INVESTIGATION FOR NATURAL EXTRACT INHIBITORS OF BOVINE LENS ALDOSE REDUCTASE RESPONSIBLE FOR THE FORMATION OF DIABETIS DEPENDENT CATARACT

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INVESTIGATION FOR NATURAL EXTRACT INHIBITORS OF BOVINE LENS ALDOSE REDUCTASE RESPONSIBLE FOR THE FORMATION OF DIABETIS DEPENDENT CATARACT

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to the this work.

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ABSTRACT

INVESTIGATION FOR NATURAL EXTRACT INHIBITORS OF ALDOSE REDUCTASE RESPONSIBLE FOR THE FORMATION OF DIABETIS DEPENDENT CATARACT

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In the polyol pathway, Aldose reductase (AR) is an important enzyme in reduction of aldehydes and aldosugars to their suitable alcohols. AR, using NADPH as a coenzyme, has a molecular weight of 37 000 dalton. AR in its activated form, known to increase the sorbitol accumulation in lens, is responsible for the cataract formation in diabetis diseases. Therefore, the inhibition of aldose reductase is important to prevent the incedence of cataract formation in diabetus mellitus. In the treatment of diabetis dependent cataract, chemically synthetized drugs were sometimes less than beneficial due to the severe side effects they cause.

Recently a huge amount of study has been intensified on developing new drugs from natural compounds and even by utilizing plant extracts for their easily metabolizing polyphenolic compounds. In this study, BLAR, source of enzyme, was obtained as crude via differential centrifugation and ammonium sulfate precipitation. The enzyme assay conditions were optimized for the protein, substrate, coenzyme, and salt concentrations, also for the effects of pH and temperature. ***Ocimum basilicum***, ***Lavandula stoechas***, ***Melissa officinalis***, ***Glycyrrhiza* glabra L.** and ***Tilia tomentosa*** were selected as commonly used alternative medicine plants. Plant extracts were prepared in ethanol and ethyl acetate and their inhibitory effects were tested on crude bovin lens aldose reductase enzyme. Fifty percent inhibitory concentrations (IC_{50}) were found between values of 25.53 µg/mL and 54.15 µg/mL for ethanol extracts and between 41.55 µg/mL and 82.96 µg/mL for the ethyl acetate extracts of selected plants. In addition, the plant extracts were also characterized for their antioxidant activities by of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method and test of total phenolic content (TPC) .

Keywords: Aldose Reductase Inhibition, Medicinal Plants, Antioxidant Activity, Bovine Lens.

ÖZ

DİYABETE BAĞLI KATARAK OLUŞUMUNDAN SORUMLU ALDOZ REDUKTAZIN DOĞAL ÖZÜT İNHİBİTÖRLERİNİN ARAŞTIRILMASI

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Aldoz redüktaz (AR) aldehit ve aldoşekerlerin, uygun alkollere indirgenmesinde önemli bir enzimdir. NADPH'i koenzim olarak kullanan AR 37 000 dalton moleküler ağırlığa sahiptir. Göz merceğinde sorbitol birikimini arttırdığı bilinen AR'nın aktive edilmiş şekli diyabette katarakt oluşumundan sorumludur. Bu yüzden, aldoz redüktazın inhibisyonu diyabette katarakt oluşumunun ilerlemesini engellemede önemlidir. Diyabete bağlı kataraktın tedavisinde, kimyasal olarak sentezlenen ilaçlar onların neden oldukları yan etkilerden dolayı bazı zamanlar daha az yararlı olmaktadırlar.

Son zamanlarda yapılan çalışmaların büyük bir kısmı doğal bileşiklerden yeni ilaçların gelişimi ve kolaylıkla metabolize olabilen fenolik bileşikler ile bitki özütlerinin kullanımı üzerine yoğunlaştırılmıştır. Bu çalışmada, enzim kaynağı aldoz redüktaz basamaklı santrifüj ve amonyum sülfat çöktürmesi aracılığı ile ham olarak elde edildi. Enzim deney koşulları protein, substrat, koenzim, tuz konsantrasyonu, pH etkileri ve sıcaklık şartları için en uygun hale getirildi. ***Ocimum basilicum, Lavandula stoechas, Melissa officinalis, Glycyrrhiza glabra L.*** ve ***Tilia tomentosa*** yaygın olarak kullanılan halk tıbbi bitkileri olarak seçildi. Bitki özütleri etanol ve etilasetat içerisinde hazırlandı ve onların inhibitör etkileri ham sığır aldoz redüktaz enzimi üzerinde test edildi. Seçilen bitkilerin etanol özütleri için yüzde elli inhibisyon konsantrasyonu 25.53 µg/mL ile 54.15 µg/mL arasında, etil asetat özütleri için 41.55 µg/mL ile 82.96 µg/mL arasında bulundu. Buna ek olarak, birde bitki özütleri, 2,2 difenil-1- pikrilhidrazil (DPPH) radikali yakalama yeteneği methodu ve toplam fenol içeriği testi ile antioksidan özelliği için karakterize edildi.

Anahtar sözcükler: Aldoz Redüktaz İnhibisyonu, Tıbbi Bitkiler, Antioksidan Aktivite, Sığır Lensi.

To the sunshine of my life Aytun…

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ARI	Aldose reductase inhibitor
BLAR	Bovine lens aldose reductase
BSA	Bovine serum albumin
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	Fifty percent effective concentration
GAE	Gallic acid equivalent
IC_{50}	Fifty percent inhibitory concentration
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
DMSO	Dimethylsulfoxide
PBS	Phosphate Buffer Saline
RSA	Radical scavenging activity
SD	Standard deviation:
TMG	Tetramethylene glutaric acid
TPC	Total phenol content
OB	*Ocimum basilicum* L. (Sweet Basil)
LS	*Lavandula stoechas* (Lavender)
MO	*Melissa officinalis* (Sweet Balm)
GG	*Glycyrrhiza glabra* (Liquorice)
TT	*Tilia tomentosa* (Linden)

CHAPTER 1

INTRODUCTION

Aldose reductase is the first enzyme of the polyol pathway and it changes glucose to sorbitol that is formed by reduction of glucose in the presence of NADPH (Crabbe, 1991). Aldose reductase in eyes, kidney, muscle and brain can cause accumulation of sorbitol in the presence of diabetes mellitus (Kador, 1988; Nishimura-Yabe 1998; Brownlee 2001). Consequently, aldose reductase is importantly associated with the pathogenesis of symptoms of diabetes. Accumulation of sorbitol under normal physiological conditions is not much important. However, in diabetes mellitus with increased glucose levels, sorbitol would be accumulated in cells due to its slow metabolization by sorbitol dehydrogenase and it has severe effects in the formation of cataract. Sorbitol does not easily diffuse through the cell membranes, so it accumulates in the cells, causing the osmotic pressure to construct eventually to the cells burst, resulting in the tissue damage. The presence of aldose reductase in the eye lenses was reported by Heyningen (Van Heyningen, 1959). Some physical properties of the partially purified bovine lens aldose reductase have been reported such as substrate specisity, cofactor requirement, optimum pH and sulfhydryl requirement (Hayman, 1965) and it is purified (Sheaff, 1976). Sheaff displayed that aldose reductase has a broad substrate specificity for substances having aldehyde groups and shown an optimum pH of 5.5, and it has been purified at 15.000 fold with a molecular weight of 37.000 dalton. The studies including inhibition of aldose reductase that prevents the onset of cataract successfully began in the early 1960s. Tetramethylene glutaric acid (TMG) was changed the cataract progression by modifying the activity of aldose reductase (Kinoshita, 1968).

Flavonoids are often found in plants and they have diversity as a group of phytochemicals. The inhibitory properties of various flavonoids were tested and all had significant activity for inhibition of aldose reductase. In the same study it was shown that quercetin, quercitrin and myricitrin were much more effective as inhibitors than TMG (Kinoshita, 1968; Varma, 1977).

1.1 Properties of plant materials

1.1.1 Properties of *Ocimum basilicum* L.

Scientific classification of *Ocimum basilicum* L. is defined as below.

Kingdom : Plantae

Subkingdom : Tracheobionta

Division : Magnoliophyta

Class : Magnoliopsida

Subclass : Asteridae

Order : Lamiales

Family : Lamiaceae

Genus : Ocimum

Species: *Ocimum basilicum* L.

Ocimum is a genus of about 35 species of aromatic annual and perrenial herbs and shrubs in the family Lamiaceae, native to the tropical and warm temperate regions of the Old World. *Ocimum basilicum*, *O. americanum*, and *O. micranthum* are members of the family Lamiaceae. Most *Ocimum spp.* can be grown and found throughout the world. However, they are not native to all areas. For example, *O. micranthum* originated in Central America. It grows to a height of approximately 50 cm. The leaves are oval and slightly toothed, and the flowers are white or purple. In contrast, *O. basilicum* looks very similar, but grows taller , generally, it has 50-80 cm of height. *O. Basilicum* has common names in different countries. For example, it is named as fesleğen in Turkey, sweet basil in USA, hung que in Vietnam, balanoi in the Philippines.

The dried leaves of *O. basilicum* contain 0.20-1 % essential oil. The major compounds in the oil are linalool and methylchaviol. However, the exact amounts can vary considerably. For example, cineole has been found in *O. Basilicum*. There is little available literature on the toxicity of *Ocimum* spp. *O. basilicum*, the species that appears to be used the most medicinally and the one for which the most analysis has been done, contains several potentially dangerous compounds. Some of these compounds are rutin, caffeic acid, tryptophan, and quercetin. *O. basilicum* has been used as an expectorant in animals. Recently, there has been much research put into the health benefits related to the essential oils found in basil. Scientific studies have established that compounds in basil oil have potent antioxidant, anti-cancer, anti-viral, and anti-microbial properties (Bozin, 2006 ; Chiang, 2005 ; Almeida, 2007 ; Manosroi, 2006). In addition, basil has been shown to decrease the occurence of platelet aggregation and experimental thrombus in mice (Tohti, 2006).

O. Basilicum is grown near Aegean and the Mediterranean region and used for diuretic treatments, depression, expectorant and headache in Turkey.

1.1.2 Properties of *Lavandula stoechas*

Scientific classification of *Lavandula stoechas* is clarified as below.

Kingdom : Plantae

Subkingdom : Tracheobionta

Division : Magnoliophyta

Class : Magnoliopsida

Subclass : Asteridae

Order : Lamiales

Family : Lamiaceae

Genus : Lavandula

Species: *Lavandula stoechas*

Lavandula stoechas occurs naturally in the Mediterranean region. It has a perennial shrub and grows to 30-100 cm tall. The leaves are 1-4 cm long, greyish tomentose. The flowers are pinkish-purple (lavender-coloured), produced on spikes 2-3 cm long at the top of slender leafless stems 10-30 cm long; each flower is subtended by a bract 4-8 mm long. *Lavandula stoechas* has common names in different countries. For example, it is named as karabaş otu in Turkey, lavender in England, lavendel in Germany and lavande in France. *Lavandula stoechas* can be grown throughout western and southern Anatolia and it is used for cholera, antiseptic, lesion treatments and expectorant (Gülçin, 2004).

1.1.3 Properties of *Melissa officinalis*

Scientific classification of *Melissa officinalis* is defined as below.
Kingdom : Plantae
Subkingdom : Tracheobionta
Division : Magnoliophyta
Class : Magnoliopsida
Subclass : Asteridae
Order : Lamiales
Family : Lamiaceae
Genus : Melissa
Species: *Melissa officinalis*

Melissa officinalis is a perennial herb in family Lamiaceae, native to southern Europe and the Mediterranean region. It grows to 70-150 cm tall. The leaves have a gentle lemon scent. At the end of the summer, little white flowers full of nectar appear and attract bees. Hence, the genus name *Melissa* is meaned to honey bee in Greek. *Melissa officinalis* has common names in different countries. For example, it is named as oğul otu and limon otu in Turkey, lemon balm melissa and sweet balm in England, melisse Germany and mélisse in France. Its flavour comes from the terpenes citronellal, citronellol, citral, and geraniol.

Lemon balm is often used as a flavouring in ice cream and herbal teas. It is also frequently paired with fruit dishes or candies. *Melissa officinalis* can be grown throughout Thrace region in Turkey. It is very useful for heart diseases, nervous agitation, and for promoting sleep, and ameliorates functional gastrointestinal complaints (Kümel, 1991). In folk medicine, balm is recommended as a plant juice, cream or tea infusion for nervous complaints, lower abdominal disorders, gastric complaints, hysteria and melancholia, chronic bronchial catarrh, migraine, nervous debility, toothache, earache, headache and high blood pressure and, externally for rheumatism, nevre pains and stiff necks (Cohen, 1964).

1.1.4 Properties of *Glycyrrhiza glabra*

Scientific classification of *Glycyrrhiza glabra* is clarified as below.
Kingdom : Plantae
Subkingdom : Tracheobionta
Division : Magnoliophyta
Class : Magnoliopsida
Subclass : Rosidae
Order : Fabales
Family : Fabaceae
Genus : Glycyrrhiza
Species: *Glycyrrhiza glabra*

Glycyrrhiza is a genus of about 18 accepted species in the family Fabaceae, with a subcosmopolitan distribution in Asia, Australia, Europe, and the Americas. The genus is best known for liquorice (British English; licorice in American English), which is the product of *G. glabra*, a species native to the Mediterranean region. It grows to 40-200 cm tall. *Glycyrrhiza glabra* has common names in different countries. For example, it is named as meyan kökü in Turkey, sweet root and licorice in England, süssholz Germany and racine dovce in France.

It contains sterols and flavones such as liquiritin and liquiritigenin. It can be grown throughout Southern, Middle and East Anatolia region in Turkey. *Glycyrrhiza glabra* is one of the oldest and widely used herbs from the ancient medical history of Ayurveda, both as a medicine and a flavoring herb to disguise the unpleasant flavor of other medications (Biondi, 2005). The first report of medicinal use comes from Greeks, who recommended it for the treatment of gastric and peptic ulcers. In Asia and Europe, the extract is used in the treatment of psoriasis. *Glycyrrhiza glabra* is used to relieve inflammations, eye diseases, throat infections, peptic ulcers, arthritic conditions, and liver diseases in Indian Ayurveda system. Other uses of the plant include the treatment of sex-hormone imbalances and menopausal symptoms in women. Anti-*Helicobacterpylori* and antibacterial activities of flavonoids from the licorice extract were reported previously (Fukai, 2002).

1.1.5 Properties of *Tilia tomentosa*

Scientific classification of *Tilia tomentosa* is defined as below.
Kingdom : Plantae
Subkingdom : Tracheobionta
Division : Magnoliophyta
Class : Magnoliopsida
Subclass : Dilleniidae
Order : Malvales
Family : Tiliaceae
Genus : Tilia
Species: *Tilia tomentosa*

Tilia is a genus of about 30 species of trees, native throughout most of the temperate Northern Hemisphere, in Asia, Europe and eastern North America; it is not native to western North America. Under the Cronquist classification system, this genus was placed in the family Tiliaceae, but genetic research by the APG has resulted in the incorporation of this family into the Malvaceae. The trees are generally called lime in Britain and linden in North America. *Tilia* species are large deciduous trees, reaching typically 20-40 m tall, with oblique-cordate leaves 6-20 cm across, and are found through the north temperate regions. Three species of *Tilia* is known as *Tilia platyphyllos, Tilia rubra* and *Tilia tomentosa* (*Tilia argentea*) in Turkey. *Tilia* flowers have a noticable importance in phytotherapy. It is stated to possess expectorant, diuretic, antispasmodic, stomachic and sedative activities. It has been used for the treatment of flu, migraine, cough, nervous tension, ingestion, various types of spasms, liver and gall bladder disorders. Medicinal properties claimed for the drug have been attributed to its flavonoid, volatile oil and mucilage components (Baytop, 1984 ; Newall, 1996).

1.2. Properties of aldose reductase

Aldose reductase is a cytoplasmic enzyme and it consists of a group of enzymes with the $(beta/alpha)_8$ barrel class. Aldose reductase is a globular protein composed of a single polypeptide chain of 315 residues, with molecular weight of 37 000 dalton and it does not contain any metal group. Aldose reductase is an NADPH-dependent oxidoreductase with a large substrate specificity that it catalyzes the reactions of aldosugars and aldehydes to alcohols.

Single chain of aldose reductase folds in an α and β barrel with an inner core of 8 parallel β strands encircled by 8 α-helices as exhibited in figure 1.1. The red core suits to the β strands and the grey surroundings correspond to the helices.

Figure 1.1: Secondary structure of aldose reductase (Wilson, 1992).

The structure is composed of two short antiparallel β-strands. In addition, it has an extra piece of α-helix between strand 7 and helix 7 and between strand 8 and helix 8. (Wilson, 1992; Wilson 1993).

Bovine lens aldose reductase go after an ordered addition of substrates with NADPH binding first and an ordered release of products with NADP being released last. (Liu, 1992) The active site of aldose reductase is placed at COOH terminal of the β barrel. The active site of the aldose reductase has highly hydrophobic residues and some aminoacids presented in cavity are nonpolar. (Rondeau, 1992; Wilson, 1992). In the enzyme –NADPH binary complex, the C-4 of the nicotinamide of NADPH, the reactive end of the molecule is in close proximity to 3 polar residues Cys-298, Tyr-48, and His-110, and one of these residues may be the acid-base catalyst for the oxidation/reduction reactions. Also NADPH is binded to the enzyme by 19 hydrogen bonds in an extended conformation across the barrel which has the nicotinamide ring in the center of cavity (Wilson, 1992) .

NADPH-Enzyme binary complex is shown in figure 1.2. Two forms of aldose reductase have been recognized. One of which is activated form and the other form is unactivated. The activated and unactivated forms of bovine aldose reductase have different physical and kinetic properties (Grimshaw 1990; Del Corso 1989).

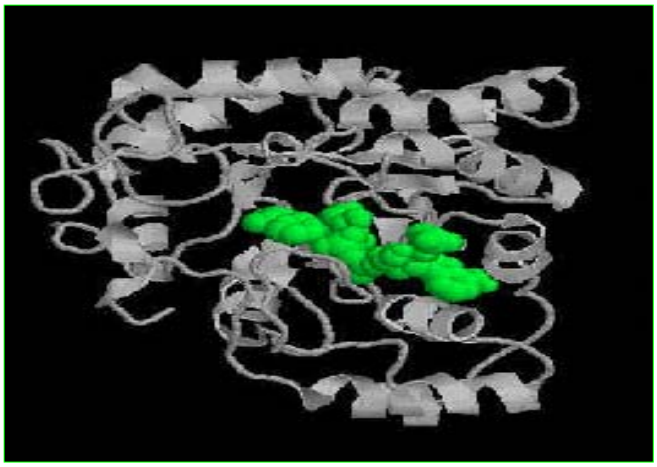


Figure 1.2 : NADPH- enzyme binary complex (Wilson, 1992).

The unactivated form can be activated in presence of glucose, glucose-6-phosphate and NADPH. The activated form of aldose reductase shows lower K_m for glucose and glyseraldehyde because V_{max} increases. In addition, activated form of aldose reductase has less sensitivity than unactivated forms for aldose reductase inhibitors. Aldose reductase with high K_m demonstrates low activity for glyceraldehyde (Das, 1985; Ohta, 1993).

One of the most functions of aldose reductase is to work as a promoter of the polyol pathway. In this pathway, glucose is converted to fructose via the sorbitol formation and aldose reductase is associated with the conversion of sugar to sugar alcohols. In addition, aldose reductase catalyzes the reduction of hexoses.

The formation of fructose via aldose reductase and sorbitol dehydrogenase is shown in figure 1.3.

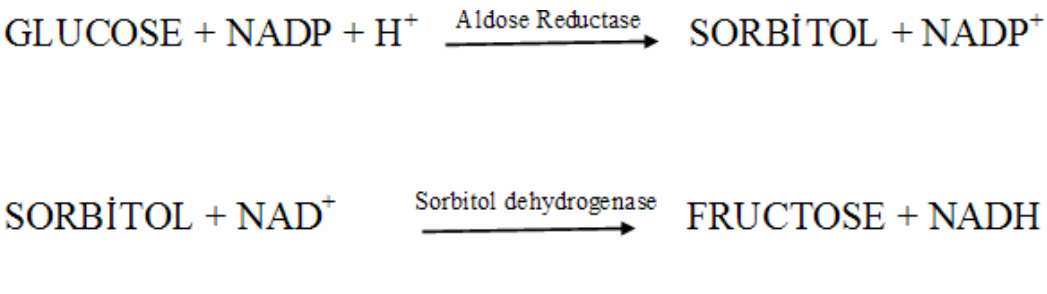


Figure 1.3 : The formation of fructose via aldose reductase and sorbitol dehydrogenase.

Fructose and sorbitol are present in the human lens. Concentration of fructose and sorbitol increase at diabetes and they may join in the pathogenesis of diabetic complications. The polyol pathway is responsible for fructose arrangement from glucose and the glucose concentration increases in tissues that are not insulin sensitive at diabetes. Glucose passing the cell is rapidly phosphorylated by the enzyme hexokinase and metabolized via the glycolytic pathway to lactate and pyruvate to supply major energy sources for the cells. Large amount of glucose is also metabolized via pentose phosphate pathway in the lens. Nevertheless, the concentrations of glucose and galactose increase in several folds in diabetes and galactosemia. The excess glucose can not be metabolized by the glycolysis pathway. Hence, the aldose reductase is activated and leads to the conversion of glucose to sorbitol. Hexokinases have higher affinity for glucose than aldose reductases, so glucose can enter the polyol pathway only when its levels increases (Clarke, 1984).

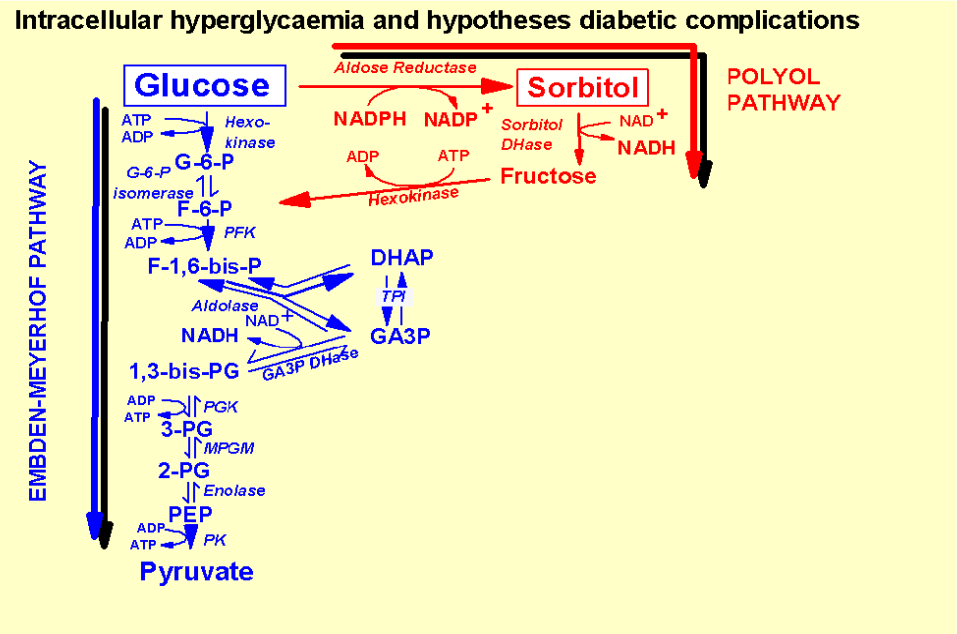


Figure 1.4 : Polyol pathway in accordance with Embden–Meyerhof pathway (Ohta, 1993 ; Clarke, 1984).

Sorbitol does not easily diffuse through the cell membranes, so it accumulates in the cells, causing the osmotic pressure to construct eventually to the cells burst. Activation of enzyme brings about a NADPH depletion altering the redox state of the cell and the depletion of antioxidants as a result an increased damage due to oxidative stress from superoxide and peroxides can be observed (Wood, 1985). The physiological properties of the polyol pathway are presented by Hers in 1956. In polyol pathway, produced sugar alcohol is oxidized to ketose by a second enzyme of the path named NAD^+-dependent sorbitol dehydrogenase (SDH). While the glucose concentration increases, aldose reductase activity raises in several folds. Nevertheless, the sorbitol dehydrogenase activity does not increase proportionally as a consequence of sorbitol accumulates in the cell (figure 1.4) (Ohta, 1993).

High concentrations of sorbitol in the lens can increase the osmotic pressure and cause cellular swelling with disruption and cell death. Finally, this process results in lenticular opacity which termed as sugar cataract. Animal experiments exhibits the dependence of the appearance of the diabetic complications associated with diabetes on the aldose reductase–initiated accumulation of sugar alcohols (Crabbe, 1991). In human cornea, aldose reductase has been found in corneal epithelium and endothelium (Kador, 1984).

The role of aldose reductase in cataract formation through *invivo* animal studies has shown by Kinoshita and Hayman (Kinoshita and Hayman, 1965). The rate of cataract formation is directly dependent upon the levels of aldose reductase present in the lens (Varma, 1977).

The pharmaceutical industry has approached the problems of diabetic complications by searching for possible common links between the various organs that are damaged. The development of potential medicines has been focused on the three areas (Raskin, P., 1987).

- Aldose reductase inhibitors (ARI s)
- Advanced glycation end-product inhibitors (AGEIs)
- Other approaches including tissue bioengineering

Table 1.1: Tissue localization of aldose reductase and related diabetic complications. (Raskin, P., 1987).

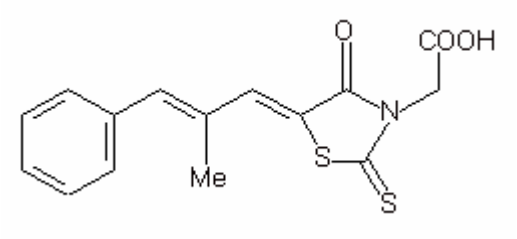
TISSUE LOCALIZATION	DIABETIC COMPLICATIONS
EYE	
Lens (Epithelium)	Cataract
Retina (pericytes)	Retinopathy
Cornea (epithelium)	Keratopathy
KIDNEY	
Loop of Henle	
Collecting tubules, interstitial	Nephropathy
Cells in inner medula	
Glomerulus,epithelial cells of the podocyte	
Mesengial cells	
Epithelial cells of the distal convuluted tubules	
PERIPHERAL NERVES	
Schwann cell, axons within myelin sheath	Neuropathy

1.3 Inhibition of aldose reductase activity

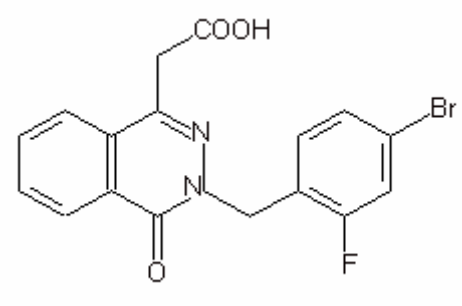
The studies including inhibition of aldose reductase began in the early 1960s when first known inhibitor of aldose reductase was determined as a long-chain fatty acid. In late 1960s, tetramethylene glutaric acid (TMG) was found that it changed the progression of cataract by modifying the activity of aldose reductase (Kinoshita, 1968). In addition, TMG minimizes the increase in lens hydration and the appearance of vacuoles but its inability to penetrate the membranes made the compound ineffective in vivo.

Types of flavonoids were tested for their inhibitory properties in 1970 and all the flavonoids studied had significant activity for inhibition of aldose reductase. Among the flavonoids, quercetin, quercitrin and myricitrin were found to be much more effective as aldose reductase inhibitors than TMG (Kinoshita 1968; Varma 1977). Although it has been known that flavonoids are non-toxic compounds, they could not made to the clinical trials. Since the mid.-70's, several studies on the inhibition of aldose reductase by flavonoids have been reported by the reseach groups of Varma and Okuda (Varma, 1975; Varma, 1976; Okuda, 1982). 94 flavonoids were examined in order to clarify the further structural requirements of flavonoids for aldose reductase inhibitory activity and the results suggested that the flavones and flavonols having the 7-hydoxyl moiety on the A ring showed the strong activity, the 2-3 double bonds enhanced the activity and the 5-hydoxyl moiety did not affect the activity (Matsuda, 2002). In the 1990s synthetic aldose reductase inhibitors were studied in two general classes. One contains a carboxylic acid moiety and other contains cyclic imide represented by a spirohydantoin or related ring system (Wrobe, 1991; Yamagish, 1992; Costantino, 1999). Both types of inhibitor have similar intrinsic activity for aldose reductase but their in vivo potencies are different. Imides are more potent in invivo because of their better pharmokinetic properties.

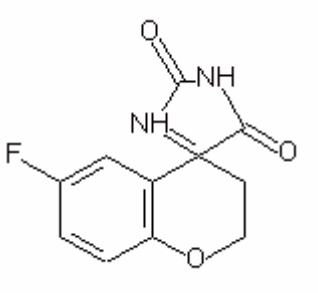
Tolrestat is one of the most effective compounds within carboxylic acid series. Although several aldose reductase inhibitors (ARIs) have been tested in clinical trials on diabetic patients for more than 20 years, they still remain effective (Preifer, 1997). Chemical structures of some of known synthetic aldose reductase inhibitors are shown in figure 1.5.



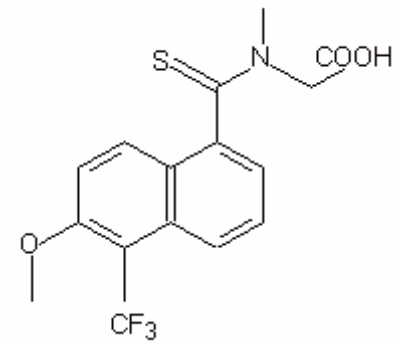
Epalrestat



Ponalrestat



Sorbinil



Tolrestat

Figure 1.5: Chemical structures of some of known synthetic aldose reductase inhibitors (Pfizer, 1978).

The majority of inhibitors that have been used for human clinical trials were the members of carboxylic acid derivatives such as epalrestat, statil, tolrestat, and ponalrestat containing carbonyl or thiocarbonyl groups. These reactive groups which tend to accept a pair of electron from the enzyme are known to lead to the inhibition.

It has also been shown that the inhibition of aldose reductase is dependent on the degree of purification of the enzyme as well as on the substrate used for the activity assays (Del Corso, 1989).

Many of compounds have inhibited the aldose reductase reaction, as measured by the change in absorbance at 340 nm (oxidation of NADPH) , when oxidizing monosaccharides are used as substrates. The aromatic hydrocarbonyl groups helps the attachment of the inhibitor (Crabbe, 1991; Kador, 1985).

1.3.1 Aldose reductase inhibitors from plant materials

Flavonoids are polyphenolic compounds that they are seperated into four groups. These are flavone, flavonol, isoflavone and flavonone. The studies shows that structural requirements of an aldose reductase inhibitor are more general. However, the shared feature of all such inhibitors has a hydrophobic region attached to an acid group. Ligand binding site of aldose reductase was shown in figure 1.6.

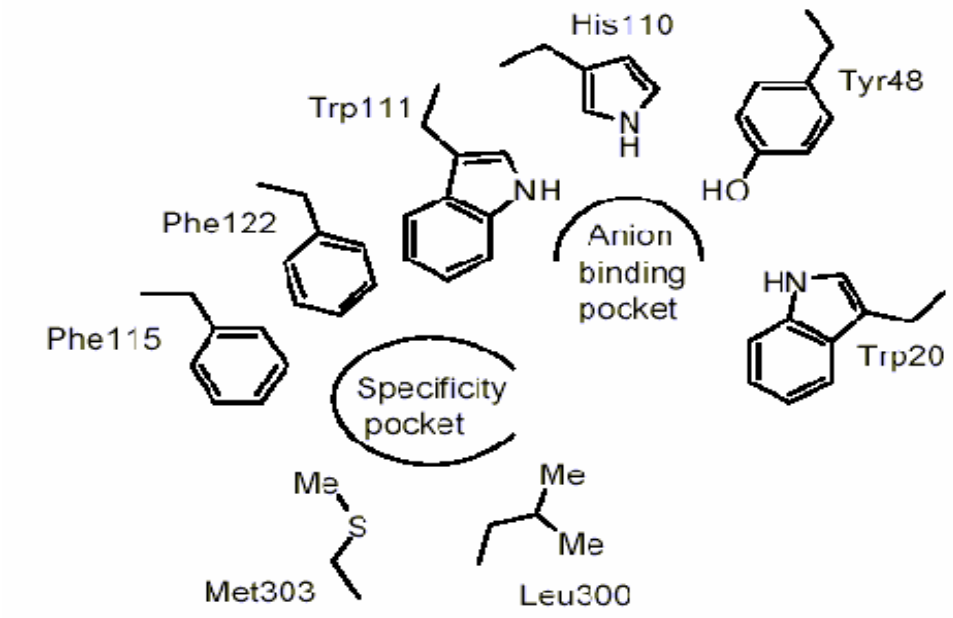


Figure 1.6 : The schematic representation of ligand binding site of aldose reductase (Miyamoto, 2002).

Flavones and flavonols having the 7-hydoxyl moiety on the A ring showed the strong activity, the 2-3 double bonds enhanced the activity and the 5-hydoxyl moiety did not affect the activity (Matsuda, 2002). Plants has a rich source of bioactive chemicals like flavonoids, many of which are largely free from adverse effects and have excellent pharmacological actions, they can lead to the development of new classes of possibly safer antidiabetic, anticataract agents. Additionally, some flavonoids and polyphenols as well as sugar derivatives are found to be effective inhibitors of aldose reductase. Hence, more effort has been focused on the plants for potentially useful products as commercial aldose reductase inhibitors. In addition, ellagic acid and its two derivatives, 4-0- methylellagic acid and 4-(α-rhamnopyranosyl) ellagic acid were shown as inhibitors of aldose reductase and these were isolated from *Myrciaria dubia* (Ueda, 2004). Effects of flavonoid compounds on aldose reductase were examined in many reviews. (Hyung, 2006; Hadler, 2003; Ueda, 2001; Guzman, 2005; Fuente 2003; Haihui 2005). The aldose reductase inhibitory activity of *Ocimum sanctum* was carried out (Gupta, 2003). Gupta studied with rat lens for inhibition activity, and determined that Ocimum sanctum has the most potential inhibitory effect for aldose reductase.

Melissa officinalis dispayed acetylcholinesterase inhibitory activity (Ferreira 2006).

1.4 Antioxidants

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols or phenolic compounds and Aldose reductase inhibitors are free radical scavengers and antioxidants. Structures of flavonoids were shown in figure 1.7.

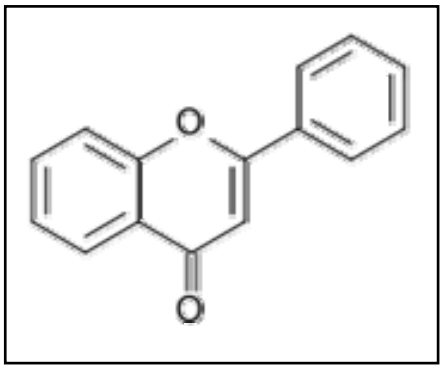


Figure 1.7 : Structure of flavonoid

Aroma compounds in basil leaves (*Ocimum basilicum L.*) were determined by gas chromatography (GC) (Lee, 2005). Lee found that 3,7-dimethyl-1,6-octadien-3-ol, 1-methoxy-4-(2-propenyl) benzene, methyl cinnamate, 4-allyl-2-methoxyphenol, and 1,8-cineole are the most aroma componenets of *Ocimum basilicum* and showed that these compounds have highly antioxidant activities. There are many reviews of *Ocimum sp*. and numerous laboratory investigation have studied that these are on radiation protection (Devi, 2000), chemopreventive activity (Prakash and Gupta, 2000), anti-inflammatory activity (Klem, 2000), a nervous system stimulant effect (Maity, 2000), bactericidal activity (Koga et al. 1999), modulatory effect on glutathione and antioxidant enzymes (Devi and Ganasoundari, 1999), antiulcer activity (Singh, 1999), antidiarrheal effects (Offiah and Chikwendu, 1999), and blood-sugar lowering (Chattopadhyay, 1999). Antioxidant capacity of free volatile aglycones from *Ocimum basilicum L*. was studied and free volatile aglycones (eugenol, chavicol, linalool and a-terpineol) were used for antioxidant activity measurements via DPPH test and found that eugenol has the most antioxidant activity (Politeo 2007).

Essential oils of *O. basilicum* display varying array of compounds, relying on variations in chemotypes, leaf and flower colors, aroma and origin of the plants (Da-Silva, 2003; Sajjadi, 2006).

1.4.1 Phenolic compounds

Phenolic compounds are often found in plants and they have diversity as a group of phytochemicals. Phenolic compounds cause different functions in plants. For example, they function as protective agents against UV light, attraction for pollinators, flavor, color and plant pigmentation. In addition, phenolic compouns have health-protecting capacities, and oxidative stability. These are advantageous for producers and consumers (Shahidi, 2004). Structures of flavonoids such as flavones, flavanones, isoflavonoids and flavonols were exhibited in figure 1.8.

Although lemon balm antioxidant studies have been carried out, studies reporting upon the antioxidant activity of polar extracts of lemon balm are limited (Hohman, Zupko, Redei, & Csanyi, 1999; Ivanova, Gerova, Chervenkov, & Yankova, 2005; Triantaphyllou, Blekas, & Boskou, 2001; Venkutonis, Gruzdiene, Trizite, & Trizite, 2005). The leaf material of lemon balm (*Melissa officinalis L.*) was extracted in aqueous ethanol and studied for antioxidant activity and total phenol content by Dastmalchi. *Melissa officinalis L.* extract included hydroxycinnamic acid derivatives and flavonoids with caffeic acid, m-coumaric acid, eriodictyol-7-O-glucoside, naringin, hesperidin, rosmarinic acid, naringenin, hesperetin. It was found that *Melissa officinalis L*. contains highly antioxidant activity (Dastmalchi, 2008).

Bouayed examined Iranian medicinal plants (leaves of *Lavandula officinalis* and of *Melissa officinalis*) and it was shown that they exhibited important antioxidant activities and present a good source of natural antioxidants. 6.18 ± 0.18 (catechinequivalent (CE) mg CE/g dw) and 10.0 ± 0.32 (catechinequivalent (CE) mg CE /g dw) respectively. (Bouayed, 2007). Total phenol content of Bulgarian *Melissa officinalis* was found as 1370.09 ± 41.38 (μM) quercetin equivalents (QE) (μM) (Ivanova, 2005).

The antioxidant activity of water and ethanol extracts of lavender showed strong total antioxidant activity. At the concentrations of 20, 40, and 60 µg/ml, water extract of lavender showed 86.9 %, 92.3%, and 94.8 % inhibition on lipid peroxidation of linoleic acid emulsion, respectively. At the same concentrations, ethanol extract of lavender exhibited 92.5 %, 93.8 %, and 96.5 % inhibition, respectively.Total phenol content of water extract of lavender was found as 153.92 µg GAE and total phenol content of ethanol extract of lavender was 226.74 µg GAE (Gulcin, 2004). Total phenol content of water extract of leaves of *Lavandula officinalis* was exhibited as 16.2 ± 0.59 (mg GAE/g dw) (gallic acid equivalent (GAE) per 1 g dry weight) (Bouayed, 2007). *Lavandula vera* (Lamiaceae) phenolic constitution was investigated by reversed phase high performance liquid chromatography. Metanolic extract of *Lavandula vera* were composed of phenolic compounds such as gallic acid, p-hydroxybenzoic acid, (+) catechin, vanillic acid, caffeic acid, ferulic acid and naringenin (Proestos, 2006). *Lavandula angustifolia* were used to identify their radical scavenging activity using DPPH and the content of total phenolic compounds. Ethyl acetate,acetone and methanol extract of *Lavandula angustifolia* were found for DPPH % inhibition as 2.5 %, 7.4 %, 35.4 %, respectively. Total phenol content of methanol extract of *Lavandula angustifolia* was shown as 5.4 mg/g plantextract(in GAE). (Miliauskas, 2004).

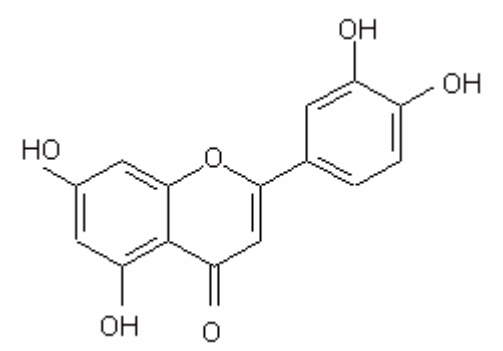
Phytochmical components of Glycyrrhiza glabra water extracts were examined and they have composed of glycyrrhizin, asparagin, sugar, starch, acid resin, flavones, coumarins (Naik, 2003).

Ethanolic extracts from the roots of wild licorice (*Glycyrrhiza lepidota*) were examined for their free-radical scavenging capacity and their antioxidant activity. Total phenolic content of *Glycyrrhiza lepidota* was found as 63 mg/g catechin equivalents/g of extract (Amarowicz, 2004).

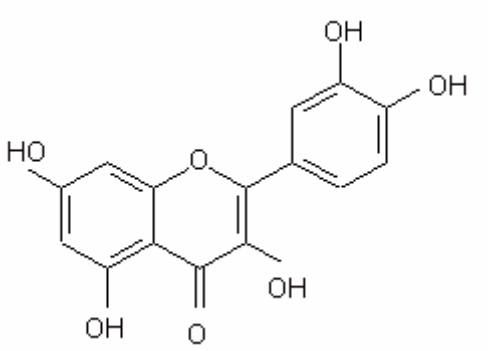
Analysis of flavonoid composition of *Tilia*. *tomentosa* (*argentea)* were studied and quercetin-3,7-dirhamnoside, isoquercitrin-rutin, quercitrin, astragalin, tiliroside from *T*. *argentea* flowers were found as flavonoid. Quercetin-3,7-dirhamnoside,isoquercitrin-rutin,kaempferol-3,7-dirhamnoside,astragalin,quercitrin, tiliroside from *T.argentea* bracts were shown as flavonoid. Quercetin-3,7-dirhamnoside, isoquercitrin-rutin, kaempferol-3,7-dirhamnoside, quercitrin, astragalin from *T*. *argentea* leaves were exhibited as flavonoid (Toker, 2001).

Flavonoid composition of *T*. *platyphyllos* were analyzed quercetin-3,7-dirhamnoside, isoquercitrin-rutin, quercitrin, astragalin from *T*. *platyphyllos* flowers were found as flavonoid. Hyperoside, quercetin-3,7-dirhamnoside, isoquercitrin-rutin, kaempferol-3,7 dirhamnoside, quercitrin, astragalin from *T*. *platyphyllos* bracts were exhibited as flavonoid. Hyperoside, quercetin-3,7-dirhamnoside, isoquercitrin-rutin, quercitrin, kaempferol-3,7 dirhamnoside, astragalin from *T*. *platyphyllos* leaves were shown as flavonoid. (Toker, 2001).

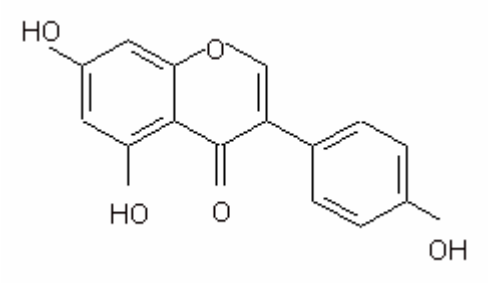
Analysis of flavonoid composition of *T*. *rubra* were investigated and quercetin-3,7-dirhamnoside, isoquercitrin-rutin, quercitrin, astragalin from *T*. *rubra* flowers were found and quercetin-3,7-dirhamnoside, isoquercitrin-rutin, astragalin, kaempferol-3,7 dirhamnoside, quercitrin from *T*. *rubra* bracts were shown also quercetin-3,7-dirhamnoside, isoquercitrin-rutin, astragalin, kaempferol-3,7 dirhamnoside, quercitrin from *T*. *rubra* leaves were exhibited as flavonoid. (Toker, 2001).



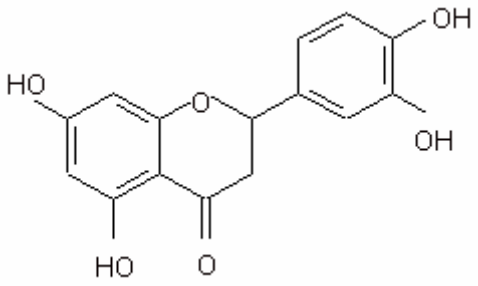
Flavone



Flavonol



Isoflavone



Flavonone

Figure 1.8: Structures of flavone, flavonol, isoflavone and flavonone.

1.4.2 Free Radicals

Free radicals act with one or more unpaired electrons in their construction (Halliwell, 1995). Some examples of free radicals are oxides of nitrogen, hydrogen peroxyl, hydroxyl and superoxide. Free radicals can naturally or artificially occur in biological systems. Artificial free radical arrangements forming from enviromental factors can induce chain reactions *in vivo* systems. For example, lipid peroxidation causes strong damages to lipid membranes and inner compositions of cells. Free radicals cause cross-linkage of macromolecules such as nucleic acids. Free radicals are highly responsible for aging and tumor production (Penzes, 1984).

1.5 Scope of the study

Diabetes has been studied to be one of the major risk factors of cataract (Lee, 2002). Aldose reductase in diabetes has been examined elaborately because inhibitors of aldose reductase are known to prevent cataractogenesis. This study was designed to investigate the bovine lens aldose reductase enzyme activity and to evaluate the inhibitory effects of plant extracts in the enzyme assay. Bovine lens was selected as the source of AR since the cattle are slaughtered mosty on daily bases in our country,so we can find them for our experiments. For this reason, bovin lens aldose reductase was isolated by centrifugation and ammonium sulfate precipitation. Assay is used for determination of aldose reductase activity by monitoring the reduction of NADPH to $NADP^+$ at 340 nm spectrophotometrically. The optimum conditions such as substrate concentration, protein amount, pH, coenzyme concentration and the effect of temprature were examined. Inhibitory effects of ***Ocimum basilicum***, ***Lavandula stoechas***, ***Melissa officinalis***, ***Glycyrrhiza* glabra L.** and ***Tilia tomentosa*** ethanol and ethylacetate extracts were determined on the crude bovine lens aldose reductase. They were expressed as % 50 inhibition concentration. Furtermore, antioxidative activities of ethanol and ethyl acetate extracts were studied

and 50 % effective concentrations of plant extracts were calculated. Also, the total phenol contents of the plant extracts were determined .

CHAPTER 2

MATERIAL AND METHOD

2.1 Materials

2.1.1 Chemicals

Bovine serum albumin (BSA), β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), DL-glyceraldehyde, lithium sulfate (Li_2S0_4), folin-phenol reagent, dimethylsulfoxide (DMSO), membrane dialysis tubing, 2,2-diphenyl-1-picrylhydrazyl (DPPH), quercetin and gallic acid as standards were all purchased from Sigma Chemical Company, Saint Louis ,Missouri,USA.

Ammonium sulfate [$(NH_4)_2SO_4$], sodium chloride (NaCl), Na –K tartarate, Cupper sulfate ($CuS0_4$), Sodium bicarbonate (Na_2C0_3), EDTA, Sodium Hydroxide (NaOH), $NaHC0_3$, ethyl acetate and ethanol of analytical grade, reagents and solvents were purchased from Merck (Darmstadt, Germany) and water distilled twice was purified by using a Milli – Q system (Millipore, Bedford, MA, USA). 125mm pore size white band filter.

Ocimum basilicum (Sweet Basil), ***Lavandula stoechas*** (Lavender), ***Melissa officinalis*** (Sweet Balm***), Glycyrrhiza* glabra L.** (Liquorice) and ***Tilia tomentosa*** (Linden).

2.1.2 Apparatus

All of the spectroscopic data as enzyme kinetic, determination of total phenolic contents and free radical scavenging activity measurements were obtained with Cary 50 Bio UV-VIS spectrophotometer (Varian).

Several instruments used for the experiments are rotary evaporator (Heidolph Laborota 4000), homogenizer (Heidolph Diax 900), lyophilizator (Heto-Holten Model Maxi-Dry Lyo), waring blender model 32BL80 (New Hartford, CT, USA); bandelin Sonorex (ultrasonic bath), Optic Ivymen System (incubator) Laborota 4000), centrifuge (Sigma 3K30 with 12159 rotor).

2.2 METHODS

2.2.1 Preparation of aldose reductase crude extract from bovine lens

The bovine eyes were obtained from abattoir (Kazan Belediye Mezbahası, Ankara) immeddiately after slaughtering, then they were put into crushed ice. As soon as the eyes were reached to laboratory, lenses were excised from the eyes and washed with cold ultra pure water. The lenses were weighted and homogenized in nearly three volumes of cold ultra pure water by the Heidolph Diax 900, and centrifuged (Sigma 3K30) by using 12159 rotor at 4 ºC, 10,000 x *g* for 30 minutes to remove insoluble material.

The supernatant was saturated with ammonium sulfate to 40 % and saturated sample was centrifuged at 10,000 x *g* for 25 minutes. Pelet was discarded and supernatant was brought to 50 % ammonium sulfate saturation to remove additional proteins. Then the saturated sample with 50 % ammonium sulfate was centrifuged at 10,000 x *g* for 25 minutes and pellet was discarded again. Supernatant was taken and saturated with 75 % ammonium sulfate at 10,000 x *g* for 25 minutes. Saturated sample was centrifuged at 10,000 x *g* for 25 minutes. In the final step, supernatant was discarded and pellet was taken. The pellet was dissolved in 50 mM NaCI

solution. The volume of the suspension was recorded and the sample was dialyzed overnight against 50 mM NaCI (double replacement of dialysis solution).

After dialysis, the volume of the sample was recorded and treated to liquid nitrogen then samples were stored in 1 mL aliquots in eppendorf tubes in deep freezer at -80 ºC for the determination of the total protein, enzyme activity and inhibition studies.

2.2.2 Preparation of crude fractions from the plant extracts

Ocimum basilicum, Lavandula stoechas, Melissa officinalis, Glycyrrhiza glabra L, and Tilia tomentosa were taken and dried at room temprature then finely grounded to increase air-surface area by Waring Blender (model 32BL80). Plant samples (20 g) were three times extracted in 1:10 ratio of ethanol and ethyl acetate for 24 hours at rocking incubator (Optic Ivymen System) at 30 °C temperature. Mixtures were filtered through a double layered cheese cloth. Then, samples were filtered with a rough filter paper. Next, solvents of plants were evaporated by rotary evaporator (Heidolph Laborota 4000) at 35 °C until mixtures remained to 50 mL of solvents. Afterwards, mixtures including 50 ml of solvent settled to lyophilizator (Heto-Holten Model Maxi-Dry Lyo) to dry plant extracts completely. Dried extracts were weight out and the percent yield of extraction was calculated then stored at 4 °C.

2.2.3 Analytical procedures

2.2.3.1 Protein determination

Protein concentration of sample was examined by means of the method of Lowry (Lowry, 1951) by using the crystalline BSA as a standard. To obtain a calibration curve, five different concentrations of standard BSA's were used. 0.02-0.2 mg BSA/mL were taken into test tubes with a final volume of 0.5ml.

The aliquots of 0.1, 0.25, 0.5 mL of diluted samples were put into test tubes and were completed to final volume of 0.5 mL with ultra pure water. All tubes were mixed with 2.5 ml alkaline-copper reagent prepared with adding 2 % copper sulfate, 2 % sodium potassium-tartarate and 0.1 M NaOH containing 2% sodium carbonate in a ratio of 1:1:100 respectively. Then all of the test tubes including alkaline-copper reagent were vortexed and waited for 10 minutes at room temprature. Finally, 0.25 ml of 1 M folin-phenol reagent prepared freshly was added to test tubes. Each tubes directly was mixed (8 second) as soon as 0.25 mL of 1 M folin-phenol reagent was added to test tubes. After the test tubes were left for 30 minutes at room temprature, the change of color was observed in each test tube and the optical density of each tube was measured at 660 nm with spectrophotometer (Cary 50 Bio UV-VIS) (Varian).

The standard calibration curve of 0-0.2 mg BSA/mL was plotted and shown in figure 2.1. Then, the protein concentration of the sample was determined with standard calibration curve.

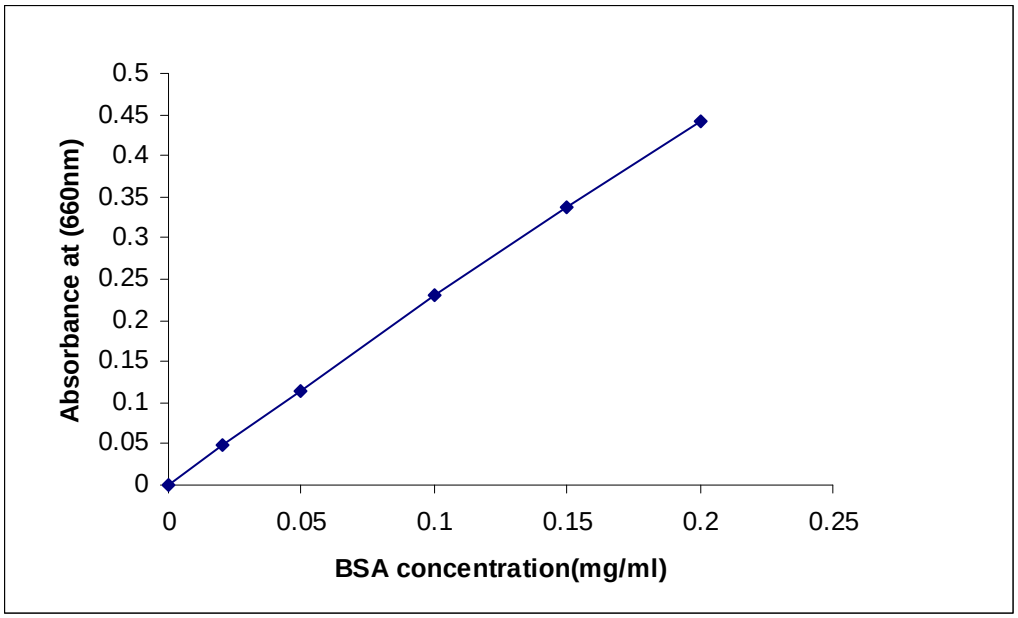


Figure 2.1 : BSA standard calibration curve for protein determination.

2.2.3.2. Determination of aldose reductase enzyme activity

The aldose reductase enzyme activity was spectrophotometrically measured by means of the method of Kinoshita (Kinoshita et al 1965). This method is based on the reduction of DL- gyceraldehyde as a substrate to glycerol by enzyme which is aldose reductase during the oxidation of Nicotinamide adenine dinucleotide phosphate (NADPH) to $NADP^+$. The oxidation of NADPH was spectrophotometrically followed at 340 nm using spectrophotometer. In our study, partially purified bovine lens extracts were taken as the enzyme source. (As described in methods section).

All of components were put into the reaction mixture with the given order was shown in Table 2.1. Determination of aldose reductase enzyme activity was applied for five minutes to each reaction cuvette. Absorbance immediately was measured at 340 nm before substrate, glyceraldehyde, was added. Then, after

substrate was added in cuvette, three minutes incubation time was maintained and the decrease in absorbance was measured once again at 340 nm. Under the same conditions, a blank solution was prepared without the substrate and the background corrections were made.

The assay mixture contained final concentrations of $6x10^{-5}$ M DL-Glyceraldehyde as substrate solution, $1x10^{-4}$ M NADPH prepared freshly, 1.43 mg/ml protein containing crude enzyme solution, 270 mM Li_2S0_4 , and 30 mM potassium phosphate buffer pH 6.7 in a final volume of 1.0 mL, is shown in table 2.1.

Table 2.1 : The components of assay mixture for aldose reductase

Components	**Volume Added (µL)**	**Final Concentration**
Glyceraldehyde	40	$6x10^{-5}$ M
NADPH	50	1 x 10^{-4} M
Enzyme Solution	*	1.43 mg/mL
Li_2S0_4	135	270mM
pH 6.7 Phosphate Buffer	600	30 mM
dH_20	To complete final volume to 1 mL	*
Total volume	1 mL	*

The specific activity was written as nmole NADPH oxidized per minute per mg protein per mL. One unit of enzyme was defined as amount of enzyme catalyzing the oxidation of 1 nmole NADPH per minute.

Background measurement, the slope of the reaction and the extinction coefficient of NADPH (6220 mM^{-1}) were taken for the calculation of aldose reductase activity.

$$\textbf{\textit{Specific Activity}}\ (\text{nmole} \cdot \text{min}^{-1} \cdot (\text{mg/mL})^{-1} = \frac{\text{Sample } \Delta \text{ OD}_{(340)} - \text{ Blank } \Delta\text{OD}_{(340)}}{\varepsilon^{340}_{(\text{NADPH})}.[\text{protein}]}$$

2.2.3.3 Inhibition of bovine lens aldose reductase

Various concentrations of extraction samples obtained in ethylacetate and ethanol solvents by extraction method were used for testing inhibitions on bovine lens aldose reductase and the volume of the test cuvette was completed to 1 mL with ultra pure water.

All of the ethylacetate and ethanol extracts were dissolved in 5 % dimethylsulfoxide (DMSO). The concentrations of these extracts were prepared by serial dilution of the stock solutions in 5 % DMSO. Various concentration of DMSO was added in the assay mixture to keep amount of DMSO constant. Each extract with DMSO content was added in test tube for measurement of bovine lens aldose reductase enzyme activity. This activity was considered as control activity. The content of final DMSO below 1 % had a little effect on the measurement of aldose reductase activity, which could be ignored on the measurement of the enzyme activity.

The standard assay mixture was used in our study. All of the components of the assay mixture and various concentrations of extract samples were put in assay cuvette and completed to 1mL with ultra pure water. Absorbances of these processes were measured at 340 nm and the results were taken as absorbances of samples at 340nm. All components of the assay mixture without dl-glyceraldehyde and various concentrations of extract sample were mixed in assay cuvette and completed to 1 mL with ultra pure water. Absorbances of these components were measured at 340 nm and the results were used as absorbances of blanks. All components of the assay mixture and various concentrations of DMSO were used in assay cuvette and completed to 1mL with ultra pure water. Absorbances of these components were measured at 340 nm and the results were taken as absorbances of controls.

The percentage inhibitions of aldose reductase activities of extract samples were calculated from the below formula:

$$\textbf{Percentage Inhibition (\%)} = \frac{(\Delta\ OD_{340\ control} - \Delta\ OD_{340\ sample}) * 100}{\Delta\ OD_{340}\ \ control}$$

The inhibitor effects of the extract samples were expressed as the 50 % inhibitory concentration (IC_{50} µg/mL).

2.2.4 Antioxidant activities of plant extracts

2.2.4.1 DPPH method (Free Radical Scavenging Activity)

2,2-diphenyl-1-picrylhydrazyl (DPPH) method was used by Blois (1958) for determining the free radical scavenging activities of samples and chemicals. DPPH is the purple-colored stable free radical reduced into the yellow colored diphenylpicryl hydrazine by giving a hydrogen from the compounds found in extracts. Reaction existing is shown in figure 2.2.

0.05 mg of DPPH was dissolved in 1 mL of ethanol.Then, 1.4 mL of DPPH ethanol solution was put into eppendorf. Absorbance of DPPH ethanol solution was recorded at 517 nm and obtained about 1.3 unit of absorbance.

Ethylacetate and ethanol extracts were dissolved in ethanol. Various concentrations of extracts were prepared and 0.1 mL of extract solutions was added to 1.4 mL of DPPH ethanol solution. Absorbances of samples were measured in 5 min-incubation time at 517 nm. In the same condition, blank incuding ethanol instead of extract solution was prepared and results were recorded at 517 nm.

Free radical scavenging activity effects of ethylacetate and ethanol extracts were calculated as formula below.

$$\textit{DPPH radical scavenging (\%)} = \frac{(\Delta\, OD_{517\ control} - \Delta\, OD_{517\ sample}) \times 100}{\Delta\, OD_{517}\ \ control}$$

$\Delta\, OD_{517\ control}$: is the absorbance of the control with ethanol

$\Delta\, OD_{517\ sample}$: is the absorbance of the ethylacetate and ethanol extracts dissolved in ethanol.

The antioxidant activities of the extract samples were expressed as the 50 % effective concentration (EC_{50} μg/mL).

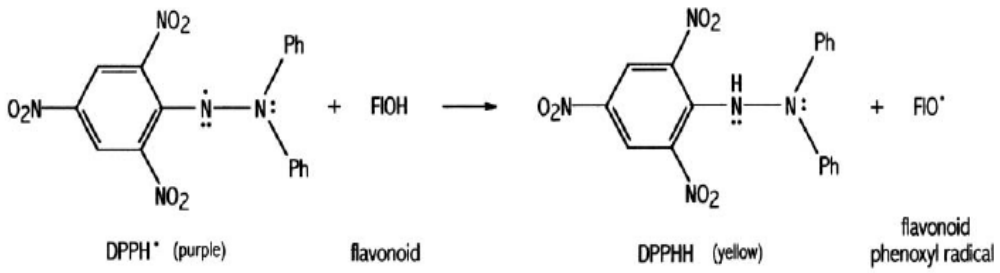


Figure 2.2: Free Radical Scavenging Activity of DPPH (2,2-Diphenyl-1 picrylhydrazyl) by a flavonoid (Dragan, 2003).

2.2.4.2 Determination of total phenol contents of plant extracts (TPC)

Total phenol contents of ethyl acetate and ethanol extracts were examined by a modified method including the method of Singleton and Rossi (1963).

Various concentrations (0.05-0.3 mg/mL) of gallic acid were used as standard. They were dissolved in ethanol and mixed with 2 mL aqueous solution of 2 % Na_2CO_3 and they were vortexed vigorously. After 3 minutes incubation time, 0.1 mL of 1 M Folin–Ciocalteu's phenol reagent was added in each gallic acid solution and vortexed. After 30 minutes for incubation time at room temperature, absorbances of gallic acid solutions were recorded at 750 nm.

Ethyl acetate and ethanol extracts were dissolved in ethanol and 0.1 mL of each ethyl acetate and ethanol extract was mixed with 2 mL aqueous solution of 2 % Na_2CO_3 and they were vortexed vigorously. We waited 3 minutes incubation time. Then, 0.1 mL of 1M Folin–Ciocalteu's phenol reagent was added in each mixture

and vortexed mixture. We waited 30 minutes for incubation time at room temperature. Absorbances of samples were recorded at 750 nm. The results obtained from absorbances were recorded as miligrams of total phenolics including in miligrams of extract as the gallic acid equivalents (GAE).

2.2.5 Statistical calculation

All of results are expressed as mean ± standard deviation (SD) and levels of significance were determined by regression analysis of variance using Minitab and plotted.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Weights of bovine lenses

Bovine lenses obtained from abattoir were excised from the eyes and washed with cold ultra pure water. Then, they were weighed and weight of average bovine lens was found as 2.06 g ± 0.04 (Mean ± SD, n=10).

3.2 Bovine lens aldose reductase activity

The bovine lens aldose reductase activity was examined according to the method of Kinoshita (Hayman; Kinoshita 1965) as described under ”Methods”. Crude extracts of the bovine lens homogenate were used as enzyme sources.

Aldose reductase activity was determined in the crude extracts prepared from 64 bovine lenses. Among the pooled lens extracts, the lowest activity was measured as 1.043 nmole NADPH oxidized /min/ mg protein and the highest activity was measured as 1.256 nmole NADPH oxidized /min/ mg protein.

The average aldose reductase activity calculated as 1.135 ± 0.058 (Mean ± SD, n=10) nmole NADPH oxidized /min/ mg protein.

3.3 Storage stability of aldose reductase activity

In order to determine effect of storage duration on the activity of lens aldose reductase, % AR activity was plotted versus duration time in weeks. Crude extracts from bovine lenses were divided into 1.5 mL of alliquots in eppendorf tubes which were treated in liquid nitrogen and stored at (-80 ^{o} C). Figure 3.1 shows the weeks of

stability of the enzyme. At the given weeks of storage, one of the alliquots was thawed and the aldose reductse activity was determined as explained under ”Methods”.

Thawing the sample once did not affect the aldose reductase activity significantly, but second thawing of the crude extract resulted in approximately 14 % loss of activity. Therefore, the measurements of the enzyme activity were performed using the samples thawed only once.

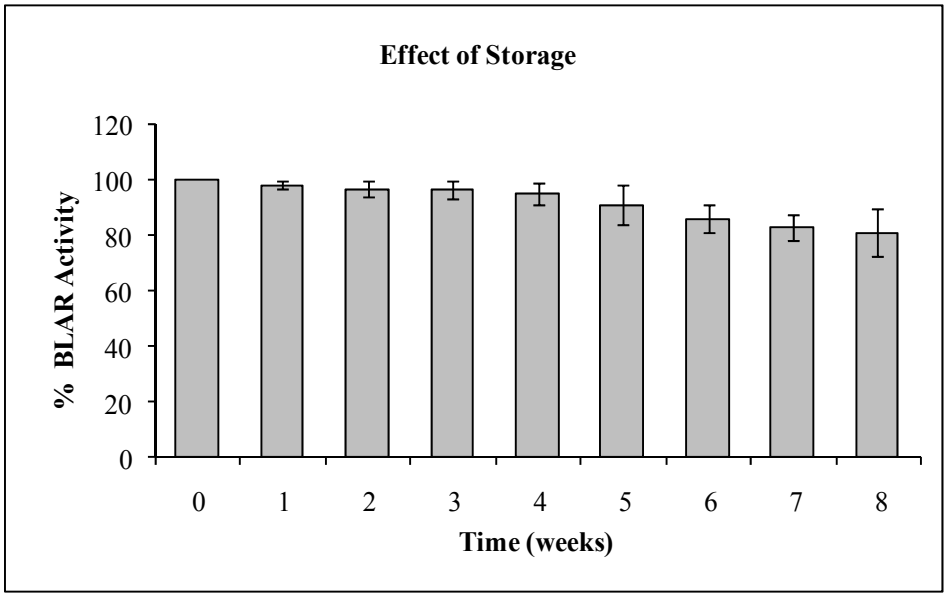


Figure 3.1: Storage stability of the bovine lens aldose reductase activity. Samples were stored in eppendorf tubes in small alliquots at -80 ºC. The data determined are mean of duplicate measurements obtained from at least three seperate experiments.

The bovine lens aldose reductase activitity was measured in the reaction medium containing 1.43 mg/mL protein containing crude enzyme solution, $6x10^{-5}$ M DL-glyceraldehyde as substrate solution, $1x10^{-4}$ M NADPH prepared freshly, 270 mM Li_2S0_4, and 30 mM potassium phosphate buffer at of pH 6.7 in a final volume of 1.0 mL.

The bovine lens aldose reductase activity was stable only for 4 weeks; afterwards, it recessed to nearly 91.1 % at the 5th week, 86.1 % at the 6th week, 82.8 % at the 7th week, and 81.2 % at the 8 th week when activities compared to the initial enzyme activity.

3.4 Characterization of bovine lens aldose reductase activity

3.4.1 The time course of bovine lens aldose reductase activity

The reaction that is catalayzed by aldose reductase exists by means of NADPH oxidation and DL-gleyceraldehyde reduction, whose, the time course of activity was shown figure 3.2.

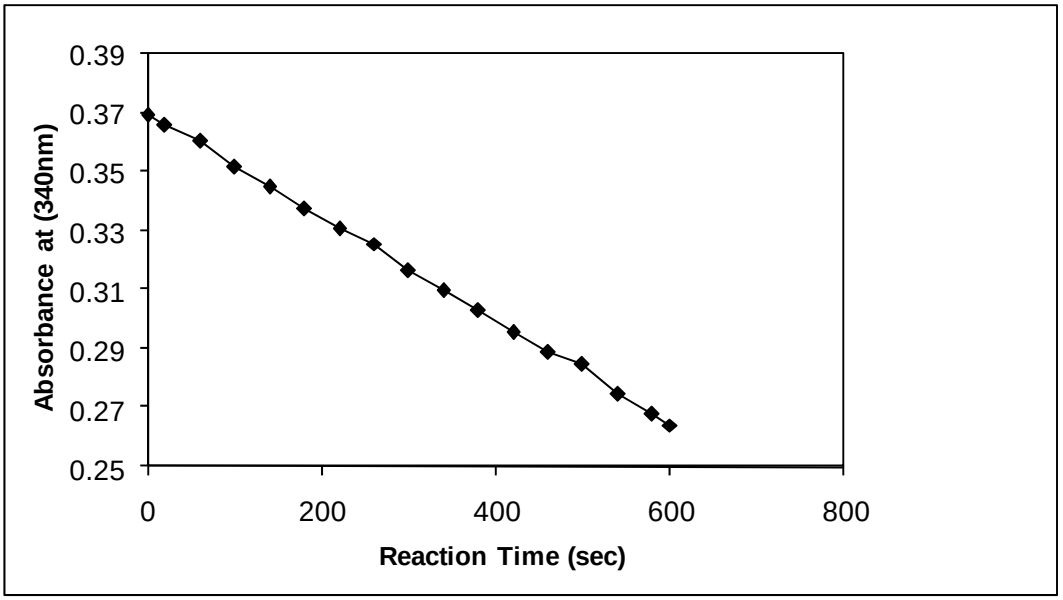


Figure 3.2: The time course of bovine lens aldose reductase enzyme activity. Reactions were examined at 25 ºC as described in the methods.

3.4.2 Effect of protein amount on the enzyme activity

The effect of the enzyme amount on BLAR activity was measured by changing the final protein concentration in the 1 mL reaction mixture. The protein amount in reaction cuvette was varied from 0.31 mg to 3.48 mg. It was found that the reaction rate was proportional with the enzyme amount up to 2.25 mg protein in 1 mL reaction mixture. In order to obtain enough quantity of product for spectrophotometric determination , 1.43 mg of protein routinely used throughout in this study. Effect of protein amount on the BLAR activity was shown in figure 3.3. The BLAR activities were measured in the reaction medium containing different concentrations of protein including crude enzyme solution, $6x10^{-5}$ M DL-glyceraldehyde as substrate solution, $1x10^{-4}$ M NADPH freshly prepared, 270 mM Li_2S0_4, and 30 mM PBS at a pH of 6.7 in the final volume of 1.0 mL.

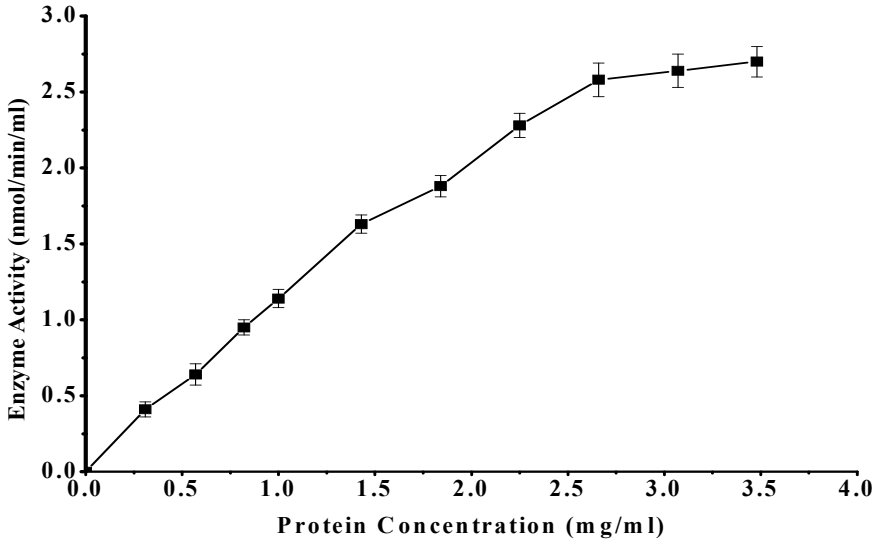


Figure 3.3: Effect of the enzyme amount on bovine lens aldose reductase activity. The values were expressed as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments.

3.4.3 Effect of pH on enzyme activity

The effect of pH on BLAR enzyme activity was shown in figure 3.4. The pH measurements were carried out by changing the pH of 30 mM acetate buffer between 4.0 and 6.0, and the pH of 30 mM phosphate buffer (PBS) between 6.0 and 8.0 in the 1 mL rection mixture. It was found that the enzyme activity increased from pH 6.0 through pH 6.7 and decreased from pH 6.7 through pH 8.0 when the PBS was used. On the other hand, when the acetate buffer was used, enzyme activity was found much lower than that of phosphate buffer. In order to obtain maximum enzyme activity for spectrophotometric determination, pH 6.7 of 30 mM PBS was routinely used throughout in this study. BLAR activity were measured in the reaction medium containing different pH values of 30 mM acetate and phosphate buffer, 1.43 mg protein as enzyme source, $6x10^{-5}$ M DL-Glyceraldehyde as substrate solution, $1x10^{-4}$ M NADPH prepared freshly and 270 mM Li_2S0_4 in a final volume of 1.0mL.

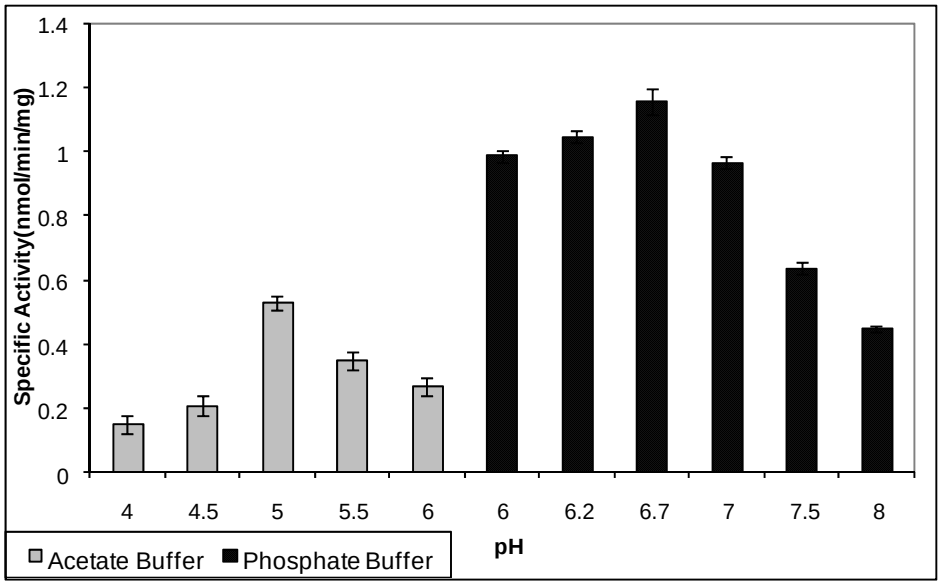


Figure 3.4: The effect of pH BLAR activity. The values were expressed as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments.

3.4.4 Effect of Li_2SO_4 amount on enzyme activity

Li_2SO_4 was used as an activator. Also, Li_2SO_4 is necessary for correct aldose reductase activity results. Aldehyde reductase II found in the crude protein mixture reduces aldehydes and oxidizes NADPH like aldose reductase (Das, 1985). The effect of Li_2SO_4 concentration on BLAR was shown figure 3.5. In absence of Li_2SO_4, BLAR activity was found 0.33±0.02 nmole/min/mg. In precence of Li_2SO_4, BLAR activity incerased while Li_2SO_4 concentration was increasing.

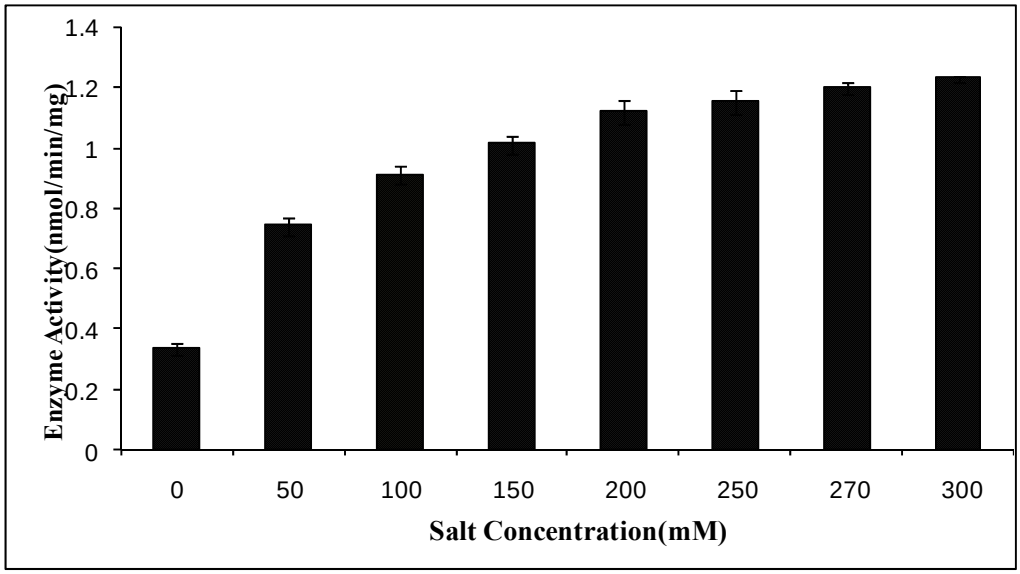


Figure 3.5: The effect of Li_2SO_4 concentration on BLAR. The values were expressed as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments.

Li_2SO_4 concentration was increased from 50 mM to 300 mM to find spesific enzyme activities and specific enzyme activities were varied from 0.74±0.03 to 1.23±0.01. When Li_2SO_4 concentration was increased from 50mM to 270 mM, specific enzyme activity raised proportionally and it was found as 1.20±0.02 at 270 mM. Then, the specific enzyme activity did not change much when Li_2SO_4 concentration was increased. Therefore, we used Li_2SO_4 concentration at 270 mM for further studies.

BLAR activity was carried out in the reaction medium containing different concentrations of Li_2SO_4, 1.43 mg protein as enzyme source, $6x10^{-5}$ M DL-Glyceraldehyde as substrate solution, $1x10^{-4}$ M NADPH prepared freshly and pH 6.7 of 30 mM PBS in a final volume of 1.0 mL.

3.4.5 The effect of glyceraldehyde concentration on enzyme activity

The effects of substrate glyceraldehyde concentration on BLAR were measured by changing glyceraldehyde concentration. Glyceraldehyde concentration was changed from 3 μM to 105 μM to find spesific enzyme activities and specific enzyme activities were varied from 0.21±0.03 to 1.22±0.06. BLAR activity reached to saturation at nearly 60 μM glyceraldehyde. As glyceraldehyde concentration was further increased, enzyme activity did not show a significant difference. Therefore, 60μM was used as the optimum concentration of substrate for further study. The effect of substrate concentration was shown in figure 3.6.

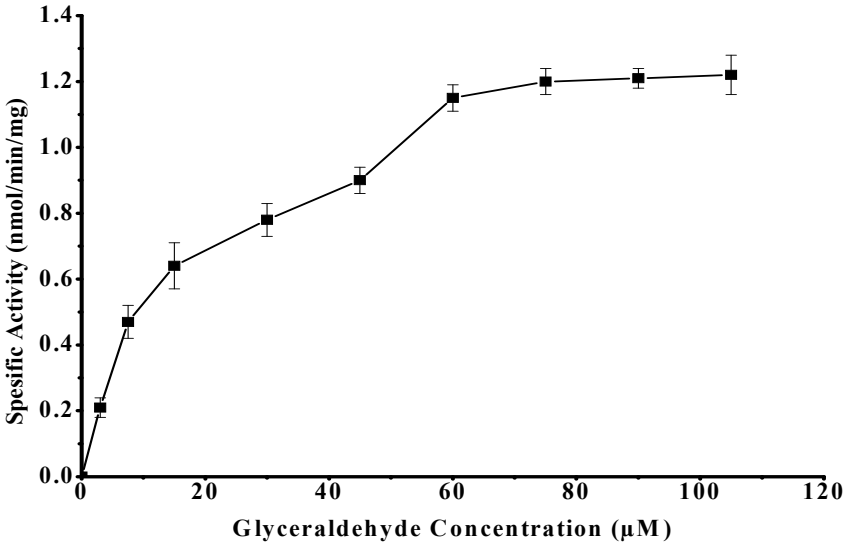


Figure 3.6: The effect of glyceraldehyde concentration on the enzyme activity. The values were expressed as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments.

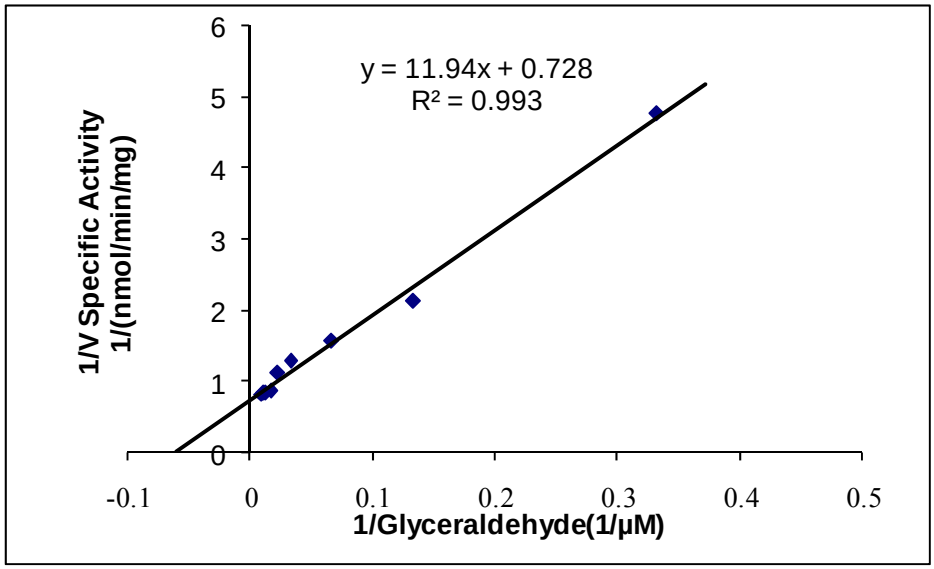


Figure 3.7: Lineweaver-Burk plot of BLAR activity against glyceraldehyde.

Meanwhile, V_{max} and K_m values of BLAR were calculated for glyceraldehyde by plotting Lineweaver-Burk plot. While V_{max} for glyceraldehyde was found as 1.372 nmol/min/mg, K_m was calculated as 16.39 µM, which was illustrated by Lineweaver-Burk plot in figure 3.7.

BLAR activity was measured in the reaction medium containing different concentrations of DL-Glyceraldehyde as substrate, 1.43 mg protein as enzyme source, $1x10^{-4}$ M NADPH prepared freshly, pH 6.7 of 30mM PBS and 270 mM Li_2S0_4 in a final volume of 1.0 mL.

3.4.6 The effect of cofactor NADPH concentration on the enzyme activity

The effect of cofactor NADPH concentration on the enzyme activity was measured by changing NADPH concentration in 1.0 mL reaction mixture. NADPH concentration was changed from 20 µM to 200 µM to find specific enzyme activity and specific enzyme activity was varied from 0.52±0.05 to 1.18±0.02. BLAR activity reached to saturation at nearly 100 µM NADPH concentration. As glyceraldehyde concentration was further increased, enzyme activity did not present a significant difference. Therefore, 100 µM was used as the concentration of optimum cofactor. The effect of cofactor concentration was shown in figure 3.8.

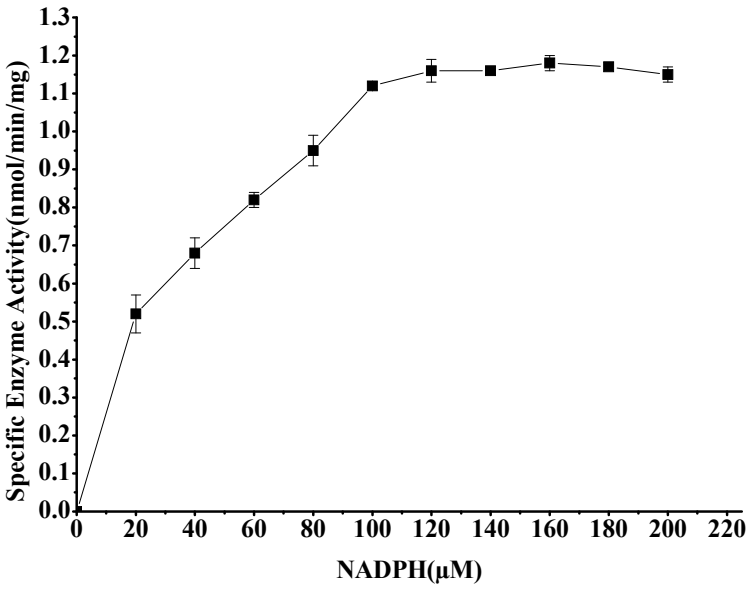


Figure 3.8: The effect of cofactor NADPH concentration on the enzyme activity. The values were expressed as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments.

Then, V_{max} and K_m values of bovine lens aldose reductase were examined for cofactor NADPH by plotting Lineweaver-Burk plot. While V_{max} for cofactor NADPH was calculated as 1.4 nmol/min/mg, K_m was found as 35.81 µM, which was illustrated by Lineweaver-Burk plot in figure 3.9.

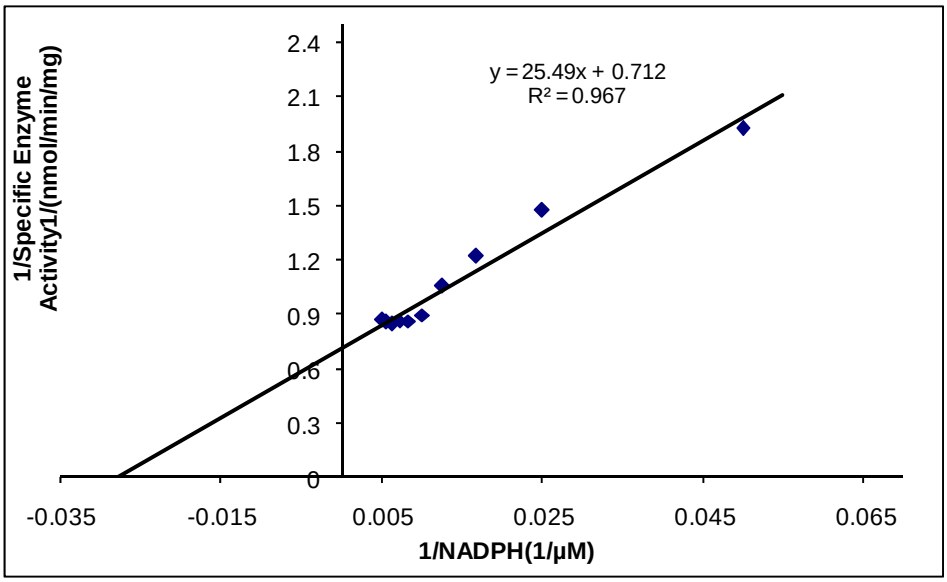


Figure 3.9: Lineweaver-Burk plot of BLAR activity against cofactor (NADPH).

BLAR activity was measured in the reaction medium containing different concentrations of NADPH as cofactor, 1.43 mg protein as enzyme source, $6x10^{-5}$ M DL-Glyceraldehyde as substrate solution, pH 6.7 of 30mM Phosphate Buffer and 270 mM Li_2S0_4 in a final volume of 1.0 mL.

3.4.7 The effect of temperature on the enzyme activity

The effect of temperature on aldose reductase enzyme activity was examined by incubating the reaction mixture at 11 different temperatures as such 10 ^{o}C, 15 ^{o}C, 20 ^{o}C, 25 ^{o}C, 30 ^{o}C, 35 ^{o}C, 40 ^{o}C, 45 ^{o}C, 50 ^{o}C, 55 ^{o}C and 60 ^{o}C. Mixtures without adding the enzyme were incubated at given tempratures for 3 minutes. Then, the enzyme was added and the enzyme activity was measured for 5 minutes. The maximum enzyme activity was found at 60 ^{o}C. However, all of the enzyme activity measurements were examined at room temperature to facilitate the study. The effect of temperature on enzyme activity was shown in figure 3.10.

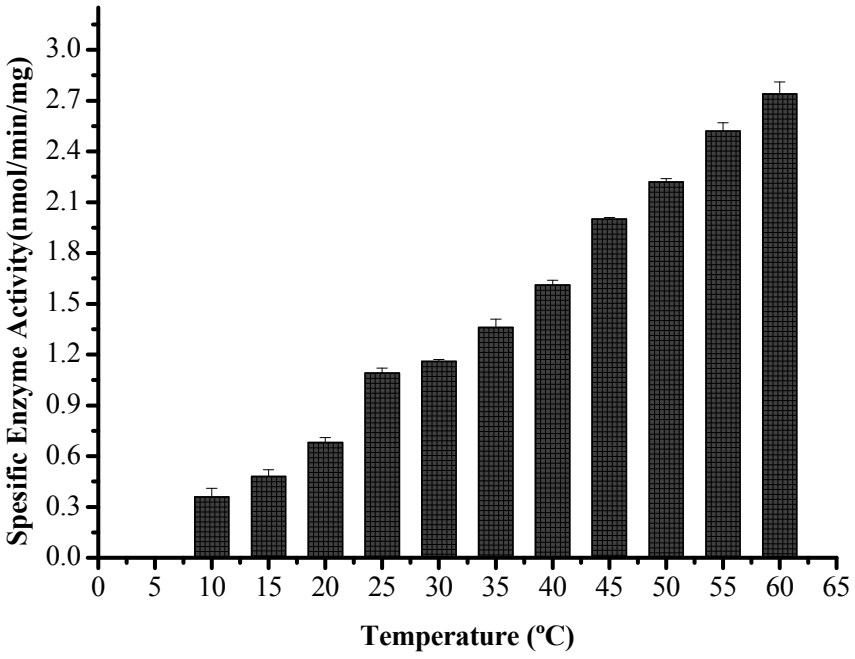


Figure 3.10: Effect of temperature on BLAR activity. The values were expressed as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments.

3.4.8 The inhibitory effect of dimethylsulfoxide (DMSO) on aldose reductase enzyme activity

The inhibitory effect of dimethylsulfoxide (DMSO) on BLAR activity was measured by changing DMSO concentration in 1.0 mL reaction mixture. DMSO concentration was changed from 0.1% to 10% to find the percent of inhibition and the percent of inhibition were varied from 0.99±0.05 to 77.23±0.24. BLAR activity was not affected importantly until 1% DMSO concentration. As DMSO concentration was further increased, enzyme activity changed notably. Hence, 1% DMSO concentration could be handled on the measurement of BLAR. The inhibitory effect of dimethylsulfoxide (DMSO) on BLAR activity was shown in figure 3.11.

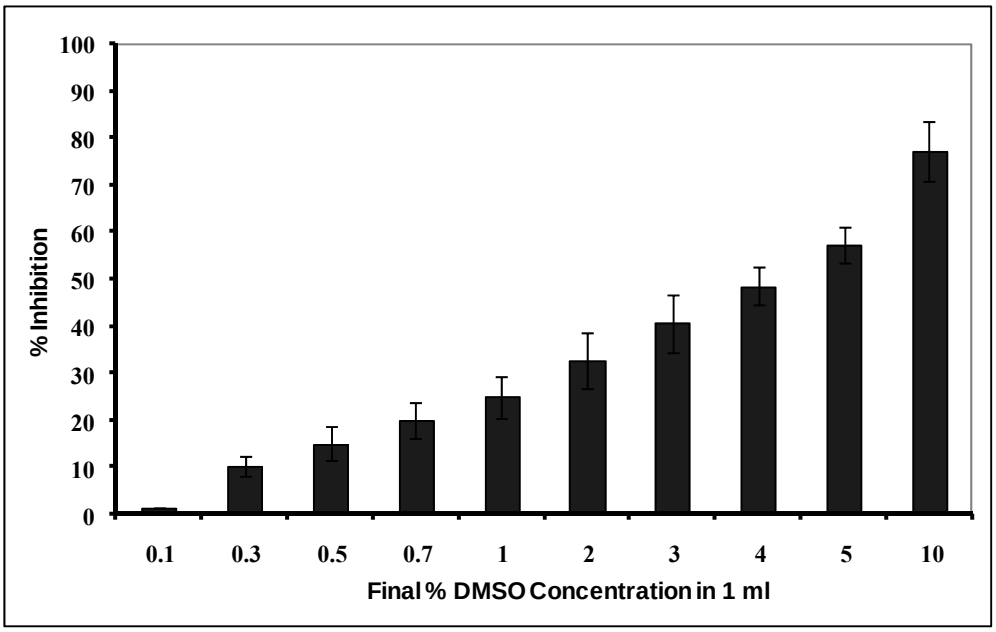


Figure 3.11: Effect of dimethylsulfoxide (DMSO) on BLAR activity. The values were expressed as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments.

3.5 Yields of ethanol and ethyl acetate extracts of plant materials

Ocimum basilicum, Lavandula stoechas, Melissa officinalis, Glycyrrhiza glabra L, and Tilia tomentosa were dried at room temprature then finely grounded to enlarge air-surface area by Waring Blender (model 32BL80). Plant samples (20 g) were three times extracted in 1:10 ratio of ethanol and ethyl acetate for 24 hours at rocking incubator (Optic Ivymen System) at 30 °C. Mixtures were filtered through a double layered cheese cloth. Then, samples were filtered with a rough filter paper. Next, solvents of plants were evaporated by rotary evaporator (Heidolph Laborota 4000) at 35 °C until mixtures remained to 50 ml of solvents. Afterwards, mixtures including 50 ml of solvent settled to lyophilizator (Heto-Holten Model Maxi-Dry Lyo) to dry plant extracts completely. Dried extracts were weight out and the percent yield of extraction was calculated and yields of ethanol and ethyl acetate extracts were shown in table 3.1 and table 3.2, respectively.

3.5.1 Yields of ethanol extracts

Dried ethanol extracts were weight out and the percent yield of extraction was calculated and yields of ethanol extracts were expressed in table 3.1.

Table 3.1 : Comparision of percent yields of ethanol extracts. The values were shown as (%) percent yield of ethanol extracts (w/w)

Ethanol Extracts	Percent Yield (w/w) (%)
Ocimum basilicum	7.76
Lavandula stoechas	11.78
Melissa officinalis	7.92
Glycyrrhiza glabra L	11.08
Tilia tomentosa	7.67

3.5.2 Yields of ethyl acetate extracts

Dried ethyl acetate extracts were weight out and the percent yield of extraction was calculated and yields of ethanol extracts were shown in table 3.2.

Table 3.2 : Comparision of percent yields of ethyl acetate extracts. The values were shown as (%) percent yield of ethyl acetate extracts (w/w).

Ethyl Acetate Extracts	Percent Yield (w/w) (%)
Ocimum basilicum	4.32
Lavandula stoechas	5.26
Melissa officinalis	3.13
Glycyrrhiza glabra L	2.55
Tilia tomentosa	4.97

3.6 Inhibitory effect of plant extracts on aldose reductase activity

Various concentrations of extraction samples obtained in ethylacetate and ethanol solvents by extraction method were used for testing inhibitions on bovine lens aldose reductase.The inhibition assay studies examined to use crude enzyme solution. Each extract was dissolved in DMSO because extracts acquired were not dissolved in water.

The concentrations of these extracts were prepared by serial dilution of the stock solutions in 5% DMSO. Various concentration of DMSO was added in assay mixture to keep amount of DMSO constant. DMSO content in each extract was added in test tube for measurement of BLAR activity. This activity was considered as control activity.

3.6.1 Inhibitory effect of ethanol extracts on aldose reductase enzyme activity

3.6.1.1 Inhibitory effect of *Ocimum Basilicum* ethanol extracts on aldose reductase enzyme activity

BLAR inhibition study was carried out by using different concentrations of *Ocimum Basilicum* ethanol extracts in 1 mL of assay solution. Assay constituents were composed of different concentrations of *Ocimum Basilicum* ethanol extracts in DMSO, 1.43 mg protein as enzyme source, $6x10^{-5}$ M DL-Glyceraldehyde as substrate solution, $1x10^{-4}$ M NADPH prepared freshly, pH 6.7 of 30 mM PBS and 270 mM Li_2S0_4 in a final volume of 1.0 mL.

The inhibitory effect results of *Ocimum Basilicum* ethanol extracts on BLAR activity was given in figure 3.12.

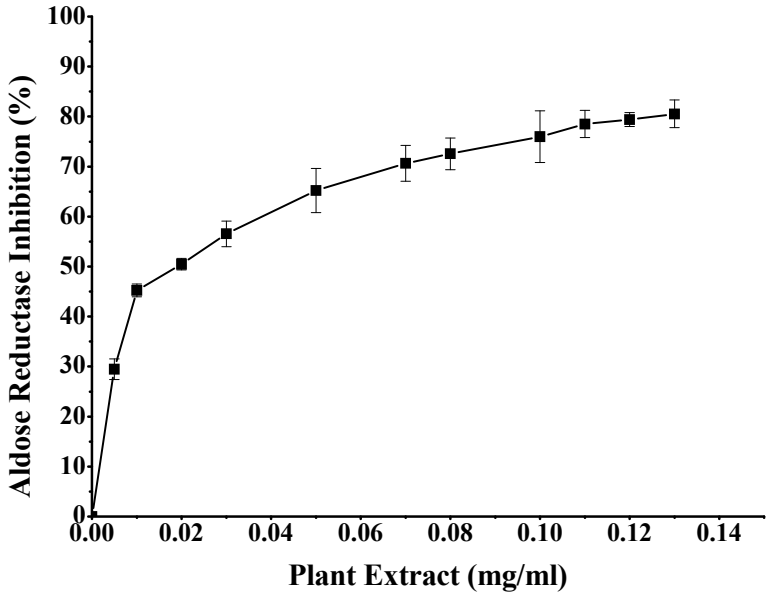


Figure 3.12: Inhibitory effect of *Ocimum Basilicum* ethanol extracts on BLAR activity. 50 % inhibitory concentration (IC_{50} µg/mL) of *Ocimum Basilicum* ethanol extracts was calculated as 45.89 ± 2.31 µg/mL.

3.6.1.2 Inhibitory effect of *Lavandula stoechas* ethanol extracts on aldose reductase enzyme activity

BLAR inhibition study was examined by using different concentrations of *Lavandula stoechas* ethanol extracts in 1 mL of assay solution. Assay constituents consist of different concentrations of *Lavandula stoechas* ethanol extracts in DMSO, 1.43 mg protein as enzyme source, $6x10^{-5}$ M DL-Glyceraldehyde as substrate solution, $1x10^{-4}$ M NADPH prepared freshly, pH 6.7 of 30 mM PBS and 270 mM Li_2S0_4 in a final volume of 1.0 mL. The inhibitory effect results of *Lavandula stoechas* ethanol extracts on BLAR activity was given in figure 3.13.

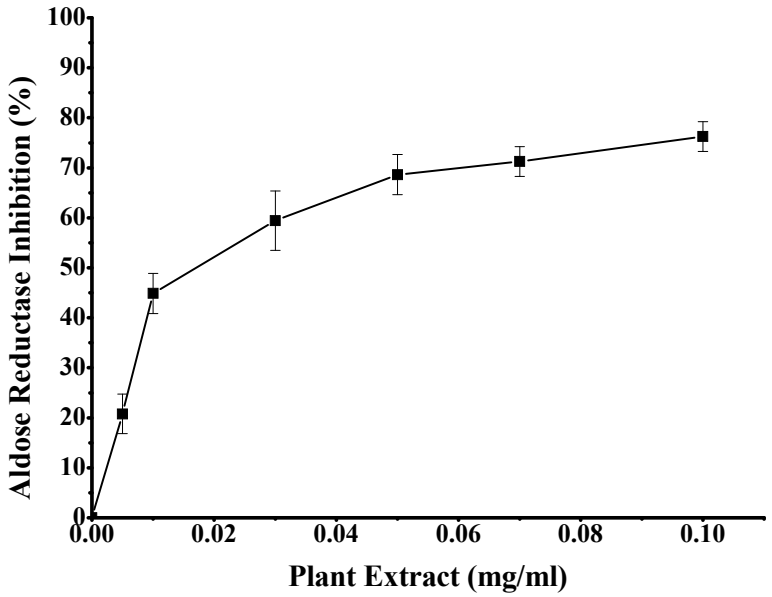


Figure 3.13: Inhibitory effect of *Lavandula stoechas* ethanol extracts on BLAR activity. The values were shown as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments. 50 % inhibitory concentration (IC_{50} µg/mL) of *Lavandula stoechas* ethanol extracts was calculated as 39.28 ± 11 µg/mL.

3.6.1.3 Inhibitory effect of *Melissa officinalis* ethanol extracts on aldose reductase enzyme activity

BLAR inhibition study was made by using different concentrations of *Melissa officinalis* ethanol extracts in 1 mL of assay medium. Assay components consist of different concentrations of *Melissa officinalis* ethanol extracts in DMSO, 1.43 mg protein as enzyme source, $6x10^{-5}$ M DL-Glyceraldehyde as substrate solution, $1x10^{-4}$ M NADPH prepared freshly, pH 6.7 of 30 mM PBS and 270 mM Li_2S0_4 in a final volume of 1.0 mL. The inhibitory effect results of *Melissa officinalis* ethanol extracts on BLAR activity was displayed in figure 3.14.

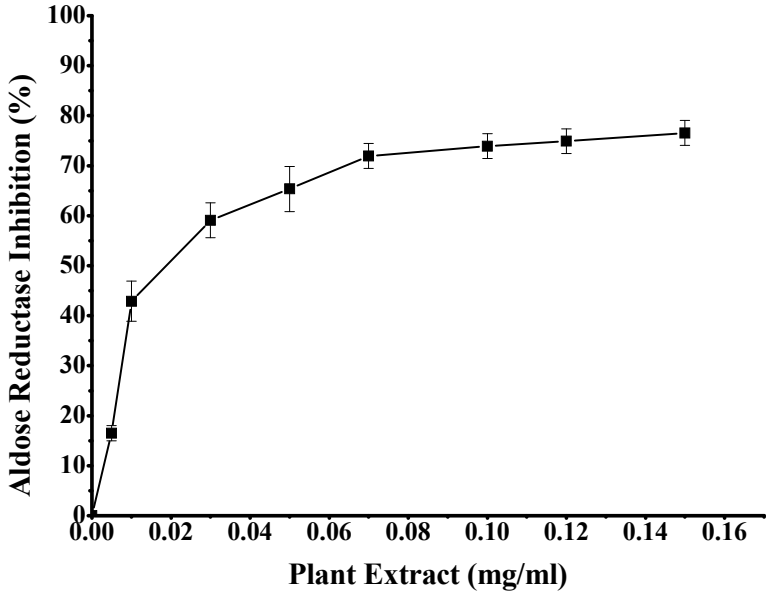


Figure 3.14: Inhibitory effect of *Melissa officinalis* ethanol extracts on BLAR activity. The values were demonstrated as Mean ± SD. Data found are mean of duplicate measurements obtained from at least three seperate experiments. 50 % inhibitory concentration (IC_{50} µg/mL) of *Melissa officinalis* ethanol extracts was found as 54.15 ± 3.26 µg/mL.

3.6.1.4 Inhibitory effect of *Glycyrrhiza glabra L* ethanol extracts on aldose reductase enzyme activity

BLAR inhibition study was carried out by using different concentrations of *Glycyrrhiza glabra L* ethanol extracts in 1 mL of assay medium. Assay constituents were composed of different concentrations of *Glycyrrhiza glabra L* ethanol extracts in DMSO, 1.43 mg protein as enzyme source, $6x10^{-5}$ M DL-Glyceraldehyde as substrate solution, $1x10^{-4}$ M NADPH prepared freshly, pH 6.7 of 30 mM phosphate buffer and 270 mM Li_2S0_4 in a final volume of 1.0 mL. The inhibitory effect results of *Glycyrrhiza glabra L* ethanol extracts on BLAR activity was shown in figure 3.15.

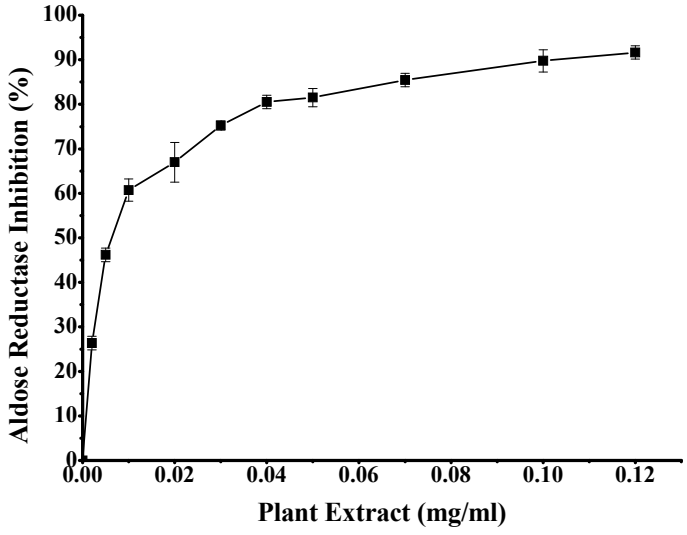


Figure 3.15: Inhibitory effect of *Glycyrrhiza glabra L* ethanol extracts on BLAR activity. The values were shown as Mean ± SD. Data obtained are mean of duplicate measurements acquired from at least three seperate experiments. 50 % inhibitory concentration (IC_{50} µg/mL) of *Glycyrrhiza glabra L* ethanol extracts was calculated as 25.53 ± 0.36 µg/mL.

3.6.1.5 Inhibitory effect of *Tilia tomentosa* ethanol extracts on aldose reductase enzyme activity

BLAR inhibition study was examined by using different concentrations of *Tilia tomentosa* ethanol extracts in 1 mL of assay solution. Assay components were combined with different concentrations of *Tilia tomentosa* ethanol extracts in DMSO, 1.43 mg protein as enzyme source, $6x10^{-5}$ M DL-Glyceraldehyde as substrate solution, $1x10^{-4}$ M NADPH prepared freshly, pH 6.7 of 30 mM PBS and 270 mM Li_2S0_4 in a final volume of 1.0 mL. The inhibitory effect results of *Tilia tomentosa* ethanol extracts on BLAR activity was given in figure 3.16.

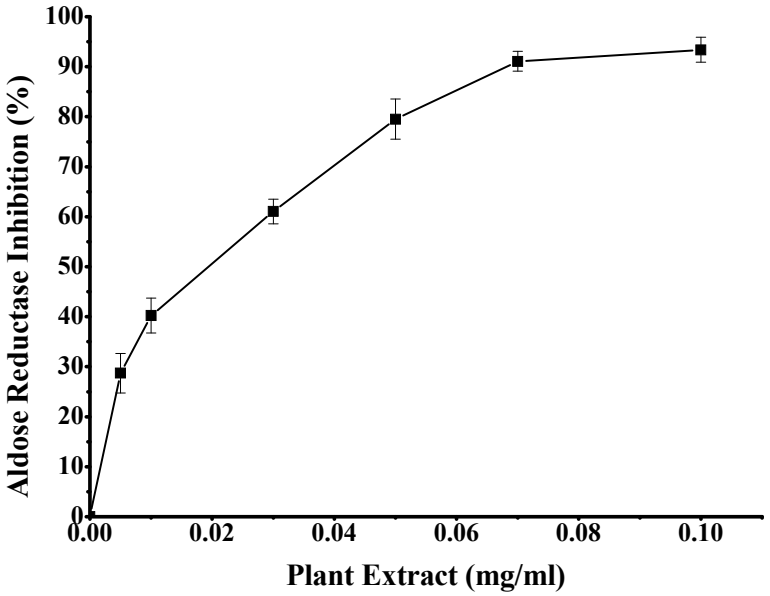


Figure 3.16: Inhibitory effect of Tilia *tomentosa* ethanol extracts on BLAR activity. The values were taken as Mean ± SD. Data given are mean of duplicate measurements obtained from at least three seperate experiments. 50 % inhibitory concentration (IC_{50} µg/mL) of *Tilia tomentosa* ethanol extracts was found as 31.76 ± 0.2 µg/mL.

3.6.1.6 Summary of inhibitory effects (IC_{50} µg/ml) of ethanol extracts on aldose reductase activity

Inhibitory effects of ethanol extracts on BLAR were expressed as 50 % inhibitory concentration (IC_{50} µg/mL) and shown in figure 3.17.

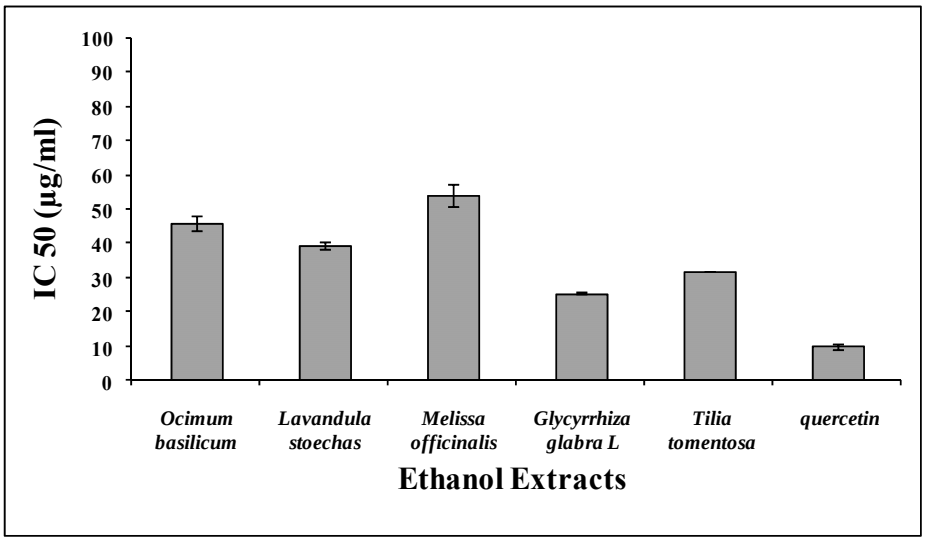


Figure 3.17 : Inhibitory Effects of Ethanol Extracts on BLAR activity. The values were taken as Mean ± SD. Data given are mean of duplicate measurements obtained from at least three seperate experiments.

3.6.2 Inhibitory effect of ethyl acetate extracts on aldose reductase enzyme activity

3.6.2.1 Inhibitory effect of *Ocimum Basilicum* ethyl acetate extracts on aldose reductase enzyme activity

BLAR inhibition study was made by using different concentrations of *Ocimum Basilicum* ethyl acetate extracts in 1 mL of assay solution.

Assay constituents consist of different concentrations of *Ocimum Basilicum* ethyl acetate extracts in DMSO, 1.43 mg protein as enzyme source, $6x10^{-5}$ M DL-Glyceraldehyde as substrate solution, $1x10^{-4}$ M NADPH prepared freshly, pH 6.7 of 30 mM PBS and 270 mM Li_2S0_4 in a final volume of 1.0 mL.

The inhibitory effect results of *Ocimum Basilicum* ethyl acetate extracts on BLAR activity was shown in figure 3.18 and the inhibitor effects of the *Ocimum Basilicum* ethyl acetate extracts were expressed as the 50 inhibitory concentration (IC_{50} µg/mL).

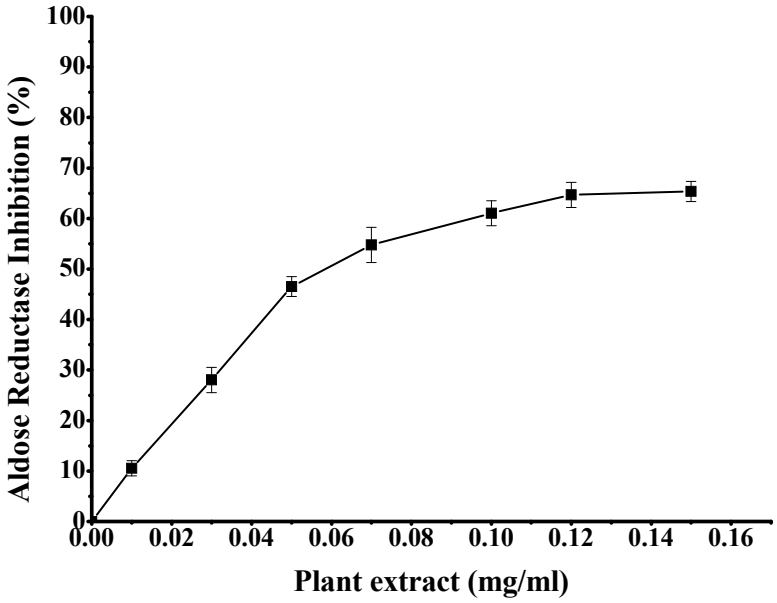


Figure 3.18: Inhibitory effect of *Ocimum basilicum* ethyl acetate extracts on BLAR activity. The values were shown as Mean ± SD. Data given are mean of duplicate measurements obtained from at least three seperate experiments. 50 % inhibitory concentration (IC_{50} µg/mL) of *Ocimum Basilicum* ethyl acetate extracts was calculated as 82.96 ± 1.88 µg/mL.

3.6.2.2 Inhibitory effect of *Lavandula stoechas* ethyl acetate extracts on aldose reductase enzyme activity

BLAR inhibition study was carried out by using different concentrations of *Lavandula stoechas* ethyl acetate extracts in 1 mL of assay solution. Assay constituents were composed of different concentrations of *Lavandula stoechas* ethyl acetate extracts in DMSO, 1.43 mg protein as enzyme source, $6x10^{-5}$ M DL-Glyceraldehyde as substrate solution, $1x10^{-4}$ M NADPH prepared freshly, pH 6.7 of 30 mM PBS and 270 mM Li_2S0_4 in a final volume of 1.0 mL. The inhibitory effect results of *Lavandula stoechas* ethyl acetate extracts on BLAR activity was shown in figure 3.19.

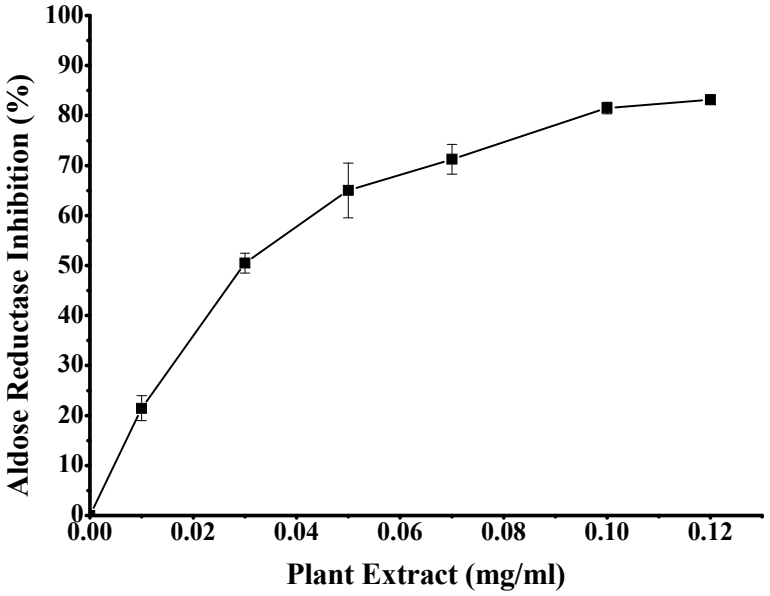


Figure 3.19: Inhibitory effect of *Lavandula stoechas* ethyl acetate extracts BLAR activity. The values were shown as Mean ± SD. 50 % inhibitory concentration (IC_{50} µg/mL) of *Lavandula stoechas* ethyl acetate extracts was found as 50.04 ± 1.5 µg/mL.

3.6.2.3 Inhibitory effect of *Melissa officinalis* ethyl acetate extracts on aldose reductase enzyme activity

BLAR inhibition study was carried out by using different concentrations of *Melissa officinalis* ethyl acetate extracts in 1 mL of assay medium. Assay components consist of different concentrations of *Melissa officinalis* ethyl acetate extracts in DMSO, 1.43 mg protein as enzyme source, $6x10^{-5}$ M DL-Glyceraldehyde as substrate solution, $1x10^{-4}$ M NADPH prepared freshly, pH 6.7 of 30 mM PBS and 270 mM Li_2S0_4 in a final volume of 1.0 mL. The inhibitory effect results of *Melissa officinalis* ethyl acetate extracts on BLAR activity was shown in figure 3.20.

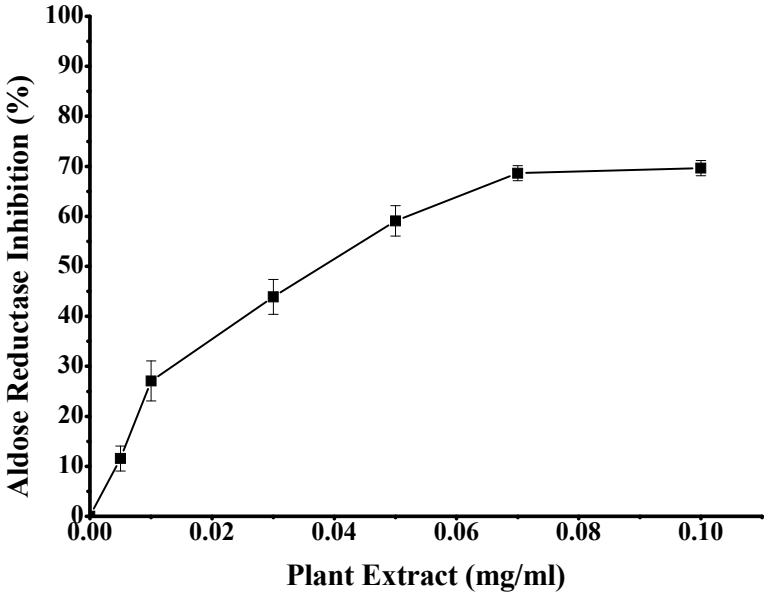


Figure 3.20: Inhibitory effect of *Melissa officinalis* ethyl acetate extracts on aldose reductase enzyme activity. 50 % inhibitory concentration (IC_{50} µg/mL) of *Melissa officinalis* ethyl acetate extracts was found as 50.32 ± 2.46 µg/mL.

3.6.2.4 Inhibitory effect of *Glycyrrhiza glabra L* ethyl acetate extracts on aldose reductase enzyme activity

BLAR inhibition study was studied by using different concentrations of *Glycyrrhiza glabra L* ethyl acetate extracts in 1 mL of assay medium. Assay components were composed of different concentrations of *Glycyrrhiza glabra L* ethyl acetate extracts in DMSO, 1.43 mg protein as enzyme source, $6x10^{-5}$ M DL-Glyceraldehyde as substrate solution, $1x10^{-4}$ M NADPH prepared freshly, pH 6.7 of 30 mM PBS and 270 mM Li_2S0_4 in a final volume of 1.0 mL. The inhibitory effect results of *Glycyrrhiza glabra L* ethyl acetate extracts on BLAR activity was given in figure 3.21.

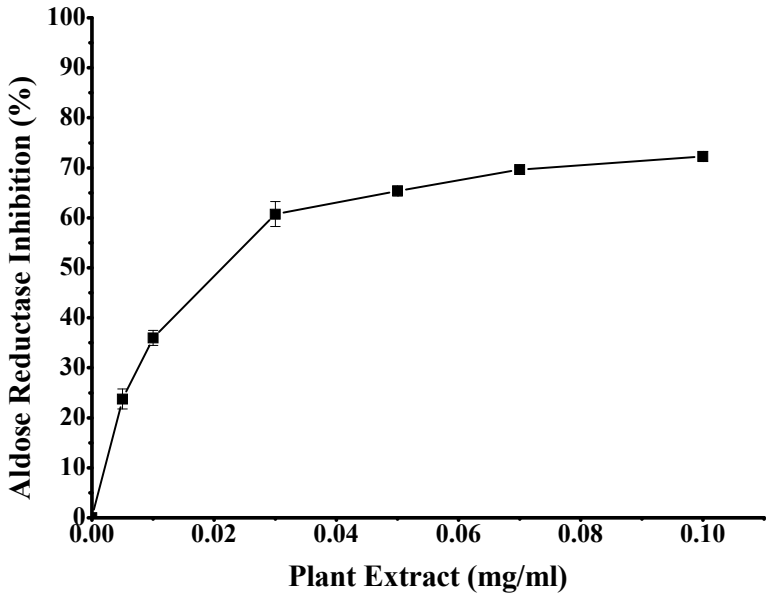


Figure 3.21: Inhibitory effect of *Glycyrrhiza glabra L* ethyl acetate extracts on BLAR activity. The values were shown as Mean ± SD. experiments. 50 % inhibitory concentration (IC_{50} µg/mL) of *Glycyrrhiza glabra L* ethyl acetate extracts was found as 41.55 ± 1.4 µg/mL.

3.6.2.5 Inhibitory effect of *Tilia tomentosa* ethyl acetate extracts on aldose reductase enzyme activity

BLAR inhibition study was examined by using different concentrations of *Tilia tomentosa* ethyl acetate extracts in 1 mL of assay solution. Assay components were added with different concentrations of *Tilia tomentosa* ethanol extracts in DMSO, 1.43 mg protein as enzyme source, $6x10^{-5}$ M DL-Glyceraldehyde as substrate solution, $1x10^{-4}$ M NADPH prepared freshly, pH 6.7 of 30 mM PBS and 270 mM Li_2S0_4 in a final volume of 1.0 mL. The inhibitory effect results of *Tilia tomentosa* ethyl acetate extracts on BLAR activity was shown in figure 3.21.

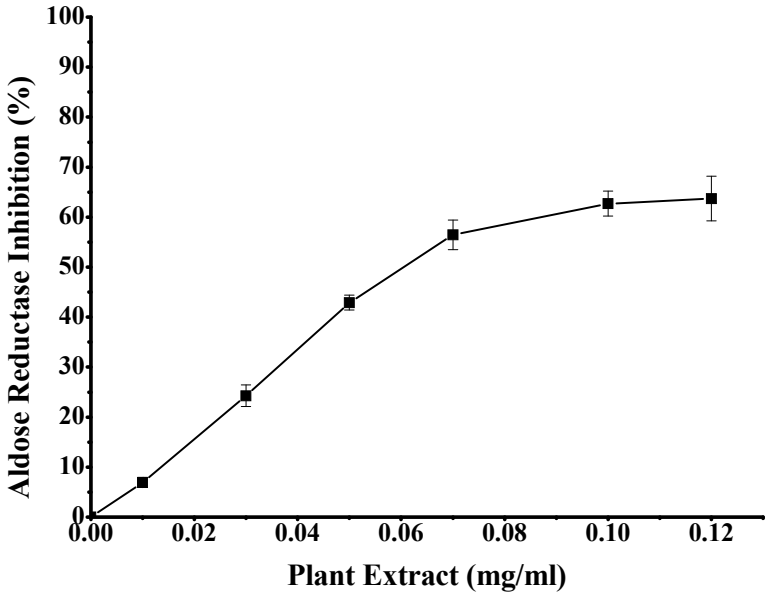


Figure 3.22: Inhibitory effect of *Tilia tomentosa* ethyl acetate extracts on BLAR activity. The values were shown as Mean ± SD. 50 % inhibitory concentration (IC_{50} µg/mL) of *Tilia tomentosa* ethyl acetate extracts was calculated as 75.96 ± 3.51 µg/mL.

3.6.3 Summary of inhibitory effects (IC_{50} µg/mL) of ethyl acetate extracts on aldose reductase activity

Inhibitory effects of ethyl acetate extracts on BLAR were expressed as 50 % inhibitory concentration (IC_{50} µg/mL) and shown in figure 3.23.

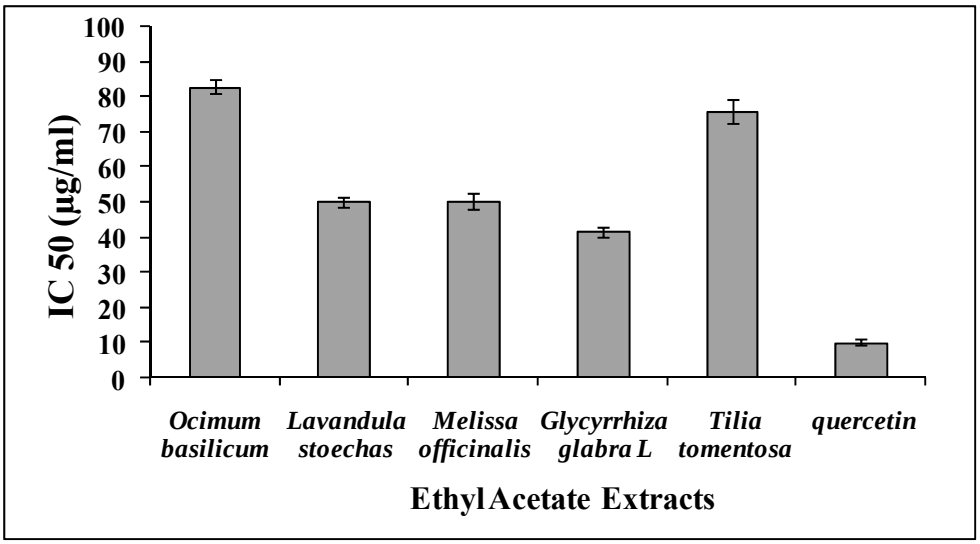


Figure 3.23 : Inhibitory effects of ethyl acetate extracts on BLAR activity. The values were shown as Mean ± SD. Data given are mean of duplicate measurements obtained from at least three seperate experiments.

3.6.4. Comparision of 50 % inhibitory concentrations (IC_{50} µg/mL) of ethanol and ethyl Acetate extracts on aldose reductase activity

50 % inhibitory concentrations of ethanol extracts on BLAR activity were compared and shown in table 3.3.

The highest aldose reductase inhibitory effect among ethanol extracts was found as 25.53 ± 0.36 µg/mL in *Glycyrrhiza glabra L* . On the other hand, the lowest aldose reductase inhibitory effect among ethanol extracts was detected as 54.15 ± 3.26 µg/mL in *Melissa officinalis.*

Table 3.3: Comparision of 50 % inhibitory concentrations (IC_{50} µg/mL) of ethanol on BLAR activity. The values were shown as Mean ± SD. Data given are mean of duplicate measurements obtained from at least three seperate experiments.

Ethanol Extracts	**(IC_{50} µg/mL) ± SD**
Ocimum basilicum	45.89 ± 2.31
Lavandula stoechas	39.28 ± 11
Melissa officinalis	54.15 ± 3.26
Glycyrrhiza glabra L	25.53 ± 0.36
Tilia tomentosa	31.76 ± 0.2
Quercetin (Standard)	9.93 ± 0.87

50 % inhibitory concentrations of ethyl acetate extracts on BLAR activity were compared and shown in Table 3.4.

The highest aldose reductase inhibitory effect among ethyl acetate extracts was observed as 41.55 ± 1.4 µg/mL in *Glycyrrhiza glabra L* . On the other hand, the lowest BLAR inhibitory effect among ethyl acetate extracts was found as 82.96 ± 1.85 µg/mL in *Ocimum basilicum*.

Table 3.4: Comparision of 50 % inhibitory concentrations (IC_{50} µg/mL) of ethyl acetate extracts on BLAR activity. The values were expressed as Mean ± SD. Data shown are mean of duplicate measurements obtained from at least three seperate experiments.

Ethyl Acetate Extracts	**(IC_{50} µg/mL) ± SD**
Ocimum basilicum	82.96 ± 1.85
Lavandula stoechas	50.04 ± 1.5
Melissa officinalis	50.32 ± 2.46
Glycyrrhiza glabra L	41.55 ± 1.4
Tilia tomentosa	75.96 ± 3.51
Quercetin (Standard)	9.93 ± 0.87

3.7 Determination of antioxidant capacities and total phenol content (TPC) of plant extracts

Antioxidant activities of *Ocimum basilicum, Lavandula stoechas, Melissa officinalis, Glycyrrhiza glabra L, and Tilia tomentosa* was examined by measuring DPPH scavenging activity and total phenolic contents.

Measurement of DPPH percent scavenging activities were made at different concentrations at plant extracts and the measurements for DPPH percent radical scavenging activities (% RSA) versus extract concentrations in mg/ml which were shown in figure 3.24- figure 3.33 and determined as EC_{50} values.

DPPH radical scavenging activities of *Ocimum basilicum, Lavandula stoechas, Melissa officinalis, Glycyrrhiza glabra L, and Tilia tomentosa* prepared ethahol and ethyl acetate were carried out at 517 nm for 5 minutes.

The effect of antioxidant for 5 minutes were examined against fifty percent effective concentration (EC_{50}) of DPPH radical scavenginig activity.

3.7.1 Determination of antioxidant capacities of plant ethanol extracts

3.7.1.1 Determination of percent DPPH scavenging activity of *Ocimum basilicum* ethanol extracts

Determination of antioxidant capacity of *Ocimum basilicum* ethanol extracts was carried out by using different concentrations of *Ocimum basilicum* ethanol extracts in 1.5 mL of the assay solution.

The antioxidant effect results of *Ocimum basilicum* ethanol extracts was given in figure 3.24 and the antioxidant effects of the *Ocimum basilicum* ethanol extracts were expressed as the 50 % effective concentration (EC_{50}).

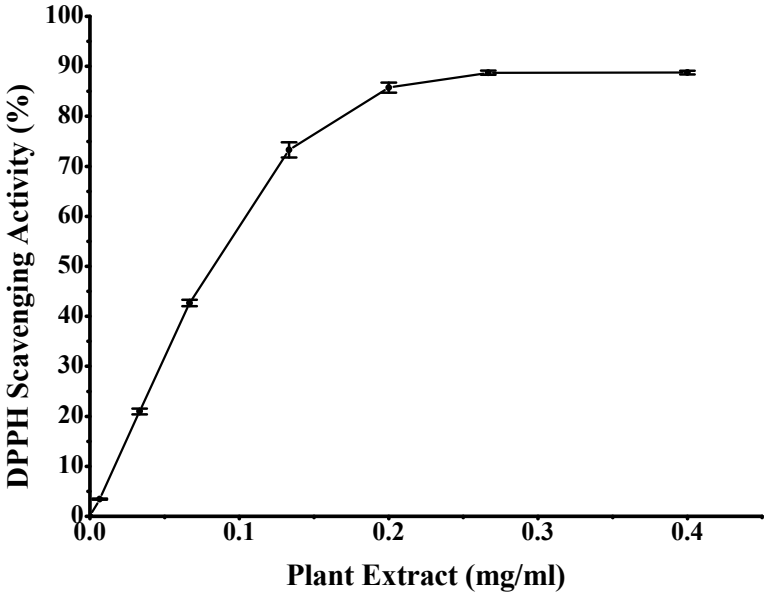


Figure 3.24: Percent DPPH scavenging activity of *Ocimum basilicum* ethanol extracts. The values were given as Mean ± SD. Data determined are mean of duplicate measurements acquired from at least three seperate experiments. 50 % effective concentration (EC_{50} µg/mL) of *Ocimum basilicum* ethanol extracts was calculated as 136.88 ± 0.72 µg/mL.

3.7.1.2 Determination of percent DPPH scavenging activity of *Lavandula stoechas* ethanol extracts

Determination of antioxidant capacity of *Lavandula stoechas* ethanol extracts was examined by using different concentrations of *Lavandula stoechas* ethanol extracts in 1.5 mL of the assay solution.

Percent DPPH scavenging activity of *Lavandula stoechas* ethanol extracts was shown in figure 3.25 and the antioxidant effects of the *Lavandula stoechas* ethanol extracts were expressed as the 50 % effective concentration (EC_{50}).

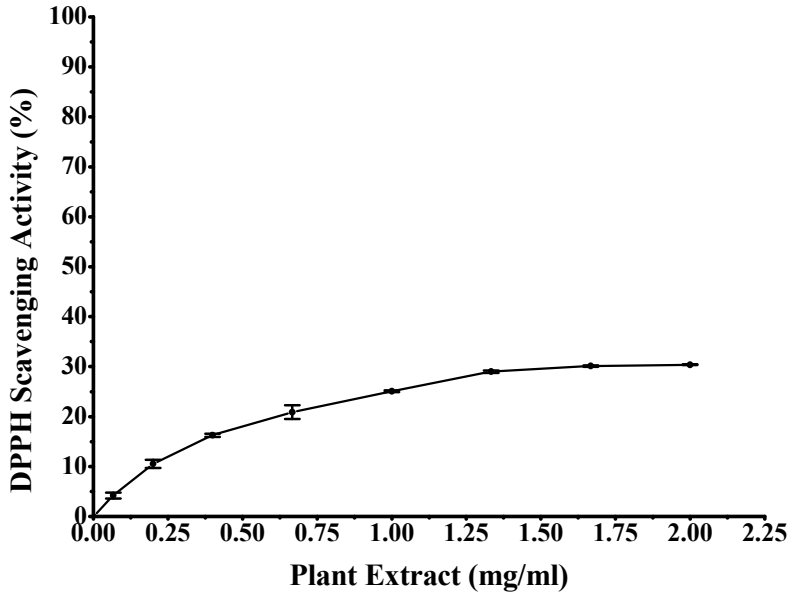


Figure 3.25: Percent DPPH scavenging activity of *Lavandula stoechas* ethanol extracts. The values were shown as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments. 50 % effective concentration (EC_{50} µg/mL) of *Lavandula stoechas* ethanol extracts was found as 2667.61 ± 13.95 µg/mL.

3.7.1.3 Determination of percent DPPH scavenging activity of *Melissa officinalis* ethanol extracts

Determination of antioxidant capacity of *Melissa officinalis* ethanol extracts was carried out by using different concentrations of *Melissa officinalis* ethanol extracts in 1.5 mL of the assay solution.

The antioxidant effect results of *Melissa officinalis* ethanol extracts was given in figure 3.26 and the antioxidant effects of the *Melissa officinalis* ethanol extracts were calculated as the 50 % effective concentration (EC_{50}).

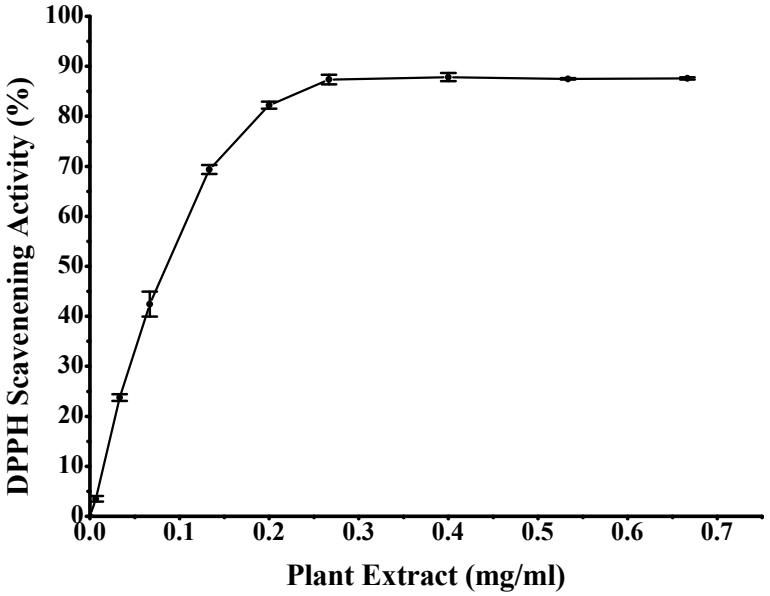


Figure 3.26: Percent DPPH scavenging activity of *Melissa officinalis* ethanol extracts. The values were expressed as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments. 50 % effective concentration (EC_{50} µg/mL) of *Melissa officinalis* ethanol extracts was found as 194.18 ± 1.91 µg/mL.

3.7.1.4 Determination of percent DPPH scavenging activity of *Glycyrrhiza glabra L* ethanol extracts

Percent DPPH Scavenging Activity of *Glycyrrhiza glabra L* ethanol extracts was examined by using different concentrations of *Glycyrrhiza glabra L* ethanol extracts in 1.5 mL of the assay solution.

The antioxidant effect results of *Glycyrrhiza glabra L* ethanol extracts was shown in figure 3.27 and the antioxidant effects of the *Glycyrrhiza glabra L* ethanol extracts were expressed as the 50 % effective concentration (EC_{50}).

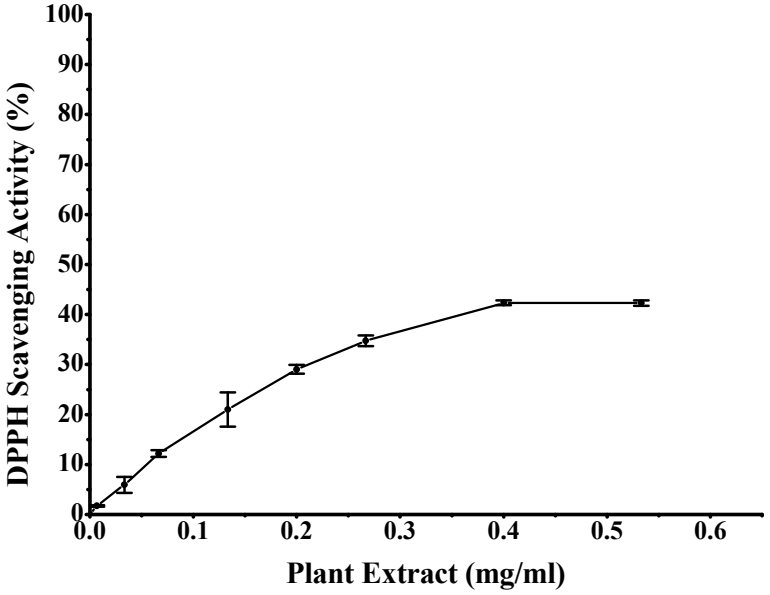


Figure 3.27: Percent DPPH scavenging activity of *Glycyrrhiza glabra L* ethanol extracts. The values were given as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments. 50 % effective concentration (EC_{50} µg/mL) of *Glycyrrhiza glabra L* ethanol extracts was expressed as 484.87 ± 8.54 µg/mL.

3.7.1.5 Determination of percent DPPH scavenging activity of *Tilia tomentosa* ethanol extracts

Percent DPPH Scavenging Activity of *Tilia tomentosa* ethanol extracts was carried out by using different concentrations of *Tilia tomentosa* ethanol extracts in 1.5 mL of the assay solution.

The antioxidant effect results of *Tilia tomentosa* ethanol extracts was given in figure 3.28 and the antioxidant effects of the *Tilia tomentosa* ethanol extracts were expressed as the 50 % effective concentration (EC_{50}).

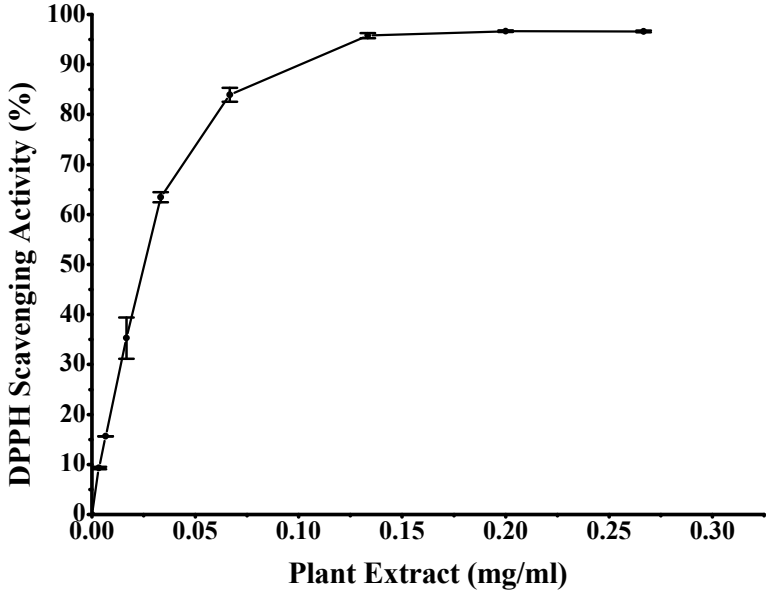


Figure 3.28: Percent DPPH scavenging activity of *Tilia tomentosa* ethanol extracts. The values were shown as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments. 50 % effective concentration (EC_{50} µg/mL) of *Tilia tomentosa* ethanol extracts was found as 70.46 ± 1.1 µg/mL.

3.7.1.6 Comparision of 50 % effective concentrations for DPPH radical scavenging activity of ethanol extracts

50 % effective concentrations for DPPH radical scavenging activity of ethanol extracts were compared and demonstrated in table 3.5.

The highest DPPH radical scavenging capacity among ethanol extracts was found as 70.46 ± 1.1µg/mL in. *Tilia tomentosa* . On the other hand, the lowest DPPH radical scavenging capacity among ethanol extracts was detected as 2667.61 ± 13.95µg/mL in *Lavandula stoechas.*

Table 3.5 : Comparision of 50 % effective concentration for DPPH radical scavenging activity of ethanol extracts. The values were shown as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments.

Ethanol Extracts	**(EC_{50} µg/mL) ± SD**
Ocimum basilicum	136.88 ± 0.72
Lavandula stoechas	2667.61 ± 13.95
Melissa officinalis	194.18 ± 1.91
Glycyrrhiza glabra L	484.87 ± 8.54
Tilia tomentosa	70.46 ± 1.1
Quercetin	8.72 ± 0.11

3.7.2 Determination of antioxidant capacities of plant ethyl acetate extracts

3.7.2.1 Determination of percent DPPH scavenging activity of *Ocimum basilicum* ethyl acetate extracts

Determination of antioxidant capacity of *Ocimum basilicum* ethyl acetate extracts was examined by using different concentrations of *Ocimum basilicum* ethyl acetate extracts in 1.5 mL of the assay solution.

The antioxidant effect results of *Ocimum basilicum* ethylacetate extracts was given in figure 3.29 and the antioxidant effects of the *Ocimum basilicum* ethyl acetate extracts were expressed as the 50 % effective concentration (EC_{50}).

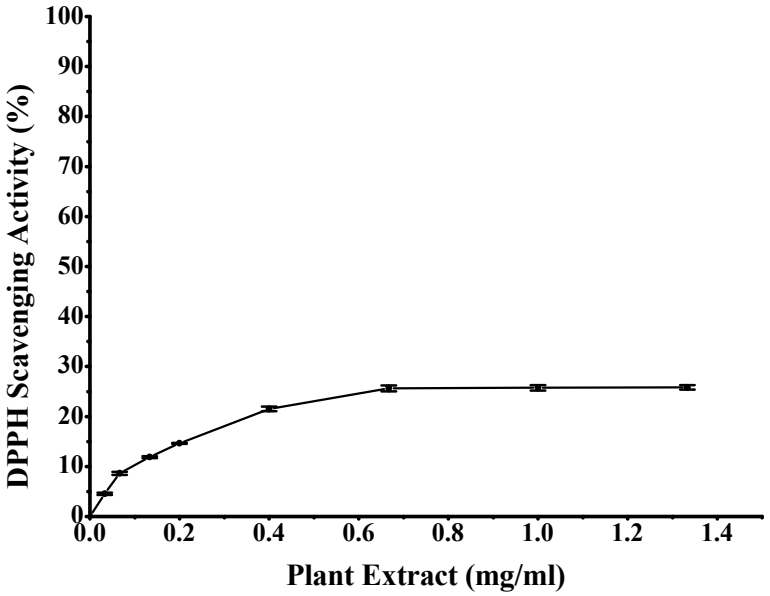


Figure 3.29: Percent DPPH scavenging activity of *Ocimum basilicum* ethyl acetate extracts. The values were given as Mean ± SD. Data determined are mean of duplicate measurements acquired from at least three seperate experiments. 50 % effective concentration (EC_{50} µg/mL) of *Ocimum basilicum* ethyl acetate extracts was calculated as 1869.01 ± 19.14 µg/mL.

3.7.2.2 Determination of percent DPPH scavenging activity of *Lavandula stoechas* ethyl acetate extracts

Determination of antioxidant capacity of *Lavandula stoechas* ethyl acetate extracts was carried out by using different concentrations of *Lavandula stoechas* ethyl acetate extracts in 1.5 mL of the assay solution.

Percent DPPH scavenging activity of *Lavandula stoechas* ethyl acetate extracts was shown in figure 3.30 and the antioxidant effects of the *Lavandula stoechas* ethyl acetate extracts were calculated as the 50 % effective concentration (EC_{50}).

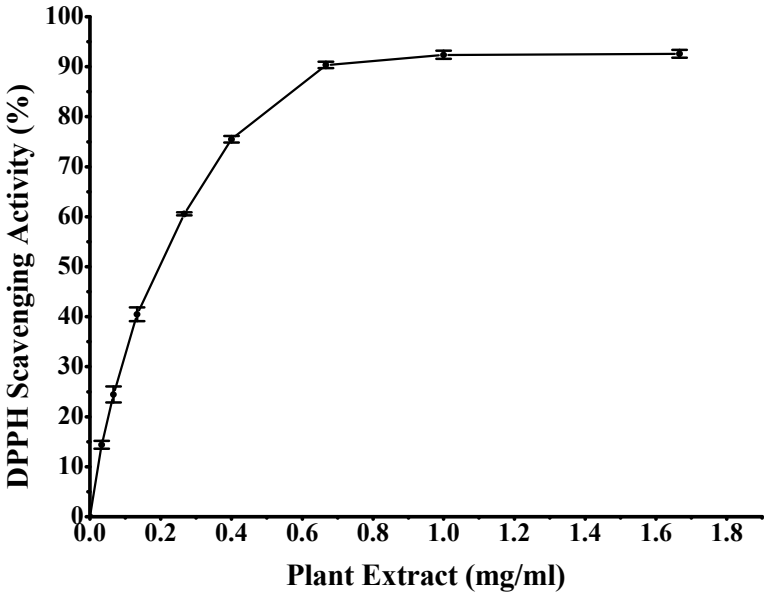


Figure 3.30: Percent DPPH scavenging activity of *Lavandula stoechas* ethyl acetate extracts. The values were given as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments. 50 % effective concentration (EC_{50} µg/mL) of *Lavandula stoechas* ethyl acetate extracts was expressed as 413.55 ± 3.46 µg/mL.

3.7.2.3 Determination of percent DPPH scavenging activity of *Melissa officinalis* ethyl acetate extracts

Determination of antioxidant capacity of *Melissa officinalis* ethyl acetate extracts was carried out by using different concentrations of *Melissa officinalis* ethyl acetate extracts in 1.5 mL of the assay solution.

The antioxidant effect results of *Melissa officinalis* ethyl acetate extracts was shown in figure 3.31 and the antioxidant effects of the *Melissa officinalis* ethyl acetate extracts were calculated as the 50 % effective concentration (EC_{50}).

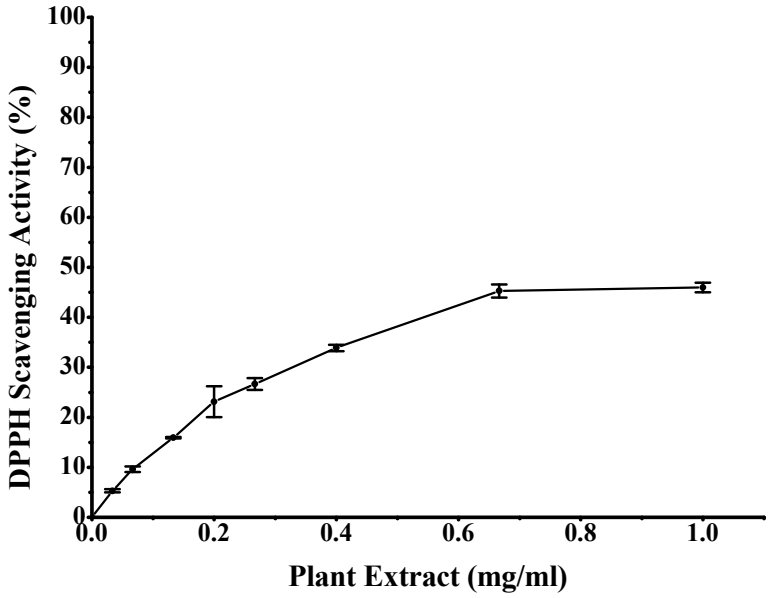


Figure 3.31: Percent DPPH scavenging activity of *Melissa officinalis* ethyl acetate extracts. The values were expressed as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments. 50 % effective concentration (EC_{50} µg/mL) of *Melissa officinalis* ethyl acetate extracts was found as 807.32 ± 14.72 µg/mL.

3.7.2.4 Determination of percent DPPH scavenging activity of *Glycyrrhiza glabra L.* ethyl acetate extracts

Percent DPPH Scavenging Activity of *Glycyrrhiza glabra L* ethyl acetate extracts was examined by using different concentrations of *Glycyrrhiza glabra L* ethyl acetate extracts in 1.5 mL of the assay solution.

The antioxidant effect results of *Glycyrrhiza glabra L*. ethyl acetate extracts was shown in figure 3.32 and the antioxidant effects of the *Glycyrrhiza glabra L* ethyl acetate extracts were expressed as the 50 % effective concentration (EC_{50}).

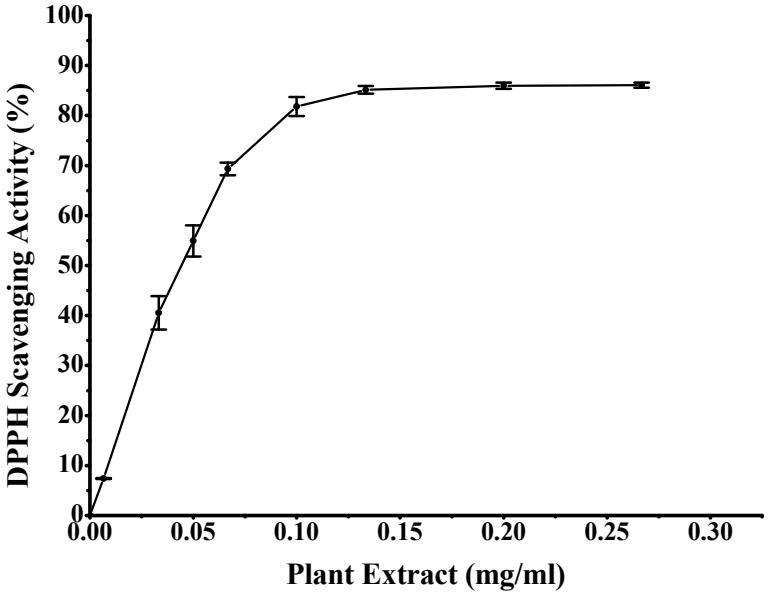


Figure 3.32: Percent DPPH scavenging activity of *Glycyrrhiza glabra L.* ethyl acetate extracts. The values were given as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments. 50 % effective concentration (EC_{50} µg/mL) of *Glycyrrhiza glabra L.* ethyl acetate extracts was expressed as 80.63 ± 0.91 µg/mL.

3.7.2.5 Determination of percent DPPH scavenging activity of *Tilia tomentosa* ethyl acetate extracts

Percent DPPH Scavenging Activity of *Tilia tomentosa* ethyl acetate extracts was carried out by using different concentrations of *Tilia tomentosa* ethyl acetate extracts in 1.5 mL of the assay solution.

The antioxidant effect results of *Tilia tomentosa* ethyl acetate extracts was given in figure 3.33 and the antioxidant effects of the *Tilia tomentosa* ethyl acetate extracts were expressed as the 50 % effective concentration (EC_{50}).

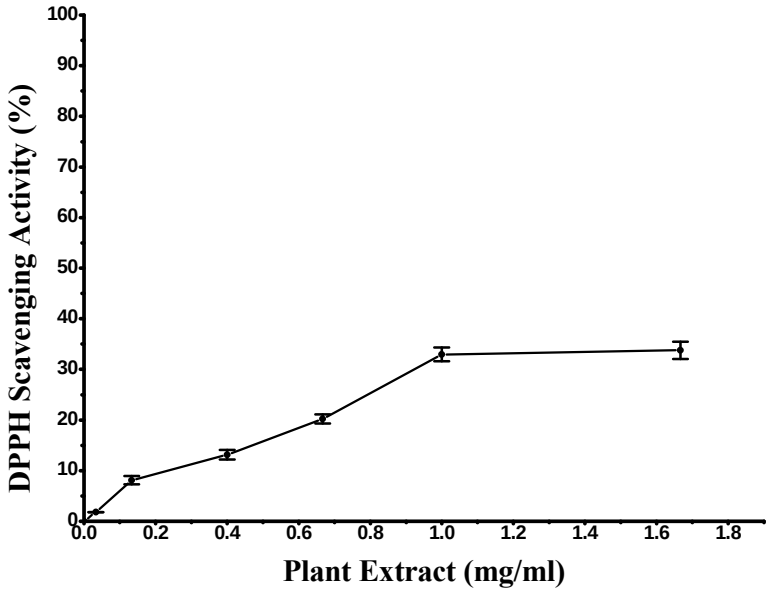


Figure 3.33: Percent DPPH scavenging activity of *Tilia tomentosa* ethyl acetate extracts. The values were shown as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments. 50 % effective concentration (EC_{50} µg/mL) of *Tilia tomentosa* ethyl acetate extracts was found as 1986.79 ± 88.81 µg/mL.

3.7.3. Comparision of 50 % effective concentration for DPPH radical scavenging activity of ethyl acetate extracts

50 % effective concentrations for DPPH radical scavenging activity of ethyl acetate extracts were compared and demonstrated in table 3.6.

The highest DPPH radical scavenging capacity among ethyl acetate extracts was found as 80.63 ± 0.91 µg/mL in *Glycyrrhiza glabra L*. On the other hand, the lowest DPPH radical scavenging capacity among ethyl acetate extracts was shown as 1986.79 ± 88.81µg/mL in *Tilia tomentosa.*

Table 3.6: Comparision of 50 % effective concentration for DPPH radical scavenging activity of ethyl acetate extracts. The values were shown as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments.

Ethyl Acetate Extracts	**(EC_{50} µg/mL) ± SD**
Ocimum basilicum	1869.01 ± 19.14
Lavandula stoechas	413.55 ± 3.46
Melissa officinalis	807.32 ± 14.72
Glycyrrhiza glabra L	80.63 ± 0.91
Tilia tomentosa	1986.79 ± 88.81
Quercetin	8.72 ± 0.11

3.8 Determination of total phenol content (TPC) of plant extracts

Total phenolic contents of *Ocimum basilicum, Lavandula stoechas, Melissa officinalis, Glycyrrhiza glabra L, and Tilia tomentosa* was examined by measuring as microgram equivalents of gallic acid per mg of crude extracts.

3.8.1 Determination of total phenol content (TPC) of ethanol extracts

Total phenolic contents of *Ocimum basilicum, Lavandula stoechas, Melissa officinalis, Glycyrrhiza glabra L, and Tilia tomentosa* ethanol extracts were shown in figure 3.34.

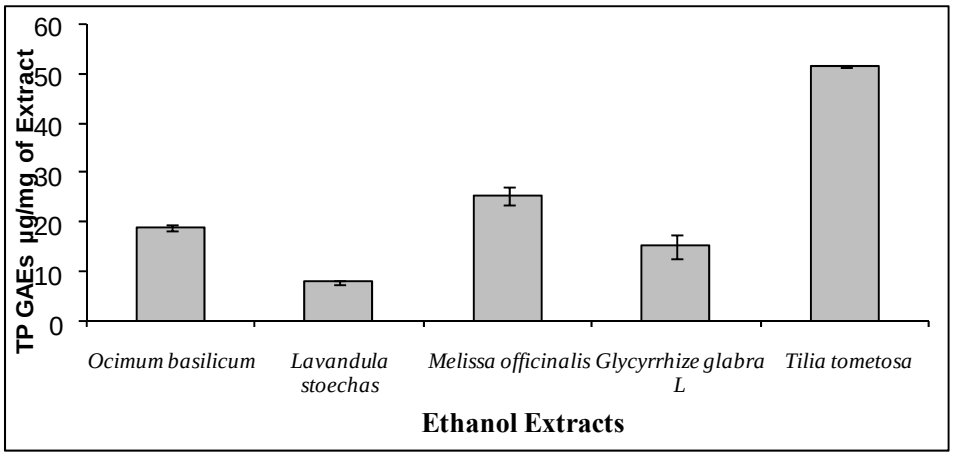


Figure 3.34: Total phenol content (TPC) of plant ethanol extracts.

The results obtained from absorbances were recorded as miligrams of total phenolics including in miligrams of extract as the gallic acid equivalents (GAE).

3.8.1.1. Comparision of total phenol content (TPC) of ethanol extracts

Results of ethanol extracts were compared as miligrams of total phenolics including in miligrams of extract as the gallic acid equivalents (GAE) and shown in table 3.7.

The highest total phenolic content among ethanol extracts was found as 51.83 ± 0.229 GAEs µg/mg of extract in *Tilia tomentosa*. On the other hand, the lowest total phenolic content among ethanol extracts was detected as 8.04 ± 0.455 GAEs µg/mg of extract *Lavandula stoechas*.

Table 3.7: Comparision of. total phenol content (TPC) of ethanol extracts. The values were shown as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments.

Ethanol Extracts	TP GAEs µg/mg of extract ± SD
Ocimum basilicum	19.07 ± 0.758
Lavandula stoechas	8.04 ± 0.455
Melissa officinalis	25.43 ± 1.694
Glycyrrhiza glabra L	15.29 ± 2.379
Tilia tomentosa	51.83 ± 0.229

3.8.2 Determination of total phenol content (TPC) of ethyl acetate extracts

Total phenolic contents of *Ocimum basilicum, Lavandula stoechas, Melissa officinalis, Glycyrrhiza glabra L, and Tilia tomentosa* ethyl acetate extracts were shown in figure 3.35.

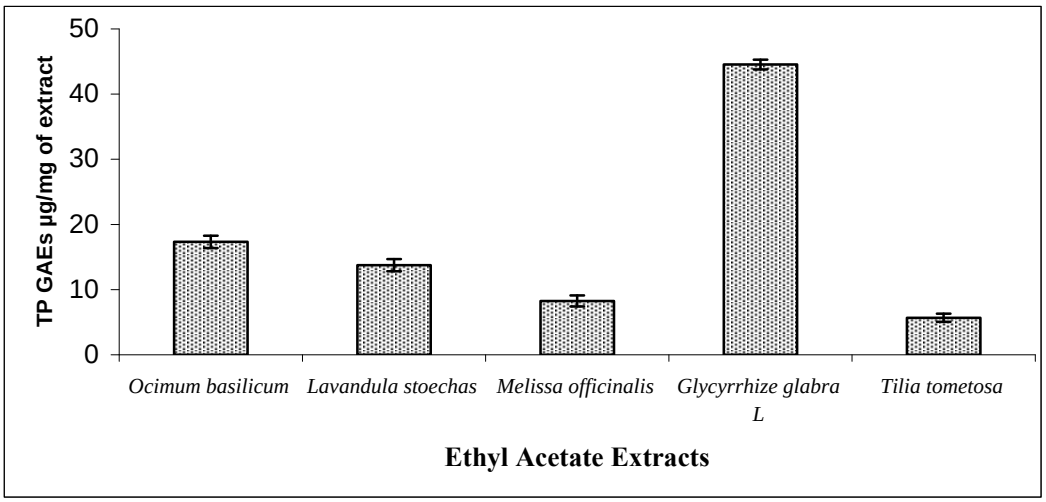


Figure 3.35: Total phenol content (TPC) of plant ethyl acetate extracts.

The results obtained from absorbances were calculated as miligrams of total phenolics including in miligrams of extract as the gallic acid equivalents (GAE).

3.8.2.1. Comparision of total phenol content (TPC) of ethyl acetate extracts

Results of ethyl acetate extracts were compared as miligrams of total phenolics including in miligrams of extract as the gallic acid equivalents (GAE) and exhibited in table 3.8.

The highest total phenolic content among ethyl acetate extracts was detected as 44.54 ± 0.754 GAEs µg/mg of extract in *Glycyrrhiza glabra L*. On the other hand, the lowest total phenolic content among ethyl acetate was shown as 5.68 ± 0.632 GAEs µg/mg of extract in *Tilia tomentosa*.

Table 3.8: Comparision of. total phenol content (TPC) of ethyl acetate extracts The values were shown as Mean ± SD. Data determined are mean of duplicate measurements obtained from at least three seperate experiments.

Ethyl Acetate Extracts	TP GAEs µg/mg of extract ± SD
Ocimum basilicum	17.33 ± 0.940
Lavandula stoechas	13.76 ± 0.930
Melissa officinalis	8.26 ± 0.840
Glycyrrhiza glabra L	44.54 ± 0.754
Tilia tomentosa	5.68 ± 0.632

CHAPTER 4

CONCLUSIONS

Average weight of bovine lens was found as 2.06 g ± 0.04. The average BLAR activity was calculated as 1.135 ± 0.058 nmole NADPH oxidized /min/ mg protein. The lowest activity was measured as 1.043 nmole NADPH oxidized /min/ mg protein and the highest activity was measured as 1.256 nmole NADPH oxidized /min/ mg protein. After optimization experiments optimum bovine lens aldose reductase (BLAR) activity was measured in the reaction medium containing 1.43 mg/mL protein containing crude enzyme solution, $6x10^{-5}$ M DL-glyceraldehyde as substrate solution, $1x10^{-4}$ M NADPH prepared freshly, 270 mM Li_2S0_4, and 30 mM PBS at of pH 6.7 in a final volume of 1.0 mL. BLAR activity was stable up to 4 weeks. After 4 weeks, BLAR activity significantly decreased.

While V_{max} for glyceraldehyde was found as 1.372 nmol/min/mg, K_m was calculated as 16.39 μM. On the other hand, while V_{max} for cofactor NADPH was calculated as 1.400 nmol/min/mg, K_m was found as 35.81 μM. Bovine lens aldose reductase activity was not affected by the addition of DMSO up to 1% concentration. As DMSO concentration was further increased, enzyme activity changed notably.

Ocimum basilicu, Melissa officinalis, Lavandula stoechas, Glycyrrhiza glabra and Tilia tomentosa were selected for the plant extract preparation to be investigated in inhibition of BLAR activity. Crude extracts were prepared using ethanol and ethyl acetate solvent system. The highest yield of ethanol extracts was found as 11.78 % in *Lavandula stoechas.* The lowest yield of ethanol extracts was shown as 7.67 % in *Tilia tomentosa.* Yield of *Glycyrrhiza glabra* was found higher than that of *Melissa officinalis.* Also, yield of *Ocimum basilicum* was displayed lower than that of *Melissa officinalis.*

The highest yield of ethyl acetate extracts was shown as 5.26 % in *Lavandula stoechas.* The lowest yield of ethyl acetate extracts was found as 2.55 % in *Glycyrrhiza glabra*. Also, *Tilia tomentosa* was displayed higher yield than that of *Ocimum basilicum.* In addition, *Melissa officinalis* was found to have lower yield than that of *Ocimum basilicum.*

Ethanol and ethyl acetate crude extracts of each selected plant were prepared 10 mg/mL in the 10% DMSO as initial stock solution. Inhibition studies were examined for the extract concentrations of 0.001-0.2 mg/mL. Among the plants prepared in ethanol extracts the highest BLAR inhibitory effect was found by *Glycyrrhiza glabra* which has the lowest 50 % inhibitory concentration of 25.53 ± 0.36 µg/mL. On the contrary, the lowest BLAR inhibitory effect was exhibited by Melissa *officinalis* with the highest 50 % inhibitory concentration of 54.15 ± 3.26 µg/mL. BLAR inhibitory effects of *Tilia tomentosa, Lavandula stoechas* and *Ocimum basilicum* were obtained as 50 % inhibitory concentration values of 31.76 ± 0.2, 39.28 ± 11, and 45.89 ± 2.31 µg/mL, respectively.

BLAR inhibitory effects for ethyl acetate extracts of same selected plants were shown a different order of the effectiveness. The highest BLAR inhibitory effect was exhibited by *Glycyrrhiza glabra* with the lowest 50 % inhibitory concentration value of 41.55 ± 1.4 µg/mL. On the other hand, the lowest BLAR inhibitory effect was displayed by *Ocimum basilicum* with 50 % inhibitory concentration of 82.96 ± 1.85 µg/mL. BLAR inhibitory effects of *Lavandula stoechas, Melissa officinalis,* and *Tilia tomentosa,* were obtained as 50 % inhibitory concentration values of 50.04 ± 1.5, 50.32 ± 2.46, and 75.96 ± 3.51 µg/mL, respectively. In addition, the 50 % inhibitory concentration of quercetin (standard) on BLAR activity was found as 9.93 ± 0.87 µg/mL.

Ethanol and ethyl acetate crude extracts of each selected plant were prepared 10 mg/mL in the pure ethanol as initial stock solution. DPPH radical scavenging activity were studied for the extract concentrations in the range of 0.001- 2.5 mg/mL.

Among the plants prepared in ethanol extracts the highest DPPH radical scavenging activity was exhibited by *Tilia tomentosa* which has the lowest 50 % inhibitory concentration of 70.46 ± 1.1µg/mL. On the other hand, *Lavandula stoechas* displayed the lowest radical scavenging activity with 50 % effective concentration value of 2667.61 ± 13.95 µg/mL. DPPH radical scavenging activities were obtained for the rest of the plants with increasing order of the activity was given by 50 % effective concentration values of 136.88 ± 0.72, 194.18 ± 1.91, and 484.87 ± 8.54 1µg/mL for *Ocimum basilicum, Melissa officinalis* and *Glycyrrhiza glabra* ethanol extracts, respectively.

DPPH radical scavenging activity for the plants extracted in ethyl acetate has shown different trend than that of ethanol extracts. On this occasion, the highest radical scavenging activity was displayed by *Glycyrrhiza glabra* with a 50 % effective concentration of 80.63 ± 0.91 µg/mL, however, the lowest activity revealed by *Tilia tomentosa* and *Ocimum basilicum* with 50 % effective concentration values of 1986.79 ± 88.81 and 1869.01 ± 19.14 µg/mL , respectively. *Melissa officinalis* and *Lavandula stoechas* had respective 50 % effective concentration values of 807.32 ± 14.72 and 413.55 ± 3.46 µg/mL. In comparison, 50 % effective concentration of quercetin used as a standard for DPPH radical scavenging activity was found as 8.72 ± 0.11 µg/mL.

Total phenol content of each plant extract was investigated to find a relationship between phenolic content of plant and its activity in terms of BLAR inhibitory activity and radical scavenging activity. The highest total phenol content (TPC) of ethanol extracts was found as 51.83 ± 0.229 µg GAE/mg of extract in *Tilia tomentosa.* The lowest total phenol content (TPC) of ethanol extracts was shown as 8.04 ± 0.455 µg GAE/mg of extract in *Lavandula stoechas. Melissa officinalis, Ocimum basilicum,* and *Glycyrrhiza glabra* exhibited respective total phenol contents of 25.43 ± 1.694, 19.07 ± 0.758, and 15.29 ± 2.379 µg GAE/mg of extract

The highest total phenol content (TPC) of ethyl acetate extracts was found as 44.54 ± 0.754 µg GAE/mg of extract in *Glycyrrhiza glabra L.* The lowest total phenol content (TPC) of ethyl acetate extracts was shown as 5.68 ± 0.632 µg GAE/mg of extract in *Tilia tomentosa. Ocimum basilicum, Lavandula stoechas*, and *Melissa officinalis* exhibited respective total phenol contents of 17.33 ± 0.940, 13.76 ± 0.930, and 8.26 ± 0.840 µg GAE/mg of extract.

In conclusion, the highest total phenol content was obtained from *Tilia tomentosa* among all the ethonolic plant extracts which was comparable with its DPPH radical scavenging activity and BLAR inhibitory activity. The lowest total phenolic content was belong to *Lavandula stoechas* which exhibited the lowest radical scavenging activity, however, BLAR inhibitory activity was not the lowest among other ethanolic extracts.

The highest total phenol content among the ethyl acetate plant extracts was presented by *Glycyrrhiza glabra* as corresponding to the highest effect on radical scavenging activity and BLAR inhibitory activity. *Tilia tomentosa* ethyl acetate extract displayed the lowest phenolic content in the list and expectedly it also displayed the lowest radical scavenging and BLAR inhibitory activity.

Glycyrrhiza glabra ethanol and ethyl acetate extracts have displayed the highest BLAR inhibitory activity and *Glycyrrhiza glabra* may be carried into further investigation for prevention of diabetic complications through pharmacologic studies of aldose reduction inhibition *in vivo* studies.

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