# THE STUDY OF ISOLATION AND ANALYSIS OF MEDICINAL POTENTIAL OF STACHYS CRETICA LAMIACEAE LEAVES METHANOLIC EXTRACT

**A MASTER'S THESIS**

**in**

**Chemical Engineering and Applied Chemistry Department**

**By**

**SAADA S.ABDULGADAR ALI**

**JULY 2017**

# THE STUDY OF ISOLATION AND ANALYSIS OF MEDICINAL POTENTIAL OF STACHYS CRETICA LAMIACEAE LEAVES METHANOLIC EXTRACT

**A THESIS SUBMITTED TO**

**THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES**
**OF**

**ATILIM UNIVERSITY**
**BY**

**SAADA S. ABDULGADER ALI**

**IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE**
**IN**

**APPLIED CHEMISTRY**
**AT**

**CHEMICAL ENGINEERING AND APPLIED CHEMISTRY DEPARTMENT**

**JULY 2017**

Approval of the Graduate School of Natural and Applied Sciences, Atılım University.

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I certify that thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that we have read the thesis "The study of isolation and analysis of medicinal potential of *stachys cretica lamiaceae* leaves methanolic extract" submitted by "Saada S. Abdulgader Ali" and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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# ABSTRACT


## THE STUDY OF ISOLATION AND ANALYSIS OF MEDICINAL POTENTIAL OF

## STACHYS CRETICA LAMIACEAE LEAVES METHANOLIC EXTRACT

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JULY 2017, 38 pages

The advantage of studying antioxidant activity of natural products is they have high protection of body from free radicals and their effects. In this study the antioxidant effect of stachys *cretica* plant leaves extract was analyzed due to their total phenolic and flavonoid contents, and their effects on antioxidant systems.The chromatographic techniques such as column chromatography (CC) and thin layer chromatography (TLC) were also used. By measuring the effect of plant on the activity of the antioxidant system; glutathione-S-transferase, superoxide dismutase and catalase enzymes are used.The plant extracts were prepared by methanol extraction. The total phenolic and flavonoid contents of the fractions were compared; the fraction with highest total phenolic compounds was used for further analyses.

The inhibitory effects of leaves of plant on Glutathione-S-transferase (GST) was 100% with half maximal inhibitory concentration value IC50 of 2.639g/L and

superoxide dismutase (SOD) activities were 80% with $IC_{50}$ of 0.7230g/L and the inhibitory effect of catalase enzyme was less than 90%with $IC_{50}$ of 0.1474g/L.

**Keywords:** *stachys cretica* plant, antioxidant enzymes, Catalase, Superoxide dismutase, Glutathione-S-transferase**,** chromatographic technique

# ÖZ

## STACHYS CRETICA LAMIACEAE BİTKİSİNİN METANOL ÖZÜTÜNÜN TIBBİ POANSİYEL ANALİZİ VE IZOLASYON ÇALIŞMASI

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JULY 2017, 38 sayfa

Dogal ürünlerin antioksidan aktivitelerinin çalışılması bu yapıları serbest radikaller ve onların etkilerine karşı yüksek koruma özelliklerinden dolayı avantajdır. Bu çalışmada Stachys cretica bitkisinin yaprakları fenolic ve flavonoid içerik açısından incelenmiş, anti oksidan enzimler üzerine etkisi araştırılmıştır. Kolon kromatagrafisi ve ince tabaka kromatografileri kullanılarak fraksiyon ayrıştırması yapılmıştır. Anti oksian enzim çalışmaları için Glutatyon-S-transferaz, süperoksit dismutaz ve katalaz enzimleri kullanılmıştır. Bitki özleri, metanol yöntemleriyle hazırlanmıştır. Fraksiyonların toplam fenolik ve flavonoid içeriği açısından karşılaştırılmış, en yüksek toplam fenolik bileşi içeren fraksiyon enzim analizlerinde kullanılmıştır..Glutatiyon-S-transferaz (GST) enzimi üzerine bitki yapraklarının inhibitör etkisi % 100 olarak bulunmuş ve bu enzim için IC50 değeri2.639 g / L olarak hesaplanmıştır.

Bitki yapraklarının superoksit dismutaz (SOD) ve Katalaz enzimleri üzerine inhibitör etkisi sırsıyla % 80 ve % 90 dan az olarak bulunmuş yine bu enzimlere ait

, yarım maksimum inhibisyon konsantrasyon değeri IC50 degerleri SOD için 0.7230 g / L iken katalaz için 0.1474g/L. olarak hesaplanmıştır.

**Anahtar kelimeler**: *stachys cretica* bitkisi, antioksidan enzimler, Katalaz, Süperoksit dismutaz, Glutatyon-S-transferaz, kromatografik teknikler

**To My family**

Words not enough to tell how grateful and thankful I am to my husband for his supporting, his sacrifice and patience throughout all my studies.

Thank you for everything, my mother and my father and thanks for your prayers. Finally, thanks for my brother, my sisters, and my children.

I would like to thank my friend's muna ELKOUHA for her support, guidance, and advices during my thesis.

# ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my thesis supervisor Assoc. Prof. Dr S.Belgin İşgör for her kind support, guidance, understanding, encouraging advices, constructive criticism and valuable discussions throughout my thesis. Her approach to the students and solving their problems will sure help me in my future academic life.

I am also grateful to Assoc. Prof. Dr Yasemin G. İşgör for her support, guidance, and advices during my thesis.

I would like to express my whole gratitude to the Head of the Department Prof. Dr Atilla Cihaner for his valuable suggestions and corrections, faculty members, and my biochemistry laboratory-mates.

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| | | |
|---|---|---|
| SOD | - | Superoxide Oxide Dismutase |
| CAT | - | Catalase |
| GSH | - | Reduced Glutathione |
| GST | - | Glutathione-S-Transferase |
| CDNB | - | 1-chloro-2,4-dinitrobenzene |
| HRP | - | Horse Reddish Peroxidase |
| 4-AP | - | 4-Amino Antipyrine |
| EDTA | - | Ethylene Diamine Tetra acetic acid Disodium Salt Dihydrate |
| NBT | - | Nitro Blue Tetrazolium Chloride |
| QE | - | Quercetin Equivalent |
| GAE | - | Gallic Acid Equivalent |
| $H_2O_2$ | - | Hydrogen Peroxide |
| TFC | - | Total Flavonoid Content |
| TPC | - | Total Phenolic Content |
| DMSO | - | Dimethyl Sulfoxide |
| XOD | - | Xanthine oxidase |
| $IC_{50}$ | - | The half maximal inhibitory concentration |
| ROS | - | Reactive Oxygen Species |
| CC | - | Column Chromatography |
| TLC | - | Thin layer chromatography |

# CHAPTER 1

# INTRODUCTION

There is certainly a rising interest in using medicinal plants as natural resources in pharmaceutical, food and cosmetic companies all over the world. The chemical material of therapeutic plants contributed them to be applied in these industries such as botanical drugs, food supplements and meals product packaging. Plants also have recently been employed in ethnopharmacy for diverse diseases such as hypertension, cholesterol, eczema and diarrhoea for decades. Today their scientific validation was provided by characterization and isolation of bioactive phytochemicals (Littleton et al., 2005).

Phytochemicals are the secondary metabolites that contain distinct subgroups with diverse bioactivities such as antioxidant, antimicrobial, antivirus, anticancer (Duffy and Power 2001). Currently, re-emerging connection between plants and human health especially determined by their antioxidant activities which may delay or reduce the hazardous effects of free radicals (Pourmorad, et al., 2006).

## 1.1.Free Radicals:

Totally free radicals are electrically incurred molecules and need to be stabilised. Holding unpaired electron causes them to chase out and gain the electrons from different materials for such stabilisation or neutralisation. Though the free radical atom is made by the initial attack to be neutralised, other radical is created within the same reaction, leading to a sequence of radical major reactions to happen. Numerous

of free radical reactions take place within a few seconds within the first reaction (initial attack) until they can be deactivated, so it is called a radical chain reaction.

### 1.2. Reactive Oxygen Species (ROS):

Oxygen is an extremely reactive atom that can change into a portion of the potentially harmful radical molecules known as reactive oxygen species (ROS).It is found that 5 % or more of the O2 which enter the body during aspiration process is ghanged again to ROS through the O2 univalent reduction (Uday Bandyopudya et al., 1999).therefore, cellular components that is in the aerobic situation is continually vulnerable to ROS attack. So, it is necessary for the cell to be prepared for ROS by extremely strong antioxidant system.

ROS are oxygen containing substances, along with free radicals, such as superoxide anion radical, nitric oxide radical, peroxides (including hydrogen peroxide, singlet oxygen, hydroxyl radical, hypochlorite radical, and numerous lipid peroxides,mostly produced endogenously by cells(Figure 1.1).

The cells produce most of the oxidants due to:

- The ordinary aerobic metabolism;
- The oxidative burst by the phagocytes (white blood cells) by which the microorganism is killed and antigens are denatured;
- Xenobiotic metabolism by which the detoxification of harmful materials takes place;

Moreover, ROS can be internally generated during various diseases, including chronic inflammation and infections, during the medical treatment or during exposure to allergens, toxin like smoke, pollution, insecticides, and pesticides and all contribute to increasing the body ‘s ROS concentration.

The types of ROS that target in cells are generally biomolecules such as nucleic acids, proteins and unsaturated fatty acid as well as carbohydrates. The properties of the intrinsic membrane will be altered by these reactions which include liquidity, ion

transport, protein synthesis and damage of deoxyribonucleic acid, enzyme activity loss, eventually leading to cell death or necrobiosis (Uday Bandyopudya et al., 1999).

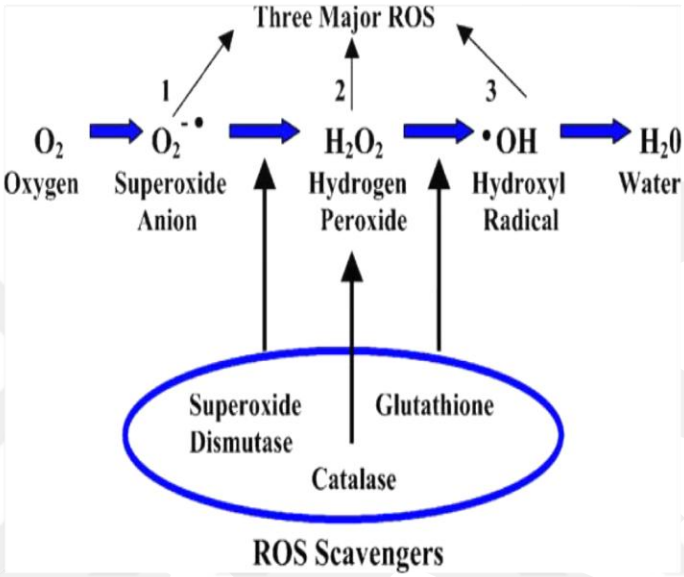



**Figure 1.1:** The ROS formation process in cells formation.

### 1.3. Antioxidant System:

Human have an excellent and sophisticated antioxidant protection and defense system. It involves a wide range of constituents (Table1), which are endogenous or exogenous in origin, both act synergistically to neutralize free radicals (Mark Percival, 1998).

**Table 1:** ROS and their corresponding neutralizing agents (antioxidants)

| **ROS** | **Neutralizing Agents** |
|---|---|
| Hydroxyl radical | Vit C, Flavonoids, Lipoic Acid, Glutathione |
| Hydrogen peroxide | Vit C, flavonoids, lipoic acid, vit E, Glutathione |
| Lipid peroxides | Flavonoid, Vit E, Beta-carotene, ubiquinone |

These protective antioxidants can be classified into:

a) Endogenous antioxidants that reduce free radical by electron donors such as:

- Bilirubin;
- Thiols, e.g., glutathione, lipoic acid, N-acetyl aminoalkanoic acid;
- NADPH and NADH;
- Ubiquinone (coenzyme Q10);
- Uric acid;
- Enzymes which catalyse ROS removal, for example, glutathione S-transferases (GST), superoxide dismutase (SOD) and catalase (CAT);

b) Exogenous dietary antioxidants that can also reduce the free radical are:

- Vitamin C and vitamin E (Halliwell B., J., Gutteridge(Eds) 1999);
- Beta-carotene and alternative carotenoids and oxy carotenoids;
- Polyphenols, e.g, flavonol's, flavonoids, flavones, and proanthocyanidins;

c) Metal binding proteins that can bind pro-oxidant metals as copper and iron, and these are:

- Copper bearing proteins;
- Albumin,ceruplasmin and metallothionein;
- Iron bearing proteins :ferritin,myoglobin and transferring;

### 1.3.1. Endogenous Antioxidants (Antioxidant Enzymes):

The entire body depends on many endogenous defensive systems which assist in the protecting the cell against radical injury produced by the endogenous free radicals other than the antioxidant. The antioxidant enzymes as part of endogenous defense system are SOD, CAT and, GPx, they are metabolized oxidative, toxic and harmful intermediates. They need certain cofactors like metal elements such as zinc, copper and iron for

optimum catalytic action in detoxifying reactions. They may also require GSH an essential soluble antioxidant, which is synthesised from the amino acids cysteine, glycine and glutamate. Glutathione directly quenches ROS like lipid peroxides and plays a substantial role in the metabolism of xenobiotics(Ursine et al., 1982).

The metabolic detoxification system inside the body turns xenobiotics such as drugs and environmental pollutants, or antibiotic like hormones from lipid soluble substances, into water soluble, less poisonous substances, which can be excreted via kidneys. This system includes phase I and phase II metabolizing enzymes and one of their activities is the biotransformation of xenobiotics.

#### 1.3.1.1. Glutathione S-Transferase Enzyme:

(GSTs) belong to phase II detoxifying enzymes and have many other important roles in normal cellular metabolism, as well as in the detoxification of a wide variety of xenobiotic compound (Lu, 2013). The main role of GST is to catalyse the conjunction of glutathione with electrophilic compounds like nitrogen, sulphur or carbon atoms, and to eliminate the foreign compounds including anti-cancer drugs (Lu, 2013).GSTs have a wide range of substrate specificity which includes nitrobenzenes, epoxides, heterocyclic amines, quinones, arene oxides, and α/ β- unsaturated carbonyl (Reinemer et al., 1996).GST correspondent in the organism is accepted as thioredoxins. GSTs are classified into 5 classes (Parde et al., 1998) related to their structure and substrates as alpha, (α), Mu (μ), Pi (π), Sigma (σ) and theta (θ). The active site in these enzymes contains two binding sites called GSH-binding site (G-site) and xenobiotic-binding site (H-site) GST consists of 25-30KDa subunits, which can be divided into two domains, domain N and domain C (Figure 1.2). The GST family consists of two superfamilies: the cytosolic and mitochondrial. Cytosolic GSTs are the largest family of transferases and have especially thiolysis, reduction and isomerization activities (Hayes et al., 2005). Besides having these activities, cytosolic GSTs can bind covalently and noncovalently to nonsubstrate ligands, and have roles in intracellular transport and disposition of

xenobiotics. These nonsubstrate hydrophobic ligands are some steroids, bilirubin and lipophilic anticancer drugs (Hayes and Pulford, 1995).

Mitochondrial GSTs prouder protection against genotoxic and cytotoxic electrophiles, which are produced within the mitochondria by the activity of mitochondrial cytochrome P450 species or might result from the decomposition of lipid peroxides produced during respiration (Harris et al., 1991).

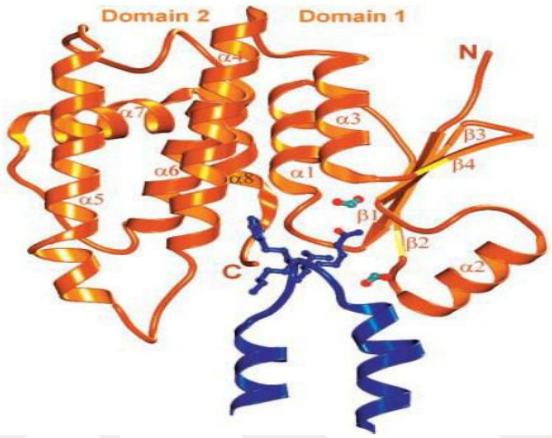



**Figure 1.2:** The structure of glutathione S-transferase (Wolf K, Backer A et al., 2003).

There is evidence that the activity of GST leads to drug resistance, in addition to the elimination of foreign compounds and has a role in the removal of free radicals, and all of these activities are resulted from natural evolution (Board and Menon, 2012).

### 1.3.1.2. Catalase (CAT) Enzyme:

(CAT) is an enzyme of the degradation of hydrogen peroxide. It is protecting enzyme present in practically all animal cells (Figure1.3). The reaction of CAT occurs in two steps: 1) A molecule of hydrogen peroxide oxidises the heme for an oxyferryl types, A porphyrin cation radical is made when one oxidation equivalent is removed from iron and one from the porphyrin diamond ring 2) A second hydrogen peroxide molecule acts as a reducing agent to create the resting state enzyme, producing a molecule of oxygen

and water. In addition, this enzyme reacts with hydrogen donors (formic acid, phenols, ethanol or methanol) with the activity of peroxides.

(Catalytic Activity) $2H_2O_2 \xrightarrow{\text{Catalase}} O_2 + 2H_2O$

(Peroxidatic Activity) $H_2O_2 + AH_2 \xrightarrow{\text{Catalase}} A + 2H_2O$

**Figure 1.3:** A Large Tetrameric Catalase Subunit. (Evans S., 1993).

### 1.3.1.3. Superoxide Dismutase Enzyme (SOD Enzyme):

Superoxide Dismutase (SOD) catalyses the dismutation of the superoxide radical ($O_2$-into hydrogen peroxide ($H_2O_2$) and molecular oxygen ($O_2$) and as such provides an important defence against the toxicity of the superoxide radical C. et al. (1993).
SOD offers an important means of cellular defence against free radical damage.

All superoxide dismutases are multimeric metalloproteins, which have scavenging effect on the superoxide radical. The Cu/ZnSODs, as most organism Mn-

SODs and FeSODs, are dimeric (Ramirez, D.C. et al. 2009), while the MnSODs from mitochondria and some thermophilic microorganism are tetrameric.

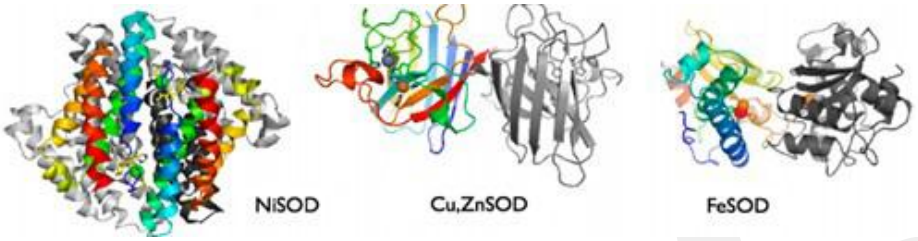



**Figure 1.4** Superoxide Dismutase Enzyme and their types.

### 1.3.2. Plants as Exogenous Antioxidant:

The exogenous antioxidants are mainly extracted from food and medicinal plants, such as fruits, vegetables, cereals, mushrooms, beverages, flowers, spices and traditional medicinal herbs (Elmastas et al., 2005, 2006; Kahkonen et al., 1999; Velioglu et al., 1998; Gulcin et al., 2002a, b). Besides the industrial treatment, agricultural by-products are also potentially important sources of natural antioxidants. These types of natural antioxidants from plant materials are typically polyphenols (phenolic acids, flavonoids, anthocyanins, lignans and stilbenes), carotenoids (xanthophylls and carotenes) and vitamins (vitamin E and C). Generally, these natural antioxidants, especially polyphenols and carotenoids, exhibit a variety of biological effects, such as anti-inflammatory, antibacterial, antiviral, anti-aging, and anticancer (Piozzi and Bruno, 2011).

Many authors have reviewed the beneficial uses of such plant species (Speroni and Scartezzini, 2000; Matkowski, 2008). Lately, Ali et al. (2008) reviewed twenty-four medicinal Indian herbs that contain great antioxidant potential. This review includes medicinal species from a variety of countries (Africa, Algeria, The United States of America, Australia, Brazil, Bulgaria, China, India, Iran, Italy, Japan, Malaysia, Poland, Portugal, Thailand and Turkey).

### 1.3.2.1. Phenolic Constituents in Plants:

Through the diverse phytochemicals as the secondary metabolites of plants, phenolic compounds are the common ones and considerably present in the plant kingdom. Phenolic constituents display several bioactivities such as antimicrobial, antioxidant, antiviral and anti-inflammatory. Dietary phenolics that have been researched deeply in the last decades are divided into various subgroups, and the leading categories of phenolic compounds are flavonoids, phenolic acids, and tannins (King and Young 1999). Some other species of phenolics are coumarins, lignans, quinones, and stilbenes (Chai, et al. 2004).

Flavonoids are the main and most studied phenolic phytochemicals that are widely distributed in plants (Chai et al., 2004). More than 6,400 flavonoid structures have been determined before (Silva et al., 2006).

Flavonoids are conntains of many subclasses such as; flavones, flavonols, flavanones, flavanonols, chalcones, isoflavonoids, anthocyanins and bioflavonoids (Chai et al., 2004).

Flavonoids are essentially split into two groups;anthocyanins and anthoxanthins. Anthocyanins have some colour pigments such as red, blue, and purple. Anthoxanthins possess colourless or white to yellow molecules (flavonols, flavones, isoflavones) (King and Young 1999).

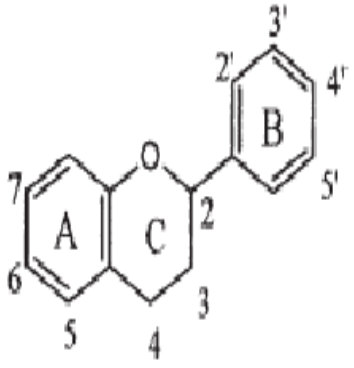



**Figure 1.5:** The generic structure of flavonoids(Source: Liu 2004).

Phenolic acids form another large class of phenolic compounds, and constitute two main groups;

**1**. Hydroxybenzoic acids (e.g. gallic acid, *p*-hydroxybenzoic acid, protocatechuic acids, vanillic acids)

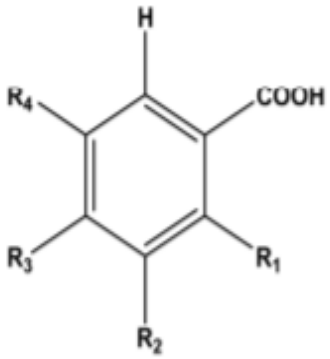



**Figure 1.6:** Hydroxybenzoic acids

**2.** Hydroxycinnamic acids (e.g. ferulic acid, caffeic acid, coumaric acid, chlorogenic acids, cinnamic acids)

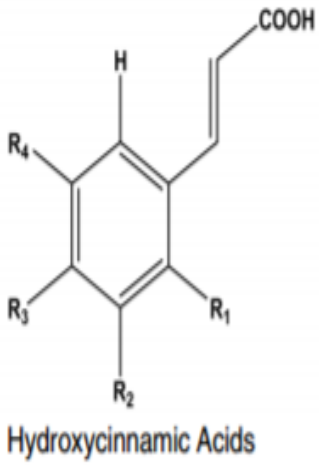



**Figure 1.7:** Hydroxycinnamic acids

### 1.3.2.2. Stachys cretica L as Exogenous Antioxidant:

Stachys cretica L. (Lamiaceae) is a significant genus of herbs and shrubs comprising about 300 species divide into temperate and tropical regions around the world, with the exclusions of Australia and New Zealand (Evans, 1996; Salmaki et al., 2012). Several initiatives have been made to define sufficient infrageneric setting up the Old-World species, while those kinds from the New World are still expecting for a satisfactory taxonomic treatment (Bhattacharjee, 1980; Nelson, 1981; Turner, 1994).

Based on the most comprehensive treatment, Old World species are arranged in two subgenera (Betonica and Stachys), with a total of 20 parts and 19 subsections (Bhattacharjee, 1980). Both the two subgenera are distinguished by several physical features-flowers/ inflorescences (Koeva-Todorovska, 1979; Rechinger, 1982). Various studies have demonstrated anti-inflammatory, cytotoxic, antitoxic, antibacterial and antioxidant activities of extracts from Stachys spp. Flavonoids, iridoids, fatty acids and phenolic(Bilušic´ - Vundac´ et al., 2007; Grujic-Jovanovic et al., 2004; Haznagy-Radnai et al., 2008; Khanavi et al., 2005).

### 1.3.2.3. Stachys cretica L in Turkey:

Stachys(figure1.8) was revised by Bhattacharjee for the flora of Turkey (1982). Since then, 18 new species have been described from Turkey. *Stachys* has 90 species (115 taxa) belonging to 15 sections and 2 subgenera in Turkey. Various species of *Stachys*, which are known as Betony, Woundwort or Mountain Tea in traditional medicine, are used as herbal teas in Iran and Turkey (Goren et al., 2011).

A

Scientific classification:

Kingdom: Plantae

Unranked: Angiosperms

Unranked: Eudicots

Unranked: Asterids

Order: lamiales

Family: lamiaceae

Sub family: lamioideae

Genus: stachys

Type species: cretica

B

**Figure 1.8:** Scientific classification (A) and Structure of Stachys cretica L (B)

### 1.4. Scope of this study:

The aim of this study was to extract, isolate and identify antioxidative capacity of extracts from *stachys cretica L* and its effect on antioxidant defence enzymes which are GST, CAT and SOD.

# CHAPTER 2

# MATERIALSAND METHODS

## 2.1. Materials

Stachys cretica (Lamiaceae), Gallic Acid (Merk), Dimethyl Sulfoxide (DMSO)(Carloerba), FolinCiocalteus Phenol Reagent (Sigma-Aldrich), Sodium Carbonate (Fisher Scientific), Quercetin (Sigma), Absolute Ethanol (Merk ), Sodium Acetate (Aklar Kimya), Aluminium Chloride (Merk), Methanol, TLC Silica gel (Merck), Silica gel(Merck), Monopotassium Phosphate and Di potassium phosphate (Riedel.dehaen), Hexane, Ethyl acetate, Reduced form of Glutathione (Sigma-Aldrich), 1-Chloro-2,4-Dinitrobenzene (CDNB) (Fluka), Bovine Liver Cytosol was extracted in our laboratory from the bovine liver was brought from slaughter house in Kazan-Ankara, Hydrogen Peroxide (H2O2) (J.T. Baker), Dichlorohydroxy Benzene Sulfonic Acid (DHBS) (Sigma), 4-Amino Antipyrine (AP) (Acros), Horse Reddish Peroxidase (HRP), Sodium Azaide (Acros), Catalase was purchased from its company Sigma- Aldrich, Ethylene Diamine Tetra acetic acid Disodium Salt Dihydrate (EDTA)(Sigma), Nitro Blue Tetrazolium Chloride (NBT) (Thermo), Xanthine Oxidase (Calbiochem), Xanthine (Sigma-Aldrich).

## 2.2. Methods

### 2.2.1. Collection of Plant Sample:

The plants were collected from Ankara, and authenticated by Prof. Dr. Fatmagül Geven, Department of Biology, Ankara University, Ankara.

### 2.2.2. Extraction Methods of *stachys cretica L* medicinal plants:

The extraction method started by drying the leaves of the plant at room temperature for about one week. Then the plant was grounded .2.0756g of grinded leaves were placed into filter paper and extracted with methanol by using Soxhlet apparatus with (150ml for 24hours). The next day the solvent was evaporated at 40 $^{o}$C, the dried extract finally dissolved in 15ml methanol and kept at -20 $^{o}$C for further analysis.


**Figure 2.1:** Experimental set-up the Extraction of plant leaves with Soxhlet Apparatus

### 2.2.3. Determination of Total Phenolic Content (TPC) of *stachys cretica L*:

Total phenolic contents of the plant material were measured by using the Folin-Ciocalteu assay (Singleton and Rossi, 1965). Briefly, the total phenolic content of the extract was determined by using calibration curve of gallic acid standard which was prepared from different concentrations (25-200µg/mL) in DMSO. The total phenolic content in the extract was expressed as µg of gallic acid equivalent (GAE)/mL of plant extract solution.

The blank of the assay was DMSO instead of plant extract; the absorbance was measured at 750 nm. The TPC of the extract were measured by using the calibration curve of the reaction components of the phenolic content given in Table2.

**Table 2:** The reaction components of TPC assay

| Components | Volumes (µL) |
|---|---|
| **Methanol extract** | 100 µL of plant extract |
| **Gallic acid** | 100µL from standard |
| **Folin–Ciocalteu's reagent (1:10 diluted with distilled water)** | 1mL |
| **Incubation for 5 minutes in dark** | |
| **2 % (w/v) sodium carbonate solution** | 1mL |
| **Incubation for one an hour at dark at room temperature** | |
| **Read at 750 nm** | |

*The reaction mixture without plant extract was read as a blank.

### 2.2.4. Determination of Total Flavonoid Content (TFC) of *stachys cretica L*:

The total flavonoid contents of the extract were measured by using the aluminium chloride colorimetric method (Chang et al., 2002). The standard curve was given from

quercetin solutions with different concentrations (50–200 µg/mL) that were given in Table 3.

**Table 3:** The reaction components of flavonoid contents determination

| Components | Volumes(µL) |
|---|---|
| **Methanol extract** | 250 µL of plant extract |
| **Quercetin standard** | 250 µL from standard |
| **95% ethanol** | 750 µL |
| **10 % (w/v) Aluminum chloride**<br>**1M Sodium acetate**<br>**DMSO** | 50µL<br>50µL<br>1 mL |
| **Incubation for 30 minutes in dark** | |
| **Read at 415 nm** | |

*The reaction mixture without plant extract was read as a blank.

The total flavonoid contents of the extracts were expressed as µg of quercetin equivalent (QE) / mL of plant extract (µg QE/mL). The ultraviolet-visible Spectrophotometer was used to measure TFC.

**2.2.5. Column Chromatography:**

In this method, silica gel chromatography was applied to separate extracts into their fractions. By wet column chromatography filled with silica gel as stationary phase and the mixture of hexane : ethyl acetate in the various ratio (1:10) was the mobile phase.

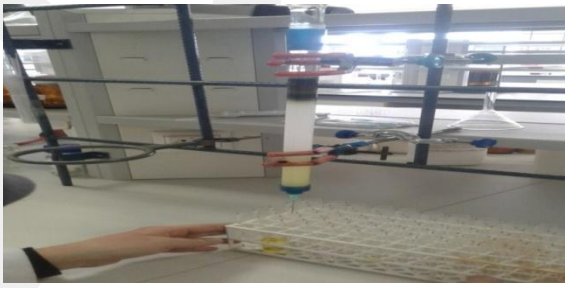


**Figure 2.2:** Purification of plant extract by using column chromatography- Sigma.

By using a column with 29.5 cm of diameter and 24 cm of height ,completely dried plant extract sample 0.2397 g of extract should be mixed with silica gel to make a fine powder form for easy distribution of the sample in the column. The column was washed

with the ratio of 1:10 hexane-ethyl acetate ,six fractions were collected. The final fraction was collected by washing the remaining material in the column with using methanol, each fraction was collected separately in a test tube and checked it by TLC (thin layer chromatography) for checking the purity of fractions. All fractions were evaporated with using rotary evaporator and dissolved in a small amount of methanol and saved by nitrogen gas and stored at -20 $^{o}$C.

### 2.2.6. Determination of TPC assay to the Fractions:

Total phenolic concentration (TPC) was measured using Folin-Ciocalteu reagent as described previously (Molan et al., 2009). By using microplate, we optimise this protocol with Assoc. Prof. Dr Belgin İşgör. The protocol was optimised in our laboratory by using microplate applications.

Briefly, as total phenolic content in microplate 50 µL of extract (F1-F5) and 25µL of F6 was diluted with 25µL of distilled water mixed with 50µL of Folin-Ciocalteu reagent (10%) keep them incubation for 5 minutes in dark after that add 50 µL of a 2 % (w/v) sodium carbonate the mixture was incubated for an hour in dark. Then the absorbance was measured at 750 nm using micro plate 96 well. The total phenolic content in the extract was expressed as µg of gallic acid equivalent (GAE)/mL of (F1-F6).

### 2.2.7. Determination of (TFC) assay of the fraction:

Total flavonoid concentration (TFC) was determined by using aluminium chloride colorimetric assay (Hatatikun and chiabchalard, 2013) with some modifications. In 96-96-good plates, 50 µL of F1-F5 and 5µL of F6 diluted with 95 µL methanol were mixed with 10 µL of 10% AlCl3 than adding 150 µLof 95% ethanol and add 10µL of 1M $NaNO_2$. Incubation for 30 minutes in dark, the absorbance of the mixture was measured at 415 nm.

### 2.2.8. Effect of Plant Extract on Glutathione S-Transferase (GST) Enzyme Activity:

The bovine liver cytosol was used as GST enzyme source and the activity was measured by conjugation of 1-chloro-2, 4-dinitrobenzene (CDNB) with reduced glutathione (GSH) (Habig and Jacoby 1981). The final concentrations were given in Table 4.

**Table 4:** Concentrations of plant extract that used in GST assay

| Dilution s | Stock plant Concentrations (mg/mL) | Final plant concentrations (mg/mL) |
|---|---|---|
| **1** | 71.8 | 3.452 |
| **2** | 23.9 | 1.149 |
| **3** | 7.9 | 0.379 |
| **4** | 2.66 | 0.127 |
| **5** | 0.89 | 0.042 |
| **6** | 0.29 | 0.014 |
| **7** | 0.098 | 0.004 |

GST activity was measured by using multi-mode plate reader (Spectra maxM2) at 340 nm and ultraviolet spectrophotometer was used to follow GST enzyme assay.

**Table5:** The components of assay mixture that used in GST enzyme assay

| Components | Volumes(µL) |
|---|---|
| **100mM phosphate buffer (pH 6.5)** | 7mL |
| **200mM GSH** | 150µL |
| **50mM CDNB** | 450µL |
| **Add 200µL mixture from above to plate reader** | |
| **Plant extract** | 12µL |
| **Buffer assay** | 200µL |
| **Bovine Liver Cytosol** | 20µL |

*Assay without plant was read as a control.

### 2.2.9. The Effect of plant extract on Superoxide Dismutase (SOD) Enzyme Activity:

The principle of SOD activity is based on the inhibition of nitroblue tetrazolium (NBT) reduction by using the method of Isgor, B.S., (2013).Since the reduction of NBT results from the action of superoxide radicals to blue coloured formazan, the absorbance was carried out at 550 nm, the concentrations of plant extract are the as used in GST and CAT assay.

**Table 6:** Concentrations of plant extract that used in SOD assay

| Dilution s | Stock plant Concentrations (mg/mL) | Final plant concentrations (mg/mL) |
|---|---|---|
| **1** | 71.8 | 3.59 |
| **2** | 23.9 | 1.195 |
| **3** | 7.9 | 0.395 |
| **4** | 2.66 | 0.133 |
| **5** | 0.89 | 0.044 |
| **6** | 0.29 | 0.014 |
| **7** | 0.098 | 0.004 |

**Table 7:** Reaction components in SOD assay

| Reagents | Volumes(µL) |
|---|---|
| **Assay Buffer** | |
| **200mM sodium carbonate buffer (pH10)** **10 mM EDTA+BSA** | 75 µL |
| **25mM NBT** | 3 µL |
| **0.3 mM xanthine** | 150 µL |
| Take 213 µL from mixture above | |
| **Plant extract** | 15µL |
| **Cytosol SOD** | From stock directly 10µL |
| **DDW** | 72µL |
| **XOD** | 5µL |
| Incubation for 40 minutes and read at 550nm | |

*the blank was reaction mixture with SOD

*As control reaction mixture with XOD without SOD

*The last one reaction mixture without SOD, XOD

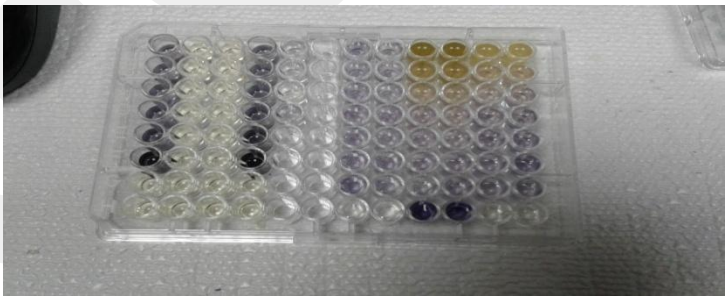


**Figure 2.3:** microplate reader show the reaction of SOD assay started after 40min.

### 2.2.10. The Effect of *Stachys cretica* on Catalase Enzyme Activity:

This method used for studying the CAT enzyme activity. This assay count on the measuring the hydrogen peroxide substrate remaining when the action of the enzyme was stopped by sodium azide, and the remaining H2O2 was determined by colorimetric method that counts on the forming of red quinonimine dye (Aebi, 1984); (Bai et al., 1999); (Fossati et al., 1980). The assay was miniaturised for micro plate application (Isgor et al., 2008) and the absorbance was read at 520 nm.

**Table 8:** Concentrations of plant extract that used in CAT assay

| Dilution s | Stock plant Concentrations (mg/mL) | Final plant concentrations (mg/mL) |
|---|---|---|
| **1** | 71.8 | 2.872 |
| **2** | 23.9 | 0.956 |
| **3** | 7.9 | 0.316 |
| **4** | 2.66 | 0.106 |
| **5** | 0.89 | 0.035 |
| **6** | 0.29 | 0.011 |
| **7** | 0.098 | 0.003 |

**Table 9:** The reaction components that used in a single reading of CAT assay.

| Reagents | Amount(µL) |
|---|---|
| Chromogen in total amount of 75mL | |
| **4 amino antipyrine (4 AP),1.25 mM** | 15mL |
| **Dichloro Hydroxyl Benzene Sulfonic Acid (DHBS), 10 mM** | 15mL |
| **Phosphate buffer,150 mM** | 45mL |
| **For each 75 mL chromogen, 7.5 µL HRP was added** | |
| **Assay mixture** | |
| **Phosphate buffer,50mM** | 26 µL |
| **Plant extract** | 4 µL |
| **100.1U/ml Catalase** | 20 µL |
| **Hydrogen peroxide, 10 mM** | 50 µL |
| **Phosphate buffer, 50 mM** | 26 µL |
| **Incubation for 2 minutes** | |
| **Sodium azide, 15 mM** | 50 µL |
| **Incubation for 5 minutes** | |
| **Above mixture +Chromogen** | 5 µL+255 µL |

*the reaction mixture without enzyme was read as $H_2O_2$ blank (enzyme control).

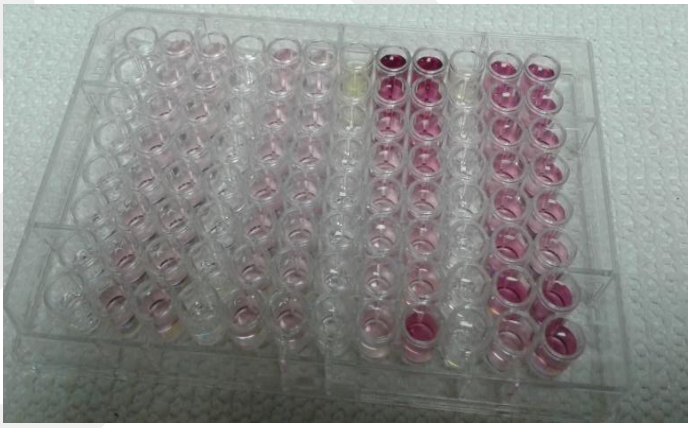


**Figure 2.4:** Microplate reader shows the reaction of CAT assay started after 40min. The colour change because of chromogen

All enzymes assay GST, SOD, CAT were done by using multi-mode plate reader.

**Figure 2.5:** Multimode plate reader (Molecular Devices Spectramax M2) and its 96 well plate.

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1. Extraction method for leaves of *stachys cretica* plant:

Extraction protocol was followed to extract the leaves of the plant sample by methanol extraction. The yield and concentrations were in Table 10.

**Table 10:** Concentration and Yield % of leaves of *stachys cretica* plant extract

| Plant Extract | Yield % | Concentrations of Extracts (mg/mL) |
|---|---|---|
| **Methanol Extract** | 23.06% | 31.96 |

## 3.2. Determination of Total Phenolic Content and Total Flavonoids Contents:

The TPC and TFC are determined in leaves of plant extracted in methanol are (TPC) 70.8794mg/g and (TFC) 73.216mg/g. The total phenol and flavonoid contents of methanol extracted plants are given in Table11.

**Table 11:** The total phenol and flavonoid contents of methanol extracted plants.

| Plant extract | Total Phenolic Contents (mg/g)* | Total Flavonoid Contents (mg/g)** |
|---|---|---|
| **Methanol Extract** | 70.8794 | 73.216 |

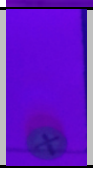
*Total phenolic contents evidenced as mg of gallic acid equivalent/g of extract

**Total flavonoid contents evidenced as mg of quercetin equivalent/g of extract

### 3.3. Determination of Total Phenolic Content and Total Flavonoids Contents for fractions from column chromatography:

As result for extract the leaves of the plant to their fractions, TLC separation was executed by using silica coated plates. After checking the fractions that acquired from column chromatography separation by using TLC six fractions were collected show different spot on TLC plate depend on the purity of them and total phenolic content,total flavonoids content were measured by using microplate 96wells and the results are given in Table 12.

**Table 12:** Total phenolic content and total flavonoids content, were measured by using microplate 96wells and TIC image.

| | TPC | TFC | D.W. | Total vol. | TLC-UV at 366nm | $IC_{50}$ GST | $IC_{50}$ SOD | $IC_{50}$ CAT |
|---|---|---|---|---|---|---|---|---|
| **Sample** | 70.869 | 73.216 | 0.4794 | 15ml | | | | |
| **Fraction I** | **ND** | 3.855 | 0.0028 | 2.5ml |  | - | - | - |
| **Fraction II** | **ND** | 5.135 | 0.0014 | 2.5ml |  | - | - | - |
| **Fraction III** | **ND** | 3.579 | 0.0254 | 2ml |  | - | - | - |
| **Fraction IV** | **ND** | 6.388 | 0.048 | 2ml |  | - | - | - |
| **Fraction V** | **ND** | 4.706 | 0.0233 | 3ml |  | - | - | - |
| **Fraction VI** | **ND** | 72.296 | 0.1795 | 2.5ml |  | 2.639 | 0.7230 | 0.1474 |

Since the fraction 6 has the highest flavonoid content, all further analysis was followed by using it.

### 3.4. The Effect of *stachys cretica* on Glutathione-S- Transferase (GST) Enzyme Activity:

Liver bovine cytosol (0.928 mg protein /mL) was applied as the source for the GST enzyme. The reaction components without plant extract were taken as control and it has maximum enzyme activity between 0.0023 and 0.0024 Micro mole/min.µL. The effect of *stachys cretica* plant on GST enzyme activity towards its substrate is given in Figure 3.2. The figure showed that the enzyme activity was inhibited by seven different concentrations of leaves extract. Since Effect of *stachys cretica* plant on glutathione-S-transferase enzyme activity has 100% inhibitory effect with respect to control, and $IC_{50}$ was 2.639 g/L.

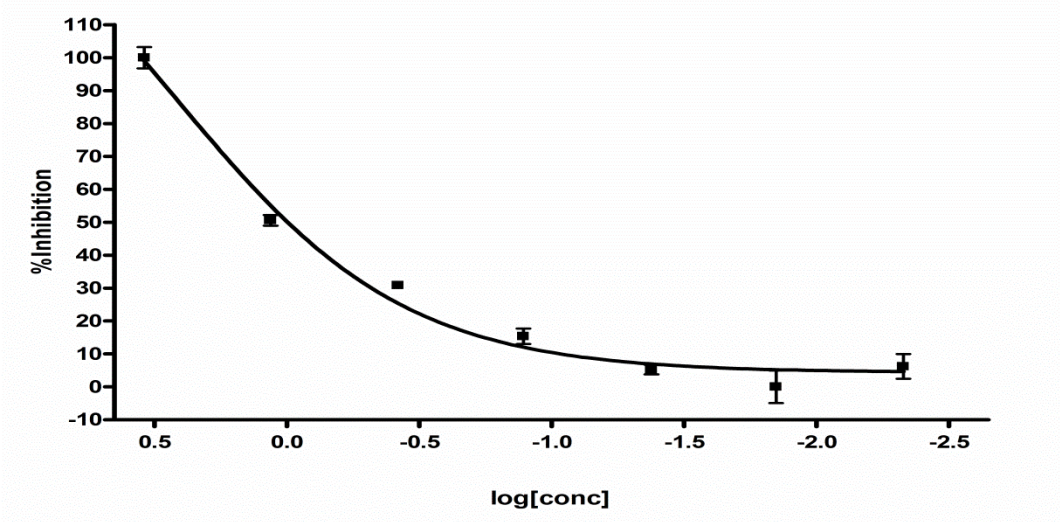



**Figure 3.1:** Effect of leaves of *stachys cretica* plant on glutathione-S-transferase enzyme activity 100 % inhibitory effect with respect to control.

### 3.5. The Effect of *stachys cretica* on Super Oxide Dismutase (SOD) Enzyme Activity:

The bovine erythrocytes (4807u/mg) Sigma Aldrich was applied as the source for superoxide dismutase enzyme assay and seven different concentrations of the plant were used to measure the effect of the extract on enzyme activity as it was mentioned under Method Table 7.

The effect of *stachys cretica* plant on superoxide dismutase activity was given in (Figure3.2).

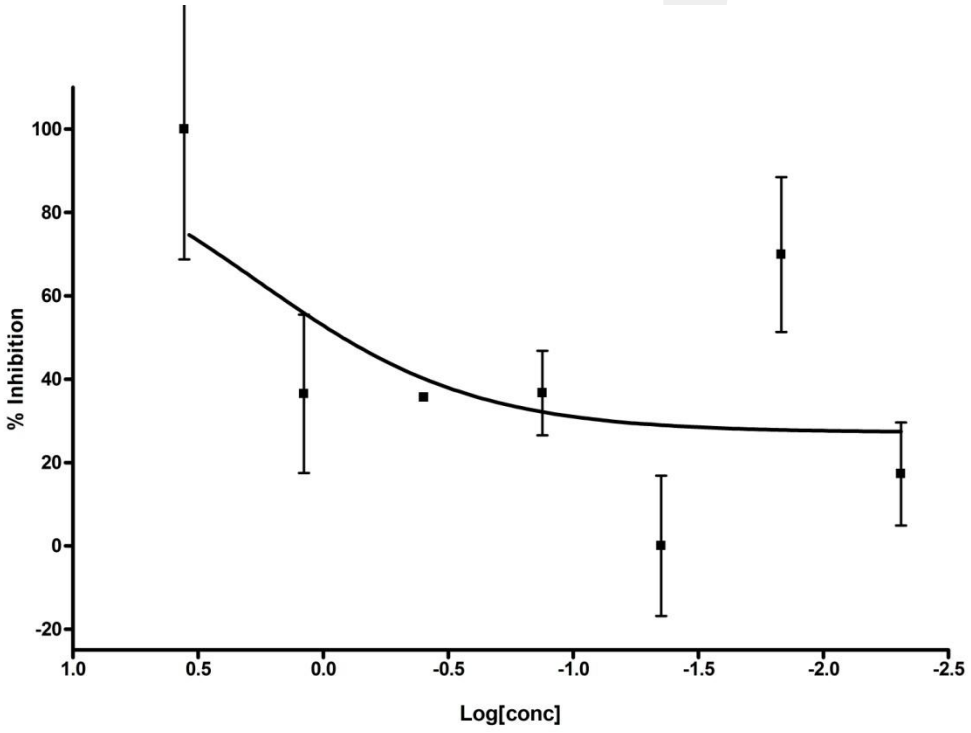



**Figure 3.2:** Effect of *stachys cretica* on superoxide dismutase enzyme activity with 80 % inhibitory effect with respect to control with $IC_{50}$ was 0.7230 g/L.

### 3.6. The Effect of stachys cretica on Catalase (CAT) Enzyme Activity:

The commercially available catalase enzyme was used in the assay which was purchased from Sigma Company and hydrogen peroxide as the substrate. In the assay,

seven concentrations of plant extract were used to measure the effect of the plant on catalase enzyme activity which was mentioned under Method Table 9.

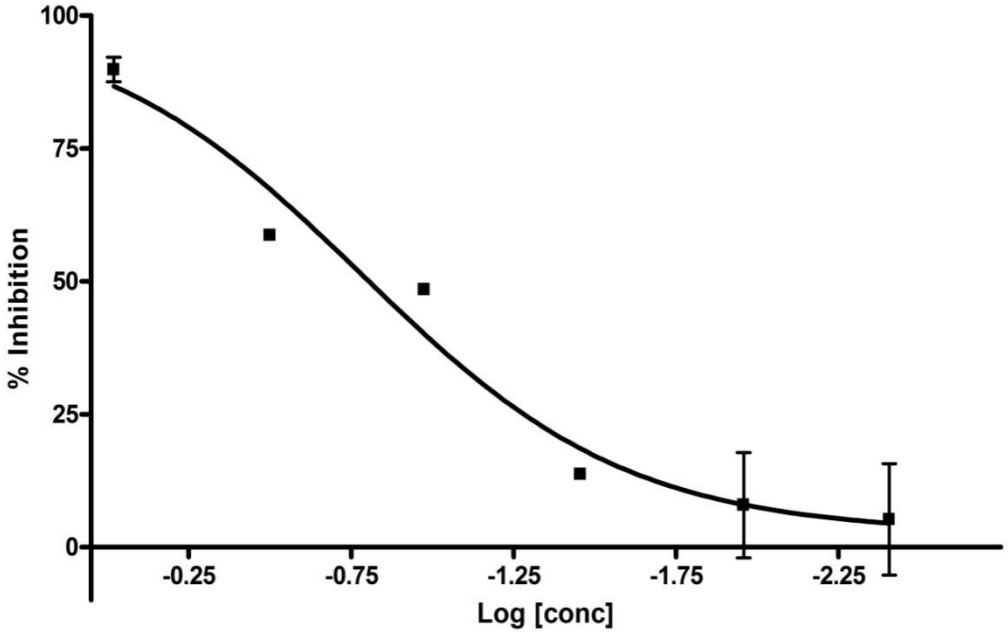



**Figure 3.3:** Effect of *stachys cretica* on catalase enzyme activity less than 90% the inhibitory effect with respect to control and $IC_{50}$ was 0.1474 g/L.

The data analysis was used with the Graph Pad Prism 6.0 program (Graph Pad Software, San Diego, CA). The kinetic results were expressed as inhibition of enzyme activity with respect to control (inhibition as % of control).

# DISCUSSION

Recently, plants play an important role in human life due to their medicinal properties (Askun et al., 2009), they were estimated in antifungal activity, antioxidant activity and anti-inflammatory activity (Digrak et al., 2001), by using different part from the family of stachys cretica in extract preparation, contain bark, leaves, root and flowers, employed in many biological activity studies (Rukayadi et al., 2009).

In the literature, there are plenty of researchers on phenolic and their biological activities (Guzman et al., 2010). Phenolic compounds obtained from plant extracts show great variety, with at least 8000 different structures (Bravo, 1998). Chun et al. (2005) reported that high phenolic and antioxidant activity was related to high antimicrobial activity against (Chen et al. (2011).

In this study, methanol extraction method was used to extract the phenolic and flavonoid contents from *stachys cretica* which were collected from Ankara region. The total phenolic content was (70.869 mg/g) of Gallic acid equivalent of the dry weight of extract, and it also has the total flavonoid content (73.216 mg/g) of quercetin equivalent of the dry weight of the extract.

In the literature, Marrubium peregrinumL amember of Lamiaceae family, general flovonoid and phenolic contents could only be compared with *Stachys Cretica* was extracted for the first time by using methanol extracts (Lluvia et al., 2014).

Plants have constantly been an exporter of a huge series of secondary metabolites with potential pharmacological properties (Russell and Duthie, 2011). Polyphenolic (flavonoids) compounds place ubiquitously in foods of plant origin have many useful

health effects due to their potential antioxidant, anti-inflammatory and cancer-preventive activities (Li et al., 2014).

In the literature, effects of synthetic antibiotics, isolation, purification and characterization of novel types of plant secondary metabolites might be a safer alternative to synthetic compounds (Bajpai and Kang, 2011). Column chromatography is one of the most common and traditionally used separation methods to characterize both organic, inorganic materials and isolation of biologically active secondary metabolites from plant sample (J.B. Harbone, 1993).

In this study, demonstration of fractionation and isolation of biologically active leaves of stachys cretica using column-chromatographic techniques, we obtained six fractions each fraction checked by TLC chromatography is most widely used in the separation of natural products (Gurib-Fakim, A. (2006). TLC especially used for check purity of isolated compounds and each fraction has different spot on TLC plate.

The total phenolic content for fraction was tested also in this study but it was taken away because it did not determine, the total flavonoid content was determined for all fraction (F1-F6) and the last fraction has the highest flavonoid content, all further analysis was followed by using it.

In general, there were not researches that studied the effect of the plant on the antioxidant system, where they studied the level of antioxidants inside the plant itself, in the literature, Thymus hirtus sp. Algerians are Boiss. Et Reut belongs to Lamiaceae family (Guesmi et al. 2014) showed the levels of antioxidants and is applied in folk medicine effect an estimate by important antioxidant activity, in this study the effect of leaves extract was shown on the antioxidant enzyme. 100% inhibition effect on the glutathione-S-transferase enzyme, 80% inhibition effect on superoxide dismutase enzymesand less than 90% inhibition effect on catalase enzyme.

# CONCLUSION

Curative plants are important natural materials in food, cosmetic, and pharmaceutical industries due to their several biological activities such as antioxidant, antimicrobial, anticancer and antiinflammatory. In this study plant leaves extracted by using methanol extraction .The total phenolic and flavonoid contents of leaves extract was determined, all assays were done by using this extract.

At first of this research was the preparation and extraction of the collected plant, extraction yields for leaves of plant were gained after a standardized solvent extraction protocol. The extraction yield was gained as 23.06%.

Secondly, the total phenolic contents and total Flavonoid contents of extracts were determined as TPC (70.8794 mg/g), TFC (73.216mg/g), Since bioactive compounds take place in plant material consist of multi-component mixtures, in particular most of them have to be courteous by the combination of many chromatographic techniques and several other purification methods to isolate bioactive compound such as CC and TLC.

The experimental results in this study showed that the inhibition effect of leaves of *S.cretica* on GST enzyme was 100%; it is advantages to use these antioxidant properties together with chemotherapeutics to avoid the drug resistance. On the other hand, the 80% inhibition effect on superoxide dismutase and less than 90%inhibition on catalase peroves the antioxidant potential of the extract and can protect the body from oxidative damage.

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