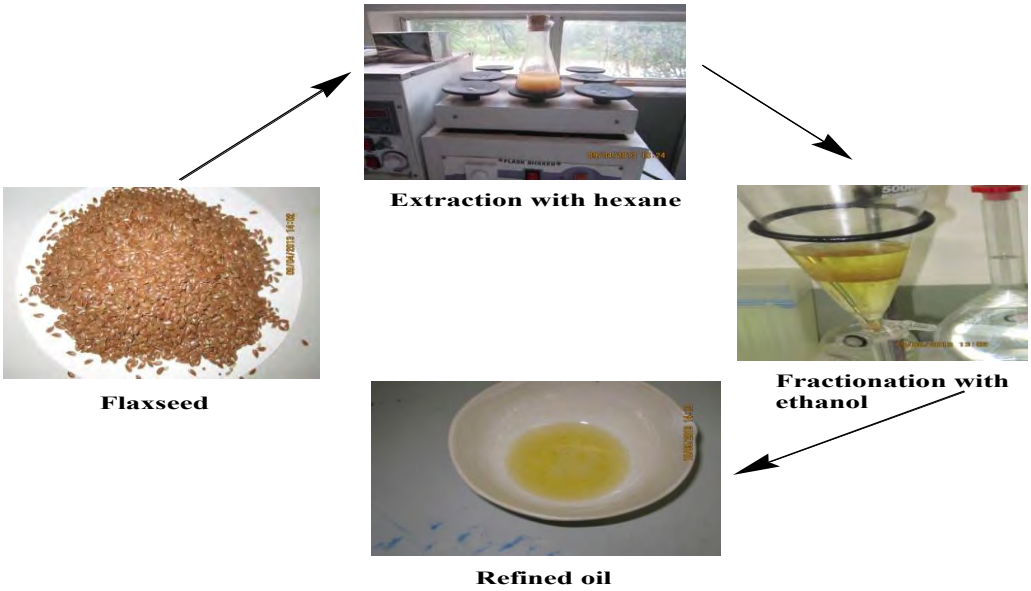


Figure 3.3 Schematic representation of extraction of flaxseed oil

Analysis of all samples were run in triplicate and averaged. Ascorbic acid and BHT were used as a reference compounds. Inhibition percent of linoleic acid peroxidation was calculated using following formula:

% Inhbitition = $(A_c - A_s)/A_c \times 100$

Where, A_c is absorbance of the control and A_s is absorbance of the sample.

3.2.3.7 Thiobarbituric acid reactive substances (TBARS) assay

During lipid peroxidation, lipid peroxides are formed, with a subsequent formation of peroxyl radicals, followed by a decomposition phase to yield aldehydes such as hexanal, malondialdehyde and 4-hydroxynonenal. The TBARS assay is based on the detection of a stable product (Figure 3.4), which is formed between malondialdehyde and thiobarbituric acid (TBA) in the aqueous phase. The production of TBARS was measured with UV-Vis spectrophotometer (JENWAY, 96500, UK) at 532 nm. After the completion of the

oxidation treatments, TBARS assay was followed. A 1 mL of sample from the previous FTC method or flaxseed oil (oxidized emulsion) was added to the TBA reagent (1mL of 15% (w/v) TCA and 2 mL of 0.375% (w/v), TBA in 0.25 M HCl). The reaction mixtures were then placed in a water bath at 85 ^{o}C for 15 min. After cooling, it was centrifuged at 3000 rpm for 20 min and absorbance of the supernatant was then measured at 532 nm using UV-Vis spectrophotometer (Bakchiche *et al*., 2013). The antioxidant activity was calculated by percentage of inhibition in this method as follows:

$$\% \text{ Inhibition} = 100 - [A_1/A_o] \times 100$$

Where A_o is the absorbance of the control and A_1 is the absorbance of the sample extracts.

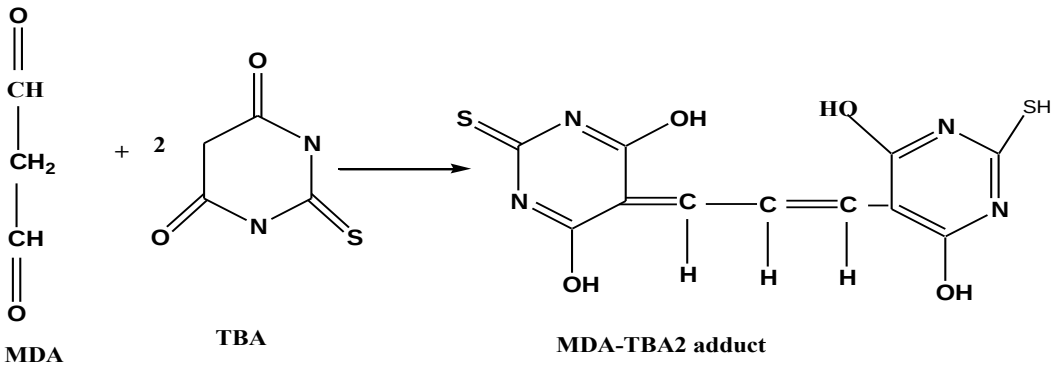


Figure 3.4 Reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA); forming a MDA-TBA2 adduct that absorbs strongly at 532 nm.

3.2.4 Statistical analysis

A triplicate data were analyzed by one way analysis of variance (ANOVA) using SPSS 20.0 statistical software. Mean separation was conducted using Duncan's multiple range tests at $p < 0.05$. The inhibitory concentration 50% (IC_{50}) was calculated from the dose–response curves (Origin 8 software) obtained by plotting the percentage of inhibition *versus* the concentrations.

3.3 Results

3.3.1 *In vitro* antioxidant activity

The antioxidant properties of different plant extracts can be evaluated using various *in vitro* assays. Antioxidant assays in foods and biological systems can be divided in two groups: (a) those that evaluate lipid peroxidation and (b) those that measure free radical scavenging ability (Sanchez-Moreno, 2002). In this case, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) methods, ferric reducing power, phosphomolybdenum assay, ferrous ion chelating, and capacity for preventing lipid peroxidation in the presence of a lipid substrate were assayed in selected dietary spices and herbs.

3.3.1.1 DPPH scavenging

The DPPH is stable free radical, which dissolves in ethanol or methanol. DPPH radical is scavenged by antioxidants through the donation of hydrogen forming the reduced DPPH. The color changes from purple 2, 2-diphenyl-1-picrylhydrazyl radical to reduced yellow diamagnetic 2, 2-diphenyl-1-picrylhydrazine molecule, which can be quantified by its absorbance reduction at wavelength 520 nm (Von Gadow *et al*., 1997).

The DPPH scavenging results were shown in Figure 3.5. As the concentration of the sample increased, the percent inhibition of DPPH radical also increased (Huang *et al*., 2005). At the concentration of 1 mg/mL used (Figure 3.5 E), the DPPH scavenging potential of the aqueous: methanol (20:80, v/v) extracts ranged from 70.19–95.17%, methanol extracts showed in the range of 75.70–92.24% (Figure 3.5 D). Whereas, the acetone extracts (Figure 3.5 C) showed the percentage inhibition in the range of 51.65–95.37%. In water and petroleum ether extracts (Figure 3.5 A and Figure 3.5 B), LAA and

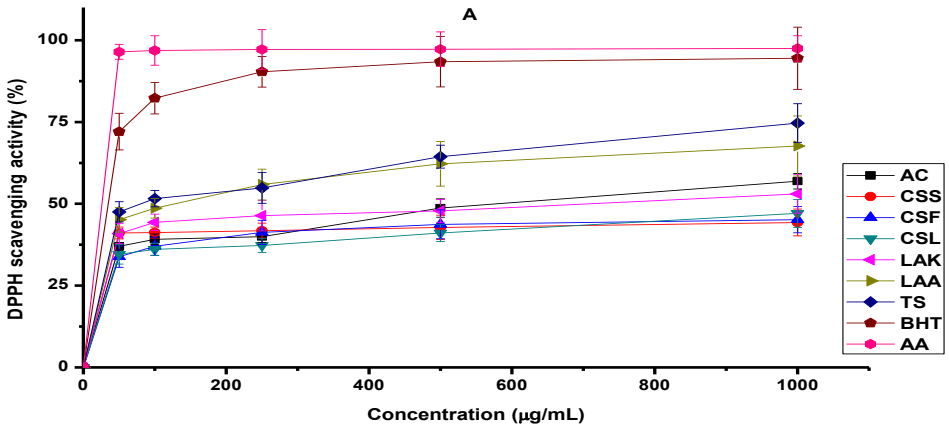
TS showed stronger DPPH scavenging activity with the values of 86.34% and 74.68%, respectively. In all plants, petroleum ether extracts showed the weakest, DPPH scavenging activity. The maximum DPPH scavenging activity (%) was offered by aqueous: methanol (20:80, v/v) and methanol extracts of all pant materials and acetone extract of TS.

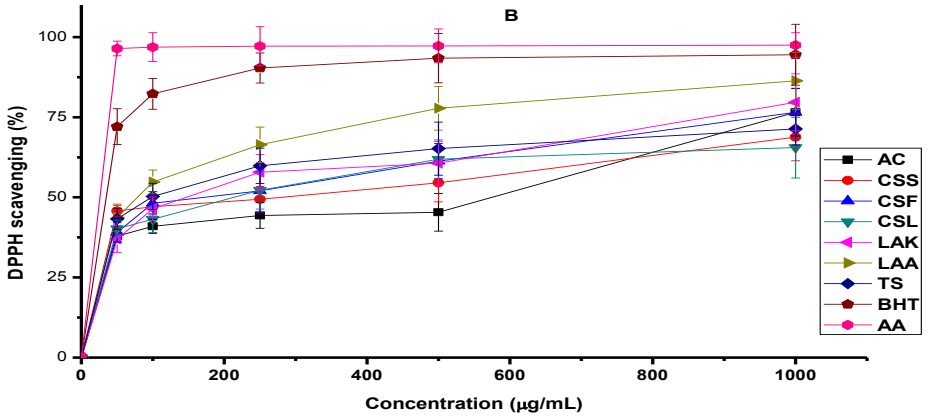
The IC_{50} values of all the extracts were calculated from plotted graph of percentage scavenging activity against concentration of the extracts (Table 3.1). The lower the IC_{50} value, the higher is the scavenging potential. In the petroleum ether extract, LAA showed the strongest DPPH scavenging (IC_{50} = 29.97 ± 1.84 µg/mL) followed by TS (73.99 ± 2.38 µg/mL), LAK (90.77 ± 5.22 µg/mL), and AC (674.74 ± 10.80 µg/mL). Whereas, CSS, CSF, and CSL showed the weakest DPPH scavenging ($p < 0.05$) with IC_{50} value > 1000 µg/mL. In water extracts, the IC_{50} values ranged from 29.33 ± 2.00 µg/mL for LAA to 827.16 ± 10.78 µg/mL for AC. For water extract, the strongest scavenging activity was recorded for LAA ($p < 0.05$) which appeared more than three times stronger than the DPPH scavenging activity of TS and one and half times stronger than that of LAK. The IC_{50} values water extracts of LAA and LAK were not found to be significantly different ($p > 0.05$), but these values were significantly lower than ($p < 0.05$) the IC_{50} values of TS, CSS, CSF, and CSL. The AC showed the weakest DPPH scavenging activity ($p < 0.05$).

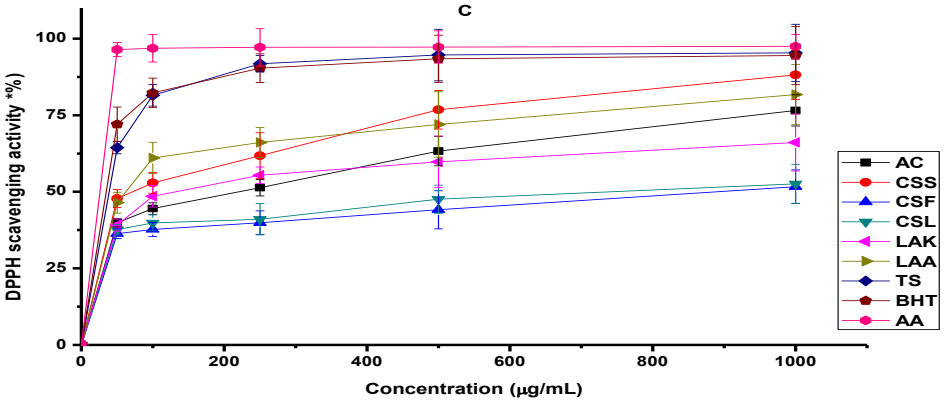
For methanol extracts, LAA and LAK exhibited the strongest DPPH scavenging activity with IC_{50} values of 11.91 ± 3.80 µg/mL and 18.60 ± 6.01 µg/mL respectively. No significant difference ($p > 0.05$) was observed between the IC_{50} values of LAA and LAK, and also among TS, CSS, and AC. The CSL and CSF exhibited the weakest DPPH scavenging activity with IC_{50} values of 161.43 ± 9.65 µg/mL and 167.75 ± 38.93 µg/mL, respectively.

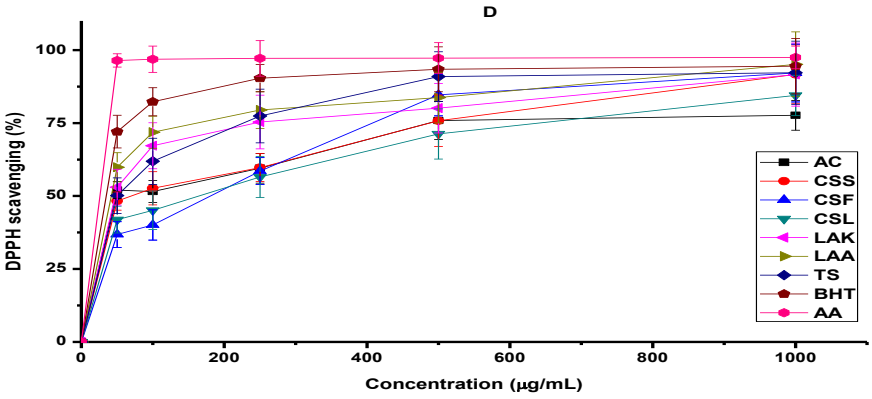
Similarly, no significant difference ($p > 0.05$) was observed between the IC_{50} values of aqueous: methanol (20:80, v/v) extracts of LAA, TS and LAK and also between CSL and CSS extracts. The CSF showed the weakest DPPH scavenging activity ($p < 0.05$) with the IC_{50} value of 123.20 ± 25.47 µg/mL.

In the acetone extracts, LAA, with IC_{50} value of IC_{50} =14.00 ± 1.08 µg/mL, exhibited the strongest free-radical scavenging activity. The LAK showed approximately three times lower activity (43.91 ± 5.00 µg/mL), whereas, the CSS showed more than five times lower activity, with IC_{50} of 78.71 ± 6.75 µg/mL, followed by AC (IC_{50} = 220.67 ± 9.78 µg/mL). CSF, and CSL showed the lowest activity ($p < 0.05$) among the plants studied in this work, since they required much high concentrations to reduce 50% of free-radical concentrations.




B
DPPH scavenging (%)
Concentration (µg/mL)
AC
CSS
CSF
CSL
LAK
LAA
TS
BHT
AA


C
DPPH scavenging activity *%)
Concentration (µg/mL)
AC
CSS
CSF
CSL
LAK
LAA
TS
BHT
AA


D
DPPH scavenging (%)
Concentration (µg/mL)
AC
CSS
CSF
CSL
LAK
LAA
TS
BHT
AA

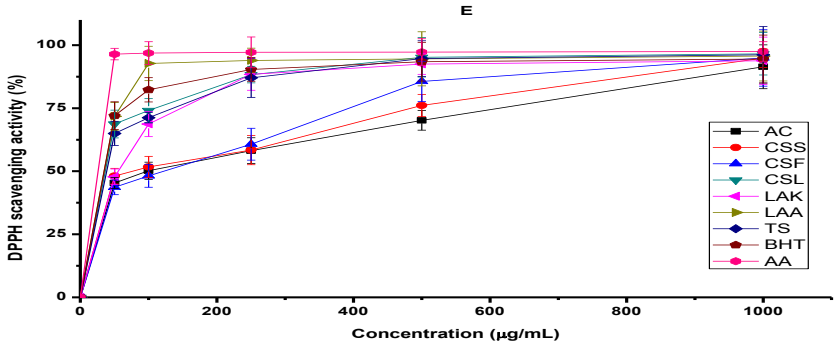


Figure 3.5 DPPH radical scavenging activity (%) of petroleum ether (A), water (B), acetone (C), methanol (D), and aqueous: methanol (20:80, v/v) (E) extracts from LAK, LAA, CSS, CSF, CSL, AC, TS and controls (L-ascorbic acid and BHT).Values are average of triplicate measurements (mean ± SD).

Comparison of extracts between different extracting solvents showed variation of DPPH (%) scavenging. For AC, methanol extract showed the strongest DPPH scavenging activity with IC_{50} value of 51.36 ± 1.14, followed by aqueous: methanol extract (IC_{50} = 97.21 ± 3.79). Water extract of AC showed weakest DPPH scavenging activity, significantly different from the IC_{50} values of petroleum ether and acetone extracts ($p < 0.05$). The IC_{50} values of aqueous: methanol (20:80, v/v) and methanol extracts were not significantly different ($p > 0.05$) but these values were significantly lower ($p < 0.05$) than the IC_{50} values of water, acetone, and petroleum ether extracts. For CSS, there was no significant difference in IC_{50} values of acetone, methanol, and aqueous: methanol (20:80, v/v) extracts. But these values were lower than the IC_{50} values of water and petroleum ether extracts of CSS. In CSF extracts, aqueous: methanol, (20:80, v/v) extract showed the strongest DPPH scavenging activity ($p < 0.05$) with the IC_{50} value of 123.20 ± 25.47 µg/mL. No significant difference ($p > 0.05$) was observed between the IC_{50} values of

water, methanol and aqueous: methanol (20:80, v/v) extracts. But these values were significantly lower ($p < 0.05$) than the IC_{50} values of acetone and petroleum ether extracts of CSF. Similarly for CSL, aqueous: methanol, (20:80, v/v) extract exhibited the strongest DPPH scavenging (IC_{50} = 43.05 ± 5.00 µg/mL).

Table 3.1 IC_{50} values of DPPH scavenging activities in various solvent extracts from AC, CSS, CSF, CSL, LAK, LAA and TS.

	IC_{50} (µg/mL) ± SD				
Plant	Pet. ether	Water	Acetone	Methanol	Aqueous: Methanol (20:80, v/v)
AC	674.74 ± 10.80^{cC}	827.23 ± 10.78^{fD}	220.67 ± 9.78^{bB}	51.36 ± 1.14^{bA}	97.21 ± 3.79^{cA}
CSS	>1000	293.60 ± 28.0^{eB}	78.71 ± 6.75^{aA}	68.61 ± 5.90^{bA}	84.33 ± 6.39^{bA}
CSF	>1000	146.22 ± 37.6^{cA}	907.05 ± 71.1^{dB}	161.43 ± 9.65^{cA}	123.20 ± 25.47^{cA}
CSL	>1000	214.56 ± 17.9^{dC}	672.71 ± 88.6^{cD}	167.75 ± 38.93^{cB}	43.05 ± 5.00^{bA}
LAK	90.77 ± 5.22^{bC}	45.88 ± 3.51^{aB}	43.91 ± 5.00^{aB}	18.60 ± 6.01^{aA}	10.96 ± 0.73^{aA}
LAA	29.97 ± 1.84^{aC}	29.33 ± 2.00^{aC}	14.00 ± 1.08^{aB}	11.91 ± 3.80^{aA}	7.96 ± 2.11^{aA}
TS	73.99 ± 2.38^{bD}	89.49 ± 2.90^{bE}	19.81 ± 2.21^{aB}	45.8 ± 3.00^{bC}	11.03 ± 1.34^{aA}

Values are expressed as mean ± SD (n = 3). Different letters after the means indicate significant differences among solvents (upper case) and plant sources (lower case) ($p < 0.05$).

For LAK, the IC_{50} values ranged from 10.96 ± 0.73 µg/mL for aqueous: methanol (20:80, v/v) extract to 90.77 ± 5.22 µg/mL for petroleum ether extract. Strongest scavenging activity was recorded for aqueous: methanol (20:80, v/v) extract which appeared more

than four times stronger than that of water and acetone extracts and more than eight times stronger than the DPPH scavenging activity of petroleum ether extract. No Significant differences ($p > 0.05$) were observed between the aqueous: methanol (20:80, v/v) and methanol extracts, and also between acetone and water extracts of LAK. Petroleum ether extract showed the weakest DPPH scavenging ($p < 0.05$). For LAA, The IC_{50} values of water and petroleum ether extracts were not found to be significantly different ($p > 0.05$), and also no significant difference ($p > 0.05$) was observed in methanol, aqueous: methanol (20:80, v/v) extracts.

In the TS extracts, the IC_{50} values were ranged from 11.03 ± 1.34 µg/mL for aqueous: methanol (20:80, v/v) extract to 89.49 ± 2.90 µg/mL for water extract. All extracts were significantly different ($p < 0.05$) and strongest scavenging activity (lower IC_{50} value) was recorded for aqueous: methanol (20:80, v/v) extract which appeared more than eight times stronger than that of water extract and four times stronger than that of methanol extract.

The IC_{50} values of L-ascorbic acid and BHT were tested as references. The IC_{50} values were 6.11 ± 0.20 µg/mL for L- ascorbic acid and BHT showed 50% inhibition at 8.70 ± 0.19 µg/mL The IC_{50} values of most of the extracts were found to be significantly higher ($p < 0.05$) than the IC_{50} values of BHT and L-ascorbic acid, while that of the aqueous: methanol (20:80, v/v) leaf extracts of LAA, LAK, TS and methanol leaf extract of LAA extract were found to be similar ($p > 0.05$). These results were in agreement with previous studies which showed that solvent nature exert a great power in phenolic extraction capacities in many species (Turkmen *et al*., 2006). According to Siddhuraju and Becker (2003), pure methanol was an effective solvent for antioxidant extraction of phenolic compounds, in contrast to pure ethanol showed the lowest extraction power. Moreover,

Zhou and Yu (2006) assayed the addition of 20% distilled water to the methanol, ethanol or acetone influenced considerably the extraction capacity of phenolic compounds in *Limoniastrum monopetalum* leaves. Later on similar research was conducted by Trabelis *et al* (2009). They showed that phenolic contents and antioxidant activities varies considerably as a function of solvent polarity, leaf extract using pure methanol showed higher TPC and TFC. Whereas, ethanol and hexane exhibited lower capacity to extract phenolic compounds and showed lower antioxidant activity.

3.3.1.2 Ferric reducing power

Fe (III) reducing power of a compound is related to its ability to transfer electron and serves as a useful indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant reaction (Rohman *et al*., 2010). The presence of antioxidants in the spice and herbal extracts causes the reduction of the $Fe^{3+/}$ferricyanide complex to the ferrous form. Therefore, the concentration of Fe^{2+} was monitored by measuring the formation of Perl's Prussian blue at 700 nm (Amarowicza *et al*., 2004). A higher value indicates a higher reduction capacity. The results (mg AAE/g of dried extract) showing the effects of extracting solvent on the reducing potential of extracts of different plant materials at concentration of 1 mg/mL, were shown in Figure 3.6.

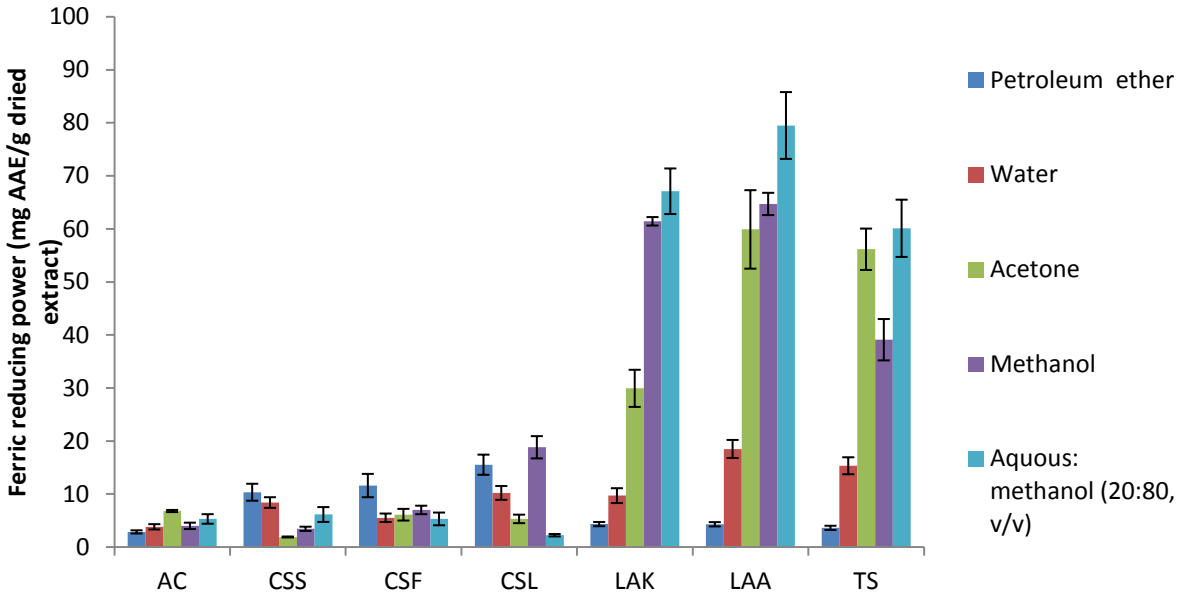


Figure 3.6 Ferric ion reducing power of petroleum ether, water, acetone, methanol, and aqueous: methanol (20:80, v/v) extracts from AC, CSS, CSF, CSL, LAK, LAA, and TS. Values expressed as mg AAE/g of dried extract. Values are average of triplicate measurements (mean ± SD).

The reducing power of methanol extracts were found to decrease in this order: LAA (64.71 ± 1.00 mg AAE/g) > LAK (61.43 ± 0.86 mg AAE/g) > TS (39.11 ± 3.45 mg AAE/g) > CSL (18.80 ± 0.67 mg AAE/g) > CSF (7.01 ± 0.60 mg AAE/g) > AC (4.03 ± 0.62 mg AAE/g) > CSS (3.42 ± 1.40 mg AAE/g. For aqueous: methanol (20:80, v/v) extract, LAA showed the strongest iron ferric reducing power (79.55 ± 6.32 mg AAE/g), followed by LAK (67.10 ± 4.32 mg AAE/g), and TS (60.1 ± 4.01 mg AAE/g). The CSS, CSF, CSL, and AC showed weakest ferric reducing power. Similarly, for acetone extracts, LAA exhibited the strongest ferric reducing power (59.9 ± 7.40 mg AAE/g), followed by TS (56.2 ± 3.94 mg AAE/g), LAK (29.93± 3.50 mg AAE/g), AC (6.83 ± 0.30 mg AAE/g), CSF (6.13 ± 1.12 mg AAE/g), and CSL (5.31± 0.82 mg AAE/g). CSS (1.90 ± 0.10 mg

AAE/g), showed weaker ferric reducing power. There is no significant difference in ferric reducing power between LAA and TS but these values were significantly different ($p < 0.05$) from the other extracts.

Among water extracts, LAA was found to have the highest reducing capacity (18.5 ± 1.7 mg AAE/g) followed by TS (15.33 ± 1.62 mg AAE/g), CSL (10.21 ± 1.30 mg AAE/g), LAK (9.71 ± 1.45 mg AAE/g), and CSS (8.44 ± 1.00 mg AAE/g). The CSF (5.52 ± 0.80 mg AAE/g) and AC (3.87 ± 0.51 mg AAE/g) showed weaker ferric reducing power. No significant difference between LAA and TS and also among CSL, LAK, and CSS were observed for their ferric ion reducing power. Also no significant difference was observed in ferric reducing power between CSF and AC. For petroleum ether extract, CSL exhibited the highest ferric reducing power (15.53 ± 1.90 mg AAE/g) followed by CSF (11.62 ± 2.24 mg AAE/g) and CSS (10.34 ± 1.60 mg AAE/g). LAK, LAA, and TS showed the weakest ferric reducing power ($p < 0.05$). For the acetone extract, the LAA also, showed the highest activity. Such great activity of ferric reducing power was accordance with the high total phenolic content (Nic´iforovic *et al*., 2010).

There was also a variation in the ferric reducing power of extracts from various extracting solvents. The reducing power of TS leaf extracts was found to decrease in the order: aqueous: methanol (60.11 ± 1.00 mg AAE/g) > acetone (56.16 ± 0.81 mg AAE/g) > methanol (39.12 ± 3.45 mg AAE/g) > water (15.33 ± 0.60 mg AAE/g) > petroleum ether (3.61 ± 0.68 mg AAE/g). Methanol, water, and petroleum ether extracts showed significant difference ($p < 0.05$) in their iron reducing power. Whereas, iron reducing power of acetone and aqueous methanol extracts were not significantly different ($p > 0.05$). Similarly for LAA, the aqueous: methanol (20:80, v/v) extract exhibited the highest

ferric reducing power, followed by methanol, acetone, water, and petroleum ether extracts. In LAK, aqueous: methanol (20:80, v/v) extract also showed the highest ferric reducing power and water extract exhibited the lowest ferric reducing power. From results, it has been observed that aqueous: methanol (20:80, v/v) extract of LAA had the highest total antioxidant activity followed by aqueous: methanol (20:80, v/v) extract of LAK, methanol extract of LAA, methanol extract of LAK, aqueous: methanol (20:80, v/v) extract of TS, acetone extract of LAA, and acetone extract of TS. This observation is in agreement with Zhao et al. (2006) who reported that various solvent extracts from the same barley variety showed significant differences in their ferric reducing power. Also Chang *et al*., (2007) reported antioxidant activities (DPPH, ferric reducing, and ABTS) on various solvent extracts of *Phellinus merrillii.* In the assays polar factions showed stronger ferric reducing power.

3.3.1.3 Total antioxidant activity by phosphomolybdenum assay

The total antioxidant activity by phosphomolybdenum assay results (Figure 3.7) showed, among the aqueous: methanol (20:80, v/v) extracts, the LAA had the highest total antioxidant activity (1.98 ± 0.14 mg BHTE/g) followed by LAK (1.81 ± 0.18 mg BHTE/g), CSL (1.36 ± 0.03 mg BHTE/g), TS (1.12 ± 0.13 mg BHTE/g) and CSF (0.30 ± 0.03 mg BHTE/g). CSS and AC had the lowest total antioxidant activity with the values of 0.19 ± 0.04 and 0.12 ± 0.01 mg BHTE/g respectively. No significant difference ($p > 0.05$) was found between the total antioxidant activity of AC and CSS ($p > 0.05$) but the total antioxidant activities of LAA, LAK, CSL, AC, and CSF were significantly different ($p < 0.05$).

Among the methanol extracts, TS showed the highest total antioxidant activity with the valued of 1.89 ± 0.14 mg BHTE/g, followed by LAA (1.66 ± 0.4 mg BHTE/g), LAK (1.5 ± 0.07 mg BHTE/g), CSL (1.00 ± 0.04 mg BHTE/g), CSF (0.83 ± 0.04 mg BHTE/g), AC (0.66 ± 0.02 mg BHTE/g), and CSS (0.55 ± 0.05 mg BHTE/g). In acetone extract, LAK showed the highest total antioxidant activity (1.08 mg BHTE/g) followed by CSF (0.89 ± 0.06 mg BHTE/g), CSL (0.79 ± 0.11 mg BHTE/g), LAA (0.78 ± 0.10 mg BHTE/g) > TS (0.72 ± 0.1 mg BHTE/g) > CSS (0.49 ± 0.06 mg BHTE/g) > AC (0.38 ± 0.04 mg BHTE/g).

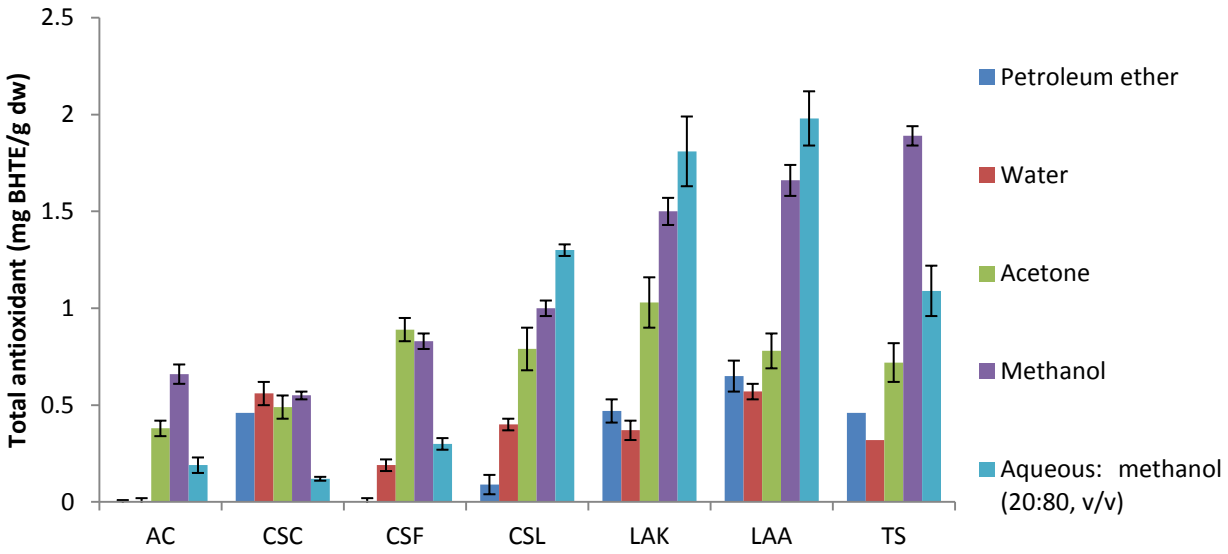


Figure 3.7 Total antioxidant capacity (mg BHTE/g of dried extract) of petroleum ether, water, acetone, methanol, and aqueous: methanol (20:80, v/v) extracts from LAK, LAA, CSS, CSF, CSL, AC, TS. Values are average of triplicate measurements (mean ± SD).

The methanol extract of TS leaf had the highest total antioxidant activity (1.89 ± 0.14 mg BHTE/g) and the lowest total antioxidant activity (0.32 ± 0.10 mg BHTE/g) was found in

the water extract. No significant difference ($p > 0.05$) was found between the total antioxidant activity of petroleum ether (0.46 ± 0.10 mg BHTE/g) and water extracts (0.32 ± 0.10 mg BHTE/g) and also between acetone (0.72 ± 0.16 mg BHTE/g) and aqueous; methanol (20: 80, v/v) (1.09 ± 0.13 mg BHTE/g) extracts of TS. However, these values were significantly lower ($p < 0.05$) than that of methanol extract.

Among the various extracts evaluated, the aqueous: methanol, 20:80, v/v) extract of LAA had the strongest phosphomolybdenum reduction [(1.98 ± 0.4 mg BHTE/g) followed by methanol extract of TS (1.89 ± 0.14 mg BHTE/g), aqueous: methanol (20:80, v/v) extract of LAK (1.81± 0.18 mg BHTE/g), methanol extract of LAA (1.66 ± 0.40 mg BHTE/g), methanol extract of LAK (1.54 ± 0.32 mg BHTE/g), aqueous: methanol (20:80, v/v) extract of CSL (1.31 ± 0.34 mg BHTE/g). The other entire sample extracts showed lower phosphomolybdenum reduction.

3.3.1.4 Ferrous ion chelating activity

Transition metals have been proposed to be the catalysts for the initial formation of radical. Chelating agents may stabilize transition metals in the living systems and inhibit radical generations, consequently reducing free radical damage. Metal chelating agents may have a dramatic effect on increasing the oxidation stability through blocking the pro-oxidant metal ions, and thus limiting the formation of chain initiators by preventing metal-assisted homolysis of hydroperoxides in lipid peroxidation (Praveen *et al*., 2012).

To better estimate the potential antioxidative properties of the extracts, chelating activity of each extract was evaluated against Fe^{2+}. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted resulting

in a decrease in the red color of the complex. Measurement of the color intensity reduction at 562 nm wavelength allows estimation of the metal chelating activity of the chelators (Yamaguchi *et al*., 2000). In this assay, both the extracts and standard compounds were assessed for their ability to compete with ferrozine for Fe^{2+} in the solution. The percentage of iron chelating activities of all extracts and references were concentration-dependent (from 100 to 800 μg/mL) (Figure 3.8). At the concentration of 800 μg/mL used, the ferrous ion chelating activity (%) of the water extracts ranged from 68.98–95.98% (Figure 3.8 A), methanol extracts ranged from 57.90–60.54% (Figure 3.8 B), aqueous; methanol (20:80, v/v) extracts showed in the range of 86.25–94.53% (Figure 3.8 C).

Table 3.2 showed the water extracts of CSS, CSL, and TS had the strongest chelating ($p < 0.05$) activity with IC_{50} value of 53.95 ± 1.22, 56.84 ± 0.25, 65.36 ± 1.06, and 56.70 ± 0.78 μg/mL, respectively. There was no significant difference among the IC_{50} values for water extracts of LAA, LAK, and CSF. The water extract of AC showed the weakest ferrous chelating ($p < 0.05$) activity (213.90 ± 17.14 μg/mL). In methanol extracts (Table 3.2), there was no significant difference ($p > 0.05$) between the IC_{50} values of CSS and CSF. Also there was no significant difference ($p > 0.05$) in the methanol extracts of LAA, LAK, and AC. Methanol extract of TS showed the weakest ferrous chelating activity (655.53 ± 13.97 μg/mL).

For aqueous: methanol (20:80, v/v) extract (Table 3.2), CSL exhibited the strongest ferrous ion chelating activity ($p < 0.05$).There was no significant difference ($p > 0.05$) in IC_{50} values between CSS and LAK and also between AC and TS but these values were significantly different ($p < 0.05$) from the rest of extracts. LAA showed the weakest ferrous chelating activity ($p < 0.05$).

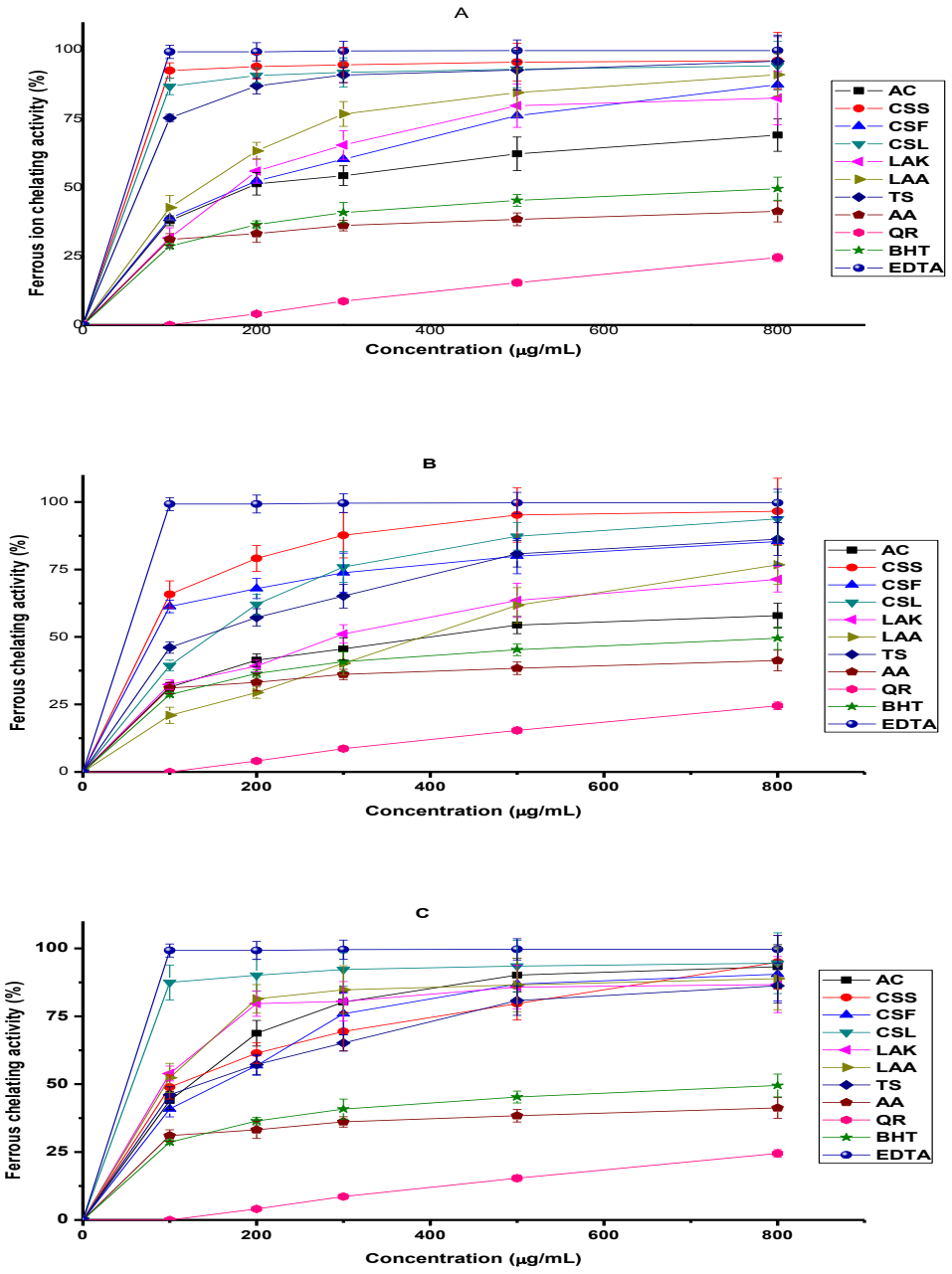


Figure 3.8 Ferrous ion chelating activity (%) of water (A), methanol (B), and aqueous: methanol (20:80, v/v) (C) extracts from LAK, LAA, CSS, CSF, CSL, AC, TS and controls (L-ascorbic acid, BHT, EDTA, and quercetin). Values are average of triplicate measurements (mean ± SD).

Table 3.2 IC_{50} (µg/mL) of ferrous ion chelating activity of various solvent extracts of *Aframomum corrorima* (AC), *Coriandrum sativum* seed (CSS), *Coriandrum sativum* fruit (CSF), *Coriandrum sativum* leaf (CSL), *Lippia adoensis* var koseret (LAK), *Lippia adoensis* var. adoensis (LAA) and *Thymus schimperi* (TS).

Plant material	Water	Methanol	Aqueous: methanol (20:80, v/v)
AC	213.90 ± 17.14^{cB}	398.14 ± 21.22^{cC}	124.74 ± 7.42^{cA}
CSS	53.95 ± 4.22^{aA}	78.25 ± 1.62^{aB}	109.14 ± 12.27^{bC}
CSF	183.99 ± 28.68^{cC}	80.69 ± 3.48^{aA}	157.59 ± 11.21^{dB}
CSL	56.84 ± 0.25^{aA}	147.42 ± 4.84^{bB}	56.70 ± 5.78^{aA}
LAK	191.13 ± 16.11^{cB}	429.35 ± 8.43^{cC}	92.79 ± 4.63^{bA}
LAA	135.74 ± 3.59^{bA}	391.34 ± 5.30^{cC}	199.93 ± 12.36^{eB}
TS	65.36 ± 7.06^{aA}	655.53 ± 13.60^{dC}	136.56 ± 14.00^{cB}

Values are expressed as mean ± standard deviation (n = 3). Different letters after the means indicate significant differences among solvents (lower case) and plant sources (upper case) ($p < 0.05$).

Extracting solvents also affected the chelating capacity of the selected spice and herbs (Boulekbache-Makhlouf, *et al*., 2013). In AC, aqueous: methanol (20:80, v/v) extract showed the strongest ferrous chelating activity, significantly different ($p < 0.05$) from the IC_{50} values of water and methanol extracts. Methanol extract exhibited the lowest potential of ferrous ion chelating activity. Among the CSS extracts, water extract had the strongest chelating capacity (IC_{50} = 53.95 ± 4.22 µg/mL), followed by methanol (IC_{50} = 78.25 ± 1.62 µg/mL) and aqueous: methanol, 20:80, v/v) (IC_{50} =109.14 ± 12.27 µg/mL) extracts. In CSF, methanol extract showed the strongest ferrous chelating ($p < 0.05$)

activity with IC_{50} value 80.69 ± 3.48 µg/mL. No significant difference in IC_{50} values was found between water and aqueous: methanol, 20:80, v/v) extract of CSL but these values were significantly lower than the IC_{50} value of methanol extract.

For LAK, aqueous: methanol (80:20, v/v) extract exhibited the strongest ferrous chelating activity with the IC_{50} value of 92.79 ± 4.63 µg/mL followed by water extract (191.13 ± 16.11 µg/mL) and methanol extract (429.35 ± 8.43 µg/mL). For LAA, water extract showed the strongest ferrous chelating ($p < 0.05$) activity with IC_{50} value of 135.74 ± 2.59 µg/mL followed by aqueous: methanol (20: 80, v/v) extract (199.93 ± 12.36 µg/mL and methanol extract (391.31 ± 5.70 µg/mL). For TS, distinctive difference was observed ($p < 0.05$) between the IC_{50} values of methanol (655.5 ± 13.60 µg/mL) and aqueous: methanol (136.6 ± 14.00 µg/mL) extracts, and these values were significantly higher ($p < 0.05$) than the IC_{50} value of water extract.

The IC_{50} values of water extracts of CSS, CSL, and TS, methanol extract of CSS, aqueous: methanol (20: 80, v/v) extract of CSL, and EDTA (one of the most powerful metal chelator known, IC_{50} = 50.23 ± 4.60 µg/mL) were not significantly different ($p > 0.05$). The results suggest that these extracts were the most effective in sequestering ferrous ions by intercepting all coordination sites of metal ions.These extracts are the richest one in Flavonols (Table 2.5) and they are exactly such type of phenolics with their multiple hydroxyl groups and the carbonyl group on ring C, have several available sites for metal complexation (Leopoldini *et al*., 2006). Nevertheless, in this assay ascorbic acid, BHTand quercetin showed weaker chelating activity of iron (II) ions than water, methanol, and aqueous: methanol (80:20, v/v) extracts, which was consistent with the findings of Yen *et al* (2002).

3.3.1.5 Antioxidant activity in linoleic acid peroxidation (FTC assay)

Lipid autoxidation is a free-radical chain reaction, leading to an increase of the reactive radical and hydroxide content and proceeds initiating further transmutations with three distinguished stages: initiation, propagation and termination.The rate of the triacylglycerol oxidation process depends on the saturation degree and the double bond position in the molecules. The polyunsaturated fatty acids are known more sensitive to oxidation than saturated ones, which help to predict lipid susceptibility to oxidation processes. Due to its high amount of polyunsaturated fatty acids (PUFAs), flaxseed oil is unstable and easier to be oxidized. One effective way to ensure a high quality of lipids and lipid-containing products and to prolong their storage time is directly associated with their optimum stabilization by addition of suitable antioxidants. Moreover, growing interest has been directed to using natural antioxidants found in plants because of the world-wide trend toward the use of natural additives in foods (Yanishlieva *et al*., 2006; Arain *et al*., 2009).

The ferric thiocyanate method measures the amount of peroxide generated at the initial stage of linoleic acid emulsion during incubation. Here, peroxide reacts with ferrous chloride to form ferric chloride, which in turn reacts with ammonium thiocyanate to produce ferric thiocyanate (FTC), a reddish pigment. Low absorbance values measured *via* the FTC method indicate high antioxidant activity (Kim and Kim, 2011). The results of the peroxidation of linoleic acid in the aqueous model system at 40 ^{o}C and after the addition of 1 mL (1 mg/mL) methanol extracts of AC, CSS, CSF, CSL, LAK, LAA, BHT, and AA are shown in Figs 3.9. A sharp increase in the level of linoleic acid peroxidation was observed in the control sample at 4 days of storage, and its rate was exponentially increasing at day 8 when the study was ended (Figure 3.9). Compared with the control, the

extract samples and references (BHT and AA) showed a lower increase in peroxidation levels over 8 days of testing. At day 8 of testing, the inhibition percentages of the methanol extracts of AA, TS, LAK, LAA, AC, CSF, CSS, CSL, and BHT were 47.33 ± 0.75, 48.63 ± 1.55, 51.46 ± 0.56, 54.55 ± 0.84, 63.88 ± 2.70, 77.44 ± 0.64, 77.60 ± 1.10, 78.30 ± 2.50 and 86.63 ± 2.66% respectively. There was no significant difference ($p > 0.05$) in percentage of peroxide inhibition between AA and TS, and also no significant difference ($p > 0.05$) among CSS, CSF, and CSL. Similarly After eight days, it had been shown that except the TS all samples showed greater than fifty percent inhibition of linoleic acid oxidation.

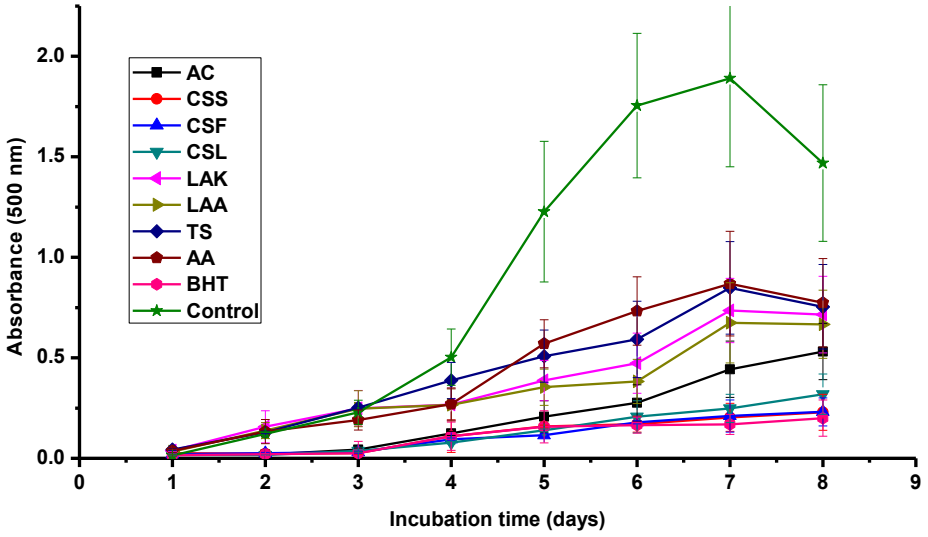


Figure 3.9 Percentage of inhibition of peroxidation of linoleic acid as measured by FTC method of AC, CSS, CSF, CSL, LAK, LAA, and TS extracts. (Mean ± SD). Each experiment was executed in triplicate.

3.3.1.6 Thiobarbituric acid reactive substances (TBARS) assay

During lipid peroxidation, lipid peroxides are formed, with a subsequent formation of peroxyl radicals, followed by a decomposition phase to yield the aldehydes such as hexanal, malondialdehyde, and 4-hydroxynonenal. This assay is based on the detection a deep orange or pink colour developed during the heating at 85 ^{o}C, which is formed between the reaction of malondialdehyde and thiobarbituric acid (TBA) at late stage of lipid oxidation, in the aqueous phase. Ketones, ketosteroids, acids, esters, sugars, proteins, pyridines, pyrimidines and vitamins can react with thiobarbituric and interfere with the assay (Devasagayan *et al*., 2003).

A comparison of secondary products of lipid peroxidation measured as percentage of inhibition of TBARS was shown in Table 3.3. The percentage of inhibition values were found to be higher in linoleic acid compared to flaxseed oil as a substrate ($p > 0.05$). In a system comprising of linoleic acid as substrate, the percentage of inhibition of methanol extract was highest for CSS, indicating the ability of this extract to inhibit lipid oxidation. The percentage of inhibition of linoleic acid was in the order BHT (86.83 ± 4.70%) > CSS (82.64 ± 2.47%) > CSL (81.62 ± 5.90%) > AC (80.83 ± 5.65%) > CSF (79.47 ± 5.74%) > LAA (77.80 ± 5.41%) > LAK (74.61 ± 6.33%) > AA (68.90 ± 4.61%), TS (60.33 ± 3.34%) respectively. Even though the percentage inhibition for CSS was the highest, the result was not statistically different ($p > 0.05$) from CSL, but significantly different ($p < 0.05$) from methanol extracts of other plant extracts. The data also showed that the methanol extracts were more effective inhibitors of linoleic acid peroxidation than the water extracts ($p < 0.05$). The comparison of antioxidant activities between the water and methanol extracts indicated that the methanol extracts were more effective than the

corresponding water extracts ($p < 0.05$). The antioxidant activities of the extracts were also compared with that of the synthetic antioxidant BHT and ascorbic acid. All the water extracts showed weaker antioxidant activity ($p < 0.05$) than BHT and ascorbic acid, while the methanol extracts showed lower activity than BHT ($p < 0.05$) but greater than ascorbic acid (except the TS). In another system, using flaxseed oil as a rich source of PUFA, methanol extract of CSF exhibited the highest percentage of inhibition of lipid peroxidation (68.13 ± 3.22%). Methanol extracts of TS, LAK, and LAA exhibited the lowest activities with the values of 52.07 ± 6.82, 53.65 ±7.13 and 55.66 ± 6.57%, respectively. Similarly the water extracts showed weaker inhibition potential of flaxseed oil than that of methanol extracts. The percentages of inhibition of TBARS (from flaxseed oil emulsion) of all extracts significantly lower ($p < 0.05$) than the percentage of inhibition of TBARS of BHT. Whereas, the inhibition potential of AA was greater than the inhibition potential of LAA, LAK, and TS ($p < 0.05$) but similar with the values of AC, CSS, CSF, and CSL ($p > 0.05$). These results indicated the relative efficiency of extracts in inhibiting lipid oxidation. Data from the current study indicated that the addition of dried spices and herbs reduced the content of malondialdehyde in the linoleic acid and flaxseed oil extracts, with the methanol extracts were more effective than the water extracts ($p < 0.05$), in reducing lipid oxidation by lowering the level of malondialdehyde and peroxide value. Phenolic compounds are considered to be one of the quick initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids (Jayathilakan *et al*., 2007).Therefore, the antioxidant activity of methanol extract is considered to be stronger antioxidant activity on lippid oxidation than the water extracts.

Our result indicated that the methanol extract contanined higher amount of TPC than the water extracts.

Table 3.3 Percentage of inhibition of thiobarbituric acid reactive substances (TBARS) by extracts of *Aframomum corrorima* (AC), *Coriandrum sativum* seed (CSS), *Coriandrum sativum* fruit (CSF), *Coriandrum sativum* leaf (CSL), *Lippia adoensis* var koseret (LAK), *Lippia adoensis* var. adoensis (LAA) and *Thymus schimperi* (TS) in linoleic acid and flaxseed oil substrates.

	Linoleic acid		Flaxseed oil	
Plant	Water extract	Methanol extract	Water extract	Methanol extract
AC	23.4 ± 3.80^{a}	80.83 ± 5.65^{cd}	23.19 ± 2.40^{a}	65.36 ± 2.93^{c}
CSS	37.91 ± 1.75^{c}	82.64 ± 2.47^{e}	32.04 ± 3.01^{c}	63.44 ± 6.90^{bc}
CSF	33.83 ± 2.88^{bc}	79.47 ± 5.74^{cd}	25.43 ± 1.82^{b}	68.13 ± 3.22^{c}
CSL	42.01 ± 6.42^{c}	81.62 ± 5.90^{e}	26.35 ± 4.41^{b}	59.64 ± 5.50^{abc}
LAK	28.78 ± 4.00^{ab}	74.61 ± 6.33^{c}	20.79 ± 3.92^{a}	53.65 ± 7.13^{a}
LAA	31.85 ± 3.96^{bc}	77.80 ± 5.41^{c}	26.94 ± 5.22^{b}	55.66 ± 6.57^{a}
TS	32.34 ± 4.11^{bc}	60.33 ± 3.34^{a}	18.66 ± 0.90^{a}	52.07 ± 6.82^{a}
AA	68.8 ± 2.60^{d}	68.90 ± 4.61^{b}	63.83 ± 4.87^{d}	63.81 ± 4.87^{bc}
BHT	86.83 ± 4.70^{e}	86.83 ± 4.70^{f}	78.11 ± 6.71^{e}	78.16 ± 6.79^{bc}

Values are expressed as mean ± standard deviation (n = 3). Different letters in columns after the means indicate significant differences ($p < 0.05$).

3.4 Correlation Analysis

It is well known that the antioxidant activity of a plant extracts are largely depending on both the composition of the extracts with variations with the test system used in the evaluation. The antioxidant activity can be influenced by a large number of factors, and cannot be fully evaluated by one single method due to the various mechanisms of antioxidant action. In this study, different assays were used to evaluate the antioxidant activity, of LAK, TS, LAA, AC, CSS, CSF, CSL extracted by various solvents. Correlation between antioxidant assay and the TPC and TFC contents are given in Table 3.4 and 3.5, respectively.

For LAK extracts (Table 3.4), the TPC correlated well with ferric reducing power (R^2 = 0.77, $p < 0.05$) and DPPH scavenging (R^2 = 0.88, $p < 0.05$), but weakly correlated with ferrous chelating activity (R^2 = 0.50, $p > 0.05$) and total antioxidant activity (R^2 = 0.32, $p > 0.1$). DPPH radical scavenging (%) (R^2 = 0.70, $p < 0.05$) and total antioxidant activity (R^2 = 0.0.73, $p < 0.05$) well corelated but ferric reducing power (0.37, $p > 0.1$) and ferrous chelating activity (%) (R^2 = 0.14, $p > 0.1$) were weakly correlated with the TFC in various solvent extracts of the LAK (Table 3.5).

Similarly, TPC (Table 3.4) strongly correlated with DPPH (R^2 = 0.93, $p < 0.01$), ferric reducing power (R^2 = 0.82, $p < 0.05$), and total antioxidant (R^2 = 0.76, $p < 0.05$) but negatively correlated with chelating activity (R^2 = -0.64, $p > 0.05$) of various extracts of LAA. The DPPH scavenging (R^2 = 0.72, $p < 0.05$), ferric ion reducing power (R^2 = 0.82, $p < 0.05$), and total antioxidant (R^2 = 0.90, $p < 0.01$) were also strongly correlated but

ferrous chelating activity (R^2 = -0.36, $p > 0.05$) was negatively correlated with TFC of LAA extracts (Table 3.5).

For TS (Table 3.4), the TPC positively and strongly correlated with ferric reducing power (R^2 = 0.95, $p < 0.001$), DPPH scavenging activity (R^2 = 0.97, $p < 0.001$) and total antioxidant activity (R^2 = 0.87, $p < 0.01$). Similarly, strong positive correlation was also found between DPPH radicals (R^2 = 0.96, $p < 0.001$), reducing power (R^2 = 0.80, $p < 0.05$) and total antioxidant (R^2 = 0.99, $P < 0.001$) with the TFC contents in TS leaf extracts (Table 3.5). But both TPC and TF contents showed negative correlation (R^2 = -0.09 and R^2 = -0.32, respectively) with ferrous chelating activity.

The dependence of antioxidant activity, obtained by different assays, in relation to the TPC and TFC of CSS, CSF, and CSL were also evaluated. The results showed good linear correlation in the cases of DPPH scavenging activity of CSS (R^2 = 0.60, $p > 0.05$), CSF (R^2 = 0.79, $p < 0.05$), and CSL (R^2 = 0.71, $p < 0.05$) but weakly correlated with total antioxidant capacity (R^2 = 0.49, $p > 0.05$) and reducing power of (R^2 = 0.40, $p > 0.05$) of CSF extracts, negatively correlated with ferric reducing power of CSS (R^2 = -0.26), and CSL (R^2 = -0.51) in relation to TPC. But ferrous chelating activity (%) of CSS, CSF, and CSL were negatively correlated with TPC (R^2 = -0.58, -0.99, -0.98), respectively. The TFC of CSF was strongly correlated with total antioxidant activity (R^2 = 0.85, $p < 0.01$), but weakly correlated with DPPH scavenging (%) (R^2 = 0.30, $p > 0.1$) and ferrous chelating activity (%) (R^2 = 0.46, $p > 0.05$). But no correlation of was observed between the TFC of CSF and ferric reducing activity. The TPC of AC was moderately correlated with DPPH scavenging (%) (R^2 = 0.55, $p > 0.05$) and ferric reducing power (R^2 = 0.52, $p > 0.05$) but no correlation was observed with total antioxidant activity and ferrous

chelating activity. Similarly The TFC was weakly correlated with DPPH scavenging (R^2 = 0.42, $p > 0.1$), ferric reducing power (R^2 = 0.27, $p > 0.1$), and total antioxidant (R^2 = 0.19, $p > 0.1$). But ferrous chelating activity (%) was negatively correlated with TFC (R^2 = -0.63).

The relationship between phenolic content and antioxidant activity was extensively investigated, and both positive and negative correlations were reported. Zhang *et al*. (2011), Bakchiche *et al*. (2013), Petra *et al*. (2012) and many other research groups stated that there was a positive correlation. Also, a few evidences of no significant correlation were confronted (Mohammed *et al*., 2008). According to Paixao *et al* (2007), strong correlation between TPC and DPPH scavenging activity, and ferric reducing power was observed. A similar study by Ling and coworkers (2010) reported that selected Malaysian plant extracts displayed strong correlations between antioxidant ability and TPC.

High coefficients of determination were found between the TPC and DPPH radical scavenging activity. The TFC exhibited moderate correlation coefficients with DPPH radical scavenging, total antioxidant and ferric reducing power activities. As the aluminum chloride method is specific only for flavones and flavonols, total flavonoid content could be underestimated by the method (Meda *et al*., 2005), which probably accounts for a lower correlation observed between antioxidant activity and TFC. Liu *et al* (2008) reported a negative correlation between TFC and antioxidant activity. Ferric reducing activity and DPPH activity (%) of LAA and TS showed a satisfactory correlation with the TFC and a very good correlation with the TPC, indicating that most phenolic compounds extracted from plant samples were capable of reacting in both methods.

Table 3.4 The coefficient of determination (R^2) between antioxidant activities and TPC of the various extracts of *Aframomum corrorima* (AC), *Coriandrum sativum* seed (CSS), *Coriandrum sativum* fruit (CSF), *Coriandrum sativum* leaf (CSL), *Lippia adoensis* var koseret (LAK), *Lippia adoensis* var. adoensis (LAA) and *Thymus schimperi* (TS).

Plants materials	DPPH (%)	FR (mg AAE/g)	TA (mg BHTE/g)	FC (%)
AC	0.55	0.52	NS	NS
CSS	0.60	-0.26	-0.13	-0.58
CSF	0.79*	0.40	0.49	-0.99
CSL	0.71*	-0.51	0.93***	-0.98
LAK	0.88**	0.77*	0.32	0.50
LAA	0.93***	0.82*	0.76*	-0.64
TS	0.95***	0.97***	0.87**	-0.09

*indicates significance at $p < 0.05$; **indicates significance at $p < 0.01$; ***statistically important difference on level $p < 0.001$; NS: no statistically important difference on level $p < 0.05$; FR ferric reducing power; TA: total antioxidant activity, FC: ferrous ion chelating activity (%).

Table 3.5 The coefficient of determination (R^2) between the total flavonoid content (TFC) and the antioxidant assays of various extracts of *Aframomum corrorima* (AC), *Coriandrum sativum* seed (CSS), *Coriandrum sativum* fruit (CSF), *Coriandrum sativum* leaf (CSL), *Lippia adoensis* var koseret (LAK), *Lippia adoensis* var. adoensis (LAA) and *Thymus schimperi* (TS).

Plants materials	DPPH (%)	FR (mg AAE/g)	TA (mg BHTE/g)	FC (%)
AC	0.42	0.27	0.19	-0.63
CSS	0.46	NS	NS	-0.39
CSF	0.30	NS	0.85**	0.46
CSL	0.60*	0.17	0.74*	-0.99
LAK	0.70*	0.37	0.73*	0.14
LAA	0.72*	0.82**	0.90**	-0.36
TS	0.96***	0.80**	0.99***	-0.32

*indicates significance at $p < 0.05$; ** indicates significance at $p < 0.01$;*** statistically important difference at $p < 0.001$; NS: no statistically important difference on level $p < 0.05$; FR: ferric reducing power; TA: total antioxidant activity; FC: ferrous chelating activity (%).

However, the correlation between antioxidant assays and TPC is usually established for TPC values obtained from different samples obtained by the same extraction method; therefore variations are quantitative rather than qualitative. The Folin–Ciocalteu assay is not specific for particular groups of phenolic compounds and is affected by interfering substances such as organic acid, proteins, sugars and organic non phenolic acids (Singleton *et al*., 1999). It may happen that these substances are good radical scavengers

but poor reducing agents, thus leading to nonlinear results. These results suggested that antioxidant activities of the extracts are not limited to phenolics and flavonoid compounds. The activity may also come from the presence of other antioxidant secondary metabolites in the extracts such as volatile oils, polysaccharides, carotenoids, and vitamins (Javanmardi *et al*., 2003).

Conclusions

The results obtained from this study revealed a substantial variability of the antioxidant activity among the selected samples (spices and herbs) and extracting solvents. Acetone, methanol, and aqueous: methanol (20:80, v/v) leaf extracts of LAA, LAK, TS, and CSL exhibited higher DPPH scavenging and ferric ion reducing antioxidant capacities than spice extracts (AC, CSS, and CSF). LAA leaves showed the most powerful antioxidant activity. Thus, herbs might be good candidates for further investigation in developing new antioxidants, and they can be used as a natural additives in food, cosmetic and pharmaceutical industries instead of more toxic synthetic antioxidants. The study indicated that these dietary herbs and spices contain a considerable amount of phenolic compounds, and have significant antioxidant activity, which can be used as easily accessible source of natural antioxidants and as a possible preventer of lipid oxidation and adverse effects of lipid peroxidation or in pharmaceutical applications. The study showed the antioxidant activities of the crude extracts were variable when extracted by different solvents indicating a high potential to be used as natural antioxidants in preventing various oxidative stresses and as food preservatives. Therefore, supplementing a balanced diet with these plant products may have beneficial health effects.

CHAPTER 4: EFFECT OF THERMAL TREATMENT ON TOTAL PHENOLIC CONTENT AND ANTIOXIDANT CAPACITY OF *Aframomum corrorima, Thymus schimperi, Lippia adoensis* AND *Coriandrum sativum*

Abstract

Spices and herbs show potential health benefits in human diets since they possess antioxidant activity. Thermal treatments may have an impact on their phenolic contents and antioxidant status. In the present study, the effect of thermal treatments (at 100 ^{o}C, 150 ^{o}C, and 180 ^{o}C for 1 and 2 h) on total phenolic contents (TPC) and antioxidant capacity of methanl extracts from leaves of *Lipia adoensis* var. adoensis (LAA), *Lippia adoensis* (LAK) var. koseret and *Thymus schimperi* (TS), and *Coriandrum sativum* fruits (CSF) and seeds of *Aframomum corrorima* and *Coriandrum sativum* were investigated. The fresh spices and herbs without any heat treatment were taken as the control group. The TPC and DPPH scavenging activity (%) of most of these dietary spices and herbs increased with increasing the temperature and prolong the heating time as compared to that of unheated samples. But LAK and LAA showed reduction when heated at high temperatures and others showed different variation in all the activities. The DPPH scavenging activity (%) was strongly correlated (R^2 = 0.86, 0.80) with TPC whereas total antioxidant activity was moderately correlated (R^2 = 0.59, 0.45) for all spices and herbs heated at different temperatures for 1 and 2 h, respectively. These results indicated that the TPC and antioxidant activities of extracts were significantly affected by heating

conditions (temperature and duration of treatment). Therefore, the heating process can be used as a tool for increasing the antioxidant activity of most of the herbs and spices.

Key words: Antioxidant; DPPH; Free radical scavenger; spices and herbs; thermal treatment; total phenolics content

4.1 Introduction

Thermal treatment is a common method used in the food industry and in consumers' homes to enhance the preservation and digestibility of food products. For this purpose, it is important to know the stability of food components during thermal treatment. Several studies were carried out on thermal stability of phenolic contents and antioxidant properties in foods. It is well known that naturally occurring antioxidants could be significantly lost during processing and storage. Paul and Ghosh (2012) reported the loss of ascorbic acid and TPC from pomegranate juice when heated over the temperature range of 70–90 ^{o}C. Another study taken on Cocoa (Olivieo *et al*., 2009) showed that the antioxidant efficiency of the roasted samples (at 180 ^{o}C) was weaker than that of unroasted samples because of the reduction of concentration of catechin antioxidant under the increased roasting times. Similarly, the study conducted by Nadja *et al* (2006), on high pressure liquid chromatography (HPLC) analysis showed the degradation of flavonols (rutin and quercetin) after heat treatment in aqueous solution. According to Raymond *et al* (2010), marinating and cooking significantly reduced the antioxidant activities of marinating sauces, and consequently reduced the amounts of antioxidant available to the consumer from this source. In the other study conducted on spices (Nisha and Arumozhi, 2013), the total antioxidant activity of cloves significantly ($p < 0.05$) increased after 1 h of cooking when compared to fresh, but total antioxidant activity of

pepper and cinnamon decreased during 1 h and significantly increased during 2 h of cooking.

Also a reduction of antioxidant activity after thermal treatment has been reported in wheat (Duh *et al*., 2001), onion bulbs and asparagus spears (Makris and Rossiter, 2001), firm tofu (Huang *et al*., 2003) and Lactobacillus plantarum-fermented cowpea (Doblado *et al*., 2005). According to Yi-Ching *et al* (2009), antioxidant activity of fermented black soybeans was stable and increased with heating at a temperature up to 80 °C for 30 min. But the extract showed a reduced antioxidant activity after heating the fermented black soybeans at 100 °C for 30 min. According to Gabriel and Ismael (2010), TPC and antioxidant activity of methanol leaf extract of *Orthosiphon stamineus* significantly decreased when heated above 60 ^{o}C. Wangcharoen and Morasuk (2009) studied the effect of heat treatment on garlic. The result showed that the antioxidant capacity of heated garlic was decreased because of the decomposition of some phenolic and sulfur-containing compounds. However, when browning pigments developed, the antioxidant capacity of the heated brown garlic increased with the degree of browning, provided that it was not too dark. Whereas, the study conducted on effect of thermal treatment on garlic ,white and red oinons was found that with blanching and frying and then microwaving no significant decrease (Shela *et al*., 2008) in the amounts of their bioactive compounds and the level of antioxidant activities were observed ($p > 0.05$).

Horváthová *et al* (2007) studied the effect of heat treatment and storage on antioxidant activity of black pepper, allspice and oregano involving the DPPH, FRAP, TBARS and TPC assays. They confirmed that thermal treatment at 130 °C for 5 min caused, significant decrease of all parameters used for the antioxidant activity evaluation, exception for the

TPC content as their increase in black pepper was noticed. The study conducted on Thai hot curry paste extract and its ingredients (Settharaksa, *et al*., 2012) heated at 100 ^{o}C caused more depletion of phenolic contents and DPPH activity compared with heating 121 ^{o}C. The study conducted on *Tylopilus alboater* wild edible mushrooms, indicated that heat treatment decreased the TPC and TFC (Nipaporn, *et al*., 2014).

However, some studies indicated that the thermal processing may not influence the antioxidant property of samples or even develop new antioxidants and increase antioxidant activity (Wonde *et al*., 2007; Michaela *et al*., 2013). Amarowicza *et al* (2009) observed that long-term storage and processing didn't alter phenolic acid and flavonoid contents of different food sources. Dewanto *et al* (2002) found that thermal processing has elevated total antioxidant activity and the bioaccessible of lycopenes in tomatoes and produced no significant changes in the TPC and TFC, although loss of vitamin C was observed. According to study conducted by Monica *et al* (1999), heating caused an increase in the overall antioxidant potential of the tomato juice. This occurred as a consequence of the formation of melanoidins during the advanced steps of the Maillard reaction. The effect of heat treatment on the antioxidant activity of the extracts from *Citrus unshiu* peels was evaluated. TPC and antioxidant activity of Citrus peels increased at different heating temperature (Seok-Moon *et al*., 2004). These observations were explained by some previous studies suggesting possible thermal degradation of naturally occurring antioxidant properties and the potential formation of Maillard reaction products with antioxidant properties may attribute to the overall increment of antioxidant activity (Arnoldi, 2002). According to Kitti (2003), cooking of yellow oinoin increased the total

amount of flavonoids, quercetin and derivatives available by disrupting the plant tissue and rendering better accessibility.

Nwaichi (2013) evaluated the effect of different heating periods on phenolic contents and antioxidant activity of the tubers of *Tetrapleura tetraptera, Xylopia aethiopica* and *Piper guineense.* Results indicated a significant increase of the antioxidant activity and flavonoid contents but the level of vitamin C decreased for all spices studied. According to Saliha (2013), TPC, TFC, anthocyanin contents and antioxidant capacity of fruit teas were higher (as compared to unheated samples) with increasing the water temperature for the extraction, and maximum values were observed at 100 °C.

Gallegos-Infante *et al* (2010) found cooking and roasting barley extracts increased the TPC as compared with the control (unprocessed) barley extracts, but the germination was found to reduce it. The effect of heating up to 180 ^{0}C, on the antioxidant effectiveness and the chemical composition of basil, cinnamon, clove, nutmeg, oregano and thyme essential oils showed significantly higher DPPH radical-scavenging activity and changes in the chemical composition only for nutmeg oil, whereas the radical scavenging activity of other essential oils under study remained unaffected (Tomaino *et al.*, 2005). Mahmuda *et al* (2006) conducted research on changes in the radical-scavenging activities and the TPC of sixteen spices heated at 100 ^{o}C for different heating times. Clove was found to have the highest radical-scavenging activity followed by allspice and cinnamon. After heating, DPPH and peroxy radical-scavenging activities as well as the TPC increased in most of the spices. A distinct increase in the activities was found in some spices such as black pepper, red pepper and turmeric. According to the study conducted by Clara *et al* (2010) on durum wheat pasta enriched with debranning fractions of wheat, the boiling water

could have enhanced the extraction of bound phenolics from the food matrix, primarily ferulic acid ester linked to cell walls. Cooking also affected the antioxidant capacity of pasta samples by enhancing its antioxidant properties *in vitro*.

Dietary spices and herbs are widely used in Ethiopia and usually consumed after thermal cooking. Therefore, it is important to consider the effect of thermal treatment on the TPC and antioxidant activity of the selected spice and herb extracts. So far, there was no report on the effect of thermal treatment on TPC and *in vitro* antioxidative activities of these dietary spices and herbs except on leaf and fruit extracts of *Coriandrum sativum* grown in India (Kaiser *et al*., 2013; Snigdha and Monika, 2014). Therefore, the objectives of this study were to investigate TPC and the *in vitro* antioxidant capacities (DPPH scavenging and total antioxidant) of the methanol extracts of the selected spices and herbs before heating and the changes of TPC and antioxidant activity after heating at different temperatures (100 ^{o}C, 150 ^{o}C, and 180 ^{o}C) for 1 and 2 h. Furthermore, the correlation between TPC and antioxidant activities was also evaluated.

4.2 Materials and Methods

To examine and compare the stabilities of samples under heat stress, 1 g of individual dried samples was placed in a light - capped test tube and stored at 100 ^{o}C, 150 ^{o}C and 180 ^{o}C in oven (Memmert model 400, Germany) in a triplicate sample treatment. For each thermal treatment, samples were heated for 1 and 2 h. After cooling to room temperature in a desiccator, the samples were immediately soaked with 10 mL methanol for 6 h on mechanical shaker. All extracts were stored in dark at -20 ^{o}C until further analysis.

4.2.1. Determination of total phenolic content (TPC)

To 0.1mL of the extract, 1 mL Folin-Ciocalteu reagent (diluted ten times) was added and the mixture was left for 5 min and then 1mL (75 g/L) of sodium carbonate was added. The absorbance of the resulting blue color was measured at 765 nm with a UV-visible spectrophotometer (JENWAY, 96500, UK) after incubation for 90 min at room temperature. The TPC was estimated from gallic acid (1-100 µg/mL) calibration curve ($y = 0.02x + 0.09$, $R^2 = 0.99$) and results were expressed as milligram gallic acid equivalent/gram of dried weight (mg GAE/g dw).

4.2.2. Determination of DPPH scavenging activity

Freshly prepared DPPH solution (2 mL, 0.006%, w/v) in methanol was added in each of the test tubes containing 1 mL of the methanol extract. The reaction mixture and the control were vortexed and left to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was then taken at 520 nm. Methanol was used as blank. The ability to scavenge the DPPH radical was calculated using the equation:

$$\text{DPPH scavenging (\%)} = [(A_c - A_s)/A_c] \times 100$$

Where A_c is the absorbance of the control and A_s is the absorbance in presence of the sample of the extracts.

4.2.3. Determination of total antioxidant using phosphmolybdenum method

The methanol extract (0.3 mL) was mixed with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The samples were incubated at 95 ^{o}C for 90 min, cooled to room temperature and absorbance was measured at 695 nm using methanol (3 mL) as a blank. The total antioxidant activity was

determined from ascorbic acid (10–250 μg/mL) expressed as milligram ascorbic acid equivalent/gram of dried sample (mg AAE/g dw) based on the calibration curve (y = 0.003x + 0.261, R^2 = 0.98).

4.3. Results

4.3.1 Effects of heating conditions on the total phenolic content (TPC)

The effect of heating temperature and time on TPC were presented in Figure 4.1. The TPC were ranged from 12.99 – 107.05 mg GAE/g dw for fresh spices and herbs and for heated samples the TPC was found in the range of 17.71–166.38 mg GAE/g dry dw.

After heating for 1h at 100 ^{o}C, the TPC of AC, significantly increased to 17.71 ± 2.76 mg GAE/g dw and further increased to 19.91 ± 3.5 mg GAE/g dw when heating for 2h. The TPC of unheated sample (12.99 ± 2.45 mg GAE/g dw) was significantly lower ($p < 0.05$) than samples heated for 1 h and 2 h. When the sample was heated at 150 ^{o}C for 1 h, the TPC significantly increased ($p < 0.05$) to 28.20 ± 3.51 mg GAE/g dw and further increased roughly by four folds ($p < 0.05$) (48.90 ± 5.52 mg GAE/g dw) when heated for 2 h as compared to unheated sample. After heating at 180 ^{o}C for 1 h the TPC significantly increased ($p < 0.05$) by more than two folds (33.08 ± 2.40 mg GAE/g dw) and further heating for 2 h increased by more than three folds (46.71 ± 2.60 mg GAE/g dw) as compared to that of unheated sample.

The TPC of CSS was significantly increased ($p < 0.05$) from 21.33 ± 3.32 mg GAE/g dw in control to 27.40 ± 4.10 mg GAE/g dw after heating at 100 ^{o}C for 1 h and reached more than two folds (56.22 ± 3.50 mg GAE/g dw) after heating for 2 h. These values were significantly higher ($p < 0.05$) than the TPC of unheated sample. After heating at 150 ^{o}C

for 1 h the TPC increased significantly to 57.02 ± 2.82 mg GAE/g dw but reduced significantly ($p < 0.05$) to 33.25 ± 3.10 mg GAE/g dw after heating for 2 h. When heated at 180 ^{o}C for 1 h the TPC increased to 57.88 ± 1.81 mg GAE/g dw but further heating for 2 h reduced slightly to 54.09 ± 4.40 mg GAE/g dw).

When heated at 100 ^{o}C for one 1 h, the TPC of CSF increased significantly ($p < 0.05$) (28.28 ± 3.64 mg GAE/g dw) and further heating for 2 h increased by more than two and half folds (60.8 ± 3.42 mg GAE/g dw) as compared to unheated sample (23.79 ± 1.60 mg GAE/g dw). When heated at 150 ^{o}C for 1 h, the TPC increased significantly ($p < 0.05$) (32.69 ± 2.24 mg GAE/g dw) and further increased significantly ($p < 0.05$) when heated for 2 h (44.84 ± 2.03 mg GAE/g dw). After heating at 180 ^{o}C for 1 h the TPC significantly increased to 47.47 ± 1.85 mg GAE/g dw as compared to that of unheated sample. Further heating for 2 h, increased to 60.94 ± 2.15 mg GAE/g dw. The TPC of CSL significantly reduced when the sample was heated at 100 ^{o}C for 1 h ($p < 0.05$) but after heating for 2 h, significantly increased ($p > 0.05$) to 40.6 ± 5.58 mg GAE/g dw) when compared with unheated sample (38.12 ± 1.11 mg GAE/g dw). Heating at 150 ^{o}C for 1 h increased the TPC significantly ($p < 0.05$) to 46.4 ± mg GAE/g dw but no significant change ($p > 0.05$) was observed after heating for 2 h. Further heating at 180 ^{o}C for 1 h increased the TPC to 53.5 ± 2.86 mg GAE/g dw but reduced to 39.20 ± 2.24 mg GAE/g dw after heating for 2 h. Similar result was observed for CSL of Indian origin (Snigdha and Monika, 2014). According to this study, the extracts of fresh leave showed the highest TPC, which was reduced significantly after treatment to 100 °C. According to the study conducted by Kaiser *et al* (2013), on fresh leaves and fruits of *Coriandrum sativum*, after steam-blanching for a min, TPC and antioxidant capacities remained virtually unchanged. In

contrast, water-blanching and extended steam-blanching even increased the TPC compared to the unheated control, whereas short-time water blanching resulted in higher values than prolonged treatment.

When heated at 100 ^{o}C for 1 h, the TPC of TS significantly reduced from 107.5 ± 4.75 mg GAE/g dw (unheated) to 86.13 ± 8.94 mg GAE/g (heated at 100 ^{o}C for 1 h) and 96.84 ± 7.63 mg GAE/g dw (heated for 2 h). Also after heating at 150 ^{o}C for 1 h, the TPC reduced to 119.93 ± 4.50 mg GAE/g dw but increased significantly ($p < 0.05$) to 166.38 ± 5.72 mg GAE/g dw when heated for 2 h. After heating at 180 ^{o}C for 1 h the TPC reduced to 130.01 ± 4.43 mg GAE/g dw and further decreased to 125.87 ± 3.10 mg GAE/g dw after heating for 2 h.

The TPC of LAK increased from 70.20 ± 6.7 mg GAE/g dw for unheated to 88.87 ± 8.82 mg GAE/g dw for heated at 100 ^{o}C for 1 h and further increased to 96.57 ± 4.30 mg GAE/g dw after heating for 2 h. After heating at 150 ^{o}C for 1 and 2 h, the TPC increased to 108.26 ± 5.10 mg GAE/g dw and 132.39 ± 8.18 mg GAE/g dw, respectively. However, when the sample was heated at 180 ^{o}C for 1 h, TPC reduced to 75.33 ± 3.04 mg GAE/g dw. Further heating for 2 h reduced ($p < 0.05$) the TPC to 56.58 ± 5.7 mg GAE/g dw.

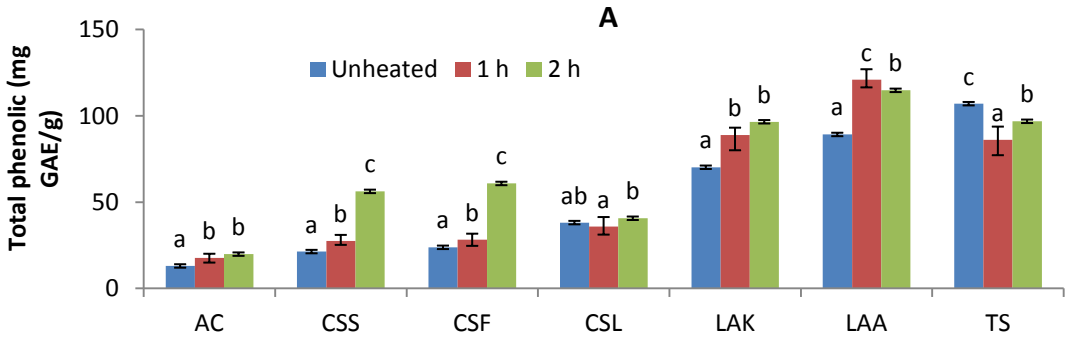
The TPC of LAA significantly ($p < 0.05$) increased from unheated sample (89.21± 6.16 mg GAE/g dw) to 120.94 ± 4.44 mg GAE/g dw for sample heated at 100 ^{o}C for 1 h and further heating for 2 h reduced the TPC to 114.78 ± 6.08 mg GAE/g dw. Heating at 150 ^{o}C for 1 h reduced the TPC to 104.4 ± 2.14 mg GAE/g dw but further heating for 2 h increased to 145.18 ± 4.77 mg GAE/g dw. The TPC of the sample heated at 180 ^{o}C for one 1 h was not significantly different ($p > 0.05$) from the TPC heated at 150 ^{o}C for 1 h.

But further heating for 2 h significantly decreased ($p < 0.05$) the TPC to 97.87 mg GAE/g dw.

The TPC of most of the samples increased when compared with unheated sample. When heated at 100 ^{o}C for 1 h, resulting in increased TPC values of AC, CSS, CSF, LAK, and LAA by 36.34, 28.46, 18.54, 26.60, and 28.66%, respectively. After heating for 2h, the TPC of AC, CSS, CSF, CSL, LAK, and LAA, increased by 52.73, 163.70, 155.61, 6.61, 37.56, and 28.66%, respectively. After heating at 150 ^{o}C for 1 and 2 h the TPC of all plant materials increased as compared to unheated samples. After heating at 150 ^{o}C for 1 h, the TPC of AC, CSS, CSF, CSL, LAK, LAA, and TS increased by 117.10, 167.32, 37.41, 21.77, 54.07, 17.07, 12.03%, respectively, and heating at 150 ^{o}C for 2 h increased by 276.37, 55.88, 88.48, 22.43, 88.60, 62.74, and 55.42%, respectively. After heating at 180 ^{o}C for 1 h the TPC of AC, CSS, CSF, CSL, LAK, LAA, and TS increased by 154.66, 171.35, 99.54, 40.35, 7.30, 17.77, and 21.41%, respectively. When heated for 2 h the TPC of AC, CSS, CSF, CSL, LAA, and TS increased by 255.74, 156.40, 156.16, 2.70, 9.71, and 17.58%, respectively. This might be attributed to the increased extractability of phenolic compounds due to the disruption of plant cell walls during high heat treatment, which might cause phenolic compounds to be released more easily than in the raw materials with different heat treatments. These results were consistent with the findings of Lo Scalzo *et al* (2004), who reported that thermal treatment generally induced an increase in the main phenolic substances of orange juice, such as anthocyanins and total cinnamates. Romson *et al* (2011) reported that heat treatment increased TPC and antioxidant activity of turmeric-chili paste and its ingredients. They suggested that bound phenolic and flavonoid compounds could be liberated by heat treatment. So-Young *et al*

(2006) also reported an increase in the TPC in heated grape seed due to the liberated phenolic compounds.

The TPC of some of the sample extracts reduced when exposed to different heating temperatures and heating periods. The TPC of LAK reduced by 19.50% when heated at 180 ^{o}C for 2 h, and after heating at 100 ^{o}C for 1 h, the TPC of CSL decreased by 6.2% and that of TS reduced by 19.54% and 10.05% after heating at 100 ^{o}C for 1 and 2 h, respectively. The reduction in TPC caused by heating at higher temperatures is consistent with that observed in rice hull (Lee *et al*., 2005). It was found that increased heating time reduced flavonoids and TPC. It meant that the active compounds were heat labile or easily destroyed and form different fragments. On the other hand, Xu *et al* (2007) reported that the total amount of phenolic acids in huyou peel extract decreased after heat treatment, which indicated that some phenolic acids probably were destroyed by heat treatment. This indicated that the phenolic compounds of plants may be present in different bound forms depending on the species So-Young *et al* (2006).



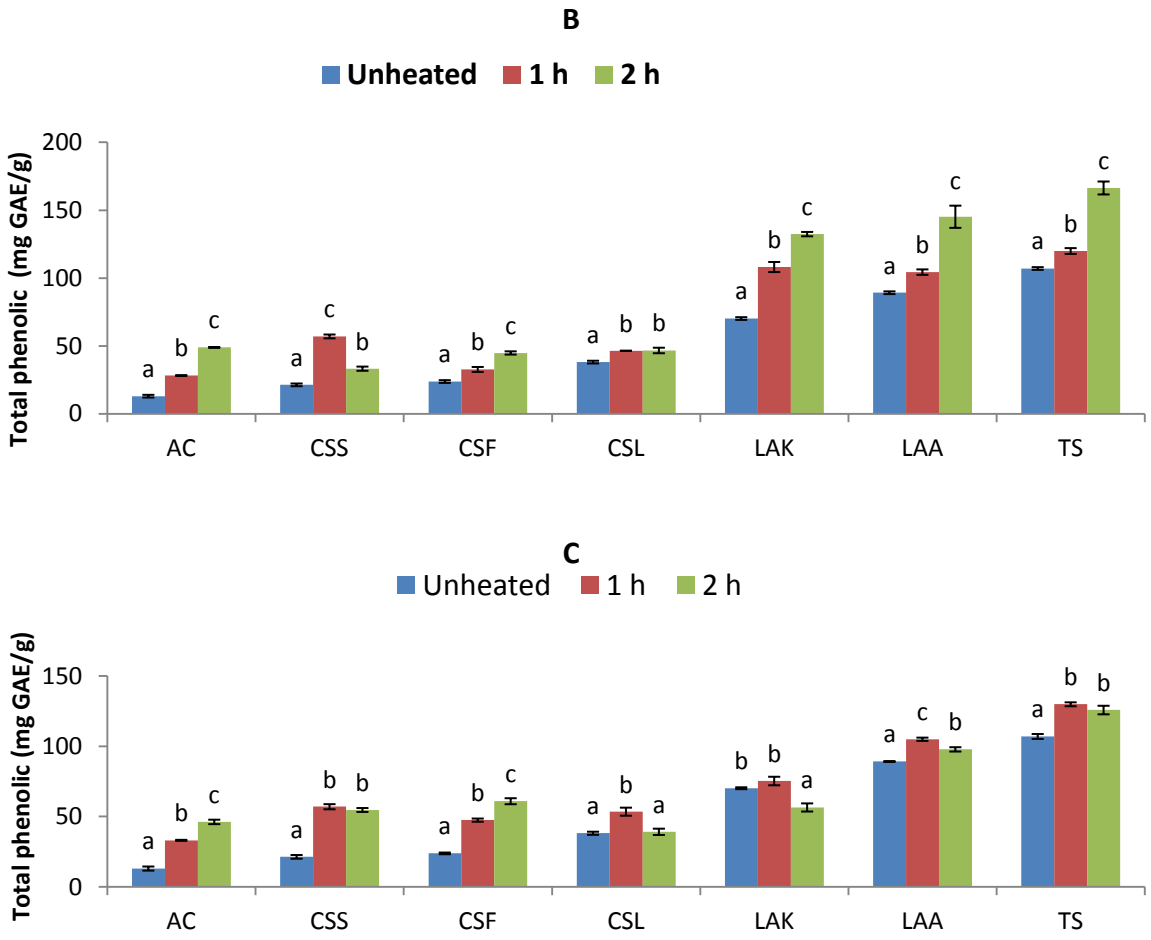


Figure 4.1 Effect of thermal treatments (unheated = kept at room temperature, 100 ^{o}C = A, 150 ^{o}C = B, and 180 ^{o}C = C heated for 1 and 2 h) on total phenolic contents (mg GAE/g dw) Values of different letters (a-c), with in the same plant materials is significantly different at $p < 0.05$.

4.3.2. Effect of heating conditions on DPPH scavenging activity

Changes in the DPPH radical scavenging activity (%) of spices and herbs for different heating times are shown in (Figure 4.2). After heating, a significant change in the activity occurred in many spices and herbs.

The DPPH scavenging (%) antioxidant activity of AC increased from 19.93 ± 3.50% for unheated to 26.52 ± 3.40% for sample heated at 100 ^{o}C for 1 h. Further heating for 2 h didn't affect ($p > 0.05$) the DPPH scavenging activity (19.52 ± 4.50%). When the sample was heated at 150 ^{o}C for 1 h, the DPPH scavenging activity (%) significantly increased by more than two folds (41.95 ± 4.03%) ($p < 0.05$) and further heating for 2 h increased slightly to 42.29 ± 3.70% as compared to sample heated 150 ^{o}C for 1 h. After heating at 180 ^{o}C for 1 h the DPPH scavenging activity (%) of AC decreased to 38.54 ± 3.34% as compared to DPPH scavenging activity of the samples heated at 150 ^{o}C but the activity of the sample heated for 2 h (40.70 ± 2.27%) was not significantly different ($p > 0.05$) from that of the sample heated at 150 ^{o}C for 1 and 2 h.

After heating at 100 ^{o}C for 1 h, DPPH scavenging activity (%) of CSS significantly reduced ($p < 0.05$) to 18.67 ± 5.91% as compared to unheated sample (24.68 ± 2.20%). But further heating for 2 h increased to 24.48 ± 6.60% and almost the same as that of unheated sample ($p > 0.05$). When the sample was heated at 150 ^{o}C for 1 h, the DPPH scavenging activity reduced slightly (22.66 ± 4.82%) ($p > 0.05$) but further heating for 2 h increased to to 24.49 ± 1.85% but no significantly different ($p > 0.05$) was obseved from that of unheated sample. However, after heating at 180 ^{o}C for 1 h the DPPH scavenging activity (%) significantly increased ($p < 0.05$) by more than two and half fold (52.31 ± 4.01%) but significantly decreased ($p < 0.05$) to 39.91 ± 2.40% after heating for 2 h as compared to unheated sample. The DPPH scavenging activity of the sample heated at 180 ^{o}C for 1 h was significantly higher ($p < 0.05$) than the DPPH scavenging activity of the sample heated for 2 h at the same heating temperature.

The DPPH scavenging activity of CSF reduced to 24.02 ± 4.11% when heated at 100 ^{o}C for 1 h as compared to unheated sample (27.62 ± 3.64%) but significantly increased ($p < 0.05$) to 32.42 ± 5.30% when heated for 2 h. Heating at 150 ^{o}C for 1 h increased the DPPH scavenging activity (%) to 31.4 ± 3.34% and heating further for 2 h increased to 39.70 ± 2.03% as compared to unheated sample. Further heating at 180 ^{o}C for 1 h increased the DPPH scavenging activity (%) (48.40 ± 3.86%) significantly ($p < 0.05$) but no significant change ($p > 0.05$) was observed when heated for 2 h (46.91 ± 2.33%). The DPPH scavenging activity (%) of CSL, reduced to 30.72 ± 3.82% after heating at 100 ^{o}C for 1 h, but significantly increased ($p < 0.05$) to 46.63 ± 5.80% when heated for 2 h as compared to unheated sample (37.80 ± 2.45%). When heated at 150 ^{o}C for 1 and 2 h, the DPPH scavenging activity significantly increased ($p < 0.05$) to 48.40 ± 3.61% and to 41.90 ± 1.64%, respectively. Heating at 180 ^{o}C for 1 h the DPPH scavenging activity (%) increased significantly ($p < 0.05$) to 57.83 ± 2.40% but the activity was reduced significantly ($p > 0.05$) when heated for 2 h (37.41 ± 2.24%). %). Similar trend was observed with antioxidant activity of CSL from India (Snigdha and Monika, 2014), where heating the leaf extract at 100 ^{o}C reduced the DPPH scavenging activity.

The DPPH scavenging activity (%) of TS extract heated at 100 ^{o}C reduced slightly when heated for 1 h (91.65 ± 6.34%) and increased slightly when heated for 2 h (93.12 ± 7.10%) as compared to the DPPH scavenging activity of unheated sample (92.70 ± 11.71%) ($p > 0.05$). The activity was significantly increased ($p < 0.05$) after heating at 150 ^{o}C for 1 h (94.14 ± 8.83%) and 2 h (95.24 ± 6.41%) as compared to that of unheated sample. There was no significant difference ($p > 0.05$) in DPPH scavenging activity (%) between samples heated at 150 ^{o}C for 1 and 2 h. Similarly, no significant difference ($p >$

0.05) was observed between the samples heated at 180 ^{o}C for 1 h (92.90 ± 5.51%) and for 2h (94.36 ± 7.74%). However, these values were significantly higher ($p < 0.05$) than the DPPH scavenging activity of unheated sample.

After heating at 100 ^{o}C for 1 and 2 h, the DPPH radical scavenging activity of LAK increased slightly to 92.04 ± 3.82% and 92.6 ± 8.40% respectively, as compared to unheated sample (90.90 ± 6.72%). But sample heated at 150 ^{o}C (for 1 and 2 h), had significantly different ($p < 0.05$) from that of unheated sample. But when heated at 180 ^{o}C for 1 and 2 h the DPPH scavenging activities (%) were significantly lower ($p < 0.05$) (73.55 ± 2.11%, 58.68 ± 1.98%, respectively), resulting in reduced DPPH scavenging activity (%) by 19.18 and 35.44% respectively, compared with the unheated sample.

For LAA, DPPH scavenging activity of the sample heated at 100 ^{o}C for 1 h (92.02 ± 4.40%) and 2 h (94.60 ± 10.75%) were significantly different ($p < 0.05$). But these values were not significantly different ($p > 0.05$) from the DPPH scavenging activity of unheated sample (93.10 ± 9.90%). The DPPH scavenging activity (%) of unheated sample and sample heated for 2 h (at 150 ^{o}C) were significantly different ($p < 0.05$). But these values were not significantly different ($p > 0.05$) from the DPPH scavenging activity (%) of sample heated for 1 h. After heating at 180 ^{o}C for 1 h the DPPH scavenging activity (%) slightly reduced (92.14 ± 6.86%) ($p > 0.05$) but after heating for 2 h the activity was significantly lower (84.70 ± 2.13%) than the DPPH scavenging activity of the sample heated at all temperatures and times.

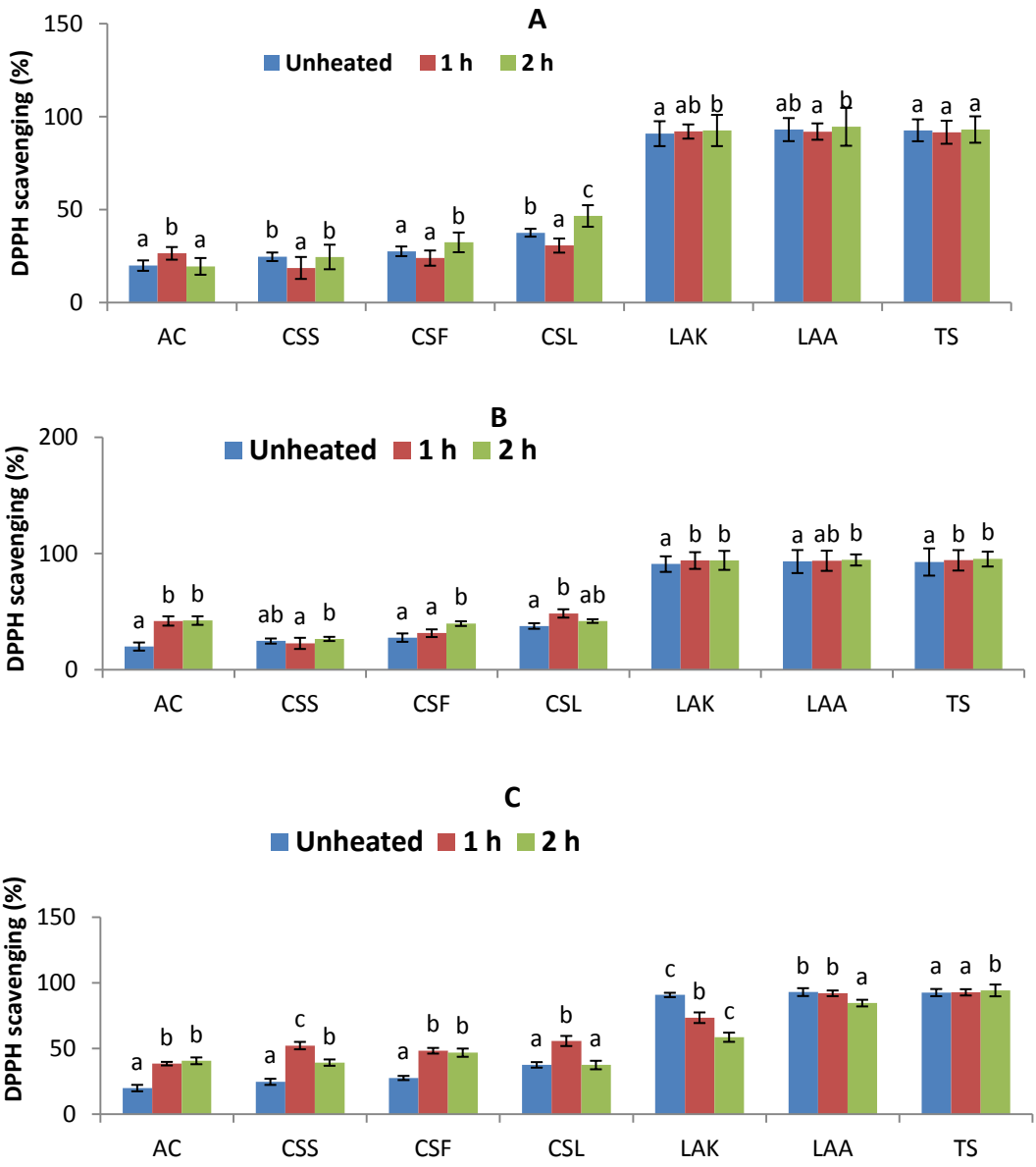


Figure 4. 2 Effect of thermal treatments (unheated = kept at room temperature, 100 ^{o}C = A, 150 ^{o}C = B, and 180 ^{o}C = C heated for 1 and 2 h) on DPPH scavenging activity (%). Values of different letters (a-c), with in the same plant materials is significantly different at $p < 0.05$.

4.3.3 Effect of heating conditions on total antioxidant activity

The power of certain antioxidants is associated with their reducing power (Jayaprakasha *et al*., 2004). Duh (2001) reported that reducing properties of antioxidants are generally associated with the presence of reductions. The total antioxidant capacity of the unheated selected spices and herbs ranged from 18.93 to 219.94 mg AAE/g dw and for the heated spices and herbs range from 18.93 to 227.84 mg AAE/g dw (Figure 4.3).

Total antioxidant activity of AC significantly ($p < 0.05$) increased after heating at 100 ^{o}C for 1 h (79.22 ± 8.22 mg AAE/g dw) when compared to unheated sample (35.42 ± 7.41 mg AAE/g dw). After heating for 2 h significantly reduced ($p < 0.05$) to 67.79 ± 5.01 mg AAE/g dw as compared to the total antioxidant activity of sample heated for 1 h. Similarly, the total antioxidant activity increased when the sample was heated at 150 ^{o}C for 1 h (64.45 ± 13.77 mg AAE/g dw) as compared to the total antioxidant activity of unheated sample. But significantly reduced ($p < 0.05$) to 52.45 ± 3.83 mg AAE/g dw when heated for 2 h, compared to the sample heated for 1 h. The total antioxidant activity reduced slightly after heating the sample at 180 ^{o}C for 1 h as compared to unheated sample but increased significantly ($p < 0.05$) to 54.42 ± 3.77 mg AAE/g dw when heated for 2 h.

The total antioxidant activity of CSS was significantly reduced ($p < 0.05$) to 5.58 ± 1.25 mg AAE/g dw when heated at 100 ^{o}C for 1 h as compared to unheated sample (18.93 ± 3.46 mg AAE/g dw). But after heating for 2 h, the total antioxidant activity slightly higher (22.03 ± 5.48 mg AAE/g dw) than that of unheated sample ($p > 0.05$). When the sample was heated at 150 ^{o}C for 1 h the reducing power increased slightly to 19.29 ± 3.86 mg AAE/g dw. But the activity increased significantly ($p < 0.05$) after heating for 2 h (33.20

± 2.55 mg AAE/g dw). After heating at 180 ^{o}C for 1 h the total antioxidant increased by two folds (39.27 ± 11.82 mg AAE/g dw) but heating for 2 h didn't affect the total antioxidant activity (39.99 ± 10.51 mg AAE/g dw) as compared to sample heated for 1 h.

After heating the dried fruit of CSF at 100 ^{o}C for 1 h, the total antioxidant activity reduced (3.05 ± 1.40 mg AAE/g dw) significantly ($p < 0.05$) as compared to the total antioxidant activity of unheated sample (62.98 ± 7.41 mg AAE/g dw). Heating for 2 h increased to 49.2 ±11.48 mg AAE/g dw but still lower than that of unheated sample. Similarly, after heating the fruits at 150 ^{o}C for 1 h, the total antioxidant reduced to 22.18 ± 5.24 mg AAE/g dw but increased significantly ($p < 0.05$) to 69.09 ± 2.3 mg AAE/g dw after heating them for 2 h as compared to unheated sample. While the total antioxidant activity of CSF decreased to 51.18 ± 10.89 mg AAE/g dw with 180 ^{o}C heating for 1 h but increased significantly ($p < 0.05$) to 96.92 ± 13.3 mg AAE/g dw after heating at 180 ^{o}C for 2 h. The total antioxidant activity of leaf extract of CSL heated at 100 ^{o}C for 1 h, reduced significantly ($p < 0.05$) to 51.79 ± 8.01 mg AAE/g dw as compared to total antioxidant activity of the unheated sample (89.69 ± 5.47 mg AAE/g dw). But after heating for 2 h, the total antioxidant activity increased significantly ($p < 0.05$) to 104.62 ± 6.30 mg AAE/g dw). When the sample was heated at 150 ^{o}C for 1 h, the total antioxidant activity was reduced to 76.57 ± 3.57 mg AAE/g dw as compared to the total antioxidant activity of unheated sample. Further heating for 2 h reduced the total antioxidant activity significantly ($p < 0.05$) to 54.08 ± 2.3 mg AAE/g dw. The activity significantly increased after heating at 180 ^{o}C for 1 h (96.31 ± 1.91 mg AAE/g dw) but significantly reduced ($p < 0.05$) to 52.63 ± 1.8 mg AAE/g dw after heating for 2 h as compared to that of unheated sample.

When heated at 100 ^{o}C for 1 and 2 h the total antioxidant activity of LAK significantly reduced ($p < 0.05$) to 122.55 ± 8.21 mg AAE/g DW and 132.59 ± 6.27 mg AAE/g dw, respectively as compared to the total antioxidant activity of unheated sample (166.71 ± 10.40 mg AAE/g dw). When heated at 150 ^{o}C for 1 h, the total antioxidant activity increased significantly (197.39 ± 22.42 mg AAE/g dw) ($p < 0.05$) but reduced slightly to 155.34 ± 7.23 mg AAE/g dw when heated for 2 h as compared to that of unheated sample. The activity significantly decreased ($p < 0.05$) when the sample was heated at 180 ^{o}C for 1 h (121.46 ± 28.0 mg AAE/g dw) and for 2 h (56.6 ± 4.72 mg AAE/g dw). The total antioxidant activity of LAA increased significantly ($p < 0.05$) to (219.95 ± 26.81 mg AAE/g dw) as compared to total antioxidant activity of unheated sample (191.14 ± 11.67 mg AAE/g dw). But after heating for 2 h, the activity significantly reduced ($p < 0.05$) to 159.95 ± 10.71 mg AAE/g dw. When the sample was heated at 150 ^{o}C for 1 and 2 h, the total antioxidant activity decreased to 159.25 ± 14.19 mg AAE/g dw and 144.30 ± 12.73 mg AAE/g dw respectively as compared to that of unheated sample. When heated at 180 ^{o}C for 1 h the activity decreased to 179.70 ± 12.27 mg AAE/g dw and heating further for 2 h, reduced to 107.74 ± 7.49 mg AAE/g dw. The total antioxidant activity of TS was significantly reduced at all heating temperatures and times ($p < 0.05$) as compared to unheated sample.

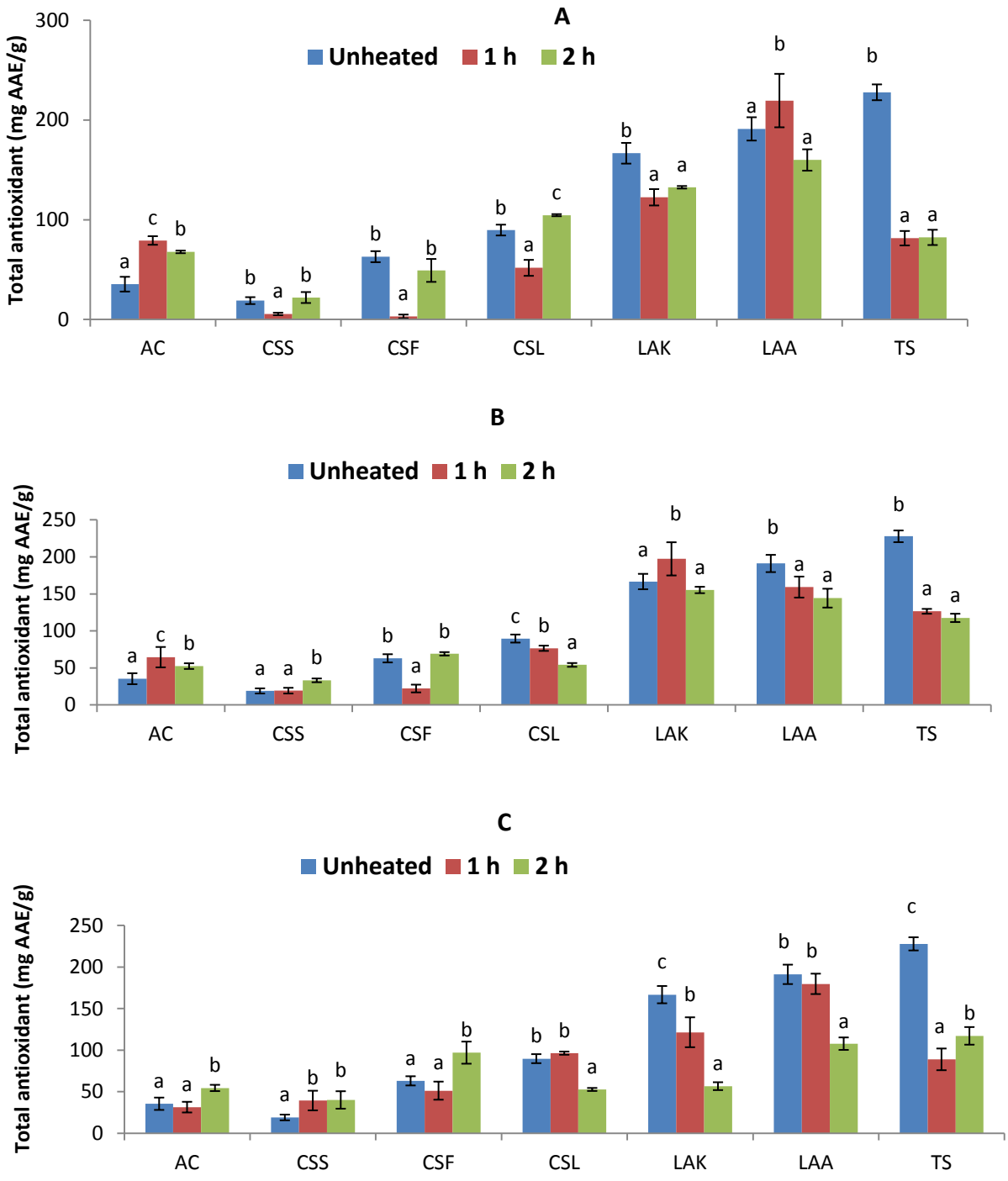


Figure 4.3 Effect of thermal treatments (unheated = kept at room temperature, 100 ^{o}C = A, 150 ^{o}C = B, and 180 ^{o}C = C heated for 1 and 2 h) on total antioxidant activity (mg AAE/g dw). Values of different letters (a-c), with in the same plant materials is significantly different at $p < 0.05$.

4.3.4 Relationship between TPC and antioxidant activities during heating conditions

As shown in Table 4.1, relationships between TPC and antioxidant activities vary with heating temperatures and heating periods. The TPC of dietary herbs and spices heated at all temperatures for 1 and 2 h strongly correlated with DPPH scavenging activity (%). The TPC of samples heated at 100 ^{o}C for 1 and 2 h, at 150 ^{o}C for 1 and 2 h, at 180 ^{o}C for 2 h moderately correlated with total antioxidant activity. But the TPC of samples heated at 100 ^{o}C for 2 h, and at 180 ^{o}C for 1 h, weakly correlated with total antioxidant activity. From the results, the changes of TPC were highly correlated with DPPH scavenging activity (%) than total antioxidant values in all cases of heating processes. In all heating temperatures (100–180 ^{o}C) heated for 1 h, TPC values seemed to be strongly correlated with DPPH (R^2 = 0.86) (Figure 4.4 A) than the values of total antioxidant (R^2 = 0.59) (Figure 4.5 A). Similarly change of TPC heated at different temperatures for 2 h, showed higher correlation with DPPH scavenging activity (R^2 = 0.80) (Figure 4.4 B) than the total antioxidant activity (R^2 = 0.45) (Figure 4.5 B). These differences might be due to the fact that DPPH scavenging method involves free radicals reacting with phenolic and browning pigment compounds, while for total antioxidant assay, it is a method for measuring total reducing power of electron donating substances, which is not directly related to free radical reactions and not as specific as DPPH assay. Form these results the active components of spices and herbs were considered to be mostly phenolic compounds. This is in agreement with several other studies that found strong correlations between TPC and DPPH scavenging activity (Raymond *et al*., 2010). Ademoyegun *et al* (2010) reported that the strong DPPH scavenging activities of different spices might be related to their phenolic antioxidant. This study reported the moderate correlation between TPC and total

antioxidant activity but other studies reported the strong relationship between the TPC and total antioxidant activity (Holasovia *et al*., 2002; Ademoyegun *et al*., 2010) of different spices under different heating conditions.

Table 4.1 Coefficient of determination of changes of antioxidant capacity with TPC of methanol extracts of *Aframomum corrorima* (AC), *Coriandrum sativum* seed (CSS), *Coriandrum sativum* fruit (CSF), *Coriandrum sativum* leaf (CSL), *Lippia adoensis* var koseret (LAK), *Lippia adoensis* var. adoensis (LAA) and *Thymus schimperi* (TS) samples during heating processes.

Heat treatment	Correlation factor	Corelation Coefficient (R^2)
Heated at 100 ^{o}C for 1 h	TPC	
	vs. DPPH radical scavenging (%)	+ 0.89
	vs. (mg AAE/g dw)	+0.67
Heated at 100 ^{o}C for 2 h	TPC	
	vs. DPPH radical scavenging (%)	+0.80
	vs. (mg AAE/g dw)	+0.30
Heated at 150 ^{o}C for 1 h	TPC	
	vs. DPPH radical scavenging (%)	+0.95
	vs. (mg AAE/g dw)	+0.75
Heated at 150 ^{o}C for 2 h	TPC	
	vs. DPPH radical scavenging (%)	+0.96
	vs. (mg AAE/g dw)	+0.78
Heated at 180 ^{o}C for 1 h	TPC	
	vs. DPPH radical scavenging (%)	+0.93
	vs. (mg AAE/g dw)	+0.27
Heated at 180 ^{o}C for 2 h	TPC	
	vs. DPPH radical scavenging (%)	+0.91
	vs. (mg AAE/g dw)	+0.68

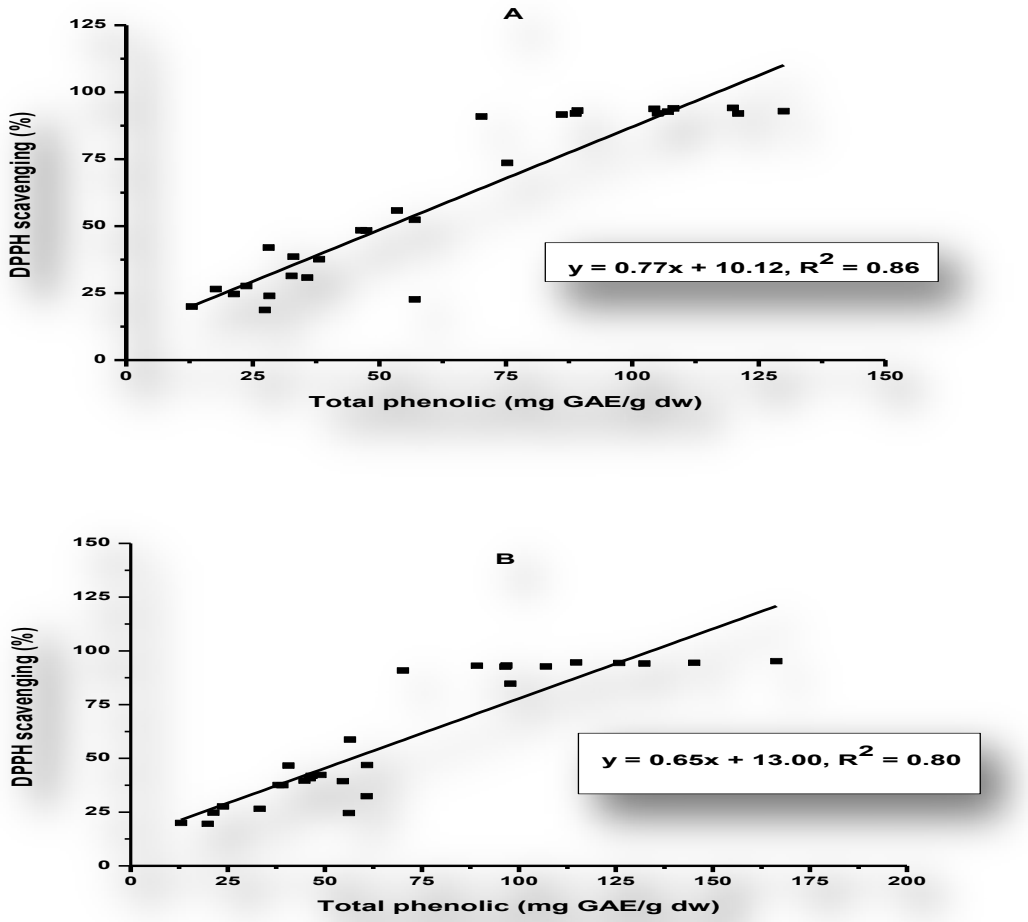


Figure 4. 4 Correlation between TPC (mg GAE/g dw) and DPPH scavenging activity (%) methanol extracts of *Aframomum corrorima* (AC), *Coriandrum sativum* seed (CSS), *Coriandrum sativum* fruit (CSF), *Coriandrum sativum* leaf (CSL), *Lippia adoensis* var koseret (LAK), *Lippia adoensis* var. adoensis (LAA) and *Thymus schimperi* (TS) heated at different temperatures (A) for 1 h, (B) for 2 h.

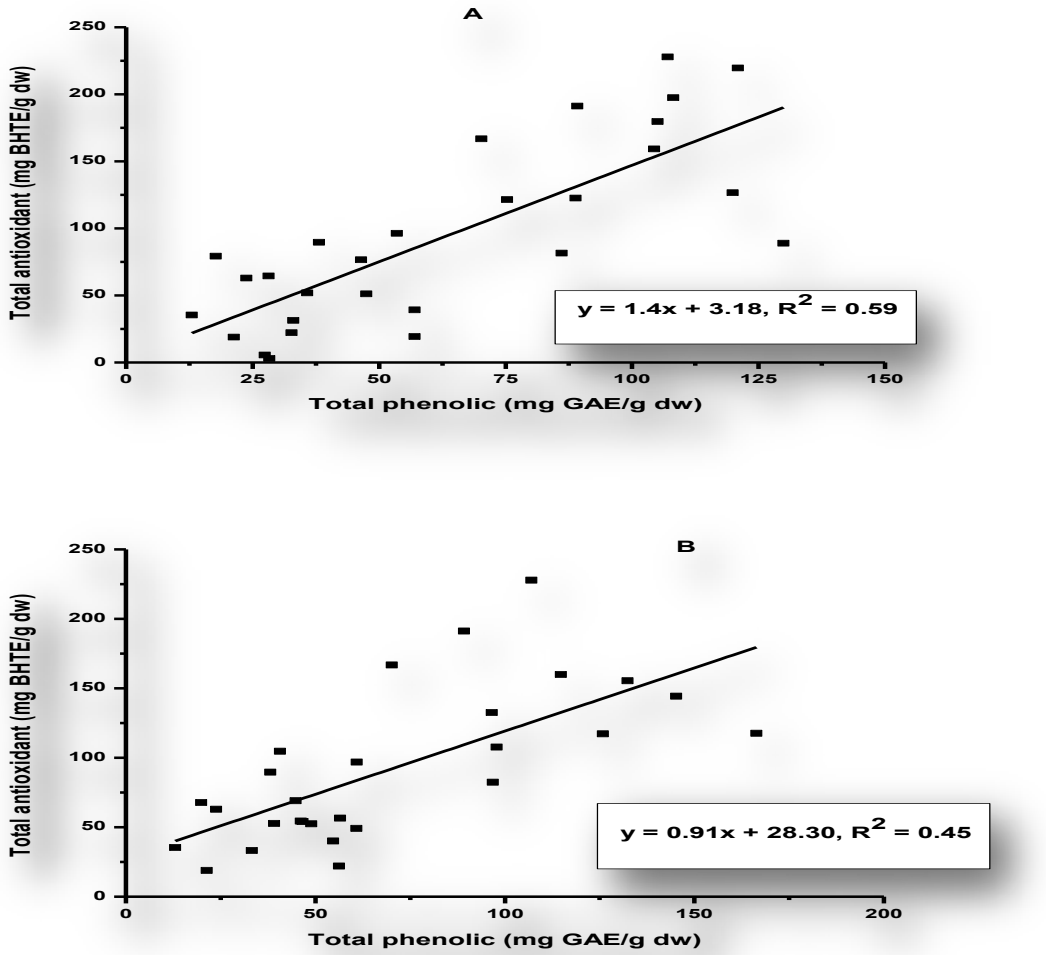


Figure 4.5 Correlation between TPC (mg GAE/g dw) and total antioxidant activity (mg AAE/g) of methanol extracts of *Aframomum corrorima*, *Coriandrum sativum* seed, *Coriandrum sativum* fruit, *Coriandrum sativum* leaf, *Lippia adoensis* var koseret, *Lippia adoensis* var. adoensis, and *Thymus schimperi* heated at different temperatures (A) for 1 h, (B) for 2 h.

Conclusions

The result showed most of the tested activities of spices and herbs increased after heating, suggesting that the bioactive components are relatively stable during thermal heating even

at higher temperatures (100–180 ^{o}C) for different heating periods (1 and 2 h). Thermal treatment caused significant increase of TPC and DPPH scavenging (%) activity but reduced the total antioxidant activity of most of spices and herbs. However, a long heating time and high temperature (180 ^{o}C) reduced the TPC and DPPH scavenging activity (%) of LAK and LAA extracts. Therefore, to maintain the antioxidant profile and activities, the optimum heating time at cooking temperature for such samples is important.

The strong correlations observed between DPPH scavenging activity (%) and TPC indicated phenolic compounds were a major contributor to the antioxidant activities. This finding about phenolics supports that an increase in TPC due to thermal treatment may partially enhance free radial scavenging activity of most of the samples heated at different temperatures for different heating periods. In conclusion, the results illustrated that the health benefits from most of the plant sources remained in the products after thermal process that is, heat do not denatured the antioxidant activities in most of the selected spices and herbs studied even increased the antioxidant activity in some of them. But further studies are required to identify the effect of thermal treatment on the individual phenolic compounds and also effect of thermal treatment on Ethiopian traditional foods that are processed by constituting different spices and herbs. Therefore, spices and herbs are expected to be a valuable food constituent for promoting good health in daily lives of the people in the country.

CHAPTER 5: EVALUATION OF *IN VITRO* ANTICANCER ACTIVITY OF KORARIMA (*Aframomum corrorima* (Braun) P.C.M. Jansen) SEED EXTRACTS AGAINST LIVER CANCER (HepG2) CELLS

Abstract

Aframomum corrorima, locally known as korarima, or the Ethiopian cardamom, is a renowned spice and medicinal crop of the family Zingiberaceae, native to Ethiopia. The spice is obtained from the plant's seeds (usually dried), and is extensively used in Ethiopian cuisine and as herbal medicine. The goal of this study was to investigate the effects of aqueous: methanol (20:80, v/v) and petroleum ether extracts of seeds of *A. corrorima* on the growth of human hepatocellular carcinoma (HepG2) cells. Viability of HepG2 cells were measured by 3-(4,5-dimethylthiazol-2-yl) -5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay after 24 h and 48 h treatment with extracts of 10–500 μg/mL concentrations. There is significant difference of IC_{50} value between petroleum ether (IC_{50} = 105.30 ± 6.92 μg/mL) and aqueous: methanol (20:80, v/v) (IC_{50} = 282.01 ± 43.40 μg/mL) extracts treated for 24 h. The result showed that phytochemicals of seeds of *A. corrorima* showed potential as natural therapeutic for hepatocellular carcinoma. This is the first report to demonstrate *in vitro* anticancer effect of seed extracts of *A. corrorima* in relation to liver cancer.

Keywords: Antioxidant activity**;** antiproliferative effect; cancer; *Aframomum corrorima*; HepG2; phenolic content

5.1. Introduction

Medicinal plants have been in use from time immemorial and their utility has been increasing day by day in the present world. Naturally obtained compounds are considered safer and easily biodegradable than synthetic compounds and the problem of drug resistance observed in synthetic drugs is also reduced (Chanchal *et al*., 2011). Plants represent a source of lead compounds for many pharmaceutical compounds that have been used in treating a number of human ailments.

Cancer is a disease that has always been a major threat and has been characterized by proliferation of abnormal cells. Though chemotherapy is now being used as a standard treatment method (Uma *et al.,* 2009) search for anticancer agents from natural plant products have been getting increased attention (Honghai *et al*., 2009). In recent years, Studies on natural products with anticancer activities has led to the discovery of many compounds, among which plant secondary metabolites include such as terpenes, phenolics and alkaloids (Mumper, 2010; Amine *et al*., 2014). Monoterpenes, sesquiterpenes and phenylpropanoids of essential oil derived from different medicinal plants exhibit antioxidant (El-Ghorab *et al.*, 2008) and anticancer properties (Hou *et al*., 2007).

Phenolics and flavonoids from various plant foods including vegetables, fruits, spices, and grains, as well as phytochemicals of diversified pharmacological efficacies have also shown a significant antioxidant activity and cytotoxicity against a variety of human cancer cell lines (Huang *et al.*, 2005; Jing *et al*., 2010; Mohammad and Randhawa, 2011). Park *et al* (2008) showed that some flavonoid components in green tea are effective in inhibiting cancer or induce mechanisms that may kill cancer cells and inhibit tumor invasion. It was found that flavonoids reduced blood-lipids and glucose, and enhanced human immunity

(Ghasemzadeh and Jaafar, 2011). The effect of flavonoids on human health is the result of their ability to induce human protective enzyme systems (Lafuente *et al*., 2009). Several studies have suggested that flavonoids such as catechin (Arts *et al*., 2002), quercetin (Priyadarsiniet *et al*., 2010; Bishayee *et al*., 2013), and rutin (Savita, 2014) are able to control cancer cell growth in the human body.

A. corrorima is a monocotyledonous flowering plant belonging to the family Zingiberaceae. It is locally known as korarima (Amharic, Oromifa, Tigrigna) or Ethiopian cardamom *and* is known only from Ethiopia where it grows naturally. It is widely cultivated in the south and south western part of Ethiopia. The seed is one of the most widely used spices in Ethiopia to flavor food and beverages. In spite of its high relative price compared with other local spices, korarima is widely consumed which shows the interest and importance that people are attached to it. The seeds (usually dried, sometimes fresh) ground with other spices, are used to flavor all kinds of traditional sauces in the country. They have been used in the preparation of hot red pepper, paste of chili pepper (datta), and to flavor butter (Jansen, 1981). It can also be used to spice coffee, tea and bread.

According to Addis *et al* (2001) traditional medicine still remains the main resource for a large majority of the people living in rural Ethiopia and has a much lower price than modern medicine. According to Abebe (1996), about 80 % of Ethiopians, depend on traditional herbal medicines. Different parts of korarima plant (leaf, seed, and pod) have been used in the traditional medicine by the people of southern Ethiopia. The leaves with or without crushing is used to rub or wrap against animal body swelling and skin wound. A decoction of the rhizomes has been used against parasitic nematodes, in ruminant

animals. The seed is effective against tonic convulsions, carminative, purgative (Jansen, 1981), headache, stomach-ache, skin wounds, and to treat sore throat when taken orally (Eyob *et al.*, 2008).

The seeds of korarima contain different types of essential oil components with a pleasant odor (Jansen, 1981; Abegaz *et al*., 1994). According to Baser and Kürkcüoglu (2001) and Hymete *et al*, (2006), the major components of the essential oil from dried seeds were 1,8-cineole and the sesquiterpene (E)-nerolidol which was most abundant in the dried pods collected from local markets According to Eyob *et al* (2007) on fresh materials, the major constituents of the oil were found to be γ-terpinene in pods and 1,8-cineole in seeds, while sesquiterpenes were the main constituents of the husks, with (*E*)-nerolidol, β-caryophyllene and caryophyllene oxide as the main representative structures of the essential oils. Later on these researchers, Eyob *et al* (2008), reported the essential oil component of leaf and rhizome. The major component of the oil of the leaf was β-caryophyllene. The rhizome oil was dominated by γ-terpinene and β-pinene.

Though, *A. corrorima* is widely used as food flavoring and traditional medicine, in Ethiopia, scarce information is available on antioxidant activity (Eyob *et al.*, 2008). So far, there is no report on anticancer effect of solvent extracts of the dried seed. Therefore, the objective of the present study was to evaluate *in vitro* antiploriferative effect of petroleum ether and aqueous: methanol (20:80), v/v) extracts of the dried seed on hepatocellular carcinoma (HepG2) cell lines.

5.2 Materials and Methods

5.2.1 Chemicals

Cell titer 96 aqueous and one solution cell proliferation (MTS) assay (Promega, Madison, WI, USA) kits and sterile dimethyl sulfoxide (DMSO) (ATCC, Rockville, USA) were purchased for the experiment. Sorafenib was purchased from sigma-Aldrich. The other chemicals and solvents used in this experiment were of analytical reagent grade.

5.2. 2 Extraction of sample

Fresh fruit of *A. corrorima* was collected from 6 km north east of Yirgalem Town Sidama Zone Southern Ethiopia in October, 2011. The fruits were air dried for 20 days and then ground to fine powder using electric grinder (FM100 model, China). The petroleum ether and aqueous: methanol (20:80, v/v) extracts were prepared by dissolving 1 g of the seeds fine powder separately in 10 mL each solvent. Samples were sonicated (model 750D, VWR Intl. Ltd., Montreal, QC, Canada) for 20 min two times at room temperature. After centrifugation (model Durafuge 300, Precision Scientific, Richmond, VA, USA) at 5000 rpm for 10 min, the supernatant was filtered using Whatman number 1 filter paper. The aqueous: methanol (20:80, v/v) and petroleum ether extracts were evaporated to dryness under N_2 and the remaining water from aqueous: methanol (20:80, v/v), dried using freeze drier (model 2085C0000, Kinetics Thermal Systems, Stone Ridge, NY, USA). Dried aqueous: methanol and petroleum ether extracts were dissolved separately in DMSO and diluted accordingly with culture medium for *in vitro* experiments. Samples were stored at −20 ^{o}C until used for the *in vitro* assays.

5.2.3 Cell lines and culture conditions

Human hepatocellular carcinoma cell lines (HepG2) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured as recommended by the ATCC. HepG2 cells were grown in Eagle's modified minimum essential media (EMEM) supplemented with 10% Fetal bovine Serum (FBS; ATCC, Rockville, MD, USA) and 1% penicillin-streptomycin. Cells were maintained at 37 °C in an incubator under 5% CO_2/95% air atmosphere at above 85% relative humidity constantly. The cells were cultured (about 80% of confluents) once a week in T-75 flasks and the media were changed one additional time a week. Experiments were conducted using cells with less than 20 passages. Cells were counted using a haemocytometer (Bright-Line Hemacytometer, Sigma-Aldrich (Mississauga, ON, Canada) and were plated according in 96-well format for 24 h prior to addition of test compounds. All the test samples were solubilized in sterile filtered DMSO (< 0.5% in the culture medium) prior to addition to the culture media. Control cells were also run in parallel and subjected to the same changes in media with < 0.5% DMSO.

5.2.4 Cell proliferation assay

Cell proliferation assay is a homogeneous, colorimetric method for determining the number of viable cells in proliferation, cytotoxicity or chemosensitivity assays. The assay is composed of solutions of a novel tetrazolium compound [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent phenazine methosulfate (PMS). MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium. This reaction (Figure 5.1)

converts the yellow salts to blue-colored formazan crystals that can be dissolved in an organic solvent and the absorbance of the formazan product can be measured at 490 nm.

Cell proliferative effect was determined according to Nair *et al* (2014). HepG2 cells (1×10^4 cells/100 μL/well) were seeded in a sterile flat bottom 96-well plate (BD Biosciences, Mississauga, ON, Canada) and stabilized by incubation for 24 h at 37 °C in a humidified incubator containing 5% CO_2 (VWR, Mississauga, ON, Canada). The aqueous: methanol (20:80, v/v) and petroleum ether extracts of *A. cororrima* seeds or sorafenib (Figure 5.2) were prepared in media and 100 μL of each treatment was added to each well, each treatment in three replications. Thereby, cells were exposed to various concentrations (10, 50, 100, 200, 300 and 500 μg/mL) of each treatment. Controls consist of cells with media containing DMSO (< 0.5%) and blank wells contained media with no cells. After 24 and 48 h of test compound incubation, 20 μL of the MTS reagent in combination with the electron coupling agent phenazine methosulfate was added to the wells and cells were incubated in a humidified CO_2 incubator for 3 h. Absorbance at 490 nm (OD490) was monitored with a plate reader (FLUO star Optima, BMG Labtech, Durham, NC, USA) to obtain the number of viable cells relative to the control population. Percentage of viability in the test compound treated cells are expressed as percentage compared to control (< 0.5% DMSO), using the following formula: % cell viability = [OD490 of treated cells / OD490 of control cells]× 100. Data were expressed as mean values ± SD and obtained from three different experiments against each cell line (n = 3 per plate per time point). Dose-response curves (% of viability vs. concentration) were constructed to obtain 50% inhibitory concentrations (IC_{50}).

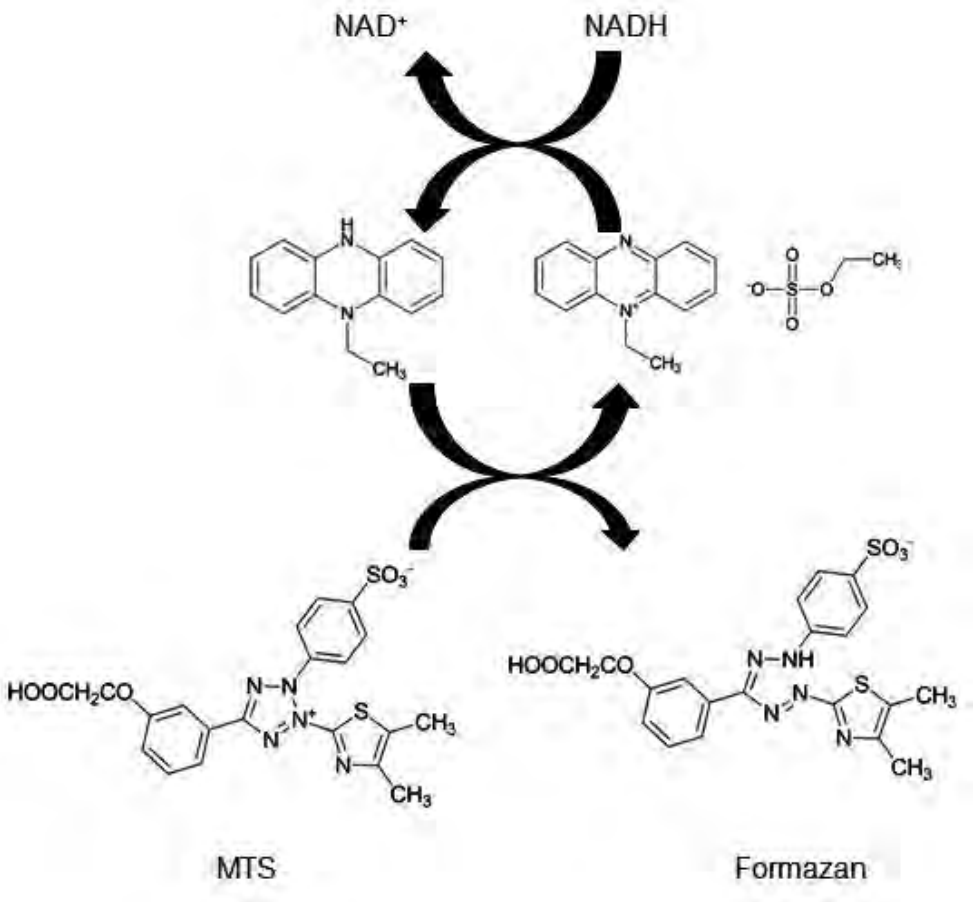


Figure 5.1 Reaction showing the reduction of MTS to Formosan.

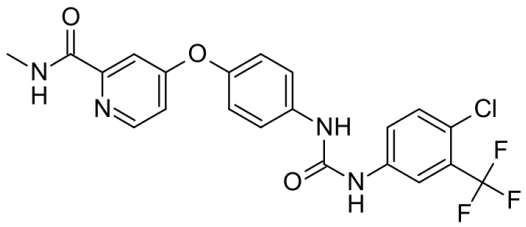


Figure 5.2 Surafenib

5.2.5 Morphological observations under inverted phase contrast microscope

HepG2 cells were equally seeded in 24-well flat bottom tissue culture treated plates (BD Biosciences) and then treated with various concentrations of the extracts, sorafenib, or DMSO (< 0.5%) control. After 24 and 48 h of treatments, the morphology of HepG2 cells

was observed under an inverted phase contrast microscope (Nikon Eclipse E 100, Nikon, ON, Canada) and images were captured at 100x magnification using infinity digital microscopy camera (Lumenera corporation, ON, Canada).

5.2.6 Statistical analyses

The data were subjected to analysis of variance (ANOVA) and Duncan's multiple range tests (SPSS, version 20) were used for mean separation at $p < 0.05$. Linear regression analysis was used to calculate IC_{50} value. All data are presented as mean ± SD.

5.3. Result

5.3.1. Microscopic evaluation of morphological changes in HepG2 cells.

To examine the effect of the petroleum ether and aqueous: methanol (20:80, v/v) extracts on cell morphology, HepG2 cells were treated with 10, 50, 100, 200, 300, and 500 μg/mL of the extract or sorafenib (positive control) for 24 and 48 h and morphological changes were observed by phase contrast microscopy. The images (Figure 5.3) showed that the petroleum ether extract induced severe morphological changes of cell death including rounding and shrinkage of cells in a dose-dependent manner. The pattern of cell death was similar to liver cancer drug sorafenib (positive control) at a concentration of 300 mg/mL and above. The control cells were well adhered, displaying the normal morphology of HepG2 cells. In contrast, majority of HepG2 cells treated with petroleum ether extract at the concentration of 300 μg/mL or higher became round and shrunken than that of aqueous: methanol (20:80, v/v) extract (Figure 5.4) and could not be affixed to the walls and cells were floating in the medium (×100 magnification).

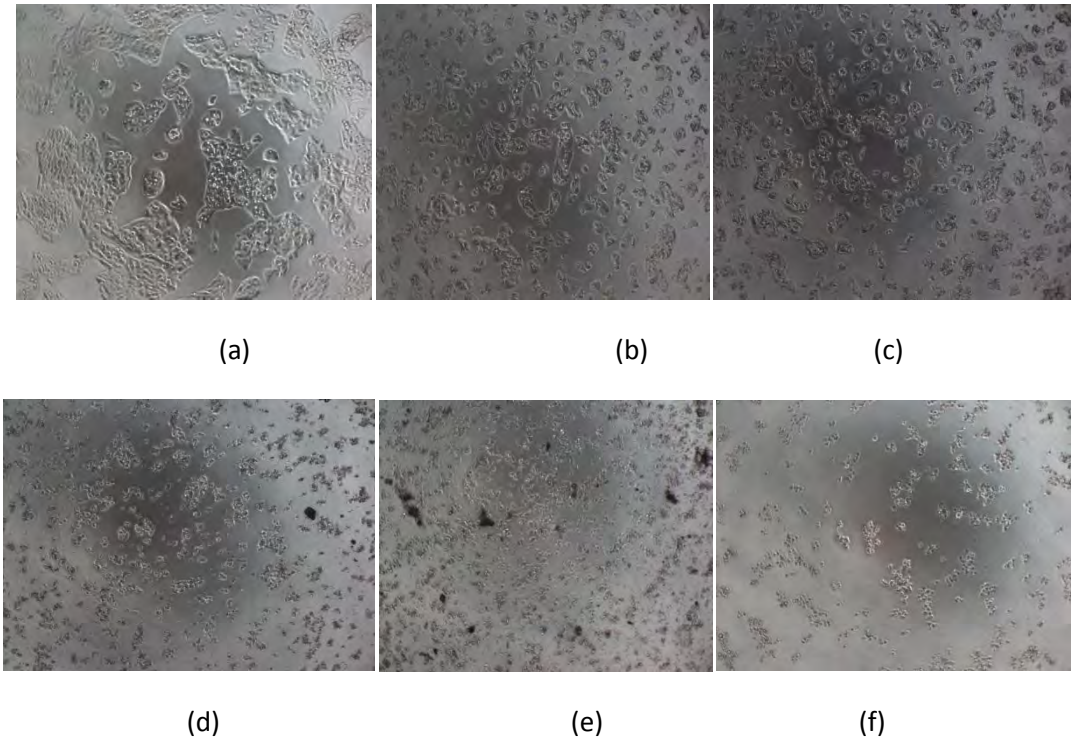


Figure 5.3 Inverted phase contrast microscopy of HepG2 cells change by petroleum ether extract of A. corrorima seed or sorafenib (positive control) exposure. (a) control; (b) 50 μg/mL extract; (c) 100 μg/mL extract; (d) 300 μg/mL extract; (e) 500 μg/mL extract; (f) 100 μg/mL sorafenib

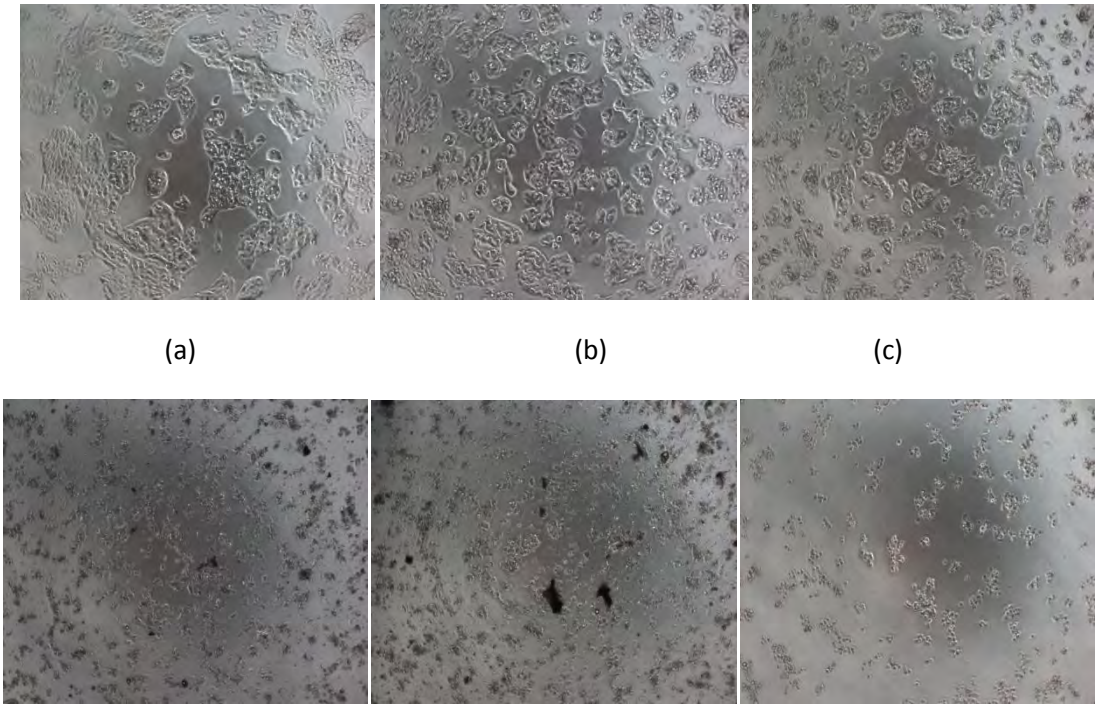


Figure 5.4 Inverted phase contrast microscopy HepG2 cells change by aqueous: methanol (20:80, v/v) extracts of *A. corrorima* seed or sorafenib (positive control) exposure. (a) control; (b) 50 μg/mL extract; (d) 100 μg/mL extract; (c) 300 μg/mL extract; (e) 500 μg/mL extract; (f) 100 μg/mL sorafenib

5.3.2 Inhibition of HepG2 cell proliferation.

HepG2 cells were treated with increasing concentrations of aqueous: methanol (20:80, v/v) extract, petroleum ether extract or sorafenib (10, 50, 100, 200, 300, and, 500 μg/mL),

and cell viability was assayed at 24 and 48 h after treatment. Cell viability decreased in a dose-dependent manner and decreased with increasing concentration of the extract. Strong negative correlation between percentage of cell viability and concentration of the extract was observed (Figure 5.6). Petroleum ether extract inhibited the proliferation of HepG2 cells more than aqueous: methanol (20:80, v/v) extract, with increasing inhibitory activity as the concentrations of extracts increased (Figure 5.5) ($p < 0.05$). After 24 and 48 h, petroleum ether extract showed 10.8 ± 1.9% and 0.5 ± 0.0% cell viability of HepG2 cells, respectively, at a concentration of 300 µg/mL, while at the same concentration aqueous: methanol (80:20, v/v) extract showed 39.8 ± 10.9% and 33.7 ± 15.0% cell viability, respectively. There is a significant difference of IC_{50} value between petroleum ether (IC_{50} = 105.30 ± 6.92 µg/mL) and aqueous: methanol (20:80, v/v) (IC_{50} = 282.01 ± 43.40 µg/mL) extracts treated for 24 h. Also the IC_{50} values of petroleum ether and aqueous: methanol (20:80, v/v) extracts were significantly different ($p < 0.05$) form IC_{50} value of sorafenib (positive control) at any time (Table 5.1). The higher anticancer activity of petroleum ether extract may be because of bioactive monoterpenes. 1,8-cineole is the major component of the essential oils of seed of *A. corrorima* (Eyob *et al*., 2007). Different studies reported the anticancer activity of 1,8-cineole. According to Hayes *et al* (1997), 1,8-cineole showed moderate cytotoxicity in HepG2 lines. Asanova and collaborators (2003) demonstrated that 1,8-cineole had moderate antioxidant and cytotoxic properties and pronounced analgesic and antitumor activity. Later on Cha and collaborators (2010) found that 1,8-cineole induced apoptosis in KB cells via mitochondrial stress and caspase activation. A number of scientific reports indicate that certain terpenoids, steroids and phenolic compounds such as tannins, coumarins and

flavonoids have chemo preventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis (Blois, 2002).

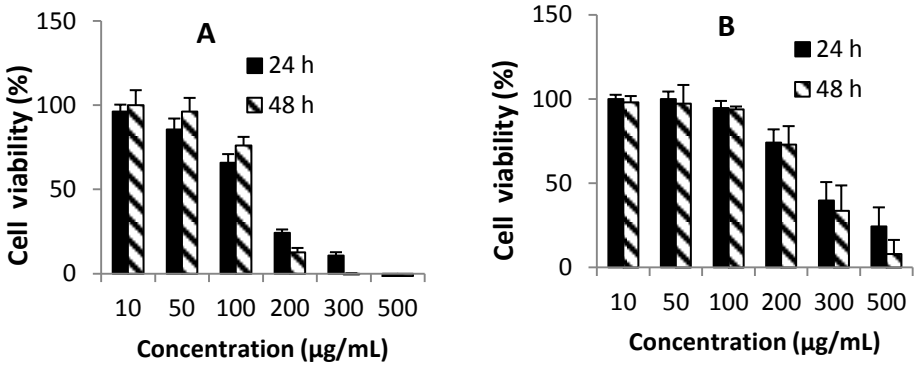


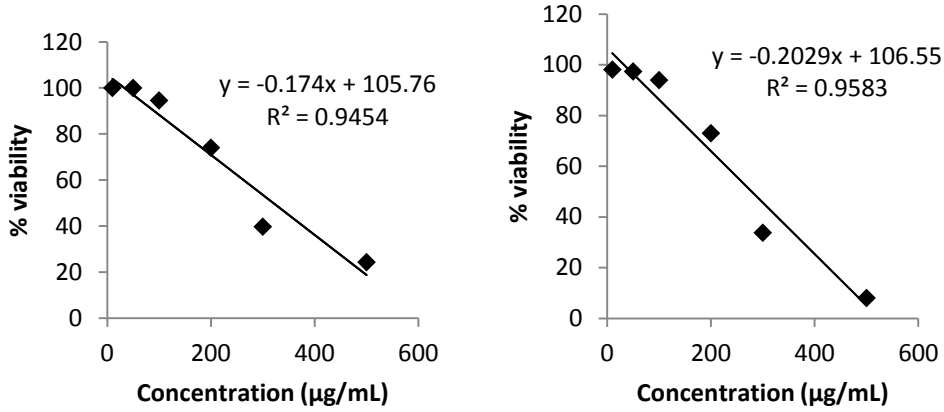
Figure 5.5 Percentage of HepG2 cells viability after exposure to petroleum ether (A) and aqueous: methanol (20:80, v/v) (B) extracts of *A. corrorima* after 24 and 48 h. The percentage of cell viability was measured by MTS assay. Data are presented as mean ± SD of three replicates from three independent experiments.

Table 5.1 IC_{50} (µg/mL) values of petroleum ether, aqueous: methanol (20:80, v/v) extracts of *A. corrorima* and sorafenib (liver cancer drug) on HepG2 cells after 24 and 48 h of exposure.

Test compounds	24 h	48 h
Petroleum ether	105.30 ± 6.92^{b}	110.9 ± 6.31^{b}
Aqueous: methanol (20:80,v/v)	282.01 ± 43.40^{c}	308.3 ± 21.73^{c}
Sorafenib (liver cancer drug)	24.8 ± 1.54^{a}	13.44 ± 2.33^{a}

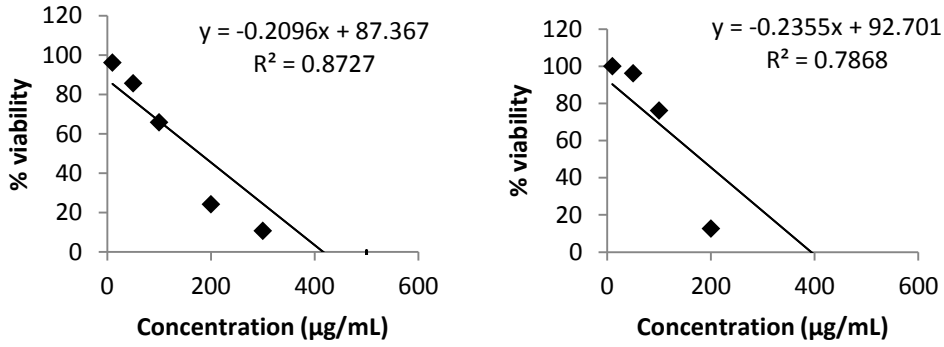
IC_{50} value of cell viability was measured by MTS assay. Data are presented as mean ± SD of three replicates from three independent experiments. Values within a column with different subscript letters are significantly different at $p < 0.05$ compared to Sorafenib (positive control).

(A)



i) 24 h ii) 48 h

(B)



i) 24 h ii) 48 h

Figure 5.6 Relationship between percentage of cell viability and concentration of aqueous: methanol (20:80, v/v) (A) and petroleum ether (B) extracts of *A. corrorima* on HepG2 cells after 24 and 48 h of exposure. The percentage of cell viability was measured by MTS assay. Data were presented as mean ± SD of three replicates from three independent experiments.

Conclusions

The result showed that the *in-vitro* studies performed using the Hepatocellular Carcinoma (HepG2) cell lines reveals that the petroleum ether seed extract of *A. corrorima* has a moderate anticancer activity but the aqueous: methanol (20:80, v/v) extract exhibited weaker anti- cancer activity. Even though there was an increase in the cell growth inhibition, when concentration of sample was increased, the IC_{50} values were more than 100 μg/ml for the cell lines studied as shown by the MTS assay method. In this study, however, the findings did not show any relationship between anticancer activity and phenolic content. Petroleum ether extract, which showed lower phenolics content, exhibited a higher anticancer activity whereas aqueous: methanol (80:20, v/v) extract with higher phenolics content, exhibited lower anticancer activity. The relatively higher anticancer activity of the petroleum ether extract containing low phenolic composition suggests that the nature of phenolic compound is the determinant for these activities rather than their amounts. Further studies toward isolation and identification of key phenolic or other compounds compounds may allow for a potential biomedical application in the therapy of liver cancer diseases. In the future study, the isolated compounds from *A.*

corrorima needs to be further evaluated in experimental animal models and clinical trials to search of lead molecule from natural resources to understand the exact molecular mechanism of action.. This study supports the anticancer property of traditional herbs and spices for their potential use as traditional medicine with minimal side effects. Therefore supplementing a balanced diet with *A. corrorima* seeds may have beneficial effect in treating liver cancer.

CHAPTER 6: EVALUATION OF *IN VITRO* ANTIDIABETIC POTENTIAL OF *Thymus schimperi* R. AND *Thymus vulgaris* L.

Abstract

Diabetes has become the most common metabolic disease worldwide. In particular, type 2 diabetes is the most commonly encountered type of diabetes, which is characterized by impaired insulin secretion and/or action. One of the effective methods to control diabetes is to inhibit the activity of α-amylase and α-glucosidase enzymes which are responsible for the breakdown of starch to more simple sugars using plant products. This study evaluated the total phenolic (TPC), total flavonoid (TFC), and antidiabetic potential of *T. schimperi* and *T. vulgaris* via *in vitro* inhibition of α-amylase and α-glucosidase, using the hot water and aqueous:methanol (20:80, v/v) extracts. The α-amylase inhibitory potentials of the extracts were investigated through reducing sugars analysis using 3, 5-dinitrosalicylic acid color reagent (DNSA) using starch solution as substrate. The α-glucosidase inhibition was determined by pre-incubating α-glucosidase with different concentrations of the extracts followed by the addition of p-nitrophenylglucopyranoside (pNPG). Aqueous: methanol (20:80, v/v) extract of *T. schimperi* contained highest TPC (46.01 ± 4.54 mg GAE/g dw) and TFC (14.72 ± 1.14 mg QE/g dw) showed the stronger α-amylase inhibition activity (IC_{50} = 0.33 ± 0.05 mg/mL) and the hot water extract exhibited the stronger α-glucosidase inhibition (IC_{50} = 0.05 ± 0.01 mg/mL) activities than that of *T. vulgaris*. The TPC and TFC were positively related ($p < 0.05$) to α-amylase inhibition activity but negatively correlated ($p > 0.05$) with α-glucosidase inhibitory activity. These results indicated that

the inhibition of these enzymes can lead to lower postprandial blood glucose. The inhibitions of α-amylase and α-glucosidase by the leaf extracts suggest that *T. schimperi* and *T. vulgaris* may be useful in the management of diabetes mellitus.

Keywords: α-Amylase, Antidiabetic, Diabetes mellitus, α-Glucosidase, Thymus, Total phenolics

6.1. Introduction

Diabetes mellitus is a metabolic disease characterized by hyperglycemia and disturbances in fat and protein metabolism that results from defects in both insulin secretion and/or insulin action (Chan e*t al*., 2010). In particular, type 2 diabetes mellitus is the most encountered form of diabetes, accounting for more than 80% of the total cases of diabetes (Chan *et al*., 2010). Various pharmacological approaches have been used to improve diabetes *via* different modes of action including stimulation of insulin release, inhibition of gluconeogenesis, increasing the number of glucose transporters and reduction of glucose absorption from the intestine (Ahmed *et al*., 2010).

Increasing evidence has shown that prolonged exposure to elevated glucose induces the production of free radicals, particularly reactive oxygen species (ROS), through glucose auto-oxidation and protein glycosylation (Bry *et al*., 2001). Oxidative injury by ROS has been suggested to explain the excess prevalence of vascular complications in diabetes mellitus, which may be mediated by oxidative stress (Peng *et al*., 2011). The non-enzymatic glycation of proteins (Maillard reaction) is a process closely linked to oxidative stress and is associated with increased production of hydrogen peroxide and other highly reactive oxidants that in turn lead to the formation of complex compounds, the advanced

glycation end-products (AGEs), which alter the structure and functions of proteins. The AGEs are involved in the pathogenesis of diabetes, and contribute to several pathophysiologies associated with aging and diabetes mellitus, such as chronic renal insufficiency, Alzheimer's disease, nephropathy, neuropathy, and cataract (Singh *et al*., 2001). Hyperglycemia accelerates the formation of AGEs and the degree of accumulation of AGEs is correlated to the severity of diabetic complications (Ahmed and Thornalley, 2007).

Both synthetic compounds and natural products have been evaluated as inhibitors against the formation of AGEs. However, despite of their inhibitory capacities against the formation of AGEs, many synthetic inhibitors of AGEs formation were withdrawn from clinical trials due to relatively low efficacies and unsatisfactory safety (Manzanaro *et al*., 2006). Moreover, a number of plant derived products have been shown to possess hypoglycemic, as well as antioxidant properties (Rajaram, 2013). Some important phytochemicals such as phenolics (Choudhary *et al*., 2010) and carotenoids (Sun *et al*., 2011) have been reported to possess anti-glycating activity. Thus, the daily consumption of dietary components, mainly from plant sources which have an antioxidant effect, is considered to be of potential benefit for prevention of diabetes and diabetic complications (Yazdanparast *et al*., 2007).

One of the most effective ways of controlling postprandial hyperglycaemia is to suppress starch digestion as it is the main contributor of glucose in the human body from diet. Suppression of starch hydrolysis is conducted through the inhibition of carbohydrate hydrolyzing enzymes such as α-amylase and α-glucosidase in the digestive organs (Mustafa *et al*., 2010). Inhibitors of these enzymes delay carbohydrate digestion and

prolong the overall time for carbohydrate digestion, resulting in a decrease in the rate of glucose absorption (Yang *et al*., 2012). Starch digestion occurs mainly in the gastrointestinal tract, and it involves the combine action of pancreatic α-amylase and intestinal α-glucosidases to release absorbable glucose (Jones *et al*., 2011). Pancreatic α-amylase catalyzes the digestion of complex starches to oligosaccharides, while α-glucosidases such as sucrases, maltases, and isomaltases hydrolyze oligosaccharides, trisaccharides, and disaccharides into monosaccharides. The inhibitory effects on the α-glucosidase and α-amylases enzymes offer ways to control the release of glucose from carbohydrates. The inhibition of α-amylase and/or α-glucosidases would retard starch digestion from our diet and reduce postprandial blood glucose levels (Gerich, 2003). Due to their high affinity for α-glucosidases, the inhibitors block the enzyme from binding to the carbohydrates, resulting in a decreased ability to metabolize the complex sugars to monosaccharides, therefore leads to decreased in the glucose concentration (Mutiu *et al*., 2013).

Modern medicine are the most effective therapeutic approaches for controlling blood-glucose level by suppressing of carbohydrate-hydrolyzing enzymes, such as *α*-amylase and *α*-glucosidase (Ann and Tshinanne, 2013; Kai *et al*., 2013). Acarbose, miglitol, and voglibose are examples of enzyme inhibitors used in the clinical treatments (Figure 6.1).They show different inhibitory effects on α-glucosidases. Acarbose is most effective on glucoamylase, followed by sucrase, maltase, and dextranase. Miglitol is a more potent inhibitor of sucrase and maltase than acarbose. It is also active on isomaltase but shows no effect on α-amylase. Voglibose has a strong α-glucosidase inhibitory effect but little effect on α-amylase. Acarbose inhibits both α-glucosidases and pancreatic α-amylases enzymes.

Voglibose has little effect on α-amylases, while miglitol shows no effect (Goldstein and Wieland, 2008).

However, these synthetic drugs currently in use for diabetes treatment sometimes have side effects (Goldstein and Wieland, 2008), such as liver disorder, flatulence, abdominal fullness, and diarrhea, have been reported. Also, excessive inhibition of pancreatic α-amylase results in the abnormal bacterial fermentation of undigested carbohydrates in the colon. Therefore, natural inhibitors from medicinal plants have been shown to have lower inhibitory effect against α-amylase activity and a stronger inhibitory activity against α-glucosidase and can be used as effective therapy for the postprandial hyperglycemia with minimal side effects (Kwon *et al*., 2008). Therefore, there is an increasing need for the development of naturally occurring, non-toxic, and readily accessible enzyme inhibitors. For this reason, natural hypoglycaemic compounds present in the diets are an attractive alternative to synthetic drugs or as reinforcements for the currently used treatments (Wongsa *et al*., 2012).

There are many plant species being used for the treatment of type 2 diabetes mellitus worldwide (Trojan-Rodrigues *et al*., 2011). In parts of the world where the population has restricted access to the healthcare system, the use of medicinal plants for the treatment of type 2 diabetes mellitus is widespread (Mamun-or-Rashid *et al*., 2014). Suppressing *α*-glucosidase and/or *α*-amylase activity delays the hydrolysis of starch or disaccharides to monosaccharides, resulting in a reduction of the blood sugar level. Therefore, development of effective food-derived enzyme inhibitors would be beneficial for the control and management of type-2 diabetes.

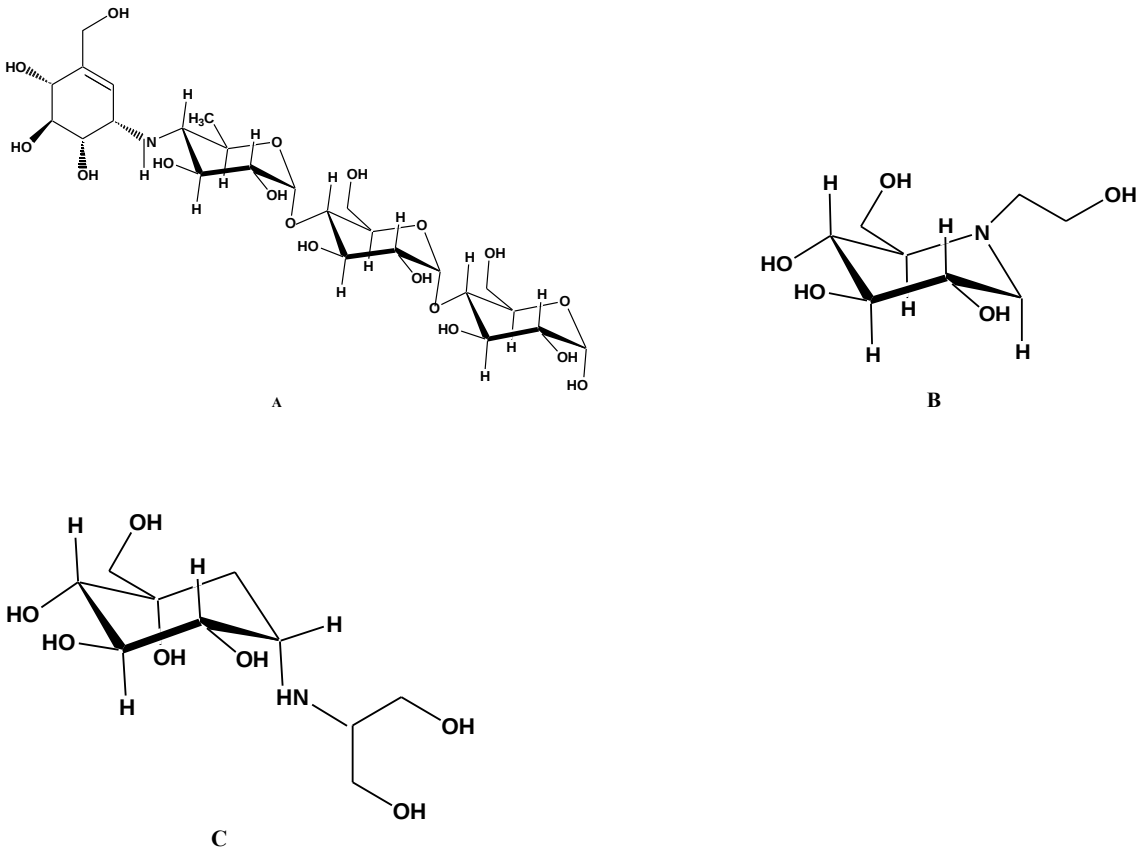


Figure 6.1 Molecular structures of the (a) acarbose, (b) miglitol, and (c) voglibose

Traditional medicinal plants have been used for many years by different cultures around the world for the management of diabetes. In recent years, investigation on herbal medicines has become progressively important in the search for a new, effective and safe therapeutic agent for the treatment of diabetes. Many pure bioactive compounds isolated from plants have been demonstrated to have blood glucose-lowering effect, several of which are flavonoids (Kati *et al*., 2010), triterpenoids (Wenli *et al*., 2009), and alkaloids (Soon *et al*., 2013). Studies indicated that some of the dietary plants possessed inhibitory effect against *α*-glycosidase and or *α*-amylase, such as sorghum, foxtail millet and proso millet, (John *et al*., 2014) guava leaves (Shakeera *et al*., 2013) and eggplant (Esther *et al*.,

2013). In addition, *in vitro* inhibitory activities have been reported for phenolic extracts of foods, including fruits (Jayaprakasam *et al*., 2005; Castañeda-Ovando *et al*., 2009; Misbah, 2013), vegetables (Oboh *et al*., 2012), medicinal herbs (Abdullah and Izabela, 2013), green, black tea (Kati *et al*., 2010), and berries (McDougall *et al*., 2005).

Even nowadays some spices and culinary herbs play an important role in primary health care and in the treatment of diabetes, especially in developing countries (Wongsa, 2012). Natural hypoglycemic compounds may be attractive alternatives to synthetic drugs or reinforcements to currently used treatments. Their huge advantage is that they can be ingested in everyday diet. *T. schimperi* Ronniger is a wild endemic herb to Ethiopia and is traditionally used for food flavoring as well as medicinal ingredient. The dried leaves are used to flavor tea, coffee, food and also boiled as a tea substitute and are believed to be good for diabetic patients (Nigist & Sebsebe, 2009). *T. vulgaris* L. is an important medicinal plant (Golmakani and Rezaei, 2008; Al-Bayati, 2008) which has been used for centuries as spice, home remedy, drug, perfume and insecticide, and also reported to have antidiabetic activity (Rime *et al*., 2014). So far, there is no report on antidiabetic activities of the dried leaf *T. schimperi*. Therefore, the objective of the present study was to compare TPC, TFC and *in vitro* antidiabetic potentials of hot water and aqueous: methanol, 20:80, v/v) extracts of the dried leaves of *T. schimperi* and *T. vulagaris*.

6.2. Materials and Methods

6.2.1. Chemicals

Gallic acid, Folin–Ciocalteu reagent, quercetin, acarbose, 3, 5-dintrosalicylic acid (DNSA), potato starch, phosphate buffer, sodium chloride, sodium carbonate, aluminum chloride, sodium potassium tartarate, α-glucosidase, α-amylase, p-nitrophenyl-α-D-

glucopyranoside were purchased from Sigma-Aldrich. The other chemicals and solvents used in this experiment were of analytical reagent grades.

6.2.2. Sample preparation and extraction

Fresh leaves of *T. schimperi* Ronniger were collected from 85 km north of Addis Ababa, on the road side to Fiche town, North Shoa, Ethiopia and fresh leaf of *Thymus vulgaris* was collected from garden in Dalhousie Agricultural College, Canada. The leaves were air dried for 10 days and then ground to fine powder using electric grinder (FM100 model, China). The hot water and aqueous: methanol (20:80, v/v) extracts were prepared by dissolving 1 g of the leaf fine powder separately in 10 mL each solvent. The hot water extract was heated for 5 min using water bath. The mixtures were then subjected to sonication (model 750D, VWR Intl. Ltd., Montreal, QC, Canada) for 15 min x 3 times, with 10 min intervals in between sonication cycles to keep the temperature below 30 ^{o}C during the extraction. After centrifugation (model Durafuge 300, Precision Scientific, Richmond, VA, USA) at 5000 rpm for 10 min, the supernatant was filtered using Whatman number 1 filter paper. The methanol was evaporated from aqueous: methanol (20:80, v/v) extract under N_2 and the remaining water (in aqueous: methanol (20:80, v/v extract) and hot water extract were freeze dried for 10 h using freeze drier (model 2085C0000, Kinetics Thermal Systems, Stone Ridge, NY, USA). Samples of each treatment were extracted and analyzed in triplicate and immediately stored in amber vials at –20 ^{o}C until used for analysis.

6.2.3. Determination of total phenolic content (TPC)

The TPC was estimated by Folin-Ciocalteu method as described in Shan *et al.* (2005) with slight modification using gallic acid as the standard. To 0.1 mL of the extract, 1 mL Folin-

Ciocalteu reagent (diluted ten times) was added and the mixture was left for 5 min and then 1 mL (75 g/L) of sodium carbonate was added. The absorbance of the resulting blue color was measured at 765 nm with a UV-Visible spectrophotometer (JENWAY, 96500, UK) after incubation for 90 min at room temperature. The TPC was estimated from gallic acid (1–100 µg/mL) calibration curve ($y = 0.015x + 0.09$, $R^2 = 0.99$) and the results were expressed as milligram gallic acid equivalent/gram of dried plant material (mg GAE/g dw).

6.2.4. Determination of total flavonoid content (TFC)

The TFC was determined as described in Ayoola *et al* (2008) with minor modifications. The analysis was based on the formation of yellow color of flavonoid-aluminum complex. Aluminum chloride (2 mL, 2%) was mixed with the same volume of the leaf extract (1 mg/mL). Individual blanks were prepared consisting of 2 mL of sample solution and 2 mL of methanol without aluminum chloride. Then absorbance readings at 415 nm were taken after 1 h of incubation at room temperature against a blank sample. The TFC was determined using a standard curve ($y = 0.24x + 0.11$, $R^2 = 0.98$) of quercetin (1- 40 µg/mL) and values were calculated as milligram quercetin equivalents/gram of dried plant material (mg QE/g dw).

6.2.5. Porcine pancreatic α-amylase inhibition assay (DNSA method)

The DNSA assay for reducing sugar was conducted using various crude extracts of the leaves and starch as a substrate for amylase enzyme as described in Kwon *et al* (2008) with minor modification. In the DNSA assay reducing sugars released by the action of enzyme will be oxidized whereas 3, 5-dinitrosalicylic acid is reduced to 3-amino-5-

nitrosalicylic acid under alkaline conditions (Figure 6.2). Test samples 200 µL (0.01- 2.5 mg/mL) in a 0.02 M sodium phosphate buffer solution (pH 6.9 with 0.006 M sodium chloride) containing 200 µL Porcine pancreatic α-amylase were incubated at 25 ^{o}C for 10 min, after which, 200 µL of 1% boiled potato starch solution in 0.02 M sodium phosphate buffer solution (pH 6.9 with 0.006 M sodium chloride) was added. After incubation of the reaction mixture at 25 °C for 10 min, the reaction was stopped by adding 400 µL of DNSA reagent (1.0 g of 3, 5- dinitrosalicyclic acid, 20 mL of 2 M NaOH and 30 g of sodium potassium tartarate in 100 mL distilled water). The sample test tubes were then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted by adding 4 mL distilled water and absorbance of 200 µL of brown solution of 3-amino-5-nitrosalicylic acid was measured at 540 nm using micro plate reader (FLUO star Optima, BMG Labtech, Durham, NC, USA). Distilled water (without amylase inhibitor) (200 µL) was used as a control.

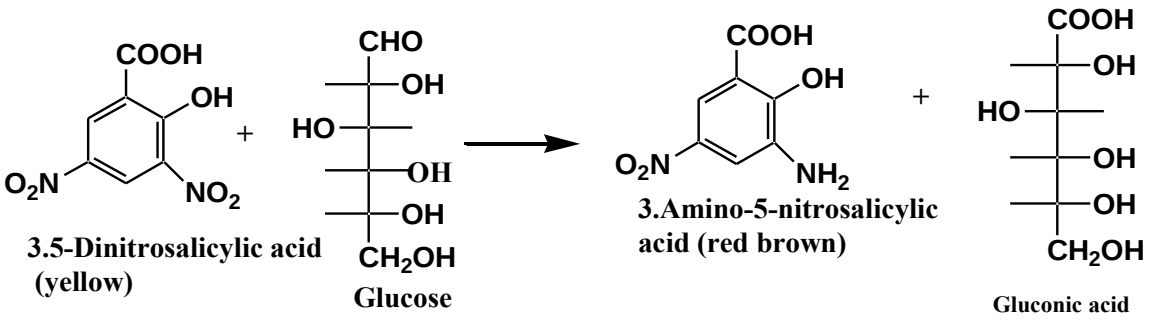


Figure 6.2 Reaction of 3, 5- Dintrosalicylic acid with reducing sugar

To remove matrix sugar interference, the absorbance of the mixture consisted of 200 µL of sample (may contain reducing sugars), 200 µL of phosphate buffer (no amylase), 200 µL of starch, 400 µL 3, 5- dinitrosalicyclic acid, and 4 mL of distilled water was recorded at

540 nm as blank. Acarbose was used as reference. The *α*-amylase inhibitory activity was expressed as % inhibition and was calculated as shown below:

$$\% \text{ inhibition} = [A_{control} - (A_{sample} - A_{blank})/A_{control}]x100$$

6.2.6 α-Glucosidase inhibition assay

α-Glucosidase inhibitory activities were evaluated according to the chromogenic method described by Ivan *et al* (2012), with some modifications. The enzyme solution contained 20 µL α-glucosidase (0.5 unit/mL), 20 µL of sample (at various concentrations) or drug (acarbose) and 60 µL 0.01 M phosphate buffer (pH 6.9). The mixture was incubated at 37 ^{o}C for 15 min. After 15 min, 20 µL of p-nitrophenyl-α-D-glucopyranoside (pNP-G) (5 mM) in the same buffer (pH 6.9) was used as a substrate solution and again incubated at 37 ^{o}C for 15 min. The reaction (Figure 6.3) was terminated by adding 80 µL of 0.2 M sodium carbonate solution. Each experiment was conducted in triplicate. The change in the absorption observed at 405 nm due to the hydrolysis of p-nitrophenyl-α-D-glucopyranoside (pNP-G) was monitored in a 96-well plate with micro plate reader (FLUO star Optima, BMG Labtech, Durham, NC, USA). Increase in absorption at 405 nm was due to enzyme activity as the enzyme hydrolyzes the pNP-G to release p-nitrophenolate ion. The temperature was maintained at 37 ^{o}C during the experiment.The positive control sample was the mixture of the enzyme (20 µL) and substrate (20 µL) without inhibitors. Instead 20 µL of working buffer was added. The sample controls and blanks were the mixtures of sample and control, respectively, except α-glucosidase which was replaced instead with buffer. The IC_{50} values of samples were calculated and reported

as the mean ± SD of the three experiments. The enzyme inhibitory rates of samples were calculated as follows:

$$\text{Inhibition\%} = [(A_S - A_{SB})/A_C - A_{CB})]x\ 100$$

Where, A_S, A_{SB}, A_C, A_{CB} are the absorbance of sample, sample blank, control, and control blank, respectively.

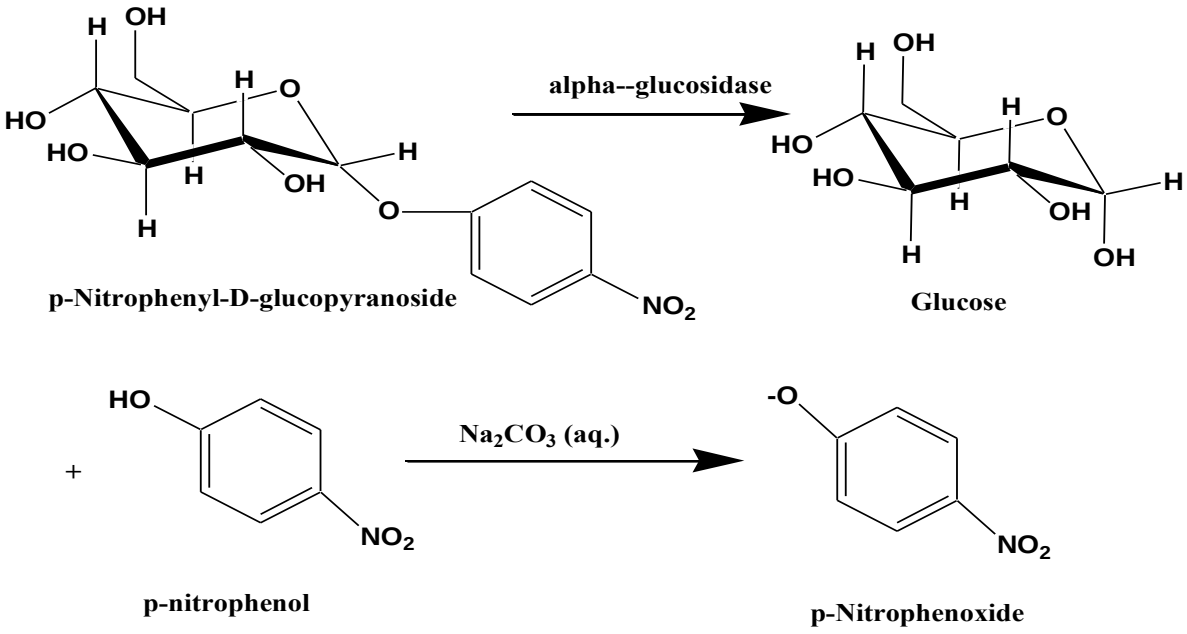


Figure 6.3 α-glucosidase catalyzed hydrolysis of p-nitrophenyl-D-α-glucopyranoside

6.3 Results

6.3.1 Total phenolic and flavonoid contents

The TPC in various solvent extracts from the leave of *T. schimperi and T. vulgaris* varied widely, ranging from 15.65 ± 4.01 to 46.0 ± 4.5 mg GAE/g dw (Table 6.1). The TPC content followed the order: aqueous: methanol (20:80, v/v) extract of *T. schimperi* > aqueous: methanol (20:80, v/v) extract of *T. vulgaris* > hot water extract of *T. schimperi* >

hot water extract of *T. vulgaris*. There was no significant difference ($p > 0.05$) in TPC between aqueous: methanol (20:80, v/v) extracts of *T. schimperi* and *T. vulgaris* but these values were significantly higher ($p < 0.05$) than the TPC of hot water extracts of *T. shimperi* and *T. vulgaris*.

Table 6.1 Total phenolic (mg GAE/ g dw) and total flavonoid (mg QE/g dw) contents of *T. schimperi and T. vulgaris*

Extract	Total phenolic (mg GAE/g dw) ± SD	Total flavonoid (mg QE/g dw) ± SD
TS (aqueous: methanol:20:80, v/v)	46.01 ± 4.54^{c}	14.72 ± 1.14^{c}
TS (hot water)	21.55 ± 3.80^{b}	3.69 ± 1.42^{a}
TV (aqueous: methanol:20:80, v/v)	45.23 ± 13.02^{c}	10.65 ± 2.15^{b}
TV (hot water)	15.65 ± 4.01^{a}	1.13 ± 0.20^{a}

The TFC (mg QE/g dw) varied from 1.13 ± 0.2 to 14.7 ± 1.1 and decreased in the order of aqueous: methanol (20:80, v/v) extract of *T. schimperi* > aqueous: methanol (20:80, v/v) extract of *T. vulgaris* > hot water extract of *T. schimperi* > hot water of *T. vulgaris* (Table 6.1). TFC in aqueous: methanol (20:80, v/v) extracts of *T. schimperi* and *T. vulgaris* were significantly different ($p < 0.05$), but in the hot water extracts were not significantly different ($p > 0.05$).

6.3.2. Porcine *α*-amylase inhibitory activity (DNSA method)

One of the effective methods to control diabetes is to inhibit the activity of α - amylase enzyme which is responsible for the breakdown of starch to more simple sugars

(Probhakar and Doble, 2011). This is contributed by α- amylase inhibitors, which delays the glucose absorption rate thereby maintaining the serum blood glucose in hyperglycemic individuals (Cazzola *et al*., 2011; Wadkar *et al*., 2008). Different studies have shown that phenolic compounds play a role in mediating α-amylase inhibition and therefore have potential to contribute to the management of type 2 diabetes (Cheplick *et al*., 2010; Ranilla *et al*., 2010).

The extracts from the crude *T. schimperi* and *T. vulgaris* leaf extracts screened for *in vitro* α-amylase enzymes inhibitory activity. The results were shown in Figure 6.4. The α-amylase enzymes inhibitory activity was concentration dependent. At 2.5 mg/mL, the porcine *α*-amylase inhibitory activity of aqueous: methanol (20:80, v/v) extract from *T. shimperi* was 68.60 ± 5.91%, and the inhibitory activity of its boiling water extract was 48.73 ± 7.17%. The inhibitory activity of aqueous: methanol (20:80, v/v) extract from *T. vulagaris* was 60.74 ± 9.20%, and the inhibitory activity of its boiling water extract reached 27.11 ± 3.90%. Aqueous: methanol (20:80, v/v) extracts of *T. shimperi* demonstrated stronger percentage of α-amylase enzyme inhibitory activities than that of *T. vulgaris* extracts. These values are lower than citronella grass, lemongrass oils (Jumepaeng *et al*., 2013) and finger millet (Shobana *et al*., 2009), but higher than cereal grains such as wheat, buckwheat, corn and oats (Randhir *et al*., 2008) and foxtail millet (Kim *et al*., 2011). As positive control, at the concentration of 2.5 mg/mL, acarbose showed the highest α-amylase inhibition activity (98.92 ± 8.86%).

The inhibitory activity was determined as the mean of triplicate measurements and expressed as the 50% inhibitory concentrations (IC_{50}) values (Table 6.2). The aqueous: methanol (20:80, v/v) extract of *T. schimperi* demonstrated stronger percentage of α-

amylase enzyme inhibitory activity than the rest of the extracts. As positive control, acarbose showed the highest α-amylase inhibition activity, five times stronger than the inhibition potential of aqueous: methanol (20: 80, v/v) extract of *T. schimperi* .Whereas, hot extract of *T. vulgaris* showed the lowest α-amylase inhibition activity (IC_{50} > 2.5 mg/ml). There were significant differences ($p < 0.05$) in the IC_{50} values among the extracts. But α-amylase enzyme inhibitory activity of these extract were significantly lower ($p < 0.05$) than the α-amylase enzyme inhibitory activity of acarbose.

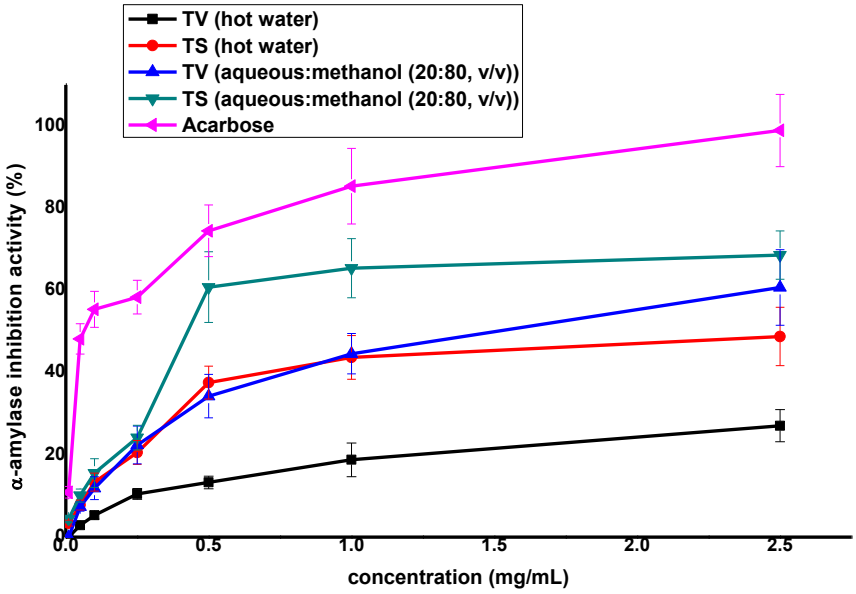


Figure 6.4 The porcine *α*-amylase inhibitory activities of aqueous: methanol (20: 80, v/v) and boiling water extracts of *T. schimperi* and *T. vulgaris*. Results were expressed as mean ± SD (n = 3).

6.3.3 In *vitro* α- glycosidase inhibition activity

The extracts were also tested through the *α*-glucosidase inhibitory assay and the results were shown in Figure 6.5. At the concentration of 2.5 mg/mL, the hot water extract of *T.*

schimperi showed the highest a-glucosidase inhibition activity (96.80 ± 10.55%) followed by hot water extract of *T. vulgaris* (86.78 ± 8.32%), aqueous: methanol (20:80, v/v) extract of *T. schimperi* (84.46 ± 8.50%), aqueous: methanol (20:80, v/v) extract of *T. vulgaris* (60.70 ± 9.22%) and acarbose (65.46 ± 5.81%).

The IC_{50} values were shown in Table 6.2. Hot water extract of *T. schimperi* showed strongest α-glucosidase inhibition activity, ten times stronger than that of aqueous: methanol (20:80, v/v) extract of *T. vulgaris a*nd more than four times stronger than the α-glucosidase inhibition activity of hot water extract of *T. vulgaris* and more than thirteen times stronger than that of α-glucosidase inhibition activity and acarbose. There was no significant difference in IC_{50} values ($p > 0.05$) between aqueous: methanol (20:80, v/v) extract of *T. schimperi* and acarbose in their α-glucosidase inhibitory effects. But these values were significantly different from that of hot water extracts and aqueous: methanol (20:80, v/v) extract of *T. vulgaris*. Similar result was reported by Toshiyuki and Miyazawa (2012) on safflower (*Carthamus tinctorius* L). According to this study the different extracts exhibited stronger α-glucosidase inhibition activity than acarbose. Similarly ethyl acetate fraction of *Thymelaea hirsute* (Abid *et al*., 2014) showed stronger α-glucosidase inhibition potential than that of acarbose. The hot water extracts of *T. schimperi* and *T. vulgaris* as natural sources thus can be potentially used to suppress glycemic load by reducing α-glucosidase activity.

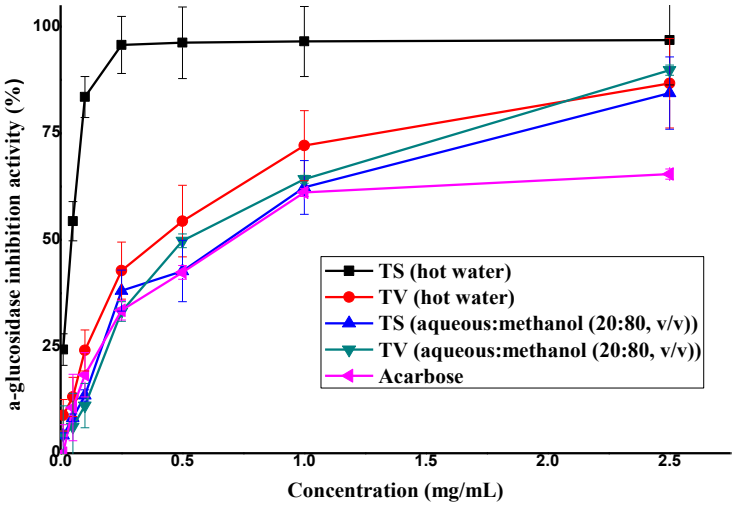


Figure 6.5 *α*-glucosidase inhibitory activity of aqueous; methanol (20:80, v/v) and boiling water extracts of *T. schimperi* and *T. vulgaris*. Results were expressed as mean ± SD (*n* = 3).

Table 6.2 IC_{50} (mg/mL) of α–amylase and α-glucosidase inhibition activity of *T. schimperi* and *T. vulgaris* leaf extracts**.**

Extract	α–amylase	*α*–glucosidase
TS (aqueous: methanol:20:80, v/v)	0.33 ± 0.05^{b}	0.69 ± 0.04^{d}
TS (hot water)	2.24 ± 0.53^{d}	0.05 ± 0.01^{a}
TV (aqueous: methanol:20:80, v/v)	1.56 ± 0.09^{c}	0.51 ± 0.02^{c}
TV (hot water)	> 2.50	0.24 ± 0.09^{b}
Acarbose	0.07 ± 0.01^{a}	0.71 ± 0.12^{d}

TS: *Thymus schimperi*; TV: *Thymus vulgaris*

6.3.4 Correlation between phenolic contents and *in vitro* antidiabetic activity

The analyses of linear correlation between enzymes' inhibitory activities and TPC and TFC showed that the inhibitory effects of samples against the activity of *α*-amylase could be due to the levels of phenolic compounds existing in the extracts. The correlation coefficients +0.78, +0.67, at $p < 0.05$, respectively. These results suggest that higher phenolic content does confer higher *α*-amylase inhibitory activity. Inhibitory activities of the extracts against *α*-glucosidase were negatively proportional to both TPC and TFC, and the correlation coefficients were $R^2 = -0.22, -0.24$, at $p > 0.05$, respectively.

Several studies have found a direct correlation between the amount of phenolic compounds in plant extracts and their capacity to inhibit α-enzymes (Patrick *et al*., 2005; Chen & Kang, 2014). However, not always plant extracts with the high phenolic content have been demonstrated to exert the inhibitory activity on α-amylase (Hairong and Baojun, 2014), which points out the importance of the nature of the different molecules and the interactions among them. Furthermore, different studies confirmed the negative correlation between phenolic contents and α-glucosidase inhibition activity. According to the result reported by Jeonga *et al* (2013), α-glucosidase inhibition activity of *Rehmannia glutinosa* tuberous root extracts was negatively correlated with TPC. To the contrary the study conducted by Hairong and Baojun (2014) on onion, reported that TPC values of samples were positively correlated to α-glucosidase and negatively correlated with *α*-amylase inhibitory activities. Silva Pinto *et al*, (2009), have also shown a positive correlation between α-glucosidase activity and TPC of *Gingko bilibo* L. leaves extracts.

Conclusions

Many of the traditional popular folk remedies have been used for the treatment of diabetes with limited information on the mechanistic basis known of their functionality. The present investigation showed that the *in vitro* antidiabetic property of *T. schimperi* and *T. vulagaris* was related with their *α*-glucosidase and α-amylase inhibitory effects. Hot water extract of *T. schimperi* had stronger α-glucosidase inhibitor and aqueous: methanol (20:80, v/v) extract showed stronger α-amylase inhibitory activity in comparison with *T. vulgaris*. These results also indicated that there was positive linear correlation between TPC and α-amylase inhibition activity and negative correlation with *α*-glucosidase inhibitory effects. However, phenolic compounds may not be the only class of active compounds to contribute to antidiabetic effects of these two herbs. The IC_{50} value of *T. schimperi* of hot water extract was much lower than that of the standard drug acarbose and thus this extract might help in the identification of new compounds for natural α-glucosidase inhibitors. However isolation and characterization of the active compounds associated with α-amylase and α-glucosidase inhibition have to be carried out to confirm these observations. It can be therefore concluded from this study that the presence of the phytochemicals in these plants might be the reason for these inhibitions and that the plants may essentially contain herbal bioactive compounds which require further structural elucidation and characterization to identify the specific bioactive constituents. Further *in vivo* and clinical investigations should be done for confirming the antidiabetic activity of these plants. The plant extracts understudy can serve as therapeutic agents and can be used as potential sources of novel bioactive compounds for treating diabetes mellitus type 2. This is the first

report on the α-amylase and α- glucosidase inhibition effect in support to ethno medicinal use of leaf extract of *T. schimperi* for treating diabetics.

CHAPTER 7: GENERAL DISCUSSIONS, CONCLUSIONS, AND RECOMMENDATIONS

7.1 General Discussions

Many spices and herbs have components that act as antioxidants and protect cells from free radicals (Amarowicza *et al*., 2004). In the present study traditional Ethiopian spices (*Aframomum corrorima, Coriandrum sativum,* seed, *Coriandrum sativum*, fruit) and herbs (*Coriandrum sativum*, *Lippia adoensis* var. koseret, *Lippia adoensis* var. adoensis, and *Thymus schimperi)* which are commonly used as food flavoring and herbal medicines in Ethiopia were investigated for their phenolic contents, various antioxidant and biological activities after extraction with various solvents. Also the effects of thermal treatments on the phenolic contents and antioxidant potentials were investigated.

The TPC was determined using Folin-Ciocalteu method. This method was chosen because it is a simple and reproducible assay which is widely used for studying phenolic antioxidant contents (Pelozo *et al*., 2008). The FTC assay is based on the reaction between flavonoids and aluminum ions to form a colored flavonoid-aluminum complex that can be monitored spectrophtometrically (Pękal and Pyrzynska, 2014). Aluminum ions react with the C4 keto group and either the C3 or C5 hydroxyl group of flavones and flavonols to form acid stable complexes and also with the hydroxyl groups in the A- and B- rings of flavonoids to form an acid labile complex (Blainski *et al*., 2013). Therefore, the assay may underestimate the TFC of the extracts. The TPC and TFC of the extracts found from the herbs were significantly higher than that of the equivalent extracts from spices. The findings of this study suggested that polar solvent extracts from the herbs were better raw

material sources for the extraction of phenolic compounds. Acetone, methanol, and aqueous: methanol (20:80, v/v) extracts of LAK, LAA, and TS were recognized as the richest sources of TPC. The methanol extract of LAK exhibited the highest source for TFC. Several studies showed that phenolic contents determined differed with solvent polarities. For instance, absolute methanol was used for the extraction of tea products (Zhao *et al*., 2004) which were found to be more effective than the water extracts. In addition, Trabelis *et al* (2009) reported that water and organic solvents used individually or in mixture such as water/methanol and water/acetone affected significantly the TPC of the extracts. On the other hand, the stability of different extracts from the same plant material may vary widely with respect to their antioxidant activities (Monica *et al*., 2011).

The analysis of individual phenolic contents of the herbs and spices studied showed that the presence of phenolic acids, flavonols, flavanols, dihydrochalcones, and aliphatic organic acids. The phenolic compounds were analyzed with HPLC coupled to mass spectrometry. The findings of this study showed that the method used to characterize and quantify the phenolic compounds in the extracts was able to identify wide range of compounds. Twenty phenolic compounds from four phenolic families (i.e. flavanols, phenolic acids, flavonols, and dihydrochalcones) and two aliphatic organic acids have been characterized and quantified in the selected spices and herbs by using HPLC-MS. The TS sample gave the highest levels of total flavonols and phenolic acids. Whereas, CSS represented the highest amount of total flavanols. Flavanols and dihydrochalcones were found in low amounts in all extracts. With regard to the individual phenolic compounds, the highest level of ferulic acid, cafeic acid, hydroxycinnamic acid, Q3-argluc, Q3-Galac, and Q3-Rut were found in TS. Whereas, 3-hydroxybenzoic acid and

synergic acid were the most abundant phenolic acids in LAK. The highest amount of quercetin was found in CSS. The CSF had the highest amount of fumaric acid and the CSL contained the highest amount of succinic acid.

So far, there is no information on the phenolic compositions of these dietary spices and herbs studied from Ethiopia. Although there are reports on phenolic contents of coriander leaves, fruits, and seeds from other countries, since different methodologies of extraction and detection were used, direct comparison of this work with such work is difficult. *C. sativum* leaves from India (Nambiar *et al*., 2010) seemed to be rich sources in quercetin, and the *C. sativum* sample from Portugal (Lillian *et al*., 2012) presented Q3-rut as the major compound in the leaf and in the fruit high C-glycosidated apigenin and anthocyains were reported. In the present work, chlorogenic acid was the highest in CSF and CSL, syringic acid was the highest in the CSS. Q3-rut was also the major compound found in the leaf part. But low amounts of flavanols and dihydrochalcones were found in leaf, fruit, and seeds. Similarly, high amount of phenolic acid were reported as majority compounds in leaf extracts of Brazilian samples (Meloa *et al*., 2005). The analysis of methanol extract of the seed from Indian local market (Rajeshwari Andallu, 2012) revealed that the presence of caffeic acid, chlorogenic acid, quercitin and rutin while rutin being predominant in the extract followed by chlorogenic acid, caffeic acid and quercetin. Other study showed that environmental factors also influence the variations of phenolic compounds between the same species (Kamel *et al*., 2013).

Phenolic compounds have an important role in the stabilizing lipid oxidation and are associated with antioxidant activity (Sakihama *et al*., 2002). They exhibit inhibitory

effects on antidiabetic and anti-carcinogenesis in humans, when ingested daily from a diet rich sources in phenolics (Yong-Seo *et al*., 2009). The findings of this study demonstrated that antioxidant activities of the extracts from the dietary herbs as measured by DPPH, ferric reducing power and phosphomolybdenum assays were higher than those of equivalent extracts from the spices. This suggests that the polar extracts from the herbs may provide higher protective effects against oxidative radical damage. The higher the antioxidant activity of the extracts from the herbs could be attributed to the higher concentration of total phenolic content compared to equivalent extracts from the spices. The number and the configuration of H-donating hydroxyl groups are both important structural features influencing the antioxidant capacity of the phenolic compounds (Amić *et al*., 2003). Among the phenolic compounds, phenolic acids appeared to have contributed significantly to the antioxidant activity of the extracts possibly because of their higher concentration. The Flavonoids were also potent antioxidants due to the presence of free hydroxyl groups. Flavonols (quercetin and its derivatives) were found to be the main flavonoids in the extracts. The flavanols may have contributed significantly to the antioxidant activity of the extracts but they were found to occur at low concentrations. According to Rice-Evans *et al* (1996), the antioxidant activity of flavanols is more dependent on the number of hydroxyl groups on their structure as there is no electron delocalization between A and B rings due to the saturation of the heterocyclic ring.

The IC_{50} values of the extracts were studied by varying the concentration of the extracts reacted with DPPH solution at room temperature and measuring absorbance at 520 nm at various concentrations to calculate the percent scavenging of DPPH radical. Then the IC_{50} of each sample was obtained by plotting scavenging of DPPH at steady state (30 min) of

the reaction against the corresponding concentration. The findings of this study demonstrated that the strong scavenging capacity of methanol, acetone, and aqueous: methanol (20:80, v/v) extracts on DPPH might possibly due to the phenolic compounds which could act as a hydrogen donor antioxidant. The antioxidant potential of phenolic compounds has been correlated to the capacity of donating hydrogen radicals. The petroleum ether extract showed the lowest level of activity while the water extracts revealed moderate activity. Interestingly, methanol and aqueous: methanol (20:80, v/v) extracts of LAK, LAA, and TS showed relatively higher antioxidant activities similar to the antioxidant activity of BHT and ascorbic acid. These findings imply that these herbs are the most important in radical scavenging antioxidant activities.

The ferric reducing method is based on the reduction of the ferricyanide complex to the intensely blue colored ferrocyanide complex by the antioxidants in the acidic medium. An increase in the absorbance at 700 nm indicates a high reducing power and the results in this work was estimated from ascorbic acid calibration curve and expressed as milligram of ascorbic acid equivalent per gram of dried exttact (mg AAE/g). The phosphomolybdenum method involves the reduction of colorless Mo (VI) to Mo (V) by the antioxidant extract and the subsequent formation of green phosphate/Mo (V) complex at acidic pH and its absorbance measurement at 695 nm. The phosphomolybdenum total antioxidant capacity was expressed as milligram of butylatedhydroxyltoluene equivalent/gram of dried extract (mg BHTE/g). Similar to the DPPH scavenging activity, variations in the antioxidant capacity of the different extracts may be attributed to differences in their phenolic contents. The reducing power indicates that the antioxidant compounds are electron donors so that they can act as primary antioxidants (Yee and Lim,

2011). Under this study, acetone, methanol, and aqueous: methanol, 20:80, v/v) extracts of LAA, LAK, and TS showed the highest ferric reducing power. Similarly, aqueous: methanol, (20:80, v/v) extracts from the LAK and LAA showed the highest total antioxidant activity. Whereas the spices exhibited lower ferric reducing power and total antioxidant activities. Herbs containing high phenolic contents have shown also strong reducing power. According to Jayaprakasha *et al* (2004), the reducing power of different extracts depends on the presence of phenolic contents which may act as reductones. Flavonoids such as Q3-arab which was found in high contents in the TS extract could be used as natural antioxidants and might substitute synthetic antioxidants that produce many undesirable secondary effects (Yanishlieva *et al*., 2006).

For most of the spices and herbs investigated in this study, the TPC and DPPH scavenging activity were found to be enhanced by increasing the temperature and prolonging the heating time as compared to that of thermally not treated samples. The result was consistent with the findings of other studies, who reported that thermal treatment enhanced the antioxidant status due to the transformation of antioxidants into more active compounds, such as the deglycosylation of onion quercetin (Kitti *et al*., 2003). The release of bound phenolic compounds by thermal treatments is known to increase the antioxidnat activities (Acosta-Estrada et al., 2014) and similar was observed in this work. The formation of certain antioxidants during thermal treatment, considered to be the primary effect of Maillard browning reactions, also can positively influence the antioxidant status of foods as well (Nicoli, *et al*., 1997). The increase in the heating temperature of most of the spices and herbs might have yielded Maillard reaction products since significant increase in the phenolic contents was observed. Also the enhanced antioxidant activity

could be due to a greater release of phenolic compounds from the cell matrix because of the thermal treatments (So-Young *et al*., 2006). However, results showed a decrease in content of free antioxidant compounds when LAK and LAA were heated at higher temperatures for 1 and 2 h. The DPPH scavenging activity of LAK, reduced by 19.18 and 35.44% when heated at 180 ^{o}C for 1 and 2 h, respectively. Similarly, the DPPH scavenging activity of LAA reduced by 19.01% when heated at 180 ^{o}C for 2 h. This might be because of the loss of bioactive phytochemicals when heated at high temperatures (Paul and Ghosh, 2012). It is well known that many antioxidant compounds are inactivated by heat treatment, thereby reducing the final antioxidant status (Michaela, *et al*., 2013). Whereas, the total antioxidant activity of most of the extracts decreased as the heating temperature and time increased as compared to unheated samples. Because the total antioxidant assay is a method for measuring total reducing power of electron donating substances, which is not directly related to the free radical reactions and not as specific as the DPPH assay (Raymond *et al*., 2010).

Herbal plants have long been used to treat diabetes, as their principal bioactive components showed good anti-diabetic and anti-oxidant properties (Mustafa *et al*., 2010). Phenolic compounds inhibit the enzyme activity that catalyzes the hydrolysis of carbohydrate because of their ability to bind with proteins (Pereanez, 2011). In the present study the *in vitro* antidiabetic activity of *T. schimpri* and *T. vulgaris* were investigated. Hot water extract of *T. schimperi* had a stronger α-glucosidase inhibitor and aqueous: methanol (20:80, v/v) extract showed stronger α-amylase inhibitory activity in comparison with *T. vulgaris*. These results indicated that there was a positive linear correlation

between TPC or TFC and α-amylase inhibition activity and negative correlation with *α*-glucosidase inhibitory effects.

The Crude extracts of petroleum ether and aqueous: methanol (20: 80, v/v) seed extracts of *Aframomoum corrorima* exhibited potent antiproliferative activity on Hepatocellular Carcinoma (HepG2) cells lines. When compared with untreated cells that were maintained as control, treated cells showed a dose and time dependent inhibitory activity. The results obtained from the *in vitro* studies revealed that the petroleum ether seed extract of *A. corrorima* has a moderate anticancer activity but the aqueous: methanol (20:80, v/v) extract exhibited weaker activity. However, the findings did not show any relationship between anticancer activity and phenolic content. Petroleum ether extract, which showed a lower phenolic composition, exhibited a higher anticancer activity whereas aqueous: methanol (80:20, v/v) extract which contained higher phenolic content, exhibited lower anticancer activity. The relatively higher anticancer activity of the petroleum ether extract containing low phenolic composition suggests that the nature of phenolic compound or other volatile monoterpenes present in the petroleum ether extract (Hayes *et al.,* 1997) may be determinant for these activities rather than their amounts.

7.2 Conclusions

The results of this study support that these spices and herbs are promising sources of natural antioxidants. The phenolic content and antioxidant capacity differs significantly among all selected spices and herbs and various extraction solvents. The acetone, methanol and aqueous: methanol (20:80, v/v) from leaf extracts of LAK, LAA, and TS showed the highest values of TPC, TFC and antioxidant activities. This suggests that the antioxidant activities of the tested extracts are closely associated with their phenolic

constituents. Moreover, phenolic acids (syringic acid, chlorogenicacid, ferulic acid, caffeic acid, isoferulic acid, 2-hydroxycinnamic acid, 3-hydroxybenzoic acid), aliphatic carboxylic acids (fumaric acid and succinnic acid) and flavonols (quercetin, quercetin-3-O-rutinoside, quercetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-rhaminoside, quercetin-3-O-arabinoglucoside) identified in these extracts as dominant phenolic compounds may contribute greatly to the high antioxidant activities of the species.

The results illustrated that the health benefits from these plant sources remained in the products after thermal process (i.e., heating have not reduced the antioxidant activities in most of the selected spices studied rather an increase was observed in most spicies and herbs).The antioxidants found in the present herbs and spices are valuable food constituent for promoting good health in the daily lives because they are much safer than the synthetic antioxidants. Thus these spices and herbs, which are widely used in Ethiopian food preparations, in addition to imparting flavor and taste to the food, they are good antioxidants and thus prevent oxidation of lipids and adverse effects of lipid peroxidation.

In conclusion, the present study shows solvent system (pure or mixture) of varying polarity used in the extraction have a significant effect on the extraction capacity and selectively for phenolic contents leading to variations in the antioxidant activities of spices and herbs. The extracts obtained using high polar solvent showed more effective than less polar solvents. The addition of 20% water to methanol has enhanced the TPC and antioxidant activity of the extract. The study showed high phenolic contents and antioxidant activities were found in the dietary herbs (TS, LAK, LAA, and CSL) than that of spices (AC, CSS, and CSS). Thus, there is a possibility of easily accessible source of

natural antioxidants, as possible food supplement or in pharmaceutical applications for dietary herbs. The selective extraction of bioactive molecules from natural sources by appropriate solvents is important for obtaining compounds with high biological activities which can be used as preservative ingredients in the food and/or pharmaceutical industry. Furthermore, the bioactive compounds present in the extracts of these dietary spices and herbs have the potential to be used as possible natural substitutes for controversial synthetic antioxidants currently used in food products. Also, this work reveals that selected Ethiopian spices and herbs, including *A. corrorima, and T. shimperi* can also be a source of anticancer and antidiabetic compounds, respectively. Further studies on isolation and identification of the key phenolic compounds may allow for a potential biomedical application in the therapy of non-communicable diseases.

7.3 Recommendations

Based on the findings of this study and the conclusion made, the following are recommended for further study:

- Thermal treatment is the common processing method to prepare spicy Ethiopian traditional foods. Hence, conducting study on effect of thermal treatment (heating temperature and heating time) on phenolic content and antioxidant capacity of different spice blends (constituted different spice and herbs) is important. Further studies are also required to identify the effect of thermal treatment on the individual phenolic compounds from each spice and herb extracts.

- Further studies are necessary on the isolation and characterization of individual compounds to elucidate their different antioxidant mechanisms and the existence of possible synergism, if any, among the compounds.
- Geographical location is one of the factors affecting the phenolic content and antioxidant activity of plant products. Study should be conducted on the effect of agro climatic location and season of harvesting variations on the antioxidant potential of spices and herbs in the country.
- *In vivo* studies should be done to investigate absorption, distribution, metabolism excretion and possible toxicity of the extracts and individual compounds.
- Under this study, *in vitro* antidiabetic activity of crude extract of TS was conducted. Further *in vivo* and clinical investigation should be conducted on isolated bioactive molecules for confirming the activity of antidiabetic agents.
- Ethiopia is one of the leading countries in Africa that exports different spices to different countries. Spices are high value and export oriented commodity crops, which play an important role in agricultural economy of the country. Storage is one of the factors that affect the quality of these products. Therefore, research should be conducted on the effect of storage on antioxidant activity and the stability of bioactive compounds so that the products are competent in the world market.
- Now days, agricultural research centers conduct research on spices to come up with high yield accessions. Besides focusing on the yield, the antioxidant potential of the new accessions should be part of the study.

- Lipid oxidation is one of the major causes of quality deterioration of processed foods, imposing an adverse effect on flavor, color, texture, nutritional value and safety. As natural antioxidants the potential of each spice and herb extracts and the mixtures in stabilizing lipid oxidation should be studied so that food factories in the country can use them (as natural preservative).
- It is timely and important to undertake investigation on extracts and essential oils so we can be able to replace the synthetic compounds by natural preservatives.
- Since there is no as such standard for producing spiced ground red pepper in the country, producers of the community are producing according to their preference and traditional knowledge. The setting of standard is crucial. Therefore, research should be conducted to set the standard in preparing spiced products in the country considering the antioxidant capacity of the products.
- It is also necessary to investigate the effect of the extracts on protein digestibility and sensory quality of food products in the view of the phenolic content and protein precipitation capacity of the extracts.
- The isolated compounds from *A. corrorima* need to be evaluated in scientific manner using scientific animal models and clinical mechanisms of action in search of bioactive molecules for treating liver cancer.

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APPENDIX

Sample HPLC chromatograms of standard compounds (Figure 1-5) and compounds extracted from seed extract of *Aframomum corrorima* (Figure 6) and leaf extract of *Lippia adoensis* var koseret (Figure 7).

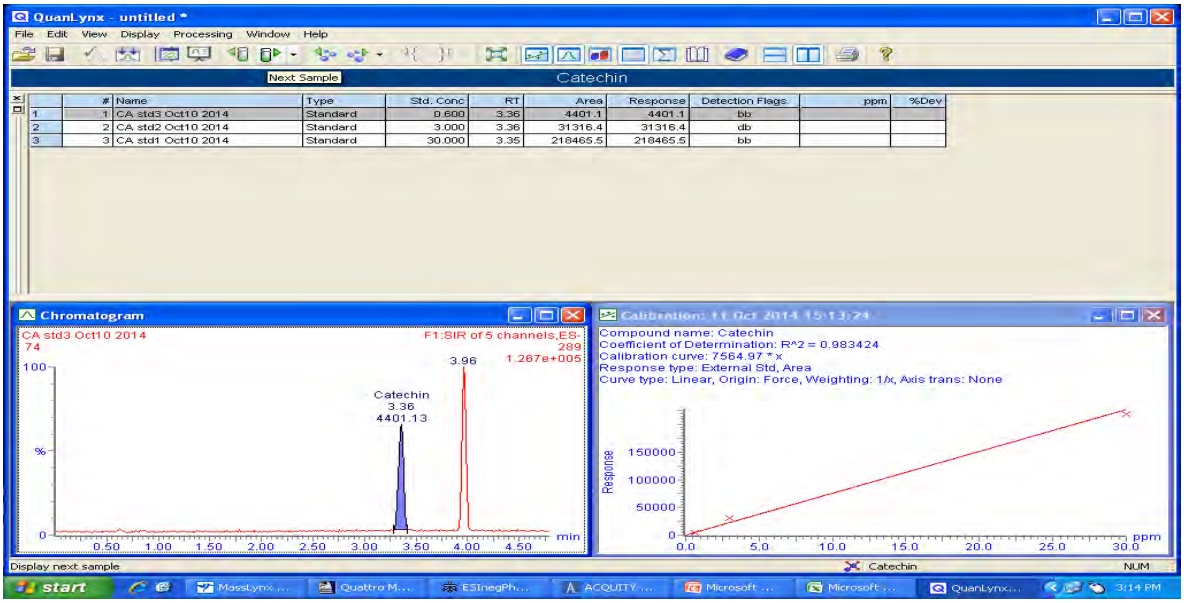

Figure 1 chromatogram and calibration curve of catechin

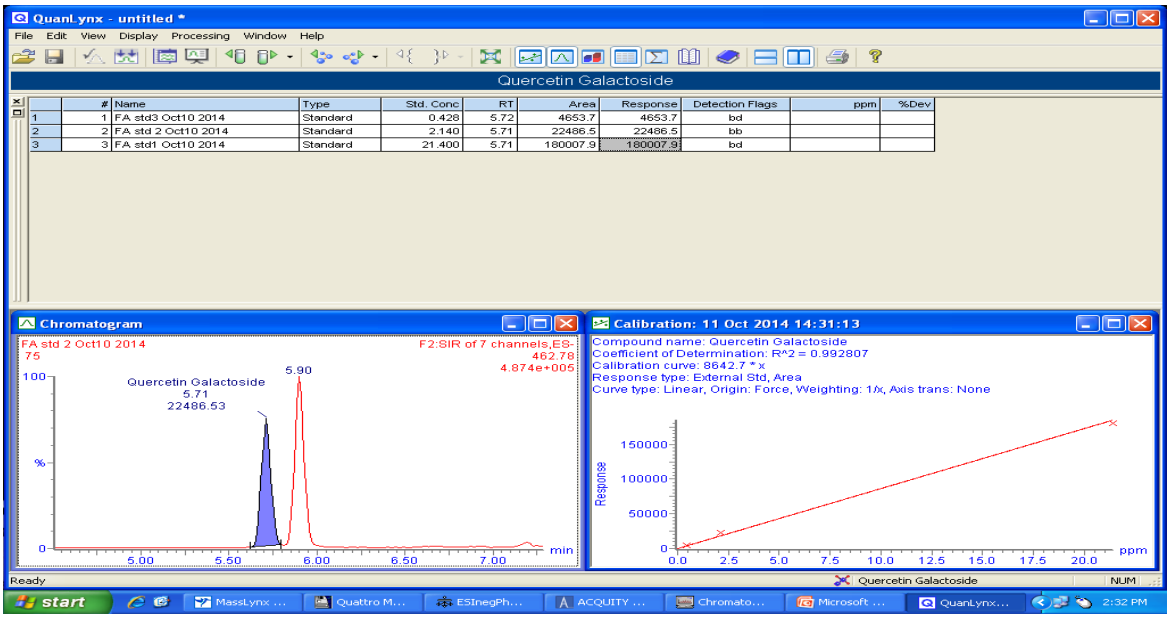

Figure 2 Chromatogram and calibration curve of quercetin-3-O- galactoside

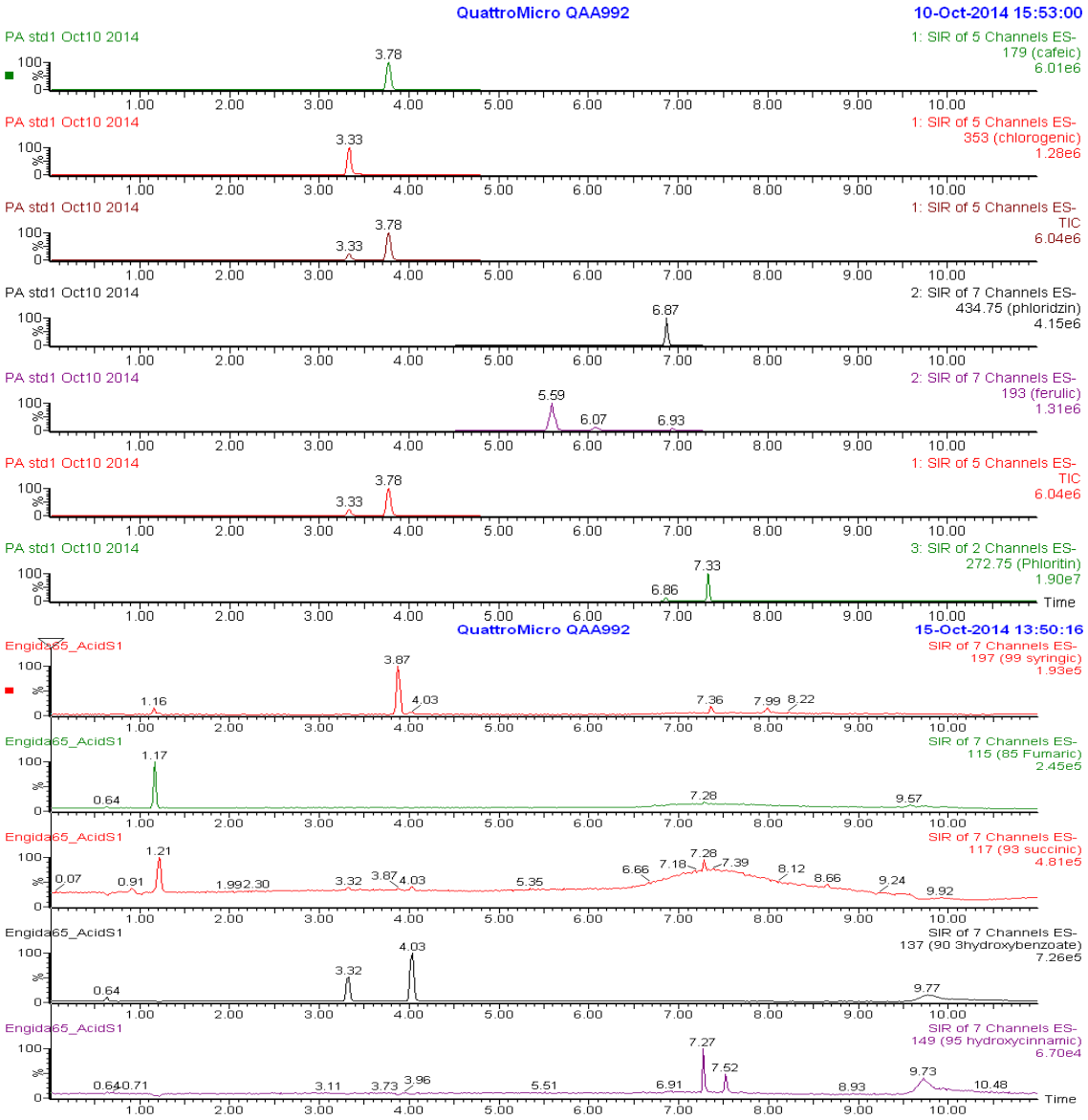

Figure 3 HPLC chromatogram of the standards (phenolic acids, aliphatic carboxylic acids and chalcones).

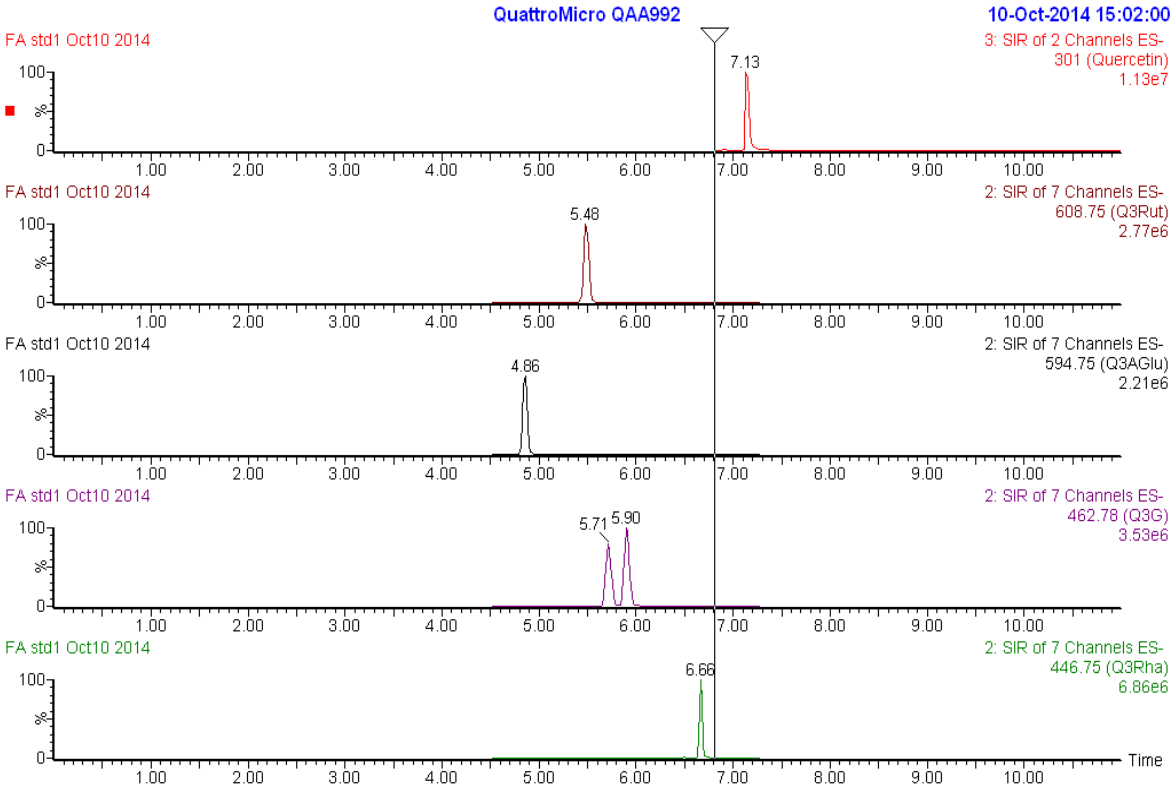

Figure 4 HPLC chromatogram of quercetin and derivative

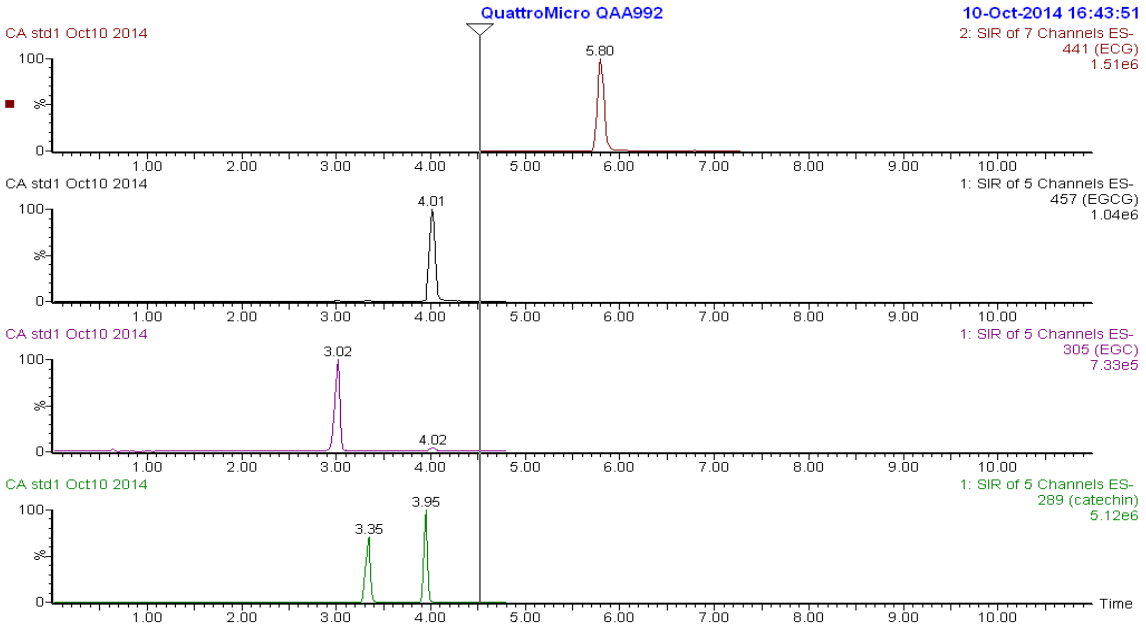

Figure 5 HPLC chromatogram of catechin and derivatives

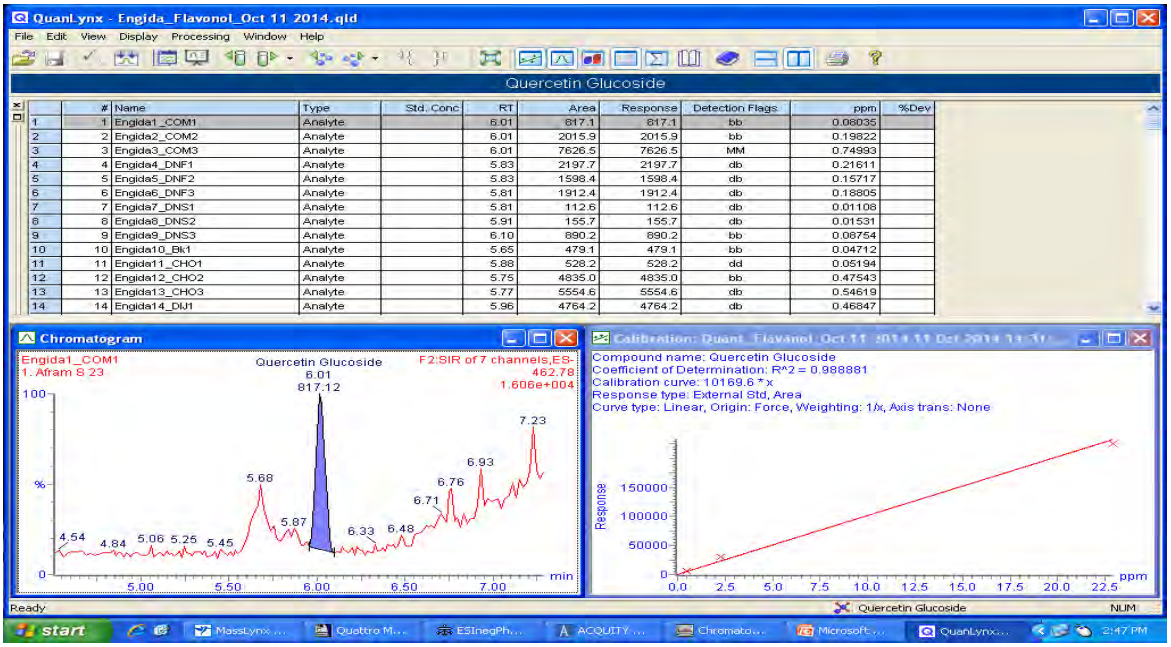

Figure 6 HPLC chromatogram of the *Aframomum corrorima* (where retention time Rt of: 4.84 = Q3-Argluc, 5.45 = Q3-rut, 5.68 =Ferulic acid, 5.97 = ECG, 6.01 = Q3-dlucoside, 6.81 = Phloridzin, 7.23 = Hydroxycinnamic acid) and calibration curve of quercetin-3-O-glucoside

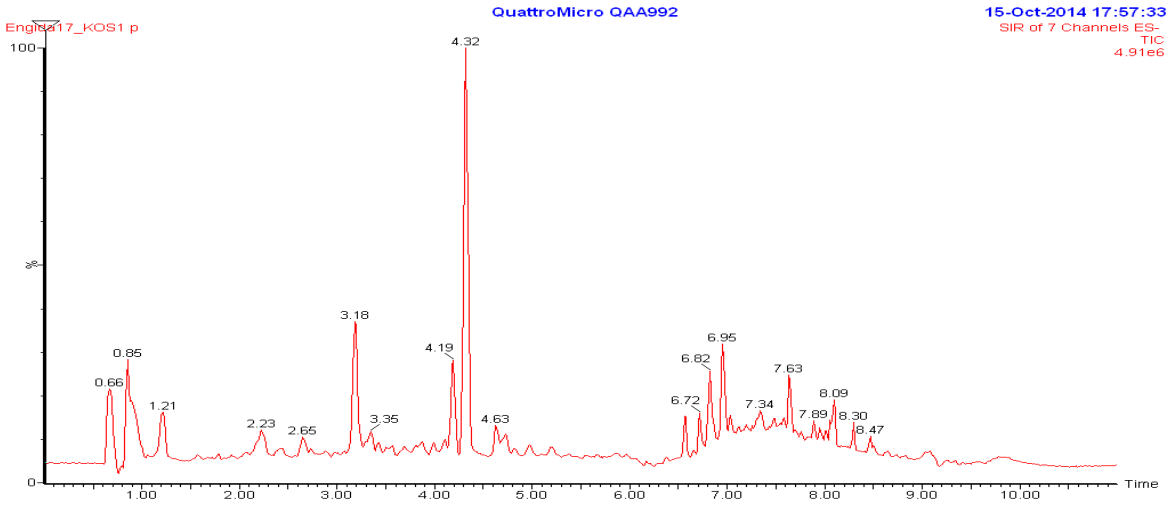

Figure 7 HPLC chromatogram of the *Lippia adoensis* var. koseret (where retention time Rt of: 1.21 = Siccunnic acid, 3.18 = EGC, 3.35 =Catechin, 4.19 = EGCG, 6.82 = Phloridzin, 7.34 = Phloritin).

Table 1. DPPH scavenging activity of plant extracts of various concentrations

Sample no.	Volume extract	Methanol (dilution)	Conc of diluted ext	DPPH (0.08%	Abscontrol (nm)	Absample (nm)	% scavenging	Mean ± SD
K11 K12 K13	1ml (triplicate)	-	1000µg/ml	2ml				
K21 K22 K23	0.5ml (triplicate)	0.5ml	500 µg/ml	2ml				
K31 K32 K33	0.25ml (triplicate)	0.75ml	250 µg/ml	2ml				
K41 K42 K43	0.1mL (triplicate)	0.9ml	100 µg/ml	2ml				
K51 K52 K53	0.05ml (triplicate)	0.95ml	50 µg/ml	2ml				
K61 K62 K63	0.01 ml (triplicate)	0.99 ml	10 µg/ml	2ml				