THE ISOLATION AND ANALYSIS OF THE MEDICINAL POTENTIAL OF METHANOLIC EXTRACT OF THE STACHYS CRETICA L. FLOWERS

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BY

MALIKA KHALIFA LATRESH

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THE ISOLATION AND ANALYSIS OF THE MEDICINAL POTENTIAL OF METHANOLIC EXTRACT OF THE STACHYS CRETICA L. FLOWERS

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

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ABSTRACT

THE ISOLATIONAND ANALYSIS OF THEMEDICINAL POTENTIAL OF METHANOLIC EXTRAC OF THE STACHYS CRETICA L. FLOWERS

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Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radicals. A variety of antioxidants exits within the body and many of them are derived from dietary sources as fruits, vegetables and plants. The plants are utilized in folkloric medicine to treat many diseases.

In this study, plant flowers was extracted with methanol and its total phenolic content (TPC) was measured as 25.32 mgGAE/g of extract and total flavonoid content (TFC) was measured as 29.60 mgQE/g of extract. This study utilized chromatography and was applied to separate extracts in to six fractions. The fraction utilized in enzyme assays was due to its high TFC as72.78 mgQE/g . The Glutathione-S-transferase (GST), catalase (CAT), superoxide dismutase (SOD) activities were measured by using multi-mode plate reader (Spectra MaxM2).

The inhibitory effects of plant on Glutathione-S-Transferase enzyme (GST) activity was about 80% with IC_{50} value of 0.6001 g/L, on SOD it was about 100 % with IC_{50} value of 0.2909g/L. The effect of *Stachys Cretica* on Catalase inhibition 75% with IC_{50} of 0.03741g/L.

Keywords: Stachys C*retica* plant, antioxidant enzymes, Catalase(CAT), Superoxide dismutase (SOD), Glutathione-S-transferase (GST), chromatographic techniques, medicinal plants, free radicals.

ÖZ

STACHYS CRETICA L. ÇİÇEKLERİNİN METANOL ÖZÜTÜNÜN HAZIRLANMASI İZOLASYONU VE TIBBİ POTANSİYELİNİN ANALİZİ

MALIKA LATRESH

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Antioksidanlar vücudu serbest radikallerin zararlı etkilerinden koruyabilecek özelliklere sahip önemli bileşiklerdir. Vücutta çok farklı antioksidanlar mevcut olupbüyük bir kısmı meyve, sebze ve bitki kaynaklı olarak diyetle alınmaktadır. Bitkiler özellikle halkarasında pekçok hastalığın tedavisinde kullanılmaktadır.

Bu çalışmada *Stachys cretica* bitkisinin çiçek özütleri metanol çözücüsü kullanlılarak hazırlanmışve hazırlanan özüte toplam fenol ve toplam flavonoid madde içeriğine bakılmıştır.Toplam fenolik madde miktarı 25.32 mg gallik asit eşdeğeri/ml özüt , toplam flavonoid madde miktarı ise 29.60 mg quercetin eşdeğiri /ml özüt olarak hesaplanmıştır. Hazırlanan özüt silika kolon ile ayrıştırlmış ve bu ayrıştırmanın sonunda 6 farklı fraksiyon elde edilmiştir. Bu fraksiyonlar arasında enyüksek flavonoid madde miktarı ile (72.78 mgQE/ml özüt) en son fraksiyon daha sonraki enzim deneylerinde kullanılmıştır.

Bitki özütlerinin glutathione-S-transferaz (GST), catalaz (CAT), superoxide dismutaz (SOD) enzim aktiviteleri üzerine etkisi multimod plaka okuyucu (Spectra MaxM2) ile tayin edilmiştir.

Bitki farksiyonu GST enzim aktivitesini & 80% oranında engellemiş ve bitki özütü için IC_{50} değeri 0.6001 g/L olarak hesaplanmıştır. Yine bitki fraksiyonu kontro ile kıyaslandığında Superoxide dismutaz (SOD) aktivitesini %100 engellemiş ve bitki fraksiyonun IC_{50} değeri SOD enzimi için 0.2909g/L olarak hesaplanmıştır. *Stachys Cretica* özütü kolon fraksiyonunun Katalaz enzimi üzerinde etkisi çalışması göstermiştir ki bitk özütü enzim aktivitesini 0.03741g/L IC_{50} değeri ile %75 engellemiştir.

Anahtar Kelimeler: *Stachys cretica* bitkisi, antiokidan enzimler, Katalaz (KAT), Superoksit dismutaz (SOD), Glutathione-S-transferaz (GST), kolon kromatografisi tibbi bitkiler, serbest radikaller.

To My Husband

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I would like to express my sincere and my thanks to God.

I would like to express my sincere gratitude to my thesis supervisor Assoc. Prof. Dr. Belgin İsgor 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.

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LIST OF ABBREVATIONS

SOD	-	Superoxide Oxide Dismutase
CAT	-	Catalase
GSH	-	Reduced Glutathione
GST	-	Glutathione-S-Transferase
CDNB	-	1-chloro-2,4-dinitrobenzene
HRP	-	Horse Reddish Peroxidase
DHBS	-	Dichlorohydroxy Benzene Sulfonic Acid
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
IC50	-	The half maximal inhibitory concentration
ROS	-	Reactive Oxygen Species
DDW	-	Double Distilled Water
TLC	-	Thin Layer chromatograph
EA	-	Ethayc Acetate

CHAPTER I

INTRODUCTION

Herbal medicine have been used as solution ubiquitously in medicinal services especially in developing countries according to World Health Organization (WHO).Around 65%-80% of the total population which lives in developing countries depends basically on plants for essential human services (Krishnamurthy and Wadhwani, 2012). Since the mid of the nineteenth century, diverse classes of bioactive mixes have been isolated and described. A large portion of these are utilized as the dynamic elements of present day medications, or as the lead mixes for new medications revelation (Soleimani et al, 2013). A few plants inferred medications are rich in phenolic mixes, flavonoids, alkaloids, tannins and so forth, utilized as a part of the treatment of various degenerative diseases. Plant-determined mixes have assumed an imperative part in treating and anticipating human diseases.

These are essential hotspots for new medications and great lead mixes reasonable for further adjustment of medication advancement to find new enzymes inhibitors (Ata et al, 2007). These chemical inhibitors might be utilized as operators to enhance chemotherapy or to treat various infections. Via reducing exposure to free radicals and expanding the intake of antioxidant enzyme rich diet or cell reinforcement compound supplements, our body's capability to diminish the danger of free radical-related medical issues are made more discernable(Krishnamurthy and Wadhwani, 2012).Cancer prevention agents are, in this manner, completely basic for keeping up ideal cell and systemic wellbeing and prosperity.

Numerous researchers have concentrated on restorative and consumable plants to find regular cancer prevention agents. The utilization of cancer prevention agents

may lessen the movement of Alzheimer's disease (AD) and limit neuronal degeneration.

1. 1. Stachys Cretica Lamiaceae (Flowers)

Stachys (Lamiaceae) as given in Figure 1.1a and Figure 1.1b, the large variant that incorporates in the vicinity of 275 and 300 species (Behravan et al., 2006). This class is primarily disseminated in subtropical and tropical areas of both sides of the equator (Piozzi et al, 2002). In Algeria, it is spoken to by 14 species including the endemic species of *Stachys Cretica*. *Stachys Cretica* Lamiaceae (figure1.1) is utilized as a part of people medication to treat genital tumors, sclerosis of the spleen, fiery illnesses, hack and ulcers, fevers, looseness of the bowels, sore mouth and throat, inside draining and shortcomings of the liver and heart (Soleimani et al., 2013).

1. 2. Classification of stachs cretica

Kingdom: Plantae

Division: Angiosperms

Class: Eudicots

Order: Lamiales

Family: Lamiaceae

Sub Family: Lamioideae

Genus: Stachys

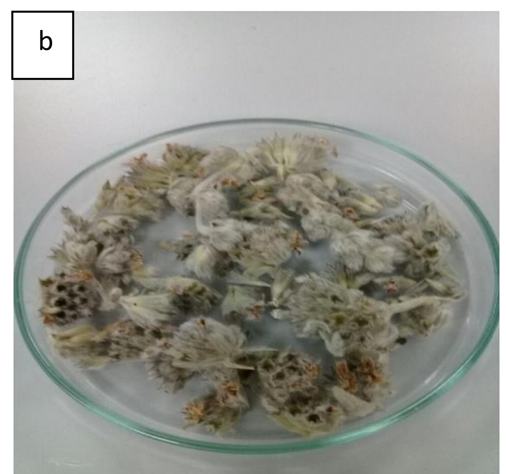

Figure. 1. 1. Structure of *Stachys Cretica L.* Flower (A) and dried *Stachys Cretica L.* Flower (B)

1.3. Antioxidant enzyme

During typical metabolic capacities, very receptive mixes called free radicals are created in the body; in any case, they may likewise be presented from nature. They respond with cell particles, for example, enzymes, lipids and starches, and denature them. Along these lines, imperative cell structures and capacities are lost and eventually bringing about different neurotic conditions (Percival, 1998).

Cancer preventing compounds are fit for balancing out, or deactivating free radicals before they assault cell segments (Percival, 1998). They act by reducing the life time of the free radicals or by surrendering some of their electrons for its utilization. Likewise, they may hinder with the oxidizing anchor response to limit the harm created by free radicals. It has been found that a considerable connection exists between free radicals and more than sixty distinctive healthcare conditions, including the maturing process, tumor, diabetes, Alzheimer's ailment, strokes, heart assaults and atherosclerosis (Percival, 1998).

1.3.1. Free Radical

Free radicals are electrically charged particles, i.e., they have an unpaired electron, that can attack to molecules of living organisms like proteins and nucleic acids.

The capacity of the cell to utilize oxygen has furnished people with the advantage of processing fats, enzymes, and starches for vitality; in any case, it doesn't come without cost. Oxygen is a profoundly responsive particle that can turn out to be a piece of possibly harming atoms normally called free radical or reactive oxygen species (ROS). Around at least 5% of the O_2in breathed air is changed over to ROS, such as superoxide, hydrogen peroxide, and hydroxyl radicals. Subsequently, cells under oxygen consuming conditions are constantly weakened with the affront of ROS, which are productively dealt with cancer prevention agent frameworks of the cells. This cancer prevention agent framework incorporates, cell reinforcement compounds (e.g., SOD, GST, CAT, and so forth.), cancer prevention agents (e.g., ascorbic corrosive, tocopherols and tocotrienols, carotenoids, glutathione and lipoic corrosive), metal restricting enzymes (e.g., ferritin, lactoferrin, egg whites, and aceruloplasminemia) and various other cancer prevention agent such as phytonutrients (Halliwell, 1999).

1.3.2. Reactive Oxygen Species

Reactive oxygen species (ROS) is a term that incorporates, oxygen-containing particles, including free radicals. Sorts of ROS incorporate the hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and different lipid peroxides. All of them are fit for responding with lipid layers, nucleic acids, enzymes and chemicals, and other little particles, bringing about cell harm. The majority of the oxidants delivered by cells are consumed by the mitochondrial electron transport system as a consequence of normal aerobic metabolism. Approximately 90% of the oxygen utilized by the cell is consumed by this way and by xenobiotic metabolism i.e., detoxification of toxic substances.

Reactive oxygen species can attack indispensable cell components like polyunsaturated and unsaturated fats, enzymes, and nucleic acids and sugars too. These responses can modify properties like particle transport, loss of catalyst movement, enzymes blend, DNA harm; at last bringing about cell passing (Bandyopadhyay et al., 1999) (Figure 1.2).

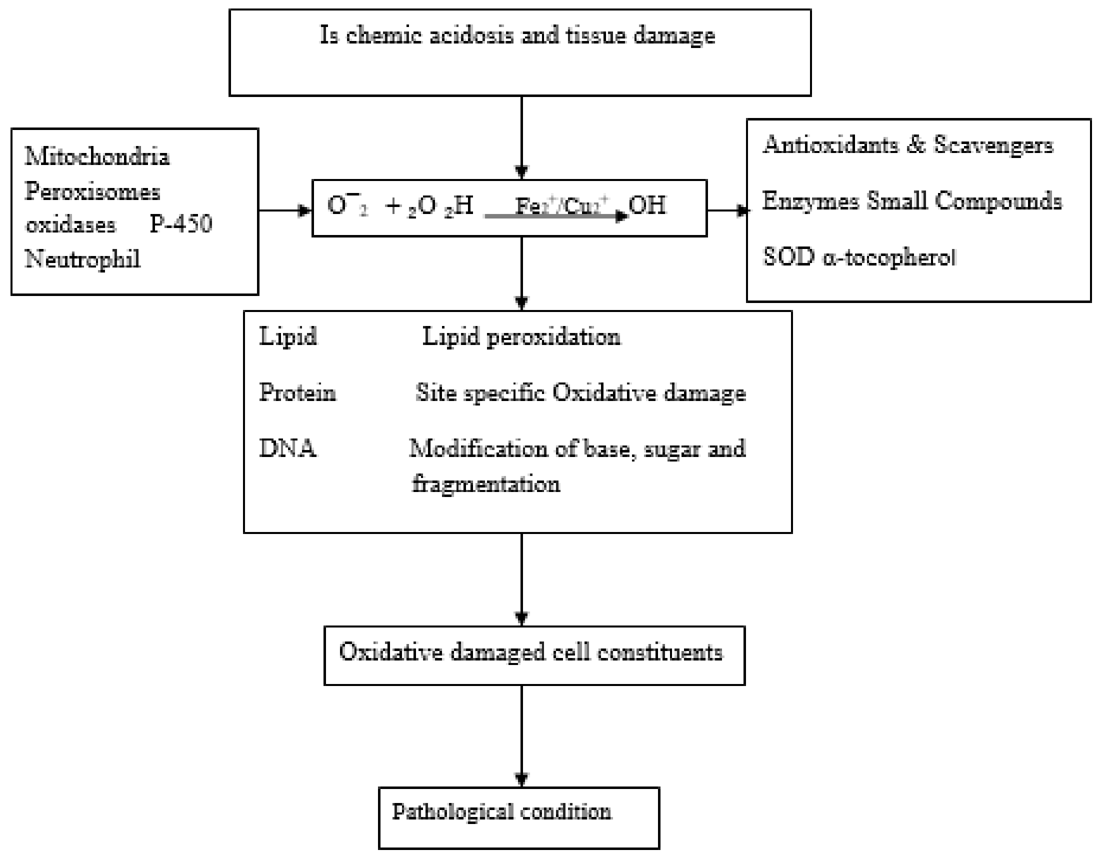

Figure. 1.2. An overall presentation of the ROS metabolism of oxidative tissue damage leading to pathological conditions.

1.3.3. Antioxidant protection system

To protect the cells and organ systems of the body against reactive oxygen species (ROS), humans have evolved a highly sophisticated and complex antioxidant protection system (Percival, 1998). It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals (Table 1.1.). These components include;

a. Endogenous Antioxidants

- Bilirubin
- Thiols, e.g., glutathione, lipoic acid, N-acetyl cysteine
- NADPH and NADH

- Ubiquinone (coenzyme Q10)
- Uric acid
- Enzymes

b. Dietary Antioxidants

- Vitamin C
- Vitamin E
- Beta carotene and other carotenoids and oxycarotenoids, e.g., lycopene and lutein.
- Polyphenols, e.g., flavonoids, flavones, flavonol's, and Proanthocyanidins.

c. Metal Binding Proteins

- Albumin (copper)
- Ceruloplasmin (copper)
- Metallothionein (copper)
- Ferritin (iron)
- Myoglobin (iron)
- Transferrin (iron)

Table 1.1: Various ROS form and their corresponding neutralizing antioxidants.

ROS	NEUTRALIZING ANTIOXIDANTS
Hydroxyl radical	Vitamin C, Glutathione Flavonoids, Lipoic acid
Superoxide radical	Vitamin C, Glutathione, Flavonoids, SOD
Hydrogen peroxide	Vitamin C, Glutathione, beta carotene, Vitamin-E, flavonoids, lipoic acid
Lipid peroxides	Beta-carotene, Vitamin-E, Ubiquinone, flavonoids, Glutathione peroxidase

1.3.3.1. Antioxidant enzymes in health

1.3.3.1.1. Super peroxide dismutase (SOD)

In 1967 biochemist chemist Irwin Fridovitch of Duke University and Joe McCord found the cancer prevention agent enzymes SOD (Figure 1.3), which gives an imperative method for cell guard against free radical harm. This leap forward made therapeutic researchers start to take a gander at free radicals. Much of the time the procedure is naturally controlled and the quantity of free radicals does not turn out to be hazardously high (Li et al., 1995). Turf (EC 1.15.1.1) is the cell reinforcement enzymes that catalyzed the debate of the exceedingly responsive superoxide anion to O_2 and to the less receptive species H_2O_2. Peroxide can be obliterated by CAT or GPX responses (Li et al., 1995).

In humans, there are three forms of SOD: cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD (EC-SOD). SOD destroys O^{-}_2 by successive oxidation and reduction of the transition metal ion at the active site in a Ping Pong type mechanism with remarkably high reaction rates (Li et al., 1995).

SOD is found in our skin and it is fundamental all together for our body to produce satisfactory measures of skin-building cells called fibroblasts. Among the normal natural sources of SOD are cabbage, brussels grows, wheat grass, grain grass and broccoli. Grass assumes a critical part in keeping the improvement of the Lou Gehrig's infection (Andersen et al.,1995).

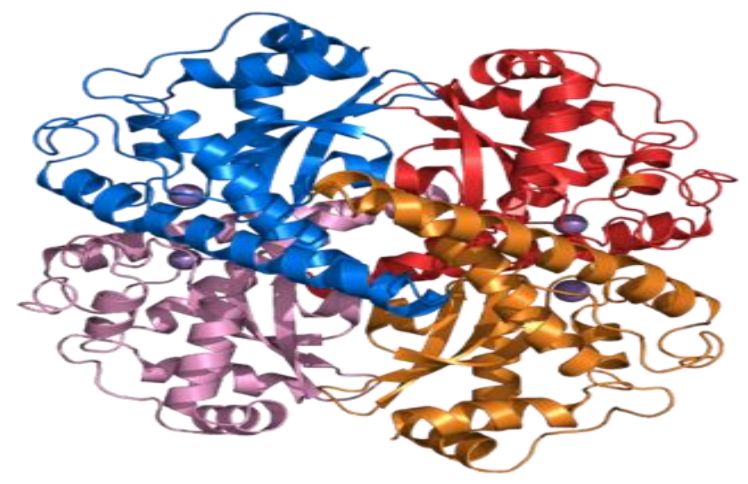

Figure. 1.3. 3D presentation of human superoxide dismutase.

1.3.3.1.2. Catalase (CAT)

Catalase (CAT) is an enzyme in charge of the corruption of hydrogen peroxide. It is a defensive enzyme introduce in about every single creature cell. CAT (EC 1.11.1.6) is a tetrameric enzyme comprising of four indistinguishable tetrahedrally organized subunits (Turrens et al., 1984). CAT responds productively with H_2O_2 to frame water and sub-atomic oxygen; and with H contributors (methanol, ethanol, formic corrosive, or phenols) with peroxidase action (Fridovich, 1998). CAT is utilized as a part of the nourishment business for expelling hydrogen peroxide from drain preceding cheddar generation. Another utilization is in nourishment wrappers where it keeps maintenance from oxidizing CAT is additionally utilized as a part of the material business (Fridovich, 1998).

1.3.3.1.3. Glutathione S-Transferase (GST)

Glutathione-S-transferases (GSTs) are enzymes that are vital in the detoxication of a wide range of xenobiotics in warm blooded creatures. The enzyme protect cells against toxicants by conjugating the thiolof the glutathione to electrophilic xenobiotics, and in this manner shield cells against the mutagenic, carcinogenic, and poisonous impacts of the mixes. GST action was found to be available in plants,

insects, yeast, microorganisms, and in most mammalian tissues, particularly in the liver, which assumes a key part in detoxification. (Harris et al., 1984).

The principle role of GST is to catalyze the conjunction of glutathione with electrophilic centers like nitrogen, sulfur or carbon particles and to kill the extra cellular chemicals including drugs. GSTs are arranged into 5 classes identified with their structure and substrates as alpha, (α), Mu (μ), Pi (π), Sigma (σ) and theta (θ). The dynamic sites in these compounds contain two sites called GSH-restricting site (G site) and xenobiotic-restricting site (H site) (Harris et al., 1984). GST comprises of 25-30 KDa subunits, which can be isolated into two areas, space N and space C appeared in the Figure 1.4.

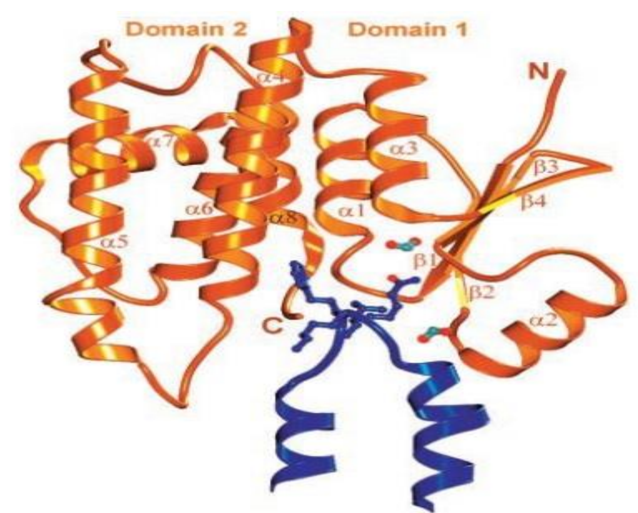

Figure.1.4. 3D presentation of glutathione S-transferase with its domains

1.3.3.2. Polyphenols

Polyphenols are phytochemicals found abundantly in plants have antioxidant properties. Organic products like grapes, apple, pear, fruits and berries contains up to 200–300 mg polyphenols per 100 grams' crisp weight. Ordinarily, some tea or espresso contains around 100 mg polyphenols. Polyphenols are auxiliary metabolites

of plants and are for the most part required in barrier against bright radiation or hostility by pathogens. More than 8,000 polyphenolic mixes have been distinguished in different plant species. Polyphenols may be classified into four different groups as a function of the number of phenol rings that they contain and based on structural elements that bind these rings to one another. The main classes include phenolic acids, flavonoids, stilbenes, lignans. (Manach et al., 2005) (Figure 1.5).

Figure.1.5. General Structure presentation of polyphenol.

Phenolic acids are secondary metabolites of plants and they have protection potentials against oxidative damage. Phenolic acids contain phenolic ring and at least one carboxylic acid function and they are separated into two classes: hydroxybenzoic acid and hydroxycinnamic acid derivatives. The hydroxycinnamic acids are more typical than hydroxybenzoic acids and comprise of coumaric, caffeic, ferulic (Parr and Bolwell, 2000).

Flavonoids include the most considered group of polyphenols. This group has a typical structure of having aromatic rings bound together by three carbons (Gróf et al., 2005). More than 4,000 assortments of flavonoids have been recognized, a large portion of which oversee the appealing shades of the blossoms, foods grown from the ground. In view of the variety in the kind of heterocycle included, flavonoids might be separated into six subclasses: flavanols', flavones, flavanones, flavanols,

anthocyanins and flavones (Crozier et al., 1997). The flavonoids have a critical role considering their potential on human wellbeing, they have been accounted for antiviral, antitumor, anti-inflammatory, and antioxidant activity.

1.4. Column chromatography

Column chromatography is one of the most useful techniques for purifying compounds. This technique utilizes a stationary phase, which is packed in a column, and a mobile phase that passes through the column. This technique uses the differences in polarity between compounds, allowing the molecules to be easily separated. The most common stationary phases for column chromatography are silica gel (SiO_2), with the most commonly utilized mobile phases being organic solvents. The solvent(s) chosen for the mobile phase are dependent on the polarity of the molecules being purified. Typically, more polar compounds require more polar solvents in order to facilitate the passage of the molecules through the stationary phase. Once the purification process has been completed the solvent can be evaporated to yield the isolated material (Sasidharan et al., 2011).

1.4.1. Tin- Layer Chromatography (TLC)

TLC is a simple, quick, and inexpensive procedure that gives the researcher a quick answer about the components in a mixture. Removed from the collected fractions using a rotary evaporator, TLC is also utilized to support the identity of a compound in a mixture when the Rf (Retardation Factor) of a compound is known. Additional tests involve the spraying of phytochemical screening reagents, which cause colour changes per the phytochemicals existing in a plant extract; or by viewing the plate under the UV light. This has also been utilized for confirmation of purity and identity of isolated compounds (Hang et al., 2004).

Thin-layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide (alumina), or cellulose. This layer of adsorbent is known as the stationary phase (Sasidharan et al., 2011).

1.5. The main objective of this study

The main objective of this study is to predict the medicinal use of Stachys *Cretica*by measuring the phenolic and flavonoid contents of methanolic extract of the plant, by analyzing free radical potential and its effect on the antioxidant defense system components such as the glutathione-S-transferase (GST), catalase (CAT), superoxide dismutase (SOD).

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Stachys cretica Flowers, Gallic Acid (Merk), Dimethyl Sulfoxide (DMSO) (Carloerba), Folin-Ciocalteus Phenol Reagent (Sigma-Aldrich), Sodium Carbonate (Fisher Scientific), Quercetin (Sigma), Absolute Ethanol (Merk), Sodium Acetate (AklarKimya), Aluminum Chloride (Merk), Methanol, Ethyelacetat (Sigma), Hexane, Mono potassium Phosphate and Di potassium phosphate (Riedel.dehaen), 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 (H_2O_2) (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 Plants 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.* Flowers Medicinal Plants

Flowers are dried at room temperature for one week and grounded into powder. For extracts, methanol was used as solvent and 1.5 g of grinding flowers were put into filter paper and the sample in filter paper put into the soxhlet with 150 ml of methanol for 24hours. After one day, the solvent was extracted at 40°C. The dried extract finally dissolved in 10 ml methanol and kept at -20°C for further analysis.

2.2.3. Determination of total Phenolic Content (TPC) of *Stachys Cretica L.* Flowers

Total phenolic contents of the plant material were measured by using Folin-Ciocalteu method. Since total phenolic content of the extract was measured by using calibration curve of standard Gallic acid which was prepared from different concentrations (50-300µg/mL) in DMSO the total phenolic content in the extract was expressed as mg of Gallic acid equivalent (GAE) per gram of plant extract solution. The blank includes DMSO instead of plant extract. The absorbance was measured at 750 nm, and total phenolic content of extract was measured by using calibration curve of the reaction components of the phenolic content given in Table 2.1.

Table 2.1: The reaction components of phenolic content determination assay

Reaction Components	Added Volumes
Methanol extract	100 µL from plant extract
Gallic acid	100 µL 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 hour in dark at room temperature	
Read at 750 nm	

2.2.4. Determination of Total Flavonoid Content (TFC) of *Stachys Cretica L.* Flowers

The total contents of flavonoids in 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 2.2. The total flavonoid contents of the extracts were expressed as mg of quercetin equivalent (QE) per gram of plant extract (mg/g QE).The blank includes Ethanol 80% instead of plant extract. Absorption was read at 415 nm.

Table 2.2: The reaction components of flavonoid contents determination.

Stock Component	Added Volumes
Methanol extract	250 µL from plant extract
Quercetin standard	250 µL from standard
95% ethanol	750 µL
10 % (w/v) Aluminium chloride 1M Sodium acetate DMSO	50µL 50µL 1 mL
Incubation for 30 minutes in dark	
Read at 415 nm	

2.2.5. Column Chromatography

Silica gel chromatography was usedin this study to separate the extracts into their fractions.Silica gel was the stationary phase and hexane:ethyl acetate solvent combination in various ratio (1:10) was used as the mobile phase. The 29.5 cm of diameter and 1.5 cm of height of column used and thedried plant extract sample of 0.3426 g was mixed with silica gel to make a fine poweder tol et the sample easily distributed in the column.The column was washed with miture (1:10) of hexane ethyleacetate and six separate fractions were collected. The the remaining sample were collected by washing the column with methanol as final fraction.Each fraction was collected in test tubes separately and checked by TLC, thin layer

chromatography with the same ration of solvent for the purity of fractions. All fractions from the first F1 to the last, F6 were evaporated by using rotary evaporator and dissolve with a small amount of methanol, saved under nitrogen gas and stored at -20°C.

2.2.6. Determination of Total Phenolic Content to the Fractions

Total phenolic concentration (TPC) was determined by Folin-Ciocalteu reagent as described previously (Chang et al., 2002). The protocol was optimized in our laboratory by using microplate application.
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%) Incubated for 5 minutes in dark after that 50 µL of a 2 % (w/v) sodium carbonate was added and the mixture was incubated an hour in dark. The absorbance was measured at 750 nm by using 96 well microplate. The total phenolic content in the extract was expressed as mg of Gallic acid equivalent (GAE)/mL of (F1-F6).

2.2.7. Determination of Total Flavonoid Content (TFC) to the Fractions

Total flavonoid concentration (TFC) was determined by using aluminium chloride colorimetric assay (Chatatikun and chiabchalard., 2013) with some modifications. In 96-well plates, 50 µL of fractions from F1 to F5 and 5µL from F6 diluted with 50µL and 95 µL methanol respectively were then mixed with 10 µL of 10% $AlCl_3$. Than 150 µL of 95% ethanol and 10µL of 1M Sodium acetate were added sequentially. After 30 minutes incubation in dark the absorbance of the mixture was measured at 415 nm.

2.2.8. Effect of *Stachys Cretica L.* Flower Extract on Glutathione S-Transferase (GST) Enzyme Activity

The bovine liver cytosol was utilized as GST enzyme source and the activity is measured by conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) according to (Habig and Jacoby, 1974). The concentration of plants and the reaction mixture were given in Table 2.3and 2.4.

Table 2.3: Concentrations of Stachys cretica extract that utilized in GST assay.

Dilutions	**Stock plant Concentrations (mg/mL)**	**Final Plant Concentrations in assay (mg/ml)**
1	56.325	2.708
2	18.775	0.903
3	6.258	0.301
4	2.086	0.100
5	0.695	0.033
6	0.230	0.011
7	0.077	0.004

Table.2.4: The components of assay mixture that utilized in GST enzyme assay.

Components	**Added Volumes**
100mM phosphate buffer (pH 6.5)	7mL
200mM GSH	150µL
50mM CDNB	450µL
Add 200µL mixture from above(Buffer assay) to plate reader	
Plant extract	12µL
200mM phosphate buffer (PH 6.5)	18µL
Bovine Liver Cytosol	20µL
Read at 340 nm	

2.2.9. The Effect of *Stachys Creitica L.* Flower Extract on Superoxide Dismutase (SOD) Enzyme Activity

The principle of SOD activity is based on the inhibition of nitroblue tetrazolium (NBT) reduction by using method of Isgor, B.S., (2013).The reduction of NBT is resulted from the action of superoxide radicals to blue colour formazan. The

absorbance was read at 550 nm. Table.2.5 and 2.6 represented the concentrations of plant extract in the assay and the reaction components of the SOD assay used in the study respectively. The color formation is shown in figure 2.1.

Table .2.5: Concentration of stachya cretica extract that utilized in SOD assay.

Dilutions	Stock Plant Concentrations (mg/mL)	Final Plant Concentrations in the assay (mg/ml)
1	56.325	2.816
2	18.775	0.938
3	6.258	0.313
4	2.086	0.104
5	0.695	0.035
6	0.230	0.012
7	0.077	0.004

Table 2.6: Reaction components in SOD assay mixture.

Reagents	Added Volumes
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
Commercial SOD	From stock directly 10µL
DDW	72µL
XOD	5µL
Incubation for 40 minutes and read at 550 nm	

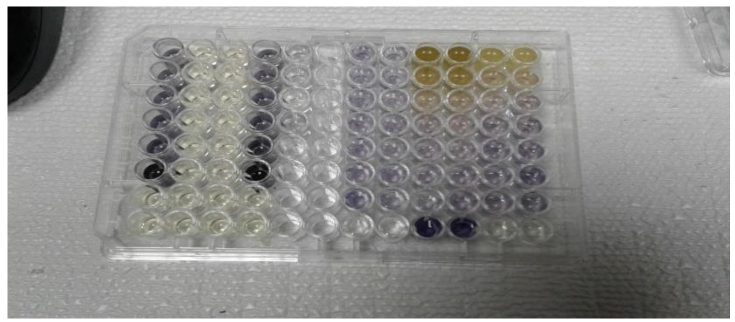

Figure.2.1. Microplate reader show the reaction of SOD assay plate after completion of reaction.

2.2.10. The Effect of *Stachys Cretica L.* Flower Extract on Catalase (CAT) Enzyme Activity

The assay depends on the measuring the remaining hydrogen peroxide substrate when the action of the enzyme was stopped by sodium aside, and the remaining H_2O_2 was determined by colorimetric method that count on the forming of red quinonimine dye (Aebi, 1984); (Fossati et al., 1980). The assay was miniaturized for micro plate application (Isgor et al., 2013) and the absorbance was read at 520 nm. The concentration of plant used in the assay and the reaction mixture compositions are given in Table 2.7 and Table 2.8 respectively. The color formation at the end of the CAT assay is shown in figure 2.2.

Table 2.7: Concentration of Stachya *cretica* extractused in CAT assay.

Dilutions	Stock plant Concentrations (mg/mL)	Final Plant Concentrations in assay (mg/ml)
1	56.325	2.253
2	18.775	0.751
3	6.258	0.250
4	2.086	0.083
5	0.695	0.027
6	0.230	0.009
7	0.077	0.003

Table 2.8: The reaction components that utilized in a single reading of CAT assay.

Reagents	Added Amount
Chromogen (in total amount of 75 mL)	
4 aminoantipyrine (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, 50 mM	26 µL
Plant extract	4 µL
Catalase 100 U/ml	20 µL
Hydrogen peroxide, 10 mM	50 µL
Incubation for 2minutes	
Sodium azide, 15 mM	50 µL
Incubation for 3 minutes	
Above mixture +Chromogen	5 µL+255 µL
Incubation for 40 minutes and read at 520nm	

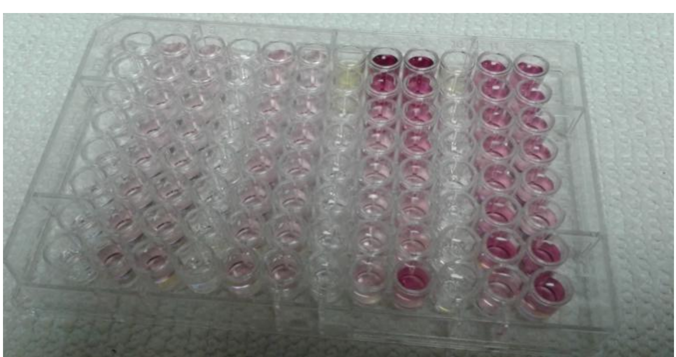

Figure.2.2: Microplate reader show the reaction of CAT assay plate after completion of reaction.

All enzyme activity measurements were completed by using multi-mode plate reader (Spectra maxM2) (Figure 2.3).

Figure. 2.3*:* Multi mode plate reader (Molecular Devices Spectramax M2) with Microplate (96-wells).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Plant extraction

The yield of 1.5 of *Stachys Cretica L.* Flowers was- determined by using following equation:

Yield %= weight of the extract / weight of plant *100

The yield and concentration of *Stachys Cretica* extract at the end of methanolic extract was calculated as 22.84% and 33.37 mg/ml respectively.

3.2. Column chromatography of Stachys Cretica extract L. Flower

Six different fractions were separated by column chromatography of *Stachys Cretica* extract. The fractions namely F1, F2, F3, F4, F5 and F6 were tested their purity by using TLC. The TLC results given in Table 3.2. Although in all fractions together with the original sample there were spots, the intensity and the places are different. The spots from original sample and from the F1 were very similar both in intensity and the place. In F2 the spots getting darker like F3 but in F4 and F5 it was seen from the TLC that one more spot was appeared and the spots were gone in F6. Due to these results the flavonoid content of each fraction were tested.

3.3. Total Phenolic (TPC) and Total Flavonoids Contents (TFC) of sample and their fractions of *Stachys Cretica L.* Flowers extract

Total phenolic content (TPC) and Total flavonoid content were determined in both sample and their fractions are given in (Table3.1). Although the TLC for fractions 1-6 ended up with spots the phenolic content could not determined from

any of the fraction. The flavonoid content measurements resulted that each fraction has very low level of flavonoid content (between 3.16-5.77 mg QE/g D.W.). The maximum value for the flavonoid content was measured in F6 as 72.78 mg QE/g D.W.

Table 3.1: TLC image, Total Phenolic Contents (TPC), Total Flavonoids Contents (TFC) and IC_{50} value of antioxidant enzyme assay of Stachys *Cretica L.* extract.

	Before column chromatography	After column chromatography					
	sample	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**
TPC mg(GAE)/g(D.W.)	25.23	ND	ND	ND	ND	ND	ND
TFC mg(QE)/g(D.W.)	29.60	3.25	5.44	3.16	5.77	3.52	72.78
D.W. (g)	0.3426	0.0282	0.0054	0.0092	0.0083	0.004	0.2253
Total vol.(ml)	10	3.5	2	2	2	1	4
IC_{50} SOD (g/L)	ND	ND	ND	ND	ND	ND	0.2909
IC_{50} GST (g/L)	ND	ND	ND	ND	ND	ND	0.6001
IC_{50} CAT (g/L)	ND	ND	ND	ND	ND	ND	0.03741
TLC (At 366nm)							

3.4. Glutathione-S-Transferase (GST) Enzyme Assay:

The bovine liver cytosol with the protein amount 0.290 mg /ml was used as a source for Glutathione-S-transferase enzyme assay. Glutathione-S-Transferase enzyme activity without Stachy C*retica* extract was taken as standard and it has a value 0.003921-0.003655 µmol/min/ml. The concentrations of Stachys *Cretica L.* used in the assay were given in (Table 2.3) under Method.

The result showed that the Glutathione-S-Transferase enzyme activity is inhibited by using seven different concentrations of Stachys *cretica L.* The Stachys *cretica L.* inhibited the Glutathione-S-Transferase enzyme activity about 80%, IC_{50} value was calculated as 0.6001 g/L. The effect of *Stachys Cretica* on Glutathione-S-Transferase activity was given in Figure 3.1.

The data analysis was applied 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).

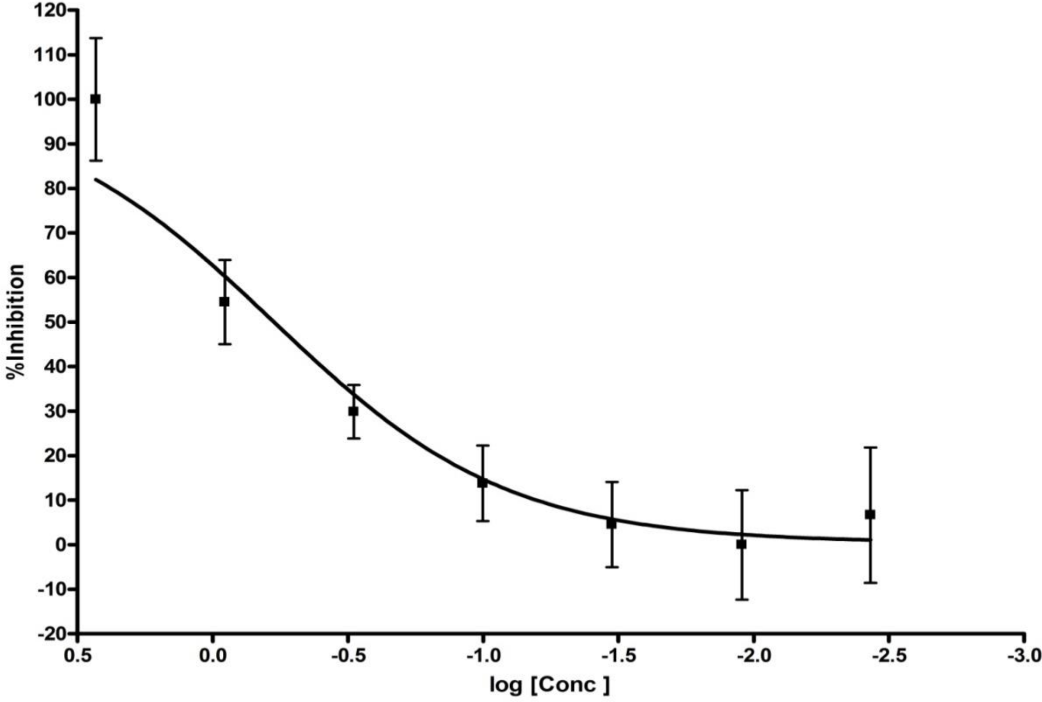

Figure.3.1: Effect of *Stachys Cretica L.* on Glutathione-S-Transferase enzyme activity with maximum 80% inhibitory effect with respect to control.

3.5. Superoxide Dismutase (SOD) Enzyme Assay:

The bovine erythrocyte with enzyme amount 4807u/mg was used as a source for SOD enzyme. Superoxide dismutase enzyme activity without Stachys *Cretica L.* extract was taken as standard and it has a value of 0.000160µmol/min/ml. Seven different concentrations of *the* Stachys *Cretica L.* extract were used to measure the effect of Stachy C*retica* extract on superoxide dismutase activity as it was mentioned under Method (Table 2.6). The Stachy *Cretica* inhibited the superoxide dismutase activity about 100 %, IC_{50} value was calculated as 0.2909g/L. The effect of Stachys *Cretica* on superoxide dismutase activity was given in Figure 3.2

The data analysis was applied 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).

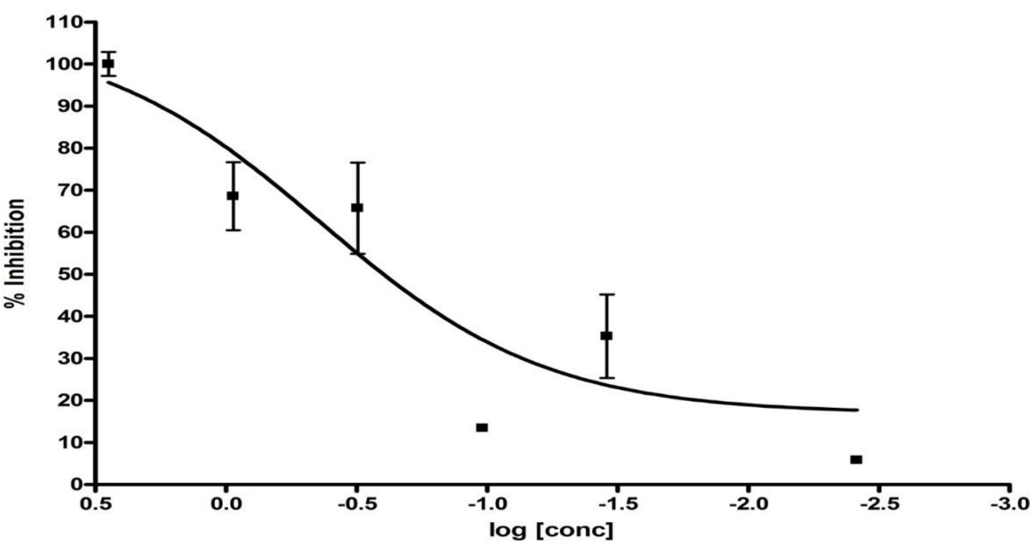

Figure. 3.2: Effect of *Stachys Cretica L. o*n superoxide dismutase enzyme activity with maximum 100% inhibitory effect with respect to control.

3.6. Catalase (CAT) Enzyme Assay:

The commercially available enzyme was used as a Catalase enzyme source. Hydrogen peroxide was used as substrate. The Catalase activity without plant extract was measured as control and has a value of 0.248703-0.107847 Mmol/min/ml. Seven different concentrations of Stachys *Cretica L.* extract were used to measure the effect of plant extract on Catalase activity as it was mentioned under Method (Table 2.8). The effect of Stachys *Cretica L.* on Catalase enzyme activity is given in Figure 3.3. and concluded that plant resulted less than 75% inhibitory effect with respect to control, with an IC_{50} of 0.03471g/L.

The data analysis was applied 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).

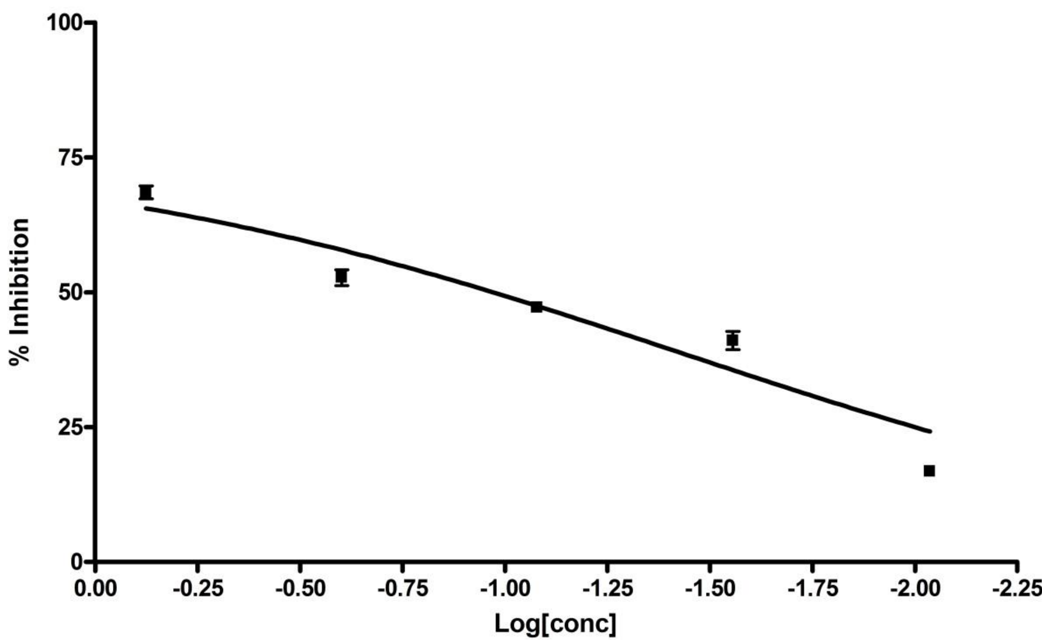

Figure3.3: Effect of *Stachys Cretica L.* on Catalase enzyme activity with approximately 70% inhibitory effect with respect to control.

DISCUSSION

Among the family of *Stachys Cretica L.,* distinctive plant parts, has been used in extract preparation, including their flower, bark, stem, root, leaves (Digrak et al.,.2001; Bouchra.et al., 2003; Tundis et al., 2014). To evaluate their medicinal value, so far they were evaluated in antibacterial, antifungal and antimycobacteriatic and chemically performed antioxidant (Alma et al., 2003).

The phenolic dissimilarities of plants has been reported (Estevinho.et al., 2008) and shown that chemically tested antioxidant activity is related with higher phenolic content of fractions analyzed (Bravo et al., 1998).

Among the solvents tested, it was found that highest phenolic content of the fractions are obtained with methanolic extraction methods (Guzman et al., 2010).By following such information provided in literature, the plant flowers were extracted in methanol.

Since it is the first time *Stachys Cretica L.* was extracted, general flavonoid and phenolic content could only be compared with Marrubium peregrinum, a member of Lamiaceae family.

Stachys Cretica L. TPC and TFC values were found consistent with current literature where 18-55 mg/g GAE and 27-49 mg/g QA was reported (Figuroa et al., 2014).

The column chromatography used to separate aqueous fractions of plant was reported (Thida Cho et al., 2013) with appropriate TLC analysis method.

By following this information, we collected 6 fractions with varying TPC and TFC values. Only the fraction with highest TFC was chosen for further evaluation for its possible antioxidant effect.

There were no reported on the effect of the plants against antioxidant system, however there were tests to define the antioxidant enzymes inside the plant itself. It was show that plants essential on contains SOD, GST at varying activating levels when this plant used in folkloric medicine, the effect assessed by significant antioxidant activity was defined with respect to the levels of GST, CAT and SOD of the plant.

For the first time, the effect of *Stachys Cretica L.* on the antioxidant system was measured in this study and the effect of plant extract was reported on the antioxidant enzymes.80% inhibition effect on the glutathione-s-transferase enzyme, 100% inhibition effect on superoxide dismutase enzymes and reported at the highest doses of extract with promising IC_{50} values.75% inhibition on Catalase enzyme.

Since no closer evaluation study is available in the literature, these promising results should be considered for further detailed shows in near future.

CHAPTER 4

CONCLUSION

Stachys Cretica L. plants obtained from wild habitats are found in different natural ecosystems of the forests, Stachys flowers extracts and analyzed by using the antioxidant activity is increasing by protecting the body from free radicals and its effect on human life.

Stachys Cretica L. flowers was found TPC and TFC rich, and upon chromatographic separation among all aqueous fractions collected, only one found with highest TFC.

The more detailed analysis as such fraction showed that flower extract is a strong GST, SOD and CAT inhibitor and therefore it can be used as drug supplement after formation studies have been performed in near future.

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