INHIBITORY EFFECTS OF PLANT ORIGINATED EXTRACTS ON BOVINE LENS ALDOSE REDUCTASE

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ABSTRACT

INHIBITIORY EFFECTS OF PLANT ORIGINATED EXTRACTS ON BOVINE LENS ALDOSE REDUCTASE

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Aldose reductase, E.C.1.1.1.21, catalyzes the reduction of different types of aldehydes to their corresponding alcohols, and especially reduces various aldo-sugars using NADPH as the coenzyme. Under hyperglycemic conditions aldose reductase is involved in the development of diabetic complications. As a result, interest has been placed over the years on the development of potent aldose reductase inhibitors for possible use in the therapy of these severe diabetic complications.

In this study, aldose reductase was isolated from bovine lens by differential centrifugation and ammonium sulfate precipitation. The conditions for the enzyme assay; such as substrate (DL-Glyceraldehyde) and coenzyme (NADPH) concentration, protein amount, effect of sulfate ions, temperature and pH on the enzyme activity were optimized.

The inhibitory effects of ***Punica granatum***, ***Spinacia olaeracea***, ***Allium cepa*** ***Allium porrum***, ***Malus flouribunda***, ***Malus domestica*** extracts were tested on crude bovine lens aldose reductase. Four different types of organic fractions from each crude plant extract were obtained by solvent fractionation. The inhibitory activity of these organic fractions was calculated considering the aldose reductase activity without extracts as 100 %. All six plants were found to inhibit aldose reductase activity to different extent. Among these fractions obtained as; petroleum ether, diethyl ether, ethyl acetate, and n-butanol. Highest inhibitory activity was found for the ethyl acetate fraction. The IC_{50} values of ethyl acetate fractions of all these plants was calculated as, 25.46 µg/ml, 20.5 µg/ml, 18.5 µg/ml, 12.32 µg/ml, 6.45 µg/ml, 5.4 µg/ml, for ***Allium porrum, Malus domestica, Spinacia olaeracea, Malus floribunda*** ***Allium cepa, Punica granatum*** respectively.

Keywords: Aldose reductase inhibition, plant extracts, flavonoids, bovine lens

ÖZ

BİTKİ KÖKENLİ EKSTRELERİN SIĞIR LENS ALDOZ REDÜKTAZ ENZİMİ ÜZERİNE İNHİBİTÖR ETKİLERİ

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Aldoz redüktaz, E.C.1.1.1.21, NADPH'i koenzim olarak kullanarak çeşitli aldehit ve bunların alkollerinin ve özellikle aldo-şekerlerin indirgenmesini katalizler. Hiperglisemik koşullarda, aldoz redüktaz enzimi diabete bağlı komplikasyonların oluşmasında etkilidir. Uzun yıllardır, aldoz redüktaz enziminin inhibitörlerine gösterilen ilginin nedeni ciddi diabetik komplikasyonların tedavisinde inhibitörlerin olası kullanımlarının olmasındandır.

Bu çalışmada, aldoz redüktaz enzimi sığır lensinden sentrifügasyon ve amonyum sülfat çöktürmesi yöntemleri kullanılarak ham olarak elde edilmiştir. Enzim çalışması için gerekli şartlar; substrat (DL-gliseraldehit) ve koenzim (NADPH) konsantrasyonları, protein miktarı, sülfat iyonu miktarı sıcaklık ve pH optimum değerleri olarak tespit edilmiştir.

Punica granatum (nar), Spinacia olaeracea (ıspanak), Allium cepa (soğan) Allium porrum (pırasa), Malus floribunda (japon elması), Malus domestica (elma) ekstrelerinin ham sığır lensi aldoz redüktaz enzimi üzerine etkileri denenmiştir. Solvent fraksiyonlaması metodu ile her ham bitki ekstresinden dört çesit organik faz elde edilmiştir. Bu organik fazların inhibitör etkisi, ekstre konulmadan elde edilen enzim aktivitesinin 100 % olarak kabul edilmesi ile hesaplanmıştır. İnhibitör özelliği araştırılan bu altı çesit bitkinin, degişik ölçülerde enzimi inhibe ettiği görülmüştür.Bu bitkilerden elde edilen petrol eter, dietil eter, etil asetat, n-butanol gibi organik fazların, en etkili olanı etil asat fazı olarak tespit edilmiştir. Etil asetat fazları için hesaplanan IC_{50} değerleri sırasıyla ***Allium porrum, Malus domestica, Spinacia olaeracea, Malus flouribunda, Allium cepa, Punica granatum*** için, 25.46 µg/ml, 20.5 µg/ml, 18.5 µg/ml, 12.32 µg/ml, 6.45 µg/ml, 5.4 µg/ml olarak bulunmuştur.

Anahtar Kelimeler : Aldoz redüktaz inhibisyonu, bitki ekstreler, flavonoidler, sığır lensi

To my family

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BSA	Bovine Serum Albumin
$NADP^{+1}$	Nicotinamide adenine dinucleotide phosphate (oxidizied)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
DMSO	Dimethylsulfoxide
TLC	Thin layer chromatography
AP	***Allium porrum*** (Leek)
AC	***Allium cepa*** (Onion)
MF	***Malus flouribunda*** (Japaneese apple)
MD	***Malus domestica*** (Apple)
SO	***Spinacia oleracae*** (Spinach)
PG	***Punica granatum*** (Pomagranate)

CHAPTER 1

INTRODUCTION

Diabetes Mellitus has an effect on many organ systems including the eye, kidney and peripheral nerves. Many of these complications develop in animal models of diabetes, which has allowed some of the mechanisms of damage in target organs to be studied. Aldose reductase is an intracellular enzyme, converts glucose to sorbitol, and it is responsible for the intracellular accumulation of sorbitol which results in irreversible damage [Lightman, S., et al. (1993)]**.** The intracellular accumulation of polar sugar alcohol can produce a hyperosmotic effect, which has been observed to lead to changes in membrane permeability and the onset of cellular pathology Therefore, the inhibition of aldose reductase may be effective in preventing diabetic complications [Kador, P. F., Robison, W. G., Kinoshita, J. H. (1985)]. The diabetic cataract is an example of this process [Kinoshita, J. H. (1979)].

Inhibitors of aldose reductase, flavonoids [Varma, S. D., et al. (1975)], and heterocyclic compounds [Peterson, M. J., et al. (1979)]. have been reported to be effective in relieving the complications of diabetes in experimental animals. Flavonoids and related compounds are the most widely studied natural product groups in aldose reductase inhibitory activity. There have been numerous reports of aldose reductase inhibitors obtained from plant originated extracts [Constantino, L., Rastelli, P., et al. (1997), Crabbe, M. J. C., and Goode, D. (1998), Rastelli, G., Antolini, S., and Constantino, L., (2000), Okuda, J., et al. (1984), Matsuda, H., Morikawa, T., Toguchida, I., Yoshkawa, M. (2002)].

The presence of aldose reductase in the eye lens has been reported by Van Heyningen in 1959. Hayman and Kinoshita, in 1965, have described some of the properties of the partially purified bovine lens aldose reductase. Then in 1976, the purification of the bovine lens aldose reductase to homogeneity has been first described by Sheaff and his group [Sheaff, C. M., et al. (1976)], who showed that the enzyme has a broad substrate specificity for the compounds having aldehyde function, exhibiting an optimum pH of 5.5. As reported in Sheaff's study, the enzyme has been purified at 15,000 fold with a molecular weight of 37,000. The α-helical content of the enzyme was found to be less than % 5 by circular dichroism data at 222 nm and this was unaltered by varying concentrations of glyceraldehyde, NADPH, ammonium sulfate, NADP or β-mercaptoethanol, and the typical isofocusing result showed the enzyme's pI as 4.85 [Sheaff, C. M., et al. (1976)].

The physical and kinetic characterization studies [Sheaff, C. M., et al. (1976), Conrad, S. D., et al. (1980)] of aldose reductase suggest that the bovine lens enzyme follows an ordered binding of substrates. First substrate binding to the enzyme is NADPH, and in an ordered release of products, NADP is released at last [Liu, S. Q., et al. (1992)]. The rate limiting step, in the reaction catalyzed by the bovine lens enzyme, has been shown to be the isomerization of enzyme-NADPH binary complex [Liu, S. Q., et al. (1992) and Kubiseski , T. J., et al. (1992)].

The x-ray structure of the enzyme as well as its binary complexes with ATP-ribose and NADPH has been published. X-ray analysis of the enzyme structure shows that the active site is highly hydrophobic and the majority of aminoacids lining the cavity are nonpolar [Rondeau, J. M., et al. (1992), Wilson, D. K., et al. (1992)].

1.1.2 Structure of Aldose Reductase

Aldose reductase has a structure that belongs to a group of enzymes with the (beta/alpha)$_8$-barrel class. Aldose reductase is a globular protein consisting of a single polypeptide chain of 315 residues with a molecular weight of 37 kD.

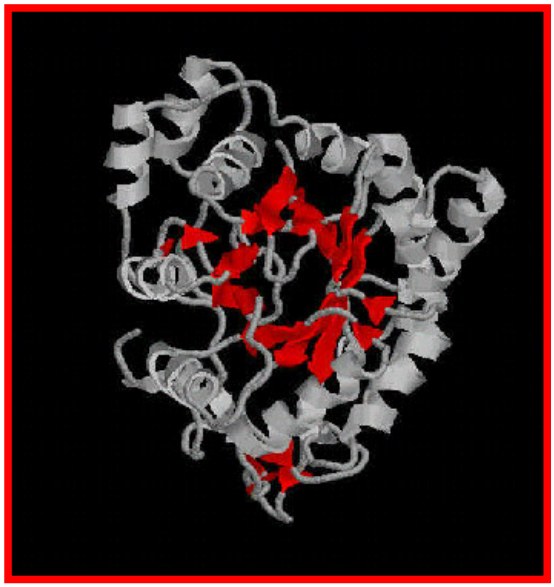


Figure 1: Secondary structure of aldose reductase

The single chain folds into a beta/ alpha barrel with an inner core of 8 parallel beta strands surrounded by 8 alpha helices as shown in Figure 1. The red core corresponds to the beta strands and the grey surroundings correspond to the helices [Wilson, K. D., et al. (1992), and Wilson, K. D., et al. (1993)]. The structure consists of two short antiparallel β-strands that are connected by a tight turn close off at the bottom of the barrel.

Also present in the structure is an extra piece of alpha helix between strand 7 and helix 7 and between strand 8 and helix 8. The COOH terminal which has 24 residues, resides at the end of helix 2 on the NH2 terminal side of the molecule to the top of the COOH terminal end of the beta barrel [Wilson, K. D., et al. (1992), and Wilson, K. D., et al. (1993)]

The active site of Aldose reductase is located at the COOH terminal of the beta barrel. The site is a large deep filled cavity of hydrophobic residues, bounded by the termini of the beta strands and loops. This site is where substrates bind along with the co-enzyme NADPH as shown in Figure 2.

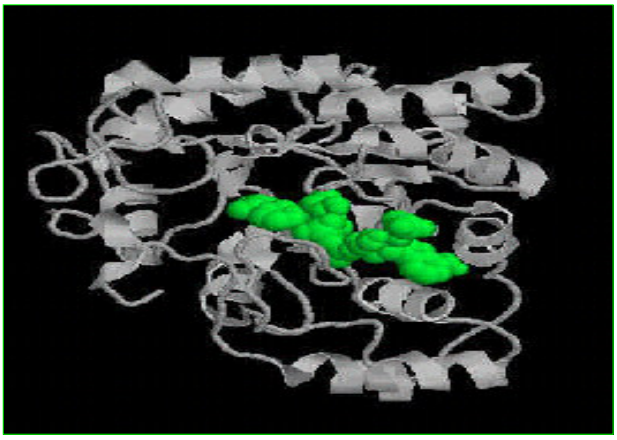


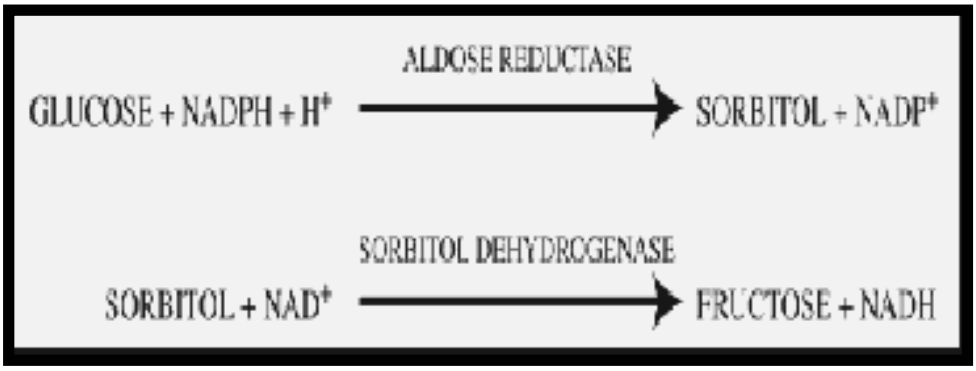
Figure 2 : NADPH-enzyme binary complex

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. One of these residues may be the acid-base catalyst for the oxidation/reduction reactions NADPH binds to the enzyme by 19 hydrogen bonds in an extended conformation across the barrel with the nicotinamide ring in the center of the deep filled cavity [Wilson, D. K., et al. (1992)].

1.1.3 Function of Aldose Reductase

The major function attributed to aldose reductase is its role as a promoter of the polyol pathway in which glucose is converted to fructose via the sorbitol formation.

The role of the enzyme is not fully understood but it has been found that it is the enzyme which takes part in occurance of complications in the retina, lens, peripheral nerves and kidneys. Investigations into the control of the enzyme such as e.g. aldose reductase inhibition by epalrestat [Wilson, K. D., et al. (1993)] have found that it is possible to inhibit the reduction to sorbitol in vitro due to the specific shape of the active site which is changed by a non competitive inhibitors like epalrestat and NADPH [Wilson, K. D., et al. (1992), and Wilson, K. D., et al. (1993)]



1.1.3.1 Polyol Pathway And Cataract Formation

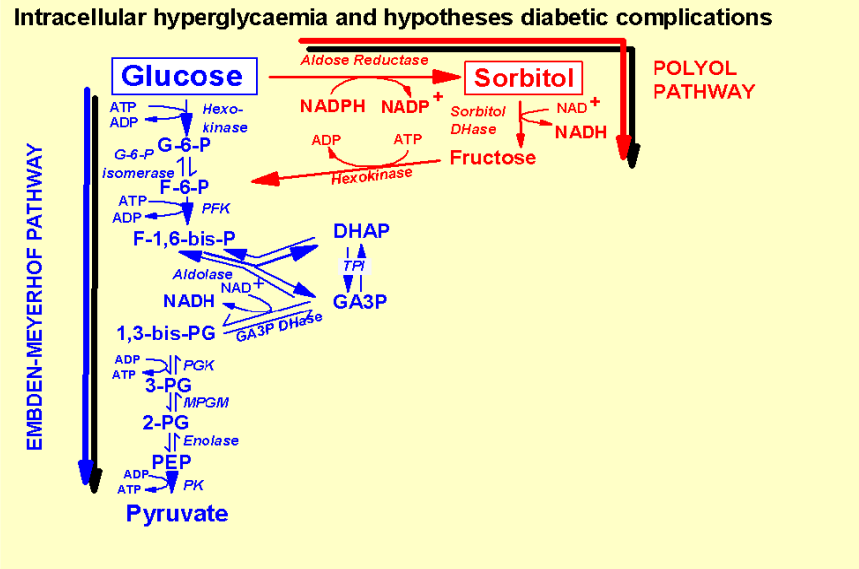


Figure 3 : Polyol pathway in accordance with Embden–Meyerhof pathway

Both fructose and sorbitol are found in the human lens, where they increase in concentration in diabetes and may involved in the pathogenesis of diabetic complications. The polyol pathway is responsible for fructose formation from glucose and increases in activity as the glucose concentration rises in diabetes in those tissues that are not insulin sensitive, i.e., lens peripheral nerves, and renal glomeruli. Because under normal conditions, the glucose entering the cell is rapidly phosphorylated by the enzyme hexokinase and metabolized mainly via the glycolytic pathway to lactate and pyruvate providing the major energy source for the cells. A proportion of glucose is also metabolized via pentose phosphate pathway in the lens. However in diabetes and galactosemia, the concentration of glucose and galactose increases in several folds. The hexokinase pathway is fully saturated even at normal glucose levels, so that if hyperglycemia occurs

as diabetes mellitus saturation of hexokinase becomes evident, the excess glucose can not be metabolized by the glycolysis pathway. Therefore the aldose reductase becomes activated and gives rise to the conversion of glucose to sorbitol (Figure 3).

Actually, aldose reductase has a much lower affinity for glucose than hexokinase, and therefore, glucose can enter the polyol pathway only when its levels are raised [Clarke, B. F., et al**.** (1984)]. Sorbitol does not easily diffuse through the cell membranes and therefore accumulates in the cells, which cause the osmotic pressure to build up and eventually the cells burst, resulting in the tissue damage. Activation of the enzyme results in a NADPH depletion which alters 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, et al. (1985)].

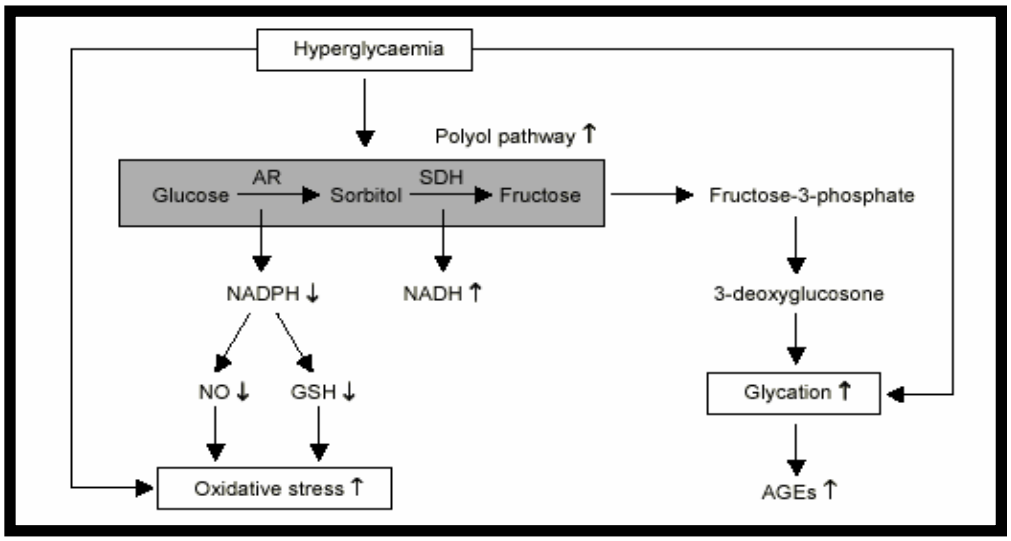


Figure 4: A schematic interaction diagram of factors related to the polyol pathway among pathogenic mechanisms of diabetic complications.
AR: Aldose reductase; **SDH:** Sorbitol dehydrogenase; **NO:** Nitric Oxide; **GSH:** Glutathione; **AGE:** Advanced glycated end products [Miyamoto, S., et al. (2001)].

Simultaneously, myoinositol levels fall which results in decrease in Na/K pump activity, and in decrease of nerve conduction velocity. All the following results of sorbitol accumulation, such as myoinositol depletion, oxidative stress increase, related high osmotic pressure, which causes diabetic complications can be prevented by the aldose reductase inhibitors [Kador, P. F., et al. (1988)].

The physiological significance of the polyol pathway is first described by Hers in 1956. In this pathway, generated sugar alcohol is oxidized to ketose by a second enzyme of the path named NAD^+-dependent Sorbitol dehydrogenase (SDH) (Figure 4). As the glucose concentration increases, aldose reductase activity increases in several folds, however the sorbitol dehydrogenase activity does not increases proportionally as a result sorbitol accumulates in the cell. Osmotic stress caused by the sorbitol accumulation as a result of impermeability through most of the membranes, results in electrolyte imbalance, and membrane streching with cell death [Ohta, M., et al. (1993)].

High concentrations of sorbitol in the lens probably increase the osmotic pressure and then cause cellular swelling with eventual disruption and cell death. Finally this process results in lenticular opacity which termed as sugar cataract. Although the etiology of ocular complications associated with diabetes still remains unknown. In the recent publications, increasingly it has been reported that aldose reductase being linked to these complications [Crabbe, J. M., et al. (1991)]. Reported animal experiments reveals the dependence of the occurance of the diabetic complications associated with diabetes on the aldose reductase–initiated accumulation of sugar alcohols.

1.1.3.2 Diabetic Complications and Their Treatments

The estimated worldwide prevalance of Diabetes mellitus was 4.0 % in 1995 and it is expected to be double by 2005 [King, H., et al. (1998)]. In spite of insulin treatment, most patients eventually experience long-term diabetic complications, such as retinopathy, neuropathy, cataract and angiopathy. Although, there is no definite pathogenic link between hyperglycemia and diabetic complications, there are several mechanisms that seem to be involved in toxic effects caused by the excess level of glucose in blood (Figure 4). [Porte, D., et al., and Schwartz, M. W., et al. (1996), and Sima, A. A. F., et al. (1999)]. Among the well–examined factors there are activation of protein kinase C [Koya, D. (1998), Ishii, H. (1965)], enhanced protein glycation with the formation of advanced glycated end products (AGEs) [Friedman, E. A. et al., (1999), Brownlee, M., et al. (2000)], rise of oxidative stress [Baynes, J. W. (1999), Betteridge, D. J., et al. (2000)], and the activation of the polyol pathway [Yabe-Nishimura, C., et al. (1998)].

Table 1: Tissue localization of aldose reductase and related diabetic complications

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

In 1989, 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 recently been focused on the three areas;

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

1.2 Inhibition of Aldose Reductase Activity

Aldose reductase has been implicated in the etiology of diabetic complications. The studies for the inhibition of aldose reductase that successfully prevent the onset of cataract, in the middle of 1960's, began with the observation that long chain fatty acids inhibit aldose reductase in lens homogenates. Therefore, the long-chain fatty acids were the first known inhibitors of aldose reductase.

In 1968, it has been shown that tetramethylene glutaric acid (TMG) could alter the cataract progression by modifying the activity of aldose reductase [Kinoshita, J. H., et al. (1968)]. Also it has been demonstrated that by reducing the synthesis and accumulation of galactitol in in vitro cultured lenses, TMG minimizes the increase in lens hydration and the appearance of vacuoles; however, its inability to penetrate the membranes made the compound ineffective in vivo.

In 1975, the inhibitory action of various flavonoids had been tested and all the flavonoids tested had significant activity, and in those studies it was shown that quercetin, quercitrin and myricitrin were much more effective as aldose reductase inhibitors than TMG [Kinoshita, J. H., et al. (1968), Varma, S. D., et al. (1977)]. Although it has been known that flavonoids are non-toxic compounds, they have not been able to reached the clinical trials. Since the mid.-70's, several studies on the inhibition of aldose reductase by flavonoids have been reported [Varma, S. D., (1975)-(1976), Okuda, J., et al. (1982)]. However, their structure–activity relationships were not discussed satisfactorily because of the limited number of compounds.

Recently, 94 flavonoids were examined in order to clarify the further structural requirements of flavonoids for aldose reductase inhibitory activity. The results suggested that the flavones and flavonols having the 7-hydoxyl moiety on the A ring exhibit the strong activity, the 2-3 double bond enhances the activity and the 5-hydoxyl moiety does not affect the activity [Matsuda, H., et al. (2002)].

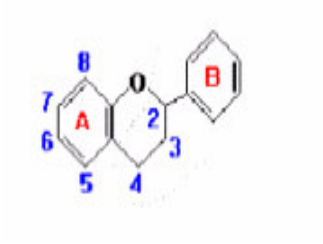


Figure 5: Basic flavonoid structure

There also exist a variety of structurally diverse orally active synthetic aldose reductase inhibitors (ARIs) (Figure 5). These compounds can be divided into two general classes, those containing a carboxylic acid moiety and those having cyclic imide represented by a spirohydantoin or related ring system [Wrobe, J., et al. (1991), Yamagish, M., et al. (1992), Costantino, L., et al. (1999)]. Both have similar intrinsic activity for aldose reductase but their in vivo potencies are quite different. Imides are more potent in in vivo because of their better pharmokinetic properties. The majority of inhibitors that have been used for human clinical trials were the members of carboxylic acid category (epalrestat, statil, tolrestat, and ponalrestat) containing carbonyl or thiocarbonyl groups. These reactive groups allow accepting a pair of electrons from the enzyme thus leading to its inhibition. Tolrestat is the most effective compound within carboxylic acid series. Although several ARIs have been tested in clinical trials on diabetic patients for more than 20 years, they still remain to be proven sufficiently effective [Preifer, M. A., et al. (1997)].

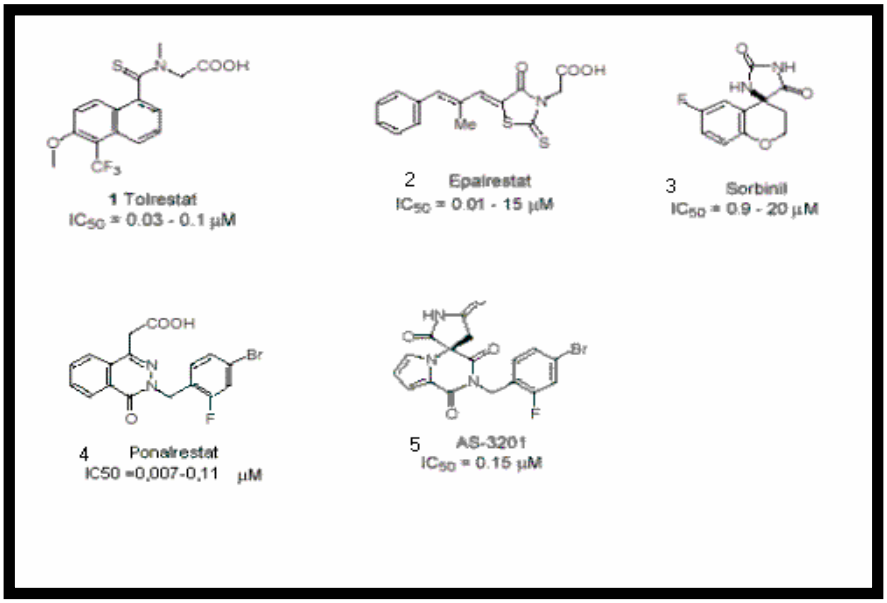


Figure 6: Chemical structures of some known synthetic aldose reductase inhibitors

Since the discovery of Sorbinil (Figure 6-3) by Pfizer in 1978, several compounds with a spirohydantoin group or closely related skeleton have been developed from the structure-activity relationship studies. Trials of sorbinil were first reported in 1983 and most have involved in small numbers of patients treated for short periods of time [Sima, A. A. F., et al. (1993), Sarges, R., et al. (1988)]. Sorbinil, which penetrates well into human lens and erythrocytes [Crabbe, M. J. C., et al. (1985)], and once thought to be a promising drug, it was plagued by toxicity problems and its performance in trials for human neuropathy was poor even where was well tolerated [Furth, A., et al. (1989)]. Unfortunately, in the early weeks of therapy, hypersensitivity reactions were induced by Sorbinil, which is oxidatively metabolized to a potentially toxic intermediate [Spielberg, S. P., et al. (1991)].

Table 2 : Summary of results of clinical trials for selected aldose reductase inhibitors

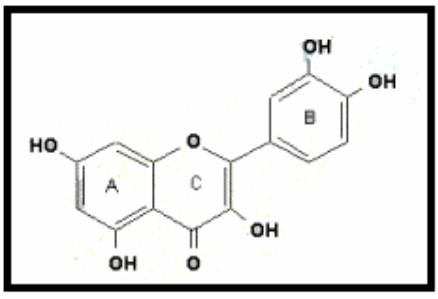
Inhibitor	No. of the patients	Outcome	Reference
Epalrestat (ONO-pharmaceuticals)	214	Less deterioration of retinopathy	**Hotta et al. (1990)**
Tolrestat (Ayerst-American home prod.)	112	Positive effect on peripheral nerve function	**Boulton et al. (1990)**
Sorbinil (Pfizer)	497 (18% dropped out of the study because of the hypersensitivity reactions evidence by effects, such as skin rash)	No improvement in early diabetic retinopathy	**Sorbinil Retinopathy Trial Research Group (1990)**
Statil (Stuart Pharmaceuticals)	47	No improvement in neuropathy	**Greene et al. (1992)**
Ponalrestat	62	No clinically significant effect on progression of retinopathy	**Arauz et al. (1992)**

Among the chemicals tested on enzyme activity, Tolrestat, improve the nerve ultra structure and function after long term treatment and is currently approved for marketing in several countries. At present, only tolrestat and (Figure 6-1), epalrestat (Figure 6-2), which was developed by Ono that launched into Japanese market in 1992, is still available [Kawamura, M. et al. (1997), Sima, A. A. F., et al. (1993), Santiago, J. V., et al. (1993), Boulton, A. J. M., et al. (1990)].

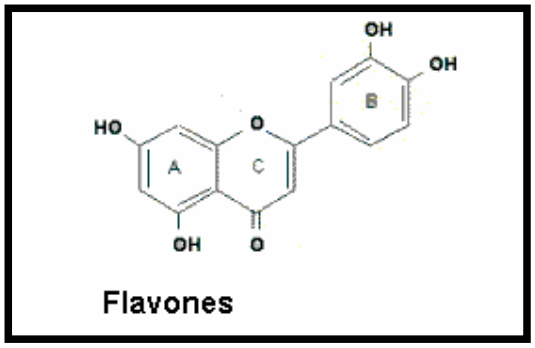
It has also been indicated that the inhibition of aldose reductase by some special chemicals is dependent on the degree of purification of the enzyme as well as on the substrate used to assay the activity [Del Corso (1989)]. A wide variety of compounds have been shown to inhibit the aldose reductase reaction, as measured by the change in absorbance at 340 nm (oxidation of NADPH), when oxidizing monosaccharides are used as substates. The aromatic hydrocarbonyl groups aid in the attachment of the inhibitor [Crabbe, J. M. C., et al. (1991), and Kador, P. F., et al. (1985)].

1.2.1 Aldose Reductase Inhibitors From Natural Sources

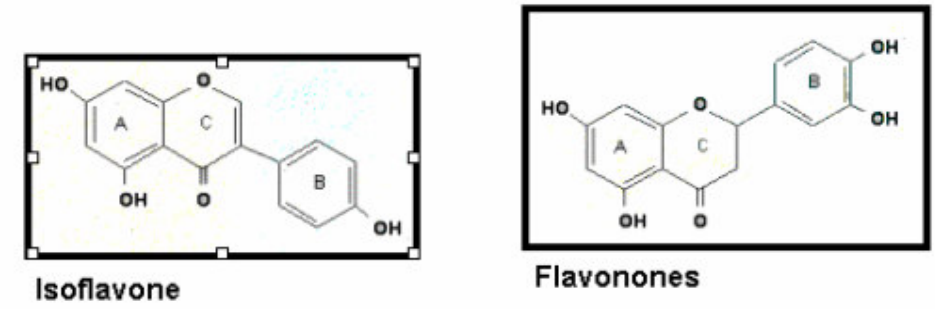
Flavonoids are polyphenolic compounds that occur ubiquitously in vegetables and fruits. They are categorized into flavonols, flavones, flavanones and isoflavonoids. These compounds, have a variety of biological effects in numerous mammalian cell systems, in vitro and as well in vivo.



Flavonol



Flavones



The studies indicate that structural requirements of an aldose reductase inhibitor are more general than specific. However, the common feature of all such inhibitors has a hydrophobic region attached to an acid group. Flavonoids meet these requirements.

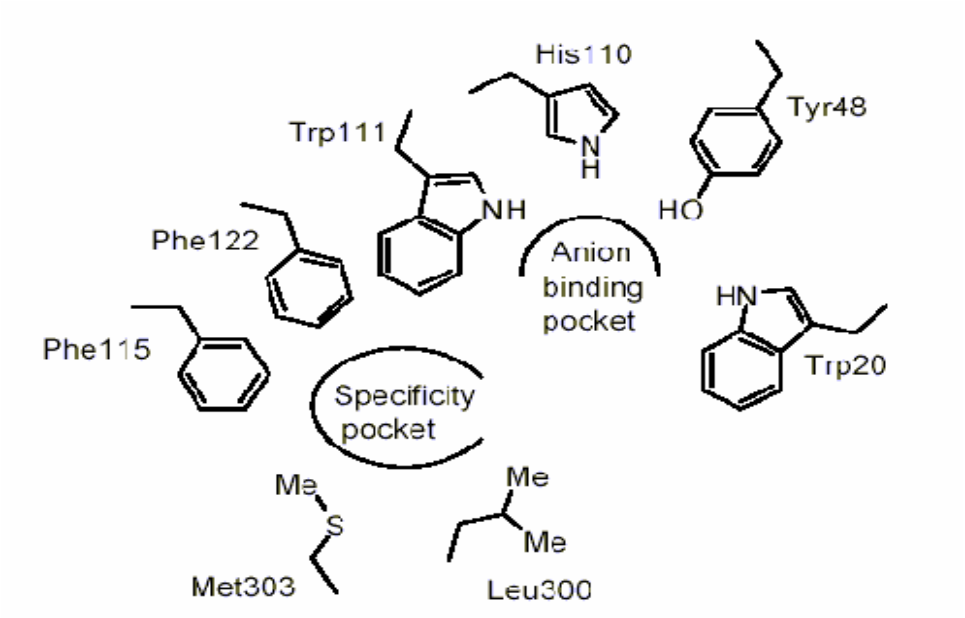


Figure 7 : The schematic representation of ligand binding site of aldose reductase [Miyamoto, S., (2001)].

One of the natural products in clinical trials, is vitamin C, which showed 81% of in vitro inhibition. Dietary supplementation with vitamin C has been shown to be effective in reducing sorbitol accumulation in the erythrocytes of diabetic patients [Cunningham, J. J., et al. (1994)].

Most of the natural inhibitors are phenolic compounds, and in addition to inhibition of the polyol pathway, they could also be involved, like the flavonoids and vitamin C, in the inhibition of the overproduction of superoxide or in metal ion chelation processes [Vincent, E. T., et al. (1998)].

Plants constitute a rich source of bioactive chemicals like flavonoids against aldose reductase. Since many of them are largely free from adverse effects and have excellent pharmacological actions, they could 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. Therefore, much effort has been focused on the plants for potentially useful products as commercial aldose reductase inhibitors or as lead compounds.

1.2.1.2 Phenolic Compounds of Some Vegetables and Fruits

There have been numerous reports of Aldose reductase inhibitors from extracts of, mostly plant material [Constantino, L., Rastelli, P., et al. (1997), Crabbe, M. J. C., and Goode, D., (1998), Rastelli, G., Antolini, S. and Constantino, L., (2000), Okuda, J., et al. (1984), Matsuda, H., Morikawa, T., Toguchida, I., Yoshkawa, M. (2002)]. Flavonoids are secondary metabolites present in fruits and vegetables. They occur in foods generally as O-glycosides bound most often at the C-3 position. Flavonoids and related compounds are the most widely studied natural product family with aldose reductase inhibitory activity.

Plants of the genus Allium have been recognized as rich sources of secondary metabolites endowed with interesting biological activities. ***Allium porrum*** and ***Allium cepa*** is the member of the genus ***Allium***. A phytochemical investigation of the extracts obtained from bulbs of leek, ***Allium porrum*** has led to the isolation of flavonol glycosides, two of which, based on a kaempferol aglycone and acylated with a 3-methoxy-4-hydroxycinnamoyl moiety [Fattorusso, E., et al. (2001)].

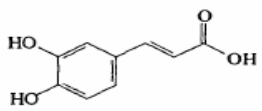
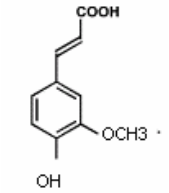
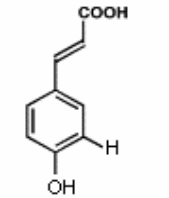
Herto and coworkers in 1993 have reported that hydrolysed samples from numerous fruits and vegatables for total aglycone content and have found the highest concentration of quercetin in ***Allium cepa***.

Earlier studies on the flavonoid content of onions (***Allium cepa***) have shown that the main flavonoids are quercetin, quercetin- 4'-glucoside, quercetin 3,4'-diglucoside, quercetin 7-4'-diglucoside and isorhamnetin glycoside [Perkin et al**.** (1896), Hermann, K. (1956), Koeppen et al**.** (1961), Park, Y., et al. (1994)]. And the minor flavonoids also found in ***Allium cepa*** are; quercetin-3-glucoside and quercetin-5-glucoside [(Brandwein, B. J. (1965), Bandyukova, V. A., et al. (1967)] (Table 3).

Major vegetable consumed in most developed countries; spinach (***Spinacia oleracea***) was also investigated for its flavonid content. Jaceidin - 4'-glucuronide; 5,3',4'-trihydroxy-3-methoxy-6:7-methylenedioxyflavone, 4'-glucuronide, and 5,4'-dihydroxy-3,3'-dimethoxy-6:7-methylenedioxyflavone 4'-glucorinide were identified from ***Spinacia oleracea*** leaves [Aritomi, M., et al. (1984), Hermann, K. (1956), Perkin, A. G., and Hummel, J. J., (1896), Koeppen, B. and Van der Spuy, J. E. (1961), Park, Y., and Lee, C. (1994), Sood, A. R., and Noshi, K. H. (1974)] (Table 4).

Table 3 : Phenolic compounds of ***Allium cepa*** **(Onion)**

	Name of the compound	**$R_{3'}$**	**$R_{4'}$**	**R_3**	**R_5**	***R_7***
1	Quercetin	OH	OH	OH	OH	*OH*
2	Quercetin-3-glucoside	OH	OH	Glucose	OH	*OH*
3	Quercetin -4'-glucoside	OH	Glucose	OH	OH	*OH*
4	Quercetin-3,4'-diglucoside	OH	Glucose	OH	OH	*OH*
5	Quercetin-7,4'-diglucoside	OH	Glucose	OH	OH	*Glucose*
6	Quercetin-3-rutinoside	OH	OH	Rutinose	OH	*OH*
7	Quercetin-3,4'-di-O-β-D-glucoside	OH	Glucose	Glucose	OH	*OH*
8	Quercetin-5-glucoside	OH	OH	OH	Glucose	*OH*
9	Quercetin 3'-methyl ether (Isorhamnetin)	CH_2CH_3	OH	OH	OH	*OH*
10	Kaempferol	X	OH	OH	OH	*OH*
11	Kaempferol-glucosides	*X*	OH	Glucose	OH	*OH*

12	Caffeic acid	
13	Ferulic acid	
14	P-coumaric acid	

** [Hermann, K. (1956), Perkin, A. G ., and Hummel, J. J. (1896), Koeppen, B., and Van der Spuy, J. E. (1961), Park,Y. and Lee, C. (1994), Sood, A. R. and Noshi, K. H. (1974)].

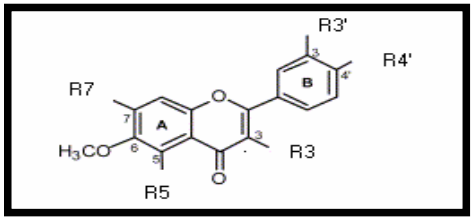
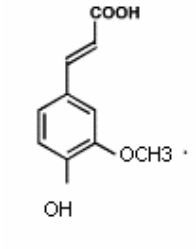
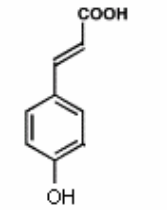


Table 4 : Phenolic compounds of ***Spinacia oleracea*** **(spinach)**

	Name of the compound	**$R_{3'}$**	**$R_{4'}$**	**R_5**	**R_6**	**R_7**	**R_3**
1	Patuletin-3-O-β-D-glucopyranosyl(1→6)-[β-D-apiofuranosyl(1→2)]-β-D-glucopyranoside	OH	OH	OH	CH_3O	OH	Glucose Apiose Glucose
2	Spinacetin-3-O-β-D-glucopyranosyl(1→6)-[β-D-apiofuranosyl(1→2)]-β-D-glucopyranoside	CH_3O	OH	OH	CH_3O	OH	Glucose Apiose Glucose
3	Patuletin-3-O-β-D-(2''feruloylglucopyranosyl)(1→6)-[β-D-apiofuranosyl(1→2)]-β-D-glucopyranoside	OH	OH	OH	CH_3O	OH	2'' feruloglucose Apiose Glucose
4	Spinacetin-3-O-β-D-(2''feruloylglucopyranosyl)(1→6)-[β-D-apiofuranosyl(1→2)]-β-D-glucopyranoside	CH_3O	OH	OH	CH_3O	OH	2'' feruloglucose Apiose Glucose

5	Spinacetin-3-O-β-D-(2''p-coumaroylglucopyranosyl)(1→6)-[β-D-apiofuranosyl(1→2)]-β-D-glucopyranoside	CH_3O	OH	OH	CH_3O	OH	2''p-coumaroglucose Apiose Glucose
6	Spinacetin-3-O-β-D-glucopyranosyl(1→6)-[β-D-glucopyranoside	CH_3O	OH	OH	CH_3O	OH	Glucose Glucose
7	Spinacetin-3-O-β-D-(2''feruloylglucopyranosyl)(1→6)-[β-D-glucopyranoside	CH_3O	OH	OH	CH_3O	OH	2'' feruloglucose Glucose
8	Quercetin	OH	OH	OH	X	OH	OH
9	Quercetin -3-rutinoside (Rutin)	OH	OH	OH	X	OH	Rutinose
10	Quercetin -3-rhamnoside (Quercitrin)	OH	OH	OH	X	OH	Rhamnose
11	Jaceidin 4'-glucuronide Jaceidin = 5,7,4'-trihydroxy-3,6,3'-trimethoxyflavone	CH_3O	Glu-	OH	CH_3O	OH	CH_3O
12	Patuletin 3-O-β-gentiobioside	OH	OH	OH	CH_3O	OH	Gentibiose
13	*Spinacetin 3-O-β-gentiobioside*	CH_3O	OH	OH	CH_3O	X	Gentibiose
14	Spinatoside(3,6-dimethoxy-5,7,3',4'-tetrahydroxyflavone-4'-O-β-D-glucopyranuronide)	OH	Glu-	OH	CH_3O	OH	CH_3O

15	Ferulic acid	
16	p-coumaric acid	

**[Aritomi, M. and Kawasaki, T. (1984), Aritomi, M., and Kawasaki, T., and Komori, T., (1986), Ferreres, F., Castaner, M. (1996)].

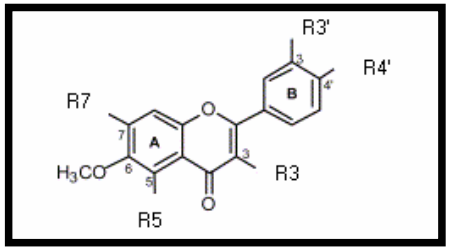
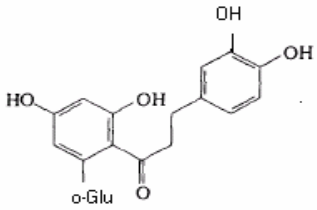
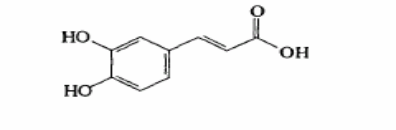
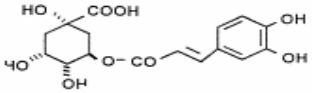
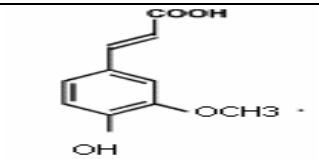
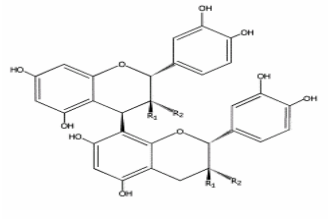
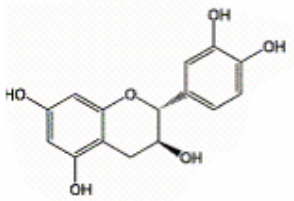
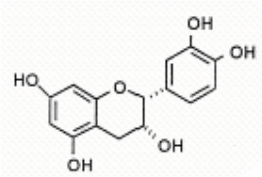
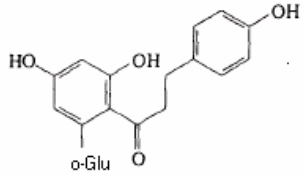


Table 5 : Phenolic compounds of ***Malus domestica*** **(Apple)**

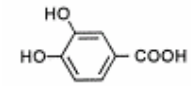
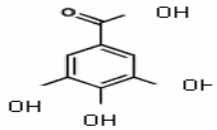
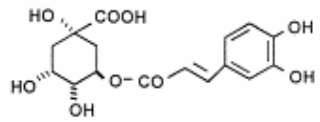
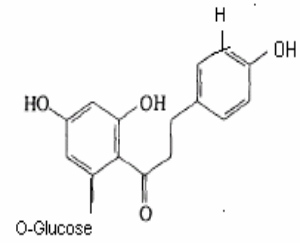
	Name of the compound	**$R_{3'}$**	**$R_{4'}$**	**R_5**	**$R_{5'}$**	**R_7**	**R_3**
1	Quercetin	OH	OH	OH	X	OH	OH
2	Quercetin-3-galactoside (Hyperoside)	OH	OH	OH	X	OH	Gal-
3	Quercetin-3-glucoside (Isoquercitrin)	OH	OH	OH	X	OH	Glu-
4	Quercetin-3-rhammnoside (Quercitrin)	OH	OH	OH	X	OH	Rham
5	Quercetin -3-xyloside	OH	OH	OH	X	OH	Xyl-
6	Quercetin-3-arabinoside	OH	OH	OH	X	OH	Arab-
7	Quercetin-3-rutinoside (Rutin)	OH	OH	OH	X	OH	Rutin-
8	Myricetin	OH	OH	OH	OH	OH	OH

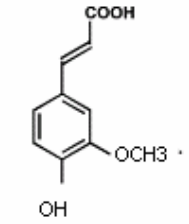
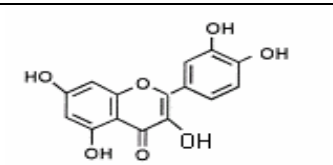
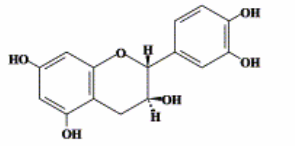
9	3-hydroxyphloridzin	
10	Caffeic acid	
11	Chlorogenic acid	
12	Ferulic acid	
13	Procyanidin	

14	(+)Catechin	
15	(-)Epicatechin	
16	Phloridzin	

**[Lister, C. E., Lancaster, J. E. and Souton, K. H. (1994), Schieber, A. K., Carle, R. (2000), Lu, l., Foo, L. Y., (1997), Scalbert, A., Williamson, G. (2000)].

Table 6: Phenolic compounds of ***Punica granatum*** **(Pomegranate*)***

No	Name of the compound	Structure
1	Protocatechuic acid	
2	Gallic acid	
3	Chlorogenic acid	
4	Phloridzin	

5	Ferulic acid	
6	Quercetin	
7	Catechin	

** [Poyrazoğlu, E., Gökmen, V., Artık, N. (2001)].

1.3 Scope of The Study

Diabetes has been considered to be one of the major risk factors of cataract [Lee and Chung (1999)]. Various pharmacological intervention strategies are underway for its prevention. Among them aldose reductase inhibitors have received considerable attention because of the proposed involvement of aldose reductase. Aldose reductase is a key enzyme of polyol pathway and has been a drug target in the clinical treatment of secondary complications of diabetes.

A vast literature exists showing that the cataract progression can be slowed down or prevented by the use of natural therapies, particularly with those plants having high flavonoid contents.

In this study, we first aimed to establish the bovine lens aldose reductase activity assay, and then to evaluate the inhibitory effects of extracts in the same assay. For this purpose, aldose reductase was isolated by means of centrifugation and ammonium sulfate precipitation. Aldose reductase enzyme activity was determined by monitoring the reduction of NADPH to $NADP^+$ at 340 nm spectrophotometrically. The optimum conditions, such as protein amount, pH of reaction mixture, coenzyme concentration, substrate concentration, effect of temperature on the reaction rate were determined. Inhibitory effect of some vegatable and fruit extracts which were obtained from solvents with different polarities were tested on the crude bovine lens aldose reductase.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) DL-Glyceraldehyde, ammonium sulfate [$(NH_4)_2SO_4$], sodium chloride (NaCl), lithium sulfate (Li_2SO_4), Dimethylsulfoxide (DMSO), 125mm pore size white band filter paper, Bovine serum albumin (BSA), Folin-Phenol reagent, Na-K tartarate, Copper sulfate ($CuSO_4$), Sodium bicarbonate (Na_2CO_3), EDTA $NaHCO_3$, flavonoid standards; Quercetin as aglycone, quercetin-3-galactoside, quercetin-3-glucoside as flavonoid glycosides, gallic acid as phenolic acids and membrane dialysis tubings were all purchased from Sigma Chemical Company, Saint Louis, Missouri, USA.

Solvents for extraction and thin layer chromotography; methanol, butanol, ethyl acetate, diethyl ether, petroleum ether, chloroform (chrotomographical grade) and thin layer chromotography plates (20x20 cm, silica gel 60 F_{254}) were all purchased from Merck chemical company.

Spinacia oleracea (spinach), ***Allium cepa*** (onion), ***Allium porrum*** (leek), ***Malus domestica*** (apple), ***Punica granatum*** (pomegranate) were purchased from the supermarket and ***Malus floribunda*** (Japaneese apple) were picked from the trees on the campus of METU, were used as the plant material.

2.2 Methods

2.2.1 Preparation of Aldose Reductase Crude Extract From Bovine Lens

The bovine eyes were obtained from the abattoir (Kazan Belediye Mezbahası-Ankara) immediately after slaughtering, and were placed in ice - bag full of crushed ice. Reaching the laboratory the lenses were excised from the eyes and washed with ice-cold distilled water. Then they were homogenized in about three volumes of cold distilled water with the Ultra-turax T-25 (Janke-Kunkel, IKA-13500 rpm) 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 then saturated with ammonium sulfate to 40 % and saturated sample was centrifuged at 10,000 x g for 25 minutes. The supernatant after second centrifugation was brought to 50 % ammonium sulfate saturation to remove additional proteins. The saturated sample with 50 % ammonium sulfate then centrifuged at 10,000 x g for 25 minutes. Pelet was discarded again and the supernatant obtained after third centrifugation was then saturated with 75 % ammonium sulfate at 10,000 x g for 25 minutes. In last step, this time the supernatant was discarded and the pellet was dissolved in 50 mM NaCl solution. The volume of the suspension was recorded before dialysis and the sample was dialyzed overnight against 50 mM NaCl solution. The replacement of dialysis solution was done after 16 hours in cold room.

After dialysis, the volume of the sample was recorded and stored in 0.5 mL aliquots in eppendorf tubes in deep freezer at -80 ºC for determination of protein and enzyme activity studies.

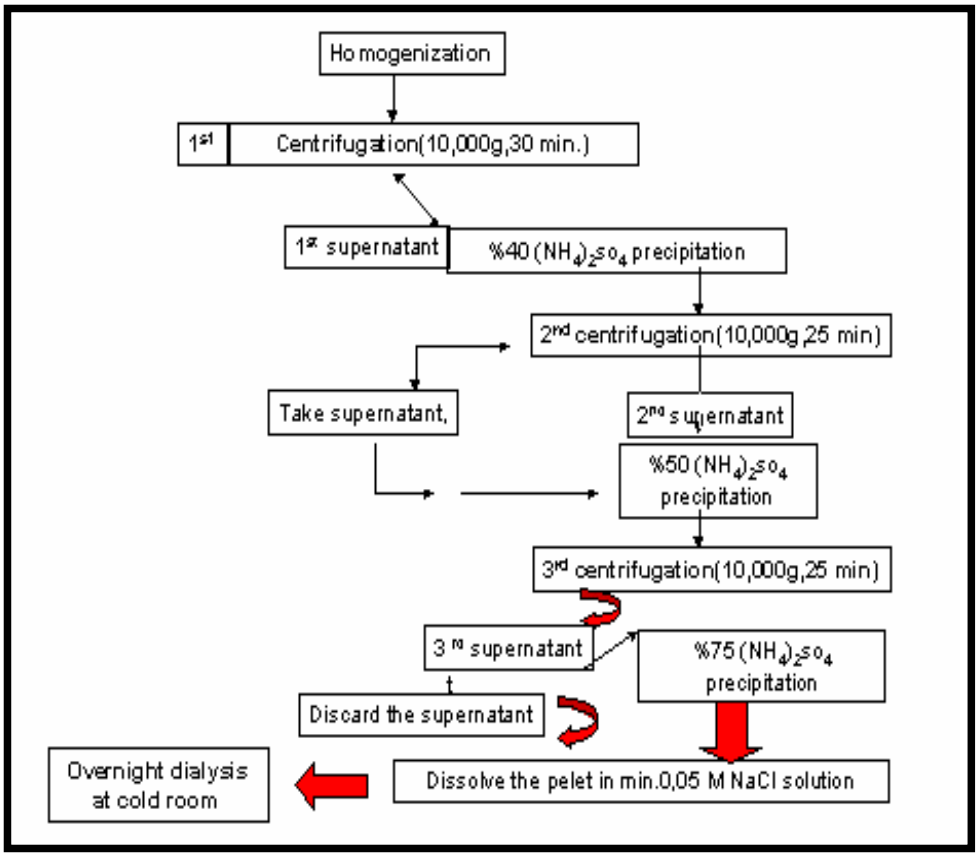


Figure 8: Preparation of crude lens aldose reductase from the bovine

2.2.2 Preparation of the Crude Fractions From The Plant Extracts

Plants were air-dried at room temperature and finely powdered. They were extracted three times with methanol at 60 ^{o}C for 4.5 hours in rotary evaporator without vacuum. Then the extracts were filtered (with 125 mm white band filter paper). The combined filtrate was concentrated into an extract in vacuum at 45 ^{o}C.

The methanolic extracts were suspended in warm distilled-water and partitioned three times with each of the following solvents in increasing polarity; petroleum ether, diethyl ether, ethyl acetate, and n-butanol respectively. The organic solvent portions were concentrated to dryness by rotary evaporator at 45 ^{o}C in order to use for the inhibition study of bovine lens aldose reductase enzyme (Figure 9).

Only the ***Punica granatum*** extract was prepared without methanol extraction under mentioned conditions above. Because fruit juice was used as the crude extract.

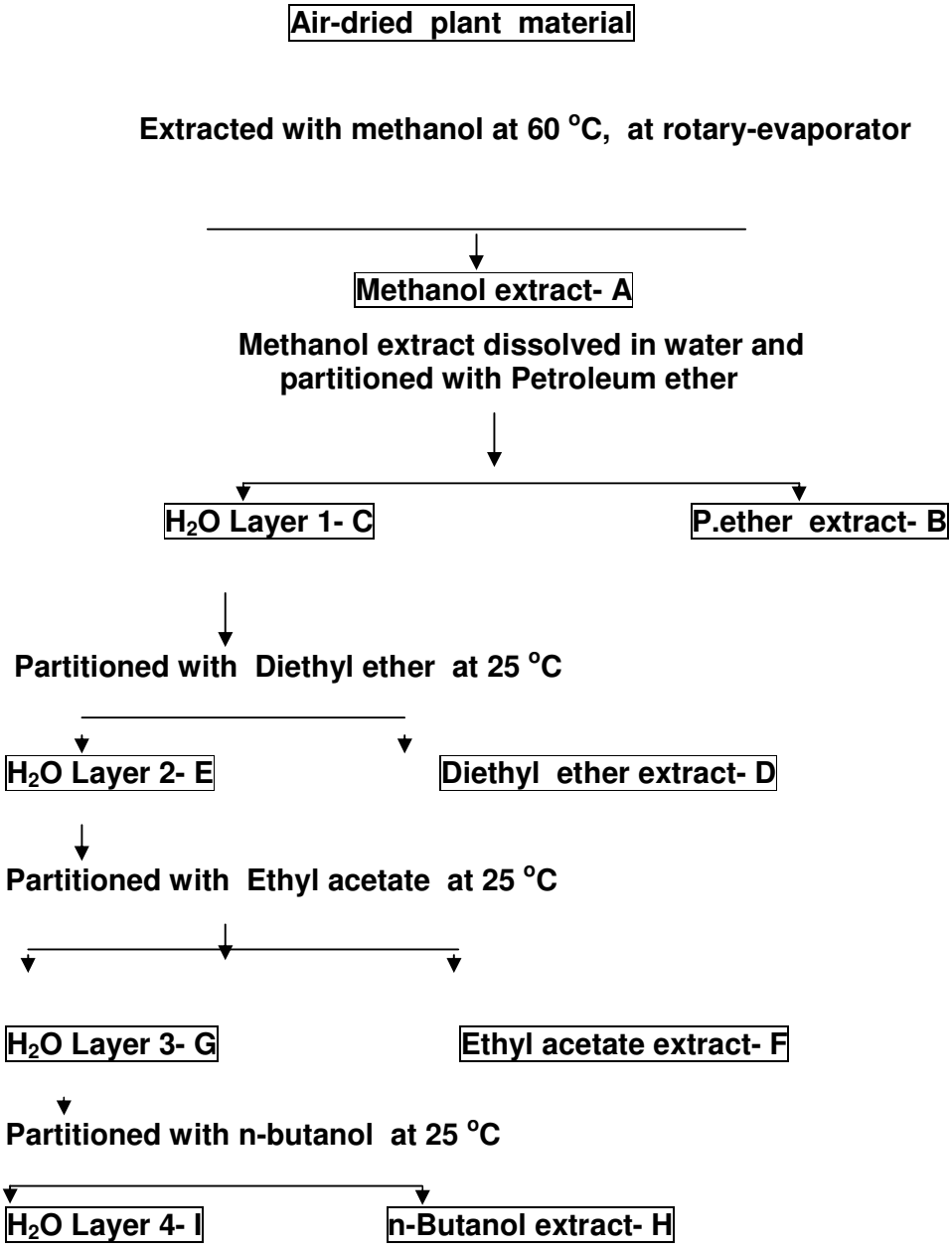


Figure 9: Schematic preparation of plant extracts by solvent fractionation

2.2.3 Thin Layer Chromotography (TLC) of The Extracts

Thin layer chromatohraphy plates coated with silica were used for the detection of groups in crude fractions of plant extracts by preparing solvent system as $CHCl_3$: CH_3OH: H_2O in proportions of 61: 32: 7 to detect groups which dissolved in ethyl acetate fractions. Quercetin as agylcone, quercetin-3-galactoside, quercetin-3-glucoside as glycosides of flavonoids and gallic acid was used as standard of phenolic acids in TLC trials.

%1 vanilin - sulphuric acid for flavonoids and $FeCl_3$ for all types of phenolic compounds such as tannins, phenolic acids etc. was applied on the TLC plates as a detection spray reagent to visualize the spots of fractions.

2.2 Analytical Procedures

2.3.1 Protein Determination

Lowry method was utilized to determine the protein concentration of samples using crystalline Bovine Serum Albumin (BSA) as the standard [Lowry, et al. (1951)]. To construct a calibration curve, four different concentrations of standard samples, 0.05 –0.2 mg BSA /mL were put into test tubes with a final volume of 0.5 mL.

The sample aliquots in portions of 0.1, 0.25 and 0.5 mL's were prepared from the stock solutions with a dilution of 1:100 and were completed to a final volume of 0.5 mL with distiled water. All the tubes were mixed with 2.5 mL alkaline-copper reagent, which was prepared by mixing 2 % sodium potassium-tartarate, 2 % copper-sulfate, and 0.1 N NaOH containing 2 % sodium carbonate in the ratios of 1:1:100 respectively.

Then all the test tubes mixed with Alkaline-Copper reagent was vortexed and allowed to stand for 10 minutes at room temperature. Finally, 0.25 mL of freshly prepared 50 % folin-phenol reagent were added to the mixture of all the test tubes and each tube immediately vortexed in 8 seconds. The samples were left for 30 minutes of waiting time at room temperature The optical density of each tube were measured at 660 nm with a Varian spectrophotometer (Carry-50). Standard calibration curve of 0-0.2 mg BSA/ mL was plotted and it was used in determining the protein concentration of the samples.

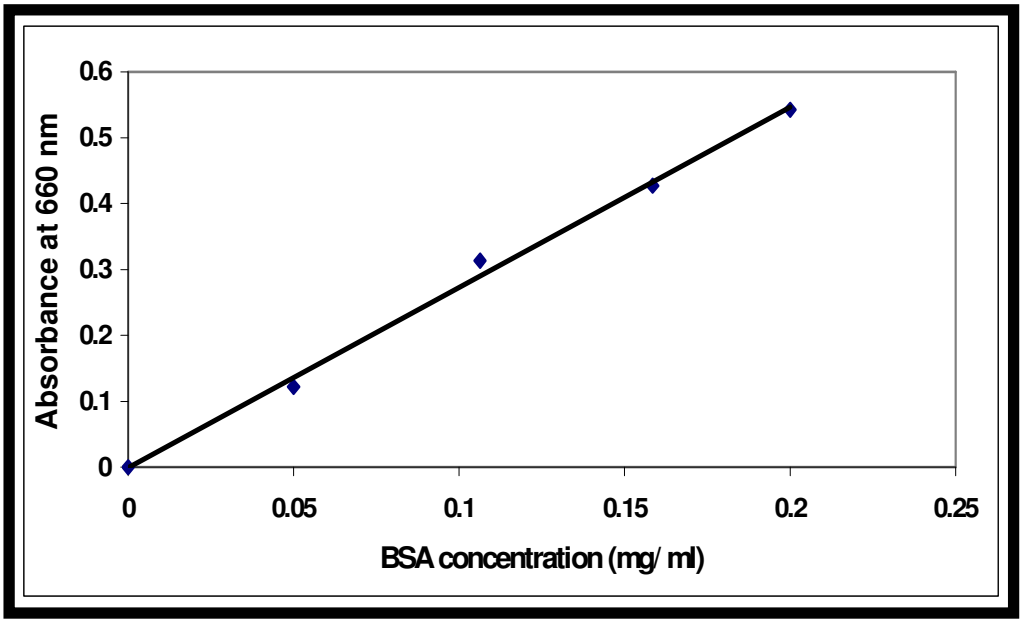


Figure 10 : BSA standard calibration curve for protein determination

2.3.2 Determination of Aldose Reductase Enzyme Activity

The enzyme activity was measured spectrophotometrically according to the method developed by Kinoshita [Kinoshita et al. (1995)]. Basically this method was based on the reduction of DL-Glyceraldehyde as a substrate to glycerol (sugar alcohol) by the enzyme aldose reductase during the oxidation of Nicotinamide adenine dinucleotide phosphate (NADPH) to $NADP^+$. This reaction was followed spectrophotometrically by monitoring the decrease in absorbance at 340 nm. In this study the crude bovine lens extracts were used as the enzyme source, which were prepared as previously described in Methods.

Table 7 : The constituents of assay mixture for aldose reductase

Constituents	**Volume Added (µL)**	**Stock Solution**	**Final Concentration**
pH 6.7 Phosphate Buffer	600	50 mM	30 mM
NADPH	30	2×10^{-3} M	6×10^{-5} M
Enzyme Solution	*	*	0.63 mg / mL
Li_2SO_4	135	2 M	270 mM
dH_2O	To complete final volume to 1 mL	*	*
Glyceraldehyde	40	1.5×10^{-3} M	6×10^{-5} M
Total volume	1 mL	*	*

All of the constituents were added into the reaction mixture with the given order of Table 7. Three minutes incubation time was carried out for every reaction cuvette before the addition of substrate, glyceraldehyde, then absorbance at 340 nm immediately was measured after the addition of substrate. The reaction have been initiated by the addition of substrate DL-Glyceraldehyde and the decrease in the absorbance was measurable. Under the same conditions, a blank solution was prepared without the substrate and the background corrections were made.

- The slope of the reaction [ΔOD at 340 (nmole/min)], background measurements and also the extinction coefficient of 6220 mM^{-1} were used for the calculation of aldose reductase activity.

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

$$\textbf{Specific activity}\ (\text{nmolex min}^{-1}\text{x (mg/mL)}^{-1} = \frac{\text{Sample } \Delta OD_{(340)} - \text{Blank } \Delta OD_{(340)}}{\varepsilon^{340}_{(NADPH)} \times [\text{protein}]}$$

2.4 Inhibition of Bovine Lens Aldose Reductase

Forthy eight different extracts were obtained by using solvent-fractionation, starting with the six methanol extracts of vegetable and fruits. The extracts that were obtained from the fractionation with petroleum ether, diethyl ether, ethyl acetate, n-butanol and their respective water fractions were tested on lens bovine aldose reductase by using 0.1 mg of extract/mL as a final concentration in 1 mL reaction mixture.

The stock extract solutions were prepared as following:

- Petroleum ether, diethyl ether, ethyl acetate and n-butanol extracts were all dissolved in 0.1% dimethylsulfoxide (DMSO).

- Water parts of plant extracts were all dissolved in distilled water

The desired concentrations of these extracts were prepared by serial aqueous dilution of the stock solutions in 0.1% DMSO. In order to keep the DMSO content constant during the activity determinations, a 10 μL of inhibitor (plant extracts) solution of varying concentrations was added into the standard assay mixture of 1mL in final volume.

The enzyme activity determined in the standard reaction mixture containing DMSO (0.1%) was considered as control activity. The presence of DMSO below 1% did not have any or little effect on blank measurements recorded in the absence of DL-glyceraldehyde.

The standard assay mixture was used in this study, all of the ingredients including 10 µl of DMSO without any extract was measured as the blank and again with the standard assay, 10 µl stock extract solution was measured as the sample of ΔOD (340)/min.

- The percentage inhibition of aldose reductase was calculated from the formula :

$$\textbf{Percentage inhibition (\%)} = \frac{\Delta\, OD_{(340)}\ \text{control} - \Delta\, OD_{(340)}\ \text{sample}}{\Delta\, OD_{(340)}\ \text{control}} \times 100$$

- The inhibitory effects of the most potent extracts were expressed as the 50% inibitory concentration [IC_{50} (µg/mL)].

Statistical calculations

Results were expressed as mean ± SD and levels of significance were assessed using ANOVA test (Analysis of Variance) with repeated measurements.

CHAPTER 3

RESULTS & DISCUSSION

3.1 Bovine Lens Aldose Reductase Activity

The bovine lens aldose reductase activity was determined according to the method of Kinoshita [Kinoshita, J. H., et al. (1965)] as described under ”Methods”. Crude enzyme solution prepared from the bovine lens homogenate was used as enzyme source.

Aldose reductase was determined in the crude enzyme solution prepared from 30 to 40 bovine lenses. Among the pooled lens solutions the lowest specific activity was measured as 1.79 nmole NADPH oxidized /min/ mg protein and the highest specific activity was measured as 2.9 nmole NADPH oxidized /min/ mg protein. The average aldose reductase specific activity calculated as 2.17 ± 0.32 (Mean ± SD, n=6) nmole NADPH oxidized /min/ mg protein.

3.2 Storage Stability of Aldose Reductase Activity

In order to determine the effect of storage on aldose reductase activity, the crude extracts prepared from bovine lenses were divided into small alliquots in eppendorf tubes and kept in -80 ^{o}C in deep freezer. At indicated days of storage one of the alliquots was thawed and the aldose reductase activity was determined as explained under "Methods". Figure 11 shows the days of stability for the enzyme.

Thawing once the sample did not affect the aldose reductase activity significantly, but a second thawing resulted in the loss of activity approximately up to 10%. Therefore, the measurements of the enzyme activity were always performed using the samples, thawed once. As shown in Figure 11, the aldose reductase activity was stable only for 10 days at -80 °C. After 10 days the enzyme activity was decreased; at 14 th day 68% and at 28th day 50 % of original activity was retained.

The enzyme activity was determined in the reaction mixture containing $6x10^{-5}$ M DL-Glyceraldehyde as substrate, 0.27 M lithium sulfate, freshly prepared $6x10^{-5}$ M NADPH, 0.63 mg/mL protein and 30 mM potassium phosphate buffer, pH 6.7 in a final volume of 1 mL. The measurements were taken in the alliquots after the first thawe as indicated above in the Figure 11.

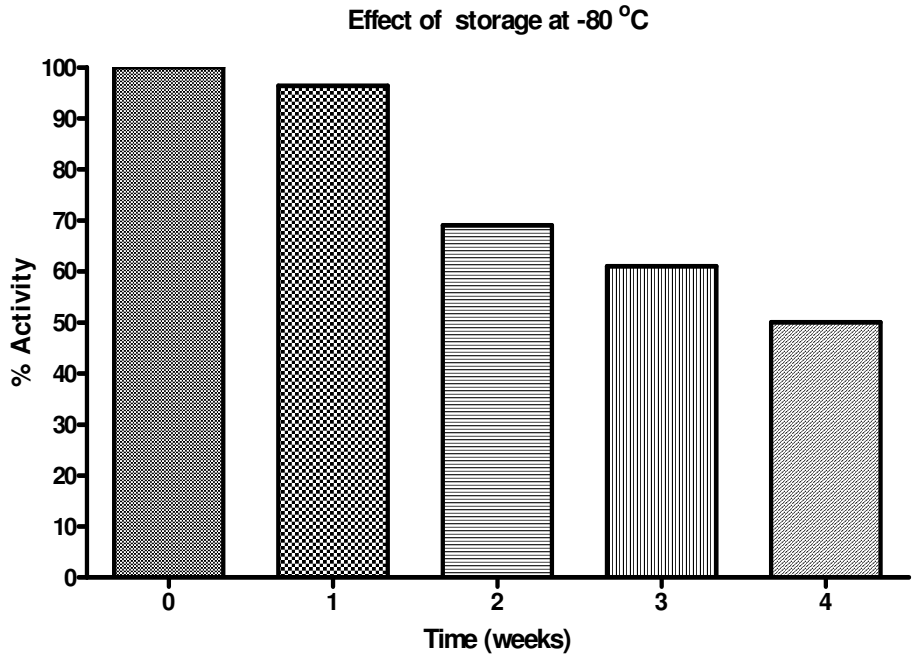


Figure 11: Storage stability of bovine lens aldose reductase crude extract. Crude extract stored in small alliquots at -80 °C.
The data shown as the means of duplicate determinations from at least two seperate experimental results.

2 mM Dithiothreitol (DTT), could have been used to improve the length of stability of the enzyme at -80 °C. Other sulfhydryl compounds such as glutathione, cysteine, β- mercaptoethanol, and were found to be less effective than DTT [Altwood, M. A., Doughty, C. C. (1974)].

3.3 Optimization Studies of Aldose Reductase Assay

3.3.1 The Time Course of Aldose Reductase Activity

The time course of a typical NADPH oxidation in the DL-Glyceraldehyde reduction catalyzed by aldose reductase is shown in Figure12. The reaction was linear up to 10 minutes. Therefore, the reactions were followed for 5 minutes, and the change in absorbance in the absence and presence of DL-Glyceraldehyde was used to calculate the aldose reductase activity by subtracting the former as a background which was also explained under "Methods".

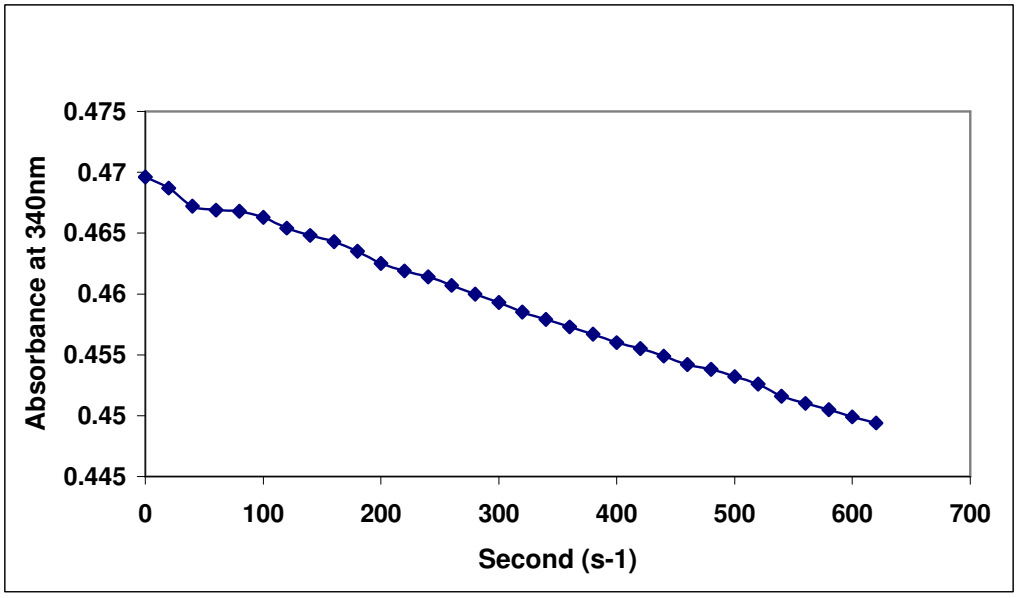


Figure 12: Time course of aldose reductase activity.
The reactions were carried out at room temperature as described under "Methods".

3.3.2 Optimization of Aldose Reductase Assay Conditions

3.3.2.1 Effect of Protein Amount on Enzyme Activity

The effect of protein amount on bovine lens aldose reductase activity was determined by the addition of different amounts of the enzyme solution into the reaction mixture. The protein amount used in the reaction mixture was varied from 0.24 mg to 1.45 mg.

Typical assay mixture containing $6x10^{-5}$ M DL-Glyceraldehyde as substrate 0.27 M Li_2SO_4, freshly prepared $6x10^{-5}$ M NADPH, varying protein concentrations of crude enzyme solution and 30 mM potassium phosphate buffer at pH 6.7 in a final volume of 1 mL.

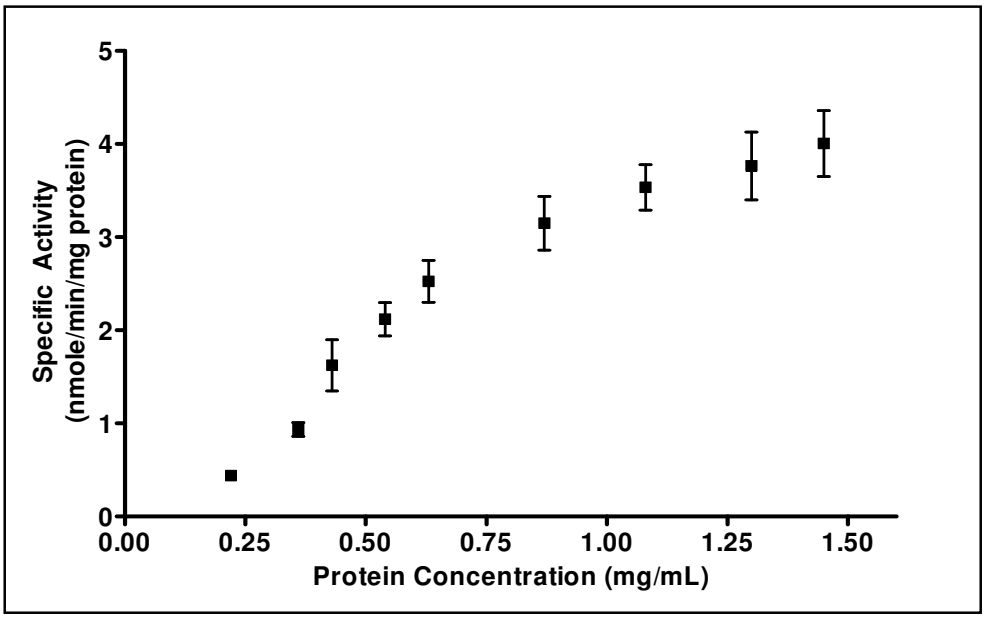


Figure 13 Effect of protein amount on the rate of bovine lens aldose reductase activity.
The enzyme activity values are the mean of triplicate measurements obtained from at least three seperate sets of data.

The reaction rate was linear with protein amount up to approximately 0.85 mg crude solution of protein per 1 mL incubation mixture. To obtain a sufficient quantity of products in the spectrophotometric determinations, the protein concentration was decided as 0.63 mg per mL**,** and was routinely used in the incubation mixture throughout this study.

3.3.2.2 Effect of Salts on Enzyme Activity

Reaction mixture contained $6x10^{-5}$ M DL-Glyceraldehyde as substrate, freshly prepared $6x10^{-5}$ M NADPH, 0.63 mg/mL protein, various concentrations of sulfate salts and 30 mM potassium phosphate buffer with a pH of 6.7 in the final volume of 1 mL.

Increasing the concentration of Li_2SO_4 beyond 400 mM did not further stimulate aldose reductase activity and addition of ammonium sulfate resulted in the nearly the same amount of aldose reductase activity as of Li_2SO_4. In the presence of Li_2SO_4, which is essential for the expression of aldose reductase activity, however it shows a complete inhibition on another type of enzyme found in the crude enzyme mixture, aldehyde reductase II that belongs to the oxidoreductase family same with the aldose reductase [Das, B., et al. (1985)]. Both of these enzymes reduce aldehydes and use NADPH as a coenzyme. They have 65 % sequence similarities in their structure. Therefore, to eliminate aldehyde reductase II activity in the reaction mixture Li_2SO_4 was chosen as an activator. For further measurements of enzyme activity, Li_2SO_4 concentration was chosen as 270 mM (Figure 14).

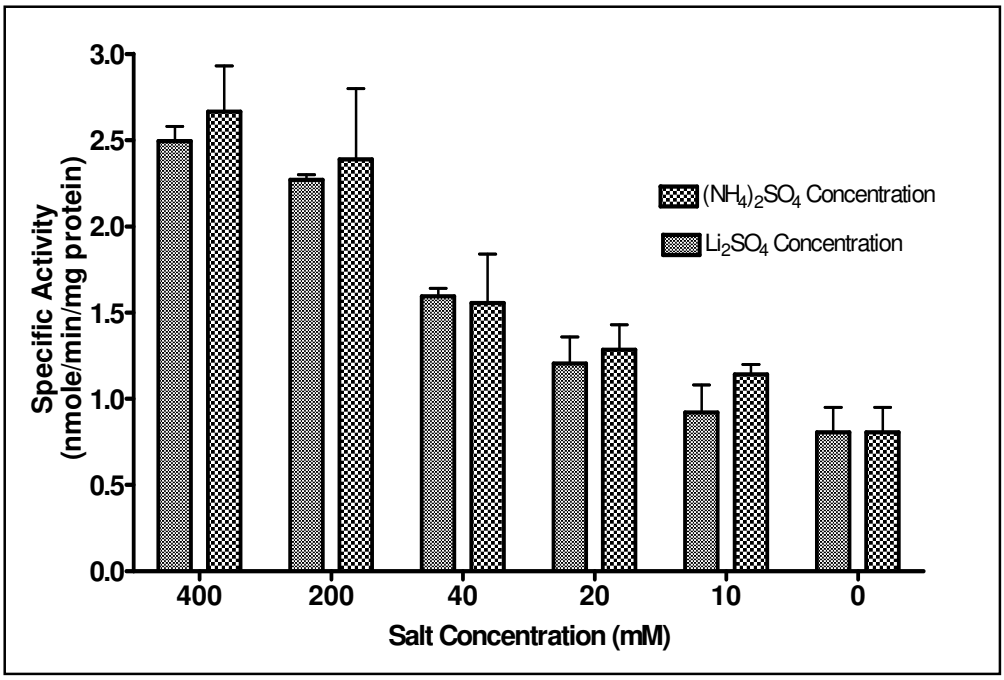


Figure 14: Effect of salts on aldose reductase activity .
The specific activity values are the mean of duplicate measurements obtained from at least three seperate experiments.

3.3.2.3 Effect of pH on Enzyme Activity

The pH measurements were carried out by using 50 mM phosphate buffer in 1 mL reaction mixture with the pH ranges varying from 5.7 to 8. Reaction mixture contained 50 mM potassium phosphate buffer, $6x10^{-5}$ M DL-Glyceraldehyde as substrate, freshly prepared 6×10^{-5} M NADPH, 0.63 mg/mL protein and 270 mM Li_2SO_4.

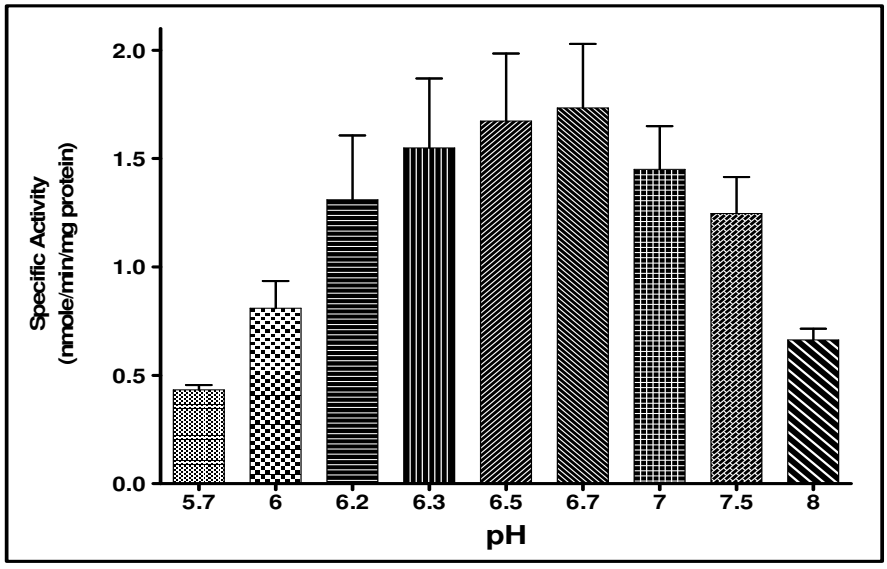


Figure 15: Effect of pH on bovine lens aldose reductase activity.
The points seen on the graph are the means of three different sets of experiments and each point is the mean of duplicate determinations.

The highest activity was observed between pH 6.2 to 6.7 when the 50 mM phosphate buffer was used. In previous studies the optiumum pH was found as 6.6 and 6.7 which were consistent with the results obtained in this study [(Dons, R. F., Doughty, C. C. (1976), Altwood, M. A., Doughty, C. C. (1974)]. Therefore phosphate buffer at pH 6.7 was used for the determination of other aldose reductase activity studies (Figure 15).

3.3.2.4 Effect of Substrate Concentration on Enzyme Activity

Figure 16 illustrates the substrate saturation curve for bovine lens aldose reductase with respect to DL-Glyceraldehyde. The enzyme activity was determined in the reaction mixture containing, 50 mM potassium phosphate buffer, pH: 6.7, freshly prepared $6x10^{-5}$ M NADPH, 0.63 mg/mL protein and 270 mm Li_2SO_4.

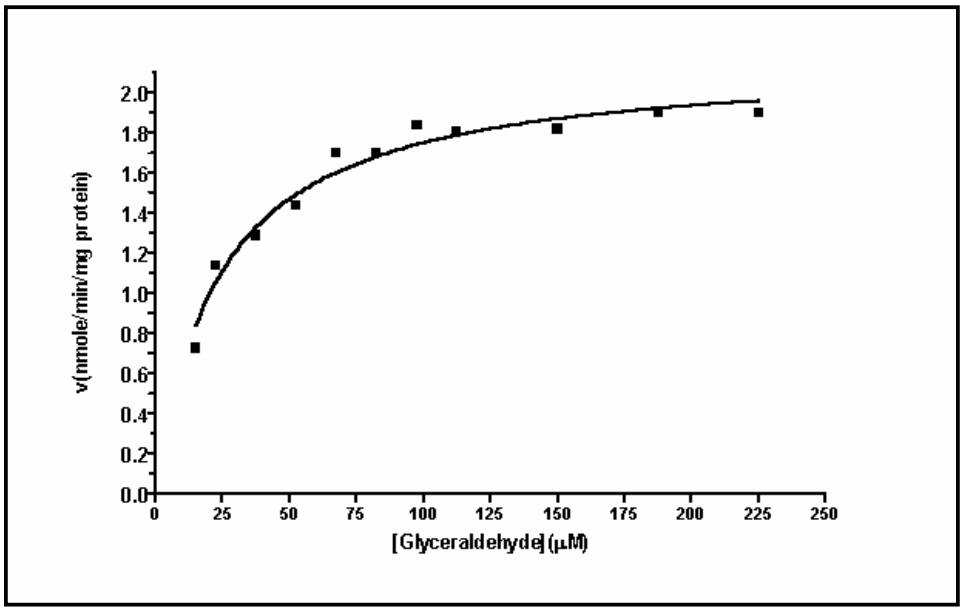


Figure 16: .Effect of Glyceraldehyde concentration on bovine lens aldose reductase activity.
The points on the graph are the means of at least three different sets of experiments and each points is the mean of triplicates.

The enzyme seemed to be saturated by DL-Glyceraldehyde at around $6x10^{-5}$ M concentration. As the substrate concentration was further increased, the enzyme activity remained constant. Therefore, $6x10^{-5}$ M as the concentration of substrate for further studies was chosen as the optimum concentration.

3.3.2.5 Effect of Coenzyme Concentration on Enzyme Activity

Figure 17 illustrates the substrate saturation curve for bovine lens aldose reductase with respect to NADPH. The enzyme activity was determined in the reaction mixture containing, 50 mM potassium phosphate buffer, pH 6.7, $6x10^{-5}$ M DL-Glyceraldehyde, 0.63 mg/mL protein and 270 mM Li_2SO_4. The reaction was carried out at room temperature.

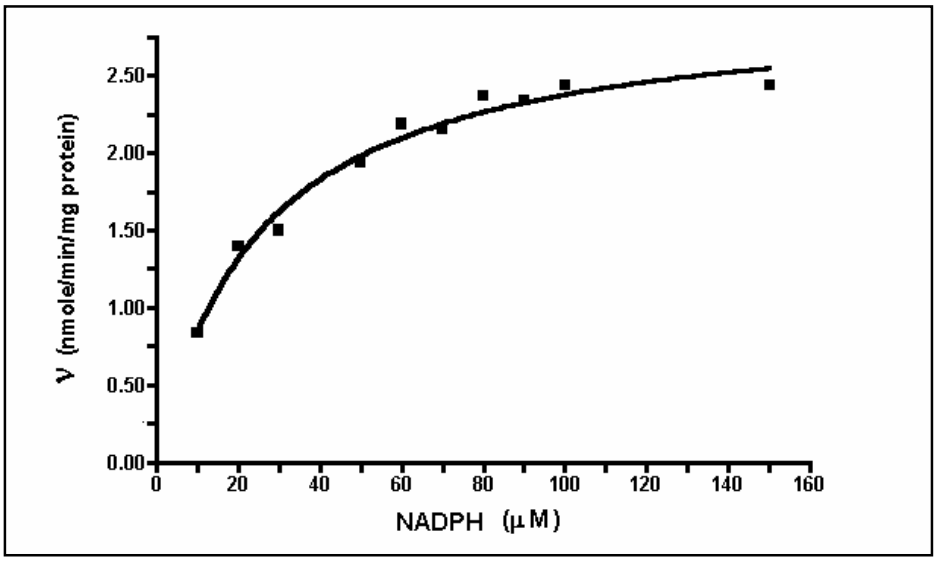


Figure 17: Effect of coenzyme (NADPH) concentration on bovine lens aldose reductase activity. The points on the graph are the means of at least three different sets experiment and each point is the mean of triplicate determinations.

The enzyme seemed to be saturated by NADPH at around $6x\ 10^{-5}$ M concentration. As the coenzyme concentration was further increased, the enzyme activity remained constant. Therefore, $6x10^{-5}$ M as the concentration of coenzyme for further studies was chosen as the optimum concentration.

3.3.2.6 Effect of Temperature on Enzyme Activity

Figure 18 shows the effect of temperature on bovine lens aldose reductase activity was detected by incubating all the reaction mixture constituents, except the enzyme at indicated temperatures for three minutes, then adding the enzyme solution and following the rate of reaction for five minutes. Six different incubation temperatures were tested namely, 10 ^{o}C, 25 ^{o}C, 37 ^{o}C, 45 ^{o}C, 55 ^{o}C, and 65 ^{o}C.

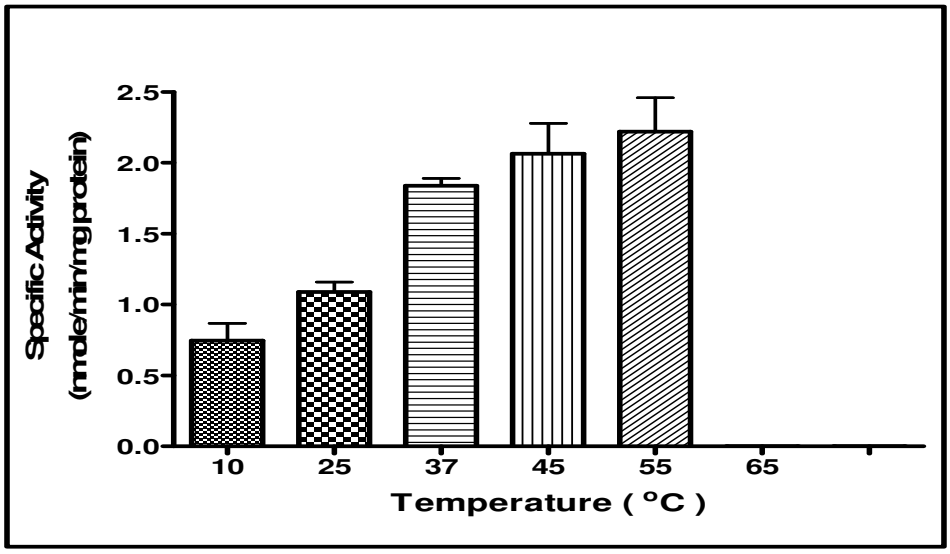


Figure 18 : Effect of temperature on bovine lens aldose reductase activity

The maximum activity were determined at 55 °C and the enzyme was denaturated at 65 ^{o}C. But all the enzyme activity measurements were carried out at room temperature for the ease of the studies**.** In an additional experiment the stability of the enzyme was tested and at 25 °C, it was found to stay stable upto 72 hrs.

3.4 Thin Layer Chromatography (TLC) of the Extracts

Ready to use, TLC aliminum sheets with silica gel 60 F_{254}, were used for the detection of groups in ethyl acetate fraction of plants using a solvent system of $CHCl_3$: CH_3OH: H_2O in 61: 32: 7 proportions. In order to detect different groups which dissolved in ethyl acetate fractions, phenolic acid and flavonoid glycosides standards were used such as gallic acid and quercetin as agylcone, quercetin-3-galactoside, quercetin-3-glucoside respectively on the TLC plates.

%1 vanilin-sulphuric acid and $FeCl_3$ were applied on the TLC plates as a detection spray reagents to visualize the spots of fractions coming from flavonoids especially and phenolics in general, respectively. Under UV radiation at 366 nm all the flavonoids were seen as yellow spots without detection reagents. In order to strengthen the colors of spots on TLC plates %1 vanilin-sulphuric acid and $FeCl_3$ were used.

Ethyl acetate fractions of ***Punica granatum, Spinacia olaeracea, Allium porrum, Malus floribunda, Malus domestica*** and ***Allium cepa*** were observed to be the highly potent extracts with an inhibitory effect on the activity of aldose reductase. Because of this reason, in the thin layer chromotography, only ethyl acetate fractions of these plants were demonstrated. (Figures 19, 20, 21)

The pink spots on TLC plates were considered as terpens and aromatic compounds. As the OH groups of the compounds increases the intensity of the yellow color increase. The dark blue color in Figure 20 corresponds to the tannin like substances.

Figure 19: TLC data of for all organic fractions of ***Punica granatum*** (pomegranate), visualization with $FeCl_3$ **Q**: Quercetin, **Q-Gal:** Quercetin-3-galactoside, **Q-Glu:** Quercetin-3-glucoside

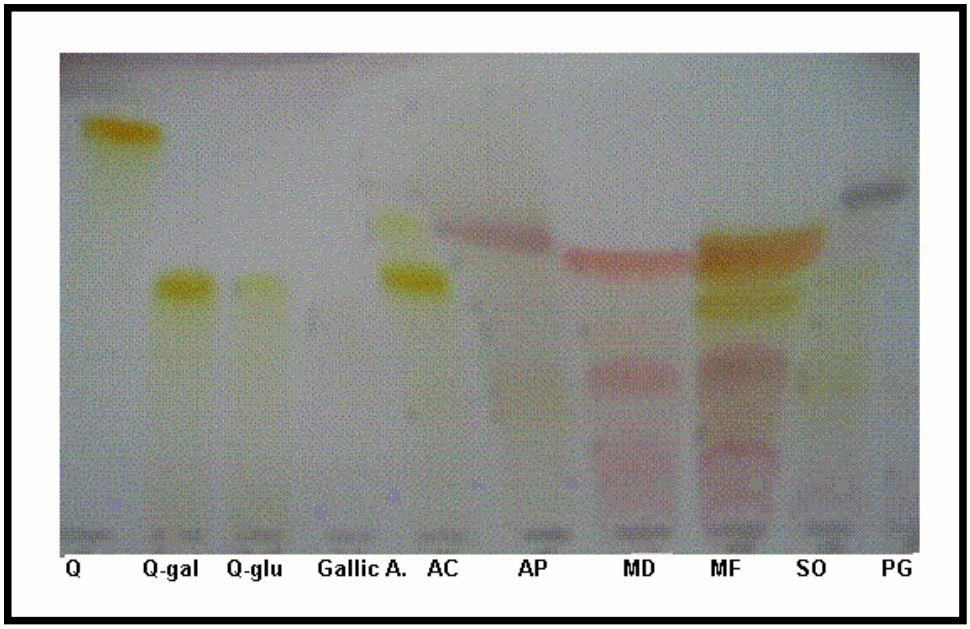


Figure 20 :TLC data of ethyl acetate extracts of all plants**-** visualization with 1% Vanilin-sulphuric acid **Q:** Quercetin, **Q-Gal:** Quercetin-3-galactoside, **Q-Glu:** Quercetin-3-glucoside, **PG:** ***Punica granatum,*** **SO:** ***Spinacia olaeracea***, **AP:** ***Allium porrum,*** **MF:** ***Malus floribunda***, **MD:** ***Malus domestica,*** and **AC:** ***Allium cepa***

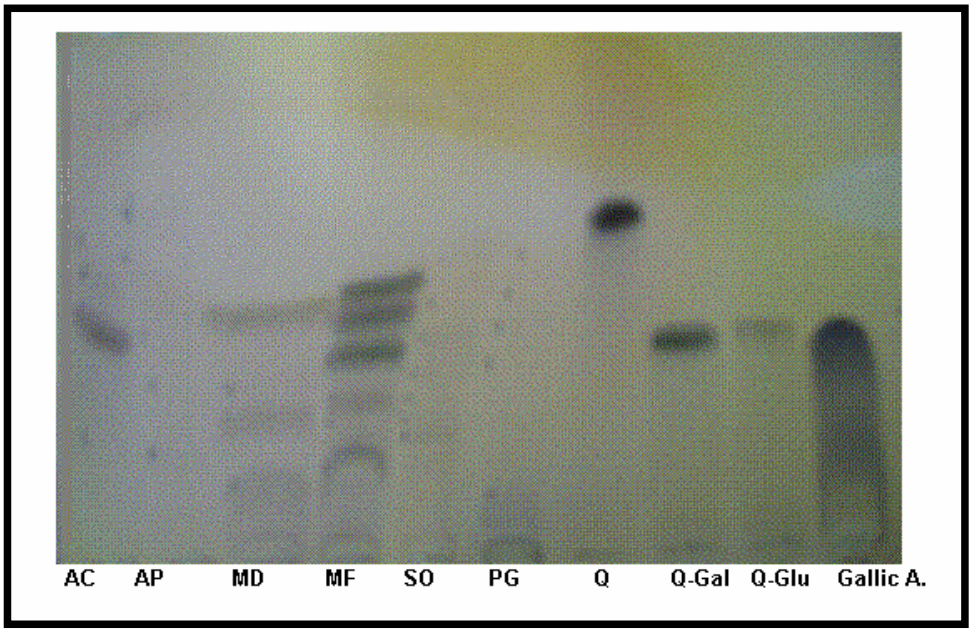


Figure 21 : TLC data of ethyl acetate extracts of all plants-visualization with $FeCl_3$ **Q:** Quercetin, **Q-Gal:** Quercetin-3-galactoside, **Q-Glu:** Quercetin-3-glucoside, **PG:** ***Punica granatum***, **SO:** ***Spinacia olaeracea***, **AP:** ***Allium porrum,*** **MF:** ***Malus floribunda***, **MD:** ***Malus domestica,*** and **AC:** ***Allium cepa***

As shown in Figure 21, $FeCl_3$ detects all the phenolic compounds. In this respect, ***Malus floribunda*** has different types of phenolics which is not supportable by the literature because there is no data available about its phenolic content. ***Punica granatum*** which is expected to be rich in phenolics showed any significant seperation on TLC plates. This result may be due to the low concentration loading, on the plate.

3.5 Inhibitory Effect of Extracts on The Enzyme Activity

The inhibition studies were carried out using crude enzyme solution of bovine lens aldose reductase. The plant extracts which are used in the aldose reductase inhibition assays, were obtained by the method of solvent-fractionation. The fractions obtained from solvent-fractionation were unable to dissolve in water, hence each extract was dissolved in dimethylsulfoxide. The DMSO was added into reaction medium used to control having any effect on the enzyme activity and showed insignificant effect using less than 1 % in the final reaction volume. The enzyme activity was determined in the reaction mixture containing $6x10^{-5}$ M DL-Glyceraldehyde as substrate, 0.27 M lithium sulfate, freshly prepared $6x10^{-5}$ M NADPH, 0.63 mg/mL protein and 30 mM potassium phosphate buffer, pH 6.7 in a final volume of 1 mL. Various concentrations of extracts were prepared by serial aqueous dilutions as explained under ''Methods''.

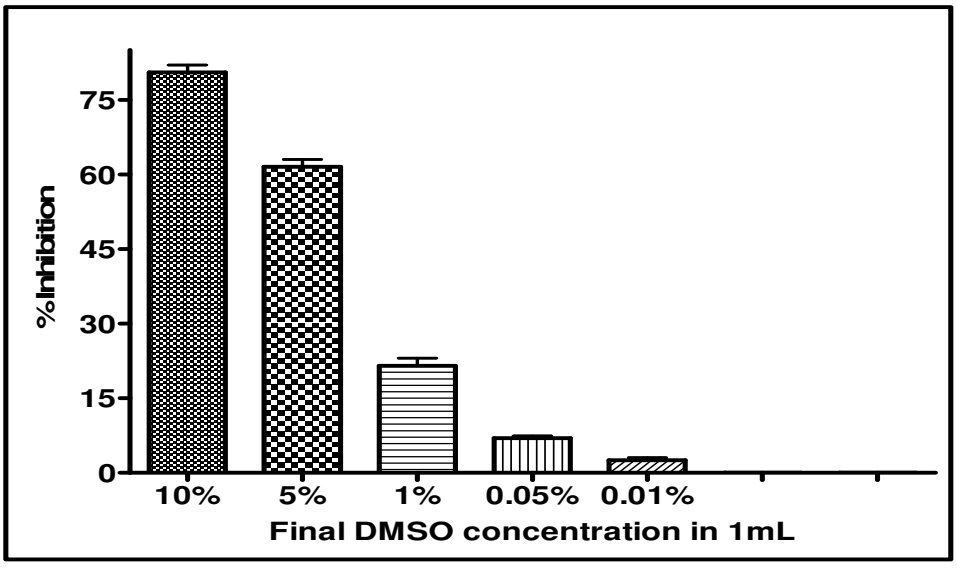


Figure 22 : Effect of DMSO on bovine lens aldose reductase.
Specific activity of control (0 % DMSO): 1.86 ± 0.05 nmole/min/mg protein.

In this study the flavonoid rich vegetable and fruit extracts were selected and tested on aldose reductase activity. Since flavonoids are well known potent inhibitors of aldose reductase [Kinoshita, J. H. et al. (1968), Varma, S. D. et al. (1977)]. Definite structural features of flavonoids which are important determinants for aldose reductase inhibition [Varma, S. D., Kinoshita, J. H. (1976), Matsuda, H., Morikawa, T., Yoshikawa, M. (2002), Chaudhry, P. S. et al. (1982)].

- Glycosylation of ring A, particulary of the 7-OH by a disaccarides, leads to a decrease in the inhibitory activity.

- In the flavonols, an ortho-dihydroxyl (catechol) orientation in ring B is more conducive to the inhibitory property as compared to meta-dihydroxyl orientation.

- 3-O-glycosilation with L-rhamnose increases its inhibitory activity.

- Doubling the number of OH groups in the B ring, also enhances the inhibitory activity.

- In the C ring, the unsaturation on carbons positioned at 2,3 also enhances the inhibitory activity.

- Phenolic hydroxyls are prefered over alcoholic hydroxyls in the flavonoid moiety.
- The 5-hydroxyl moiety does not affect the activity.
- Both 4'-O-glucosylation and 7-O-glucosylation were detrimental to the potency of inhibition.

Literature consisting the content of vegetables and fruits studied showed that they are mainly rich in flavonol glycosides, especially quercetin-3-O-glycosides, tannins and phenolic acids.

Table 8: Aldose reductase inhibitory activity of six different vegetables and fruits. **A:** Methanol extract, **B:** Petroleum ether, **D:** Diethyl ether, **F:**Ethyl acetate, **H:** n-Butanol, **I :** Water fractions

	% Inhibition					
	Methanol (crude) extract [A]	Petroleum ether [B]	D.ethyl ether extract [D]	Ethyl acetate [F]	Butanol [H]	Water fraction [I]
Allium porrum	**26 ± 1.4**	**40 ± 4.9**	**60 ± 0**	**59 ± 0.4**	**25 ± 0.7***	**7 ± 0**
Allium cepa	**42 ± 2.8**	**84.5 ± 4.9**	**54.5 ± 2.8**	**99 ± 0.2**	**74.5 ± 2.1***	**21 ± 1.4**
Malus domestica	**50 ± 4.2**	**31 ± 1.4**	**67 ± 2.8**	**81 ± 5.6**	**50.5 ± 5.6***	**19 ± 2.1**
Malus floribunda	**34.5 ± 0.7**	**39 ± 0**	**60.5 ± 0.7**	**75 ± 0.7**	**26 ± 1.4***	**7 ± 0**
Punica granatum	**67 ± 2.8**	**X**	**81 ± 2.8**	**99.9 ± 0.1**	**83.5 ± 0.7***	**12 ± 2.8**
Spinacia oleraceae	**66.5 ± 6.4**	**29.5 ± 0.7**	**68 ± 4.2**	**84.5 ± 2.1**	**66.5 ± 3.5***	**31 ± 0**

↗ Values are means ± SD, n=9 P> 0,005 and * P>0,01
The values in the table are the means of at least three different sets of experiments. There is no significant difference between the three sets of experiments.

The methanol extracts, were prepared as described under ''Methods'' and tested on crude solution of lens aldose reductase, for their inhibitory effect. Among the methanolic extracts of samples studied, ***Punica granatum*** (pomegranate) and ***spinacia oleracea*** (spinach) were found to be the most potent inhibitors as shown in Figure 23. This result could be explained by their phenolic acids and flavonoids reported in the literature [Poyrazoğlu, E., et al. (2002)]. Methanolic extracts are considered to contain all the phenolics and water soluble compounds in it.

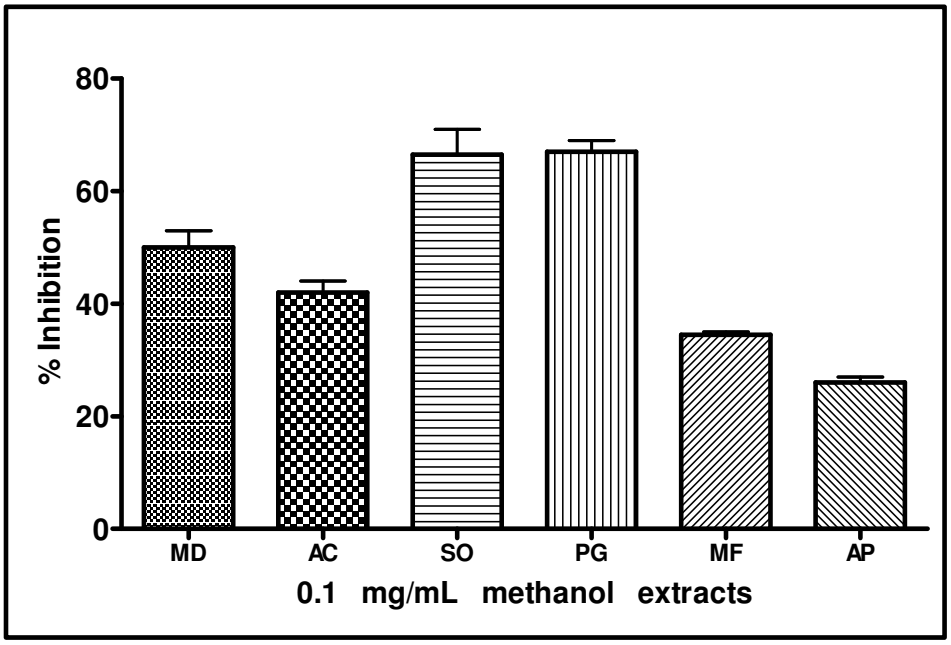


Figure 23 : Inbititory effects of methanol (crude) extracts of plants on bovine lens aldose reductase.
Specific activity of control (without extract): 1.27 ± 0.09 nmole/min/mg protein

Methanolic extracts were fractionated with different solvents of increasing polarity, such as petroleum ether, diethyl ether, ethyl acetate, and n-butanol, to collect the different types of phenolic acids and flavonoids in appropriate polarities of solvents. Described in details under ''Methods''. First, petroleum ether fraction was prepared from the methanolic extracts.

Waxes, chlorophylls, and apolar compounds were expected to get in this fraction. Highest percent inhibition was observed in the ***Allium cepa*** petroleum ether fraction as shown in Figure 24. This is a surprising result to see a high inhibitory effect in the petroleum ether fraction of ***Allium cepa*** hence petroleum ether is the most apolar solvent and evidently it is not expected to dissolve the phenolics which are virtually all polar compounds. Appearently there must be other compounds in that fraction that are good inhibitors for the aldose reductase but they dont have to be in phenolic groups.

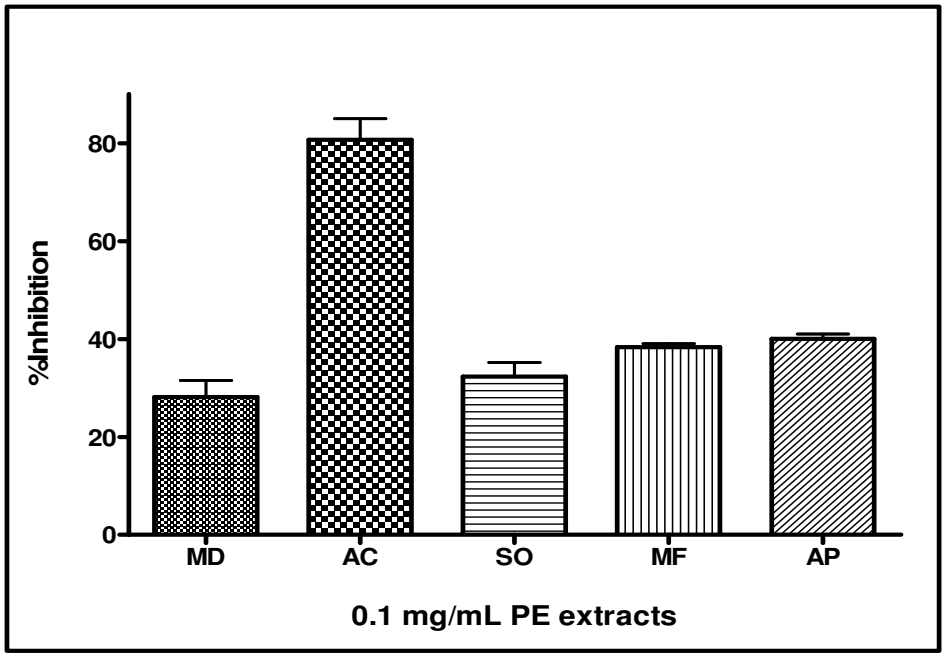


Figure 24: Inbititory effects of petroleum ether fractions of plants on bovine lens aldose reductase.
Specific activity of control (without extract): 1.19 ± 0.1 nmole/min/mg protein

Water phase separated from the petroleum ether fraction was carried out for further fractionation with diethyl ether. Diethlyl ether fractions obtained by solvent fractionation mostly contain flavonoid aglycones [Harborne T. J., Mabry, H. (1975)].

According to the data obtained from all the plants investigated, higher inhibitory activity was observed with pomegranate, apple and spinach extracts (Figure 25). This effect can explained by the phenolic acids and flavonols in pomegranate (Table 6), phenolic acids and flavonols especially the quercetin in apple (Table 5), and methoxylated flavonoids in spinach extracts (Table 4). Only the ferulic acid, among the other phenolic acids of pomegranate, has a weak inhibitory activity, is fairly soluble in diethyl ether.

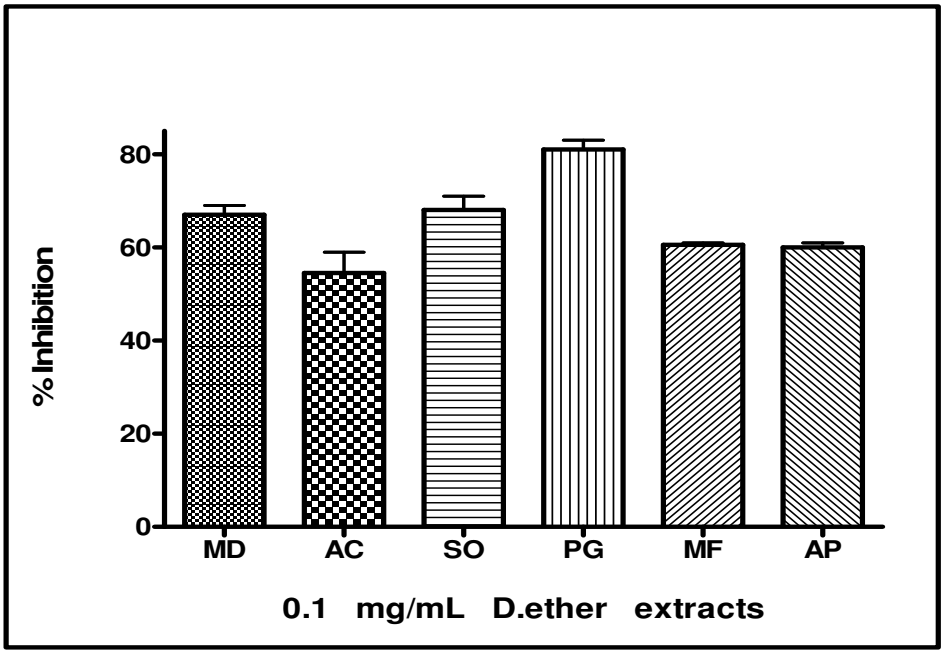


Figure 25: Inbititory effects of diethyl ether extracts of plants on bovine lens aldose reductase.
Specific activity of control (without extract): 1.5 ± 0.02 nmole/min/mg protein.

Water phase separated from the diethyl ether fraction was carried out for further fractionation with ethyl acetate. Ethyl acetate fractions investigated for their inhibitory effects on aldose reductase activity, revealed higher inhibition than all the other solvent fractions especially for onions and pomegranate (Figure 26).

This higher potency could be due to the flavonol monoglycosides, which were reported as the potent inhibitors of aldose reductase, in the previous studies [Shimizu, M., et al. (1984), Varma, S., et al. (1976)].

Most of the phenolics which are found in those selected vegatables and fruits such as chlorogenic acid, gallic acid, quercetin glycosides and caffeic acid can be dissolved in ethyl acetate [Harborne T. J., Mabry, H. (1975)] .

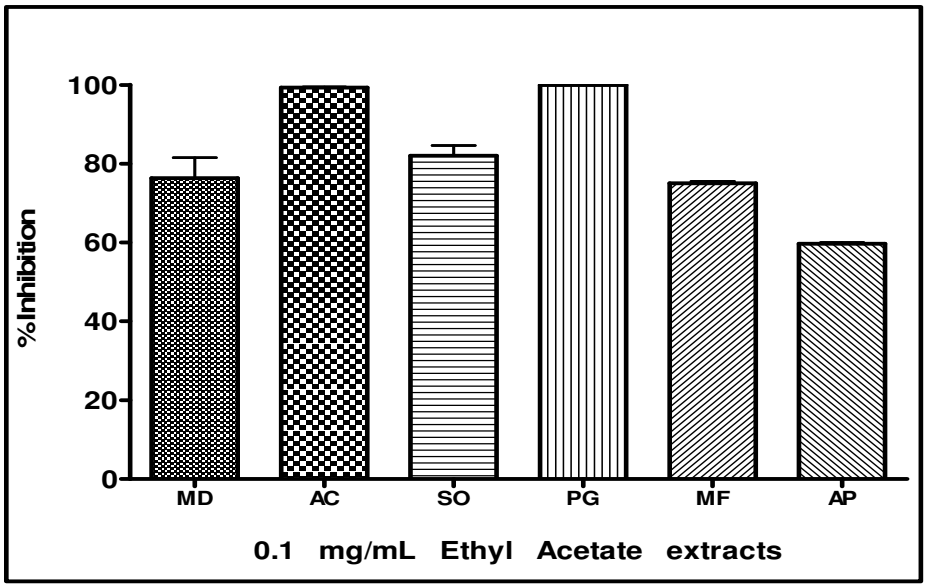


Figure 26 : Inbititory effect of ethyl acetate fractions of plants on bovine lens aldose reductase.
Specific activity of control (without extract): 1.59 ± 0.3 nmole/min/mg protein

In the last step of solvent fractionation, n-butanol and water fractions were obtained as the last two solvents. Phenolic acids, flavone and flavonol diglycosides were thought to be soluble in n-butanol fractions [Harborne T. J., Mabry, H. (1975)].

Spinacia oleraceae (spinach), ***Allium cepa*** (onion) and ***Punica granatum*** (pomegranate) have exhibited higher inhibition than the other plants as shown in Figure 27. Chlorogenic acid, catechin, gallic acid, caffeic acid, phloridzin all together could be responsible for the high potency of butanol fraction of pomegranate. Moreover, caffeic acid in onion (***Allium cepa)*** and flavone diglycosides in spinach must be the responsible inhibitory groups soluble in n-butanol. Chlorogenic acid and catechin were previously reported for their aldose reductase inhibitory activity. Results of these studies showed that chlorogenic acid is as potent as flavones, which are most effective inhibitors among all the flavonoids, however, catechins in the group of flavanols are not [Shimizu, M., et al. (1984), Varma, S., et al. (1976), Matsuda, H., Morikawa, T., Yoshikawa, M. (2002)].

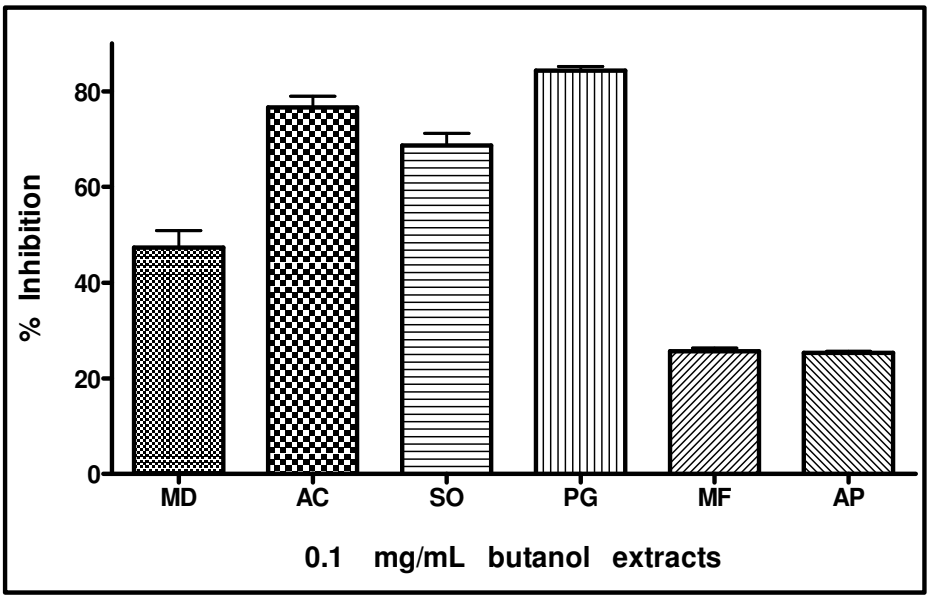


Figure 27: Inbititory effect of butanol fractions of plants on bovine lens aldose reductase.
Specific activity of control (without extract): 1.44 ±0.12 nmole/min/mg protein

It is not suprising that, the last water fraction has the least potentcy for aldose reductase inhibition among all the other organic fractions (Figure 28). Low potency of water fraction may be due to the loss of all the important inhibitor compounds in previous steps of organic solvent fractionations. The remaining compounds should only be the most polar groups, such as the free glycosides, glycosidic acids. Nevertheless, these a groups remains so insignificant to be investigated in the literature as aldose reductase inhibitors.

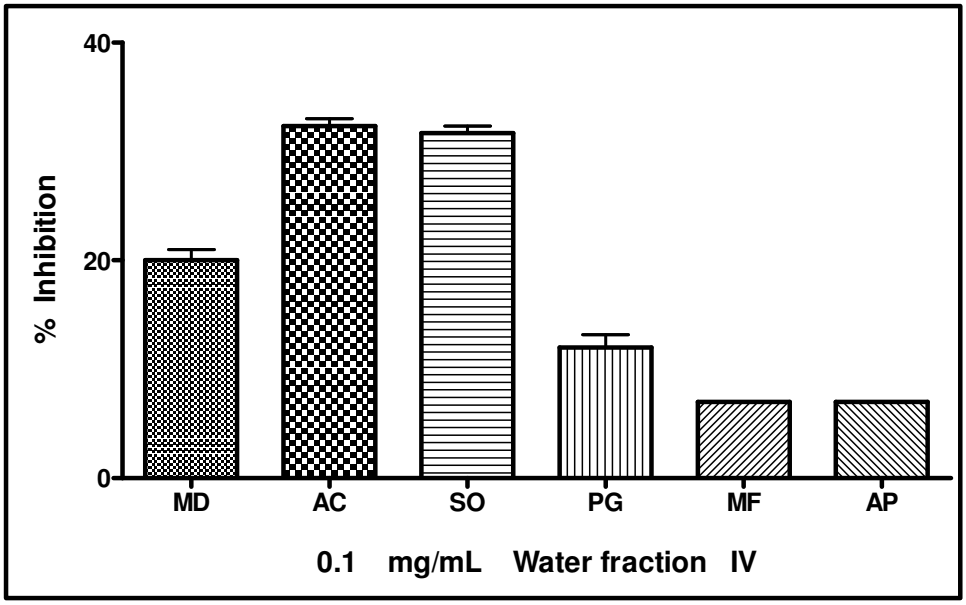


Figure 28: Inbititory effect of last water fraction of plants on bovine lens aldose reductase.
Specific activity of control (without extract): 1.44 ± 0.12 nmole/min/mg protein

3.5.1 Inhibitory Effects of *Allium porrum* (leek) Fractions on Lens Aldose Reductase

A 185.5 g of dry powdered allium porrum was extracted in methanol, dried to 57.8 g crude extract which was dissolved in 50 mL of distilled water and fractionated in solvents with increasing polarities such as, petroleum ether diethyl ether, ethyl acetate, n-butanol respectively. Each fraction was completely dried and redissolved in water to obtain approximately 1mg/mL solution before its additon to the assay mixtures. Aldose reductase inhibition study was carried out by using 0.1 mg/mL of extracts in the final assay solutions, results are given in Figure 29.

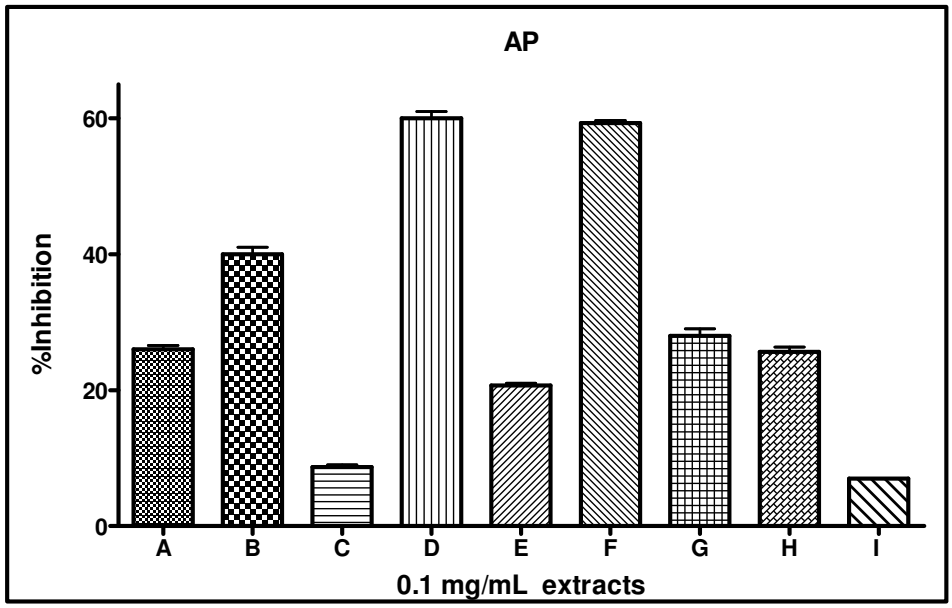


Figure 29: Aldose reductase inhibitory activity of ***Allium porrum*** (leek) **A:** Methanol extract, **B:** Petroleum ether, **D:** Diethyl ether, **F:** Ethyl acetate, **H:** n-Butanol, **C, E, G, I:** Water fractions

Specific activity of control (without extract): 1.50 ± 0.02 nmole/min/mg protein

The highest inhibitory effect on aldose reductase was observed in diethyl ether and ethyl acetate fractions. The aglycones and flavonoid monoglycosides are soluble in diethyl ether and ethyl acetate fractions respectively.

In literature we have come across with only one study by Fattorusso et al. on the flavonoid contents of ***Allium porrum***, in which kaempferol and kaempferol glycosides were reported [Fattorusso, E., Lanzotti, V. (2001)]. Kaempferol, is a well-known aglycone which is found to be soluble in diethyl ether, kaempferol glycosides on the other hand is soluble in ethyl acetate [Merck Index]. Although, in the literature kaempferol was not considered as a good aldose reductase inhibitor, which may be for the absence of ortodihydroxy (catechol) orientation in ring B [Shimizu, M., et al. (1984), Varma, S., et al. (1976)].

3.5.2 Inhibitory Effects of *Allium cepa* (Onion) Fractions on Lens Aldose Reductase

89 g Dry powdered ***Allium cepa*** was extracted in methanol, and dried to 42.8 g crude extract, which was further dissolved in 50 mL of distilled water and fractionated in solvents with increasing polarities such as, petroleum ether, diethyl ether, ethyl acetate, n-butanol respectively. Each fraction before its additon to the assay mixtures was completely dried and redissolved in water to obtain approximately 1mg/mL solution. Aldose reductase inhibition study was carried out by using 0.1 mg/mL of extracts in the final volume of assay solutions, results are given in Figure 30.

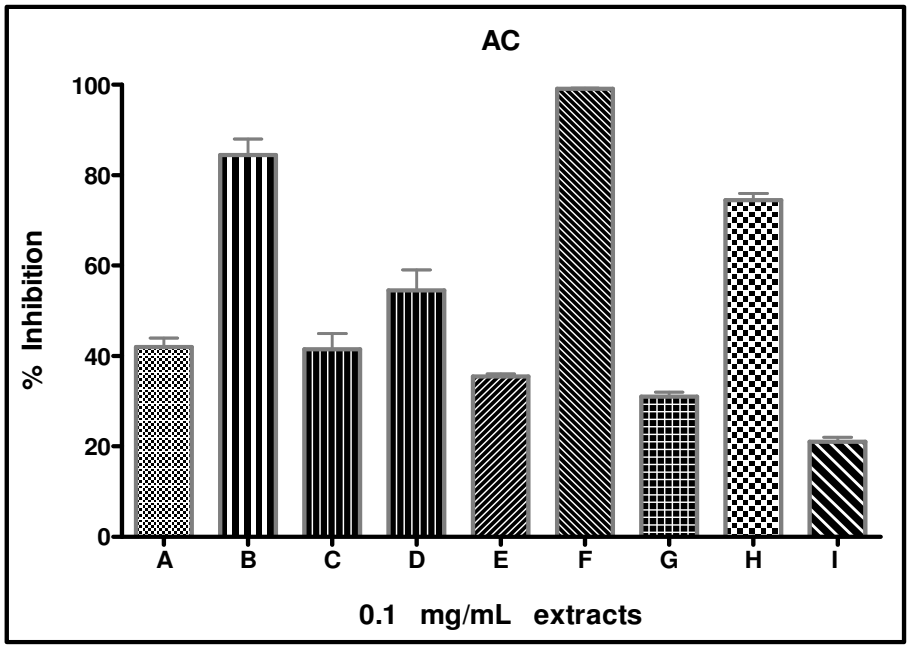


Figure 30: Aldose reductase inhibitory activity of ***Allium cepa*** (onion).
A: Methanol extract, **B:** Petroleum ether, **D:** Diethyl ether, **F:** Ethyl acetate, **H:** n-Butanol, **C, E, G, I:** Water fractions
Specific activity of control (without extract): 1.50 ± 0.02 nmole/min/mg protein

In solvent fractionation technique, important bioactive compounds must be groupped in the similar fractions, according to their polarities. In the fractionation, coumaric acid, kaempferol, quercetin must be dissolved in the diethyl ether fractions. Flavonoids quercetin and kaempferol glycosides and ferulic acid should be in ethyl acetate fractions. Caffeic acid as another phenolic acid, should be soluble in n-butanol fraction.

According to Merck Index ferulic acid is soluble in ethyl acetate and alcohol, p-coumaric acid is soluble in ether and alcohol but caffeic acid is only soluble in alcohol. On the other hand quercetin and kaempferol are soluble in diethyl ether and their glycosides are soluble in ethyl acetate [Harborne, T. J. (1975)].

Allium cepa has very high content of quercetin and kaempferol and glycosides, caffeic acid, p-coumaric acid, ferulic acid are as given in Table 3. From the results given in Figure 30, the inhibitory activity of the diethyl ether fraction of ***Allium cepa*** should be due to the dissolved phenolics as coumaric acid, kaempferol, quercetin. However very high inhibition of ethyl acetate fraction should come from a high content of quercetin and kaempferol glycosides. Quercetin and its glycosides were known to be highly effective as aldose reductase inhibitors. All the quercetin glycosides in onion is glycoslylated at C- 3 but also the type of the sugar is another important factor for determining the inhibitory potency [Varma., S. et al. (1976)].

Phenolic acids such as coumaric acid (diethyl ether soluble) and caffeic acid (alcohol soluble) were reported to have weak aldose reductase inhibitory activity which conforms our results of lower inhibitory effect of diethyl ether and butanol fractions [Koeppen, B. H., and Van der Spuy (1961), Fossen, T., Pederson, A. T., and Andersen, M. (1998)].

Onion also contains high amounts of ascorbic acid (vitamin C) which is known to inhibit aldose reductase both in vivo and in vitro studies [Vinson, J. Marienne, E., Staretz, et al. (1989), Cunningham, J. J., et al. (1994), Vincent, T., Mendiratta, S., May, J. M. (1998)]. According to the literature, ethyl acetate potency for aldose reductase inhibition can not be connected to the high vitamin C content, because of low solubility in ethyl acetate. Ascorbic acid is very fragile at high temperatures, therefore under the extract preperation conditions it can easily be decomposed.

3.5.3 Inhibitory Effects of *Malus Domestica* (Apple) Fractions on Lens Aldose Reductase

159.4 g Dry powdered ***Malus domestica*** (apple) was extracted in methanol, dried to 65.0 g crude extract which was dissolved in 50 mL of distilled water and fractionated in solvents with increasing polarities such as, petroleum ether, diethyl ether, ethyl acetate, n-butanol respectively. Each fraction was completely dried and redissolved in water to obtain approximately 1mg/mL solution before its additon to the assay mixtures. Aldose reductase inhibition study was carried out by using 0.1 mg/mL of extracts in the final assay solution, results are given in Figure 31.

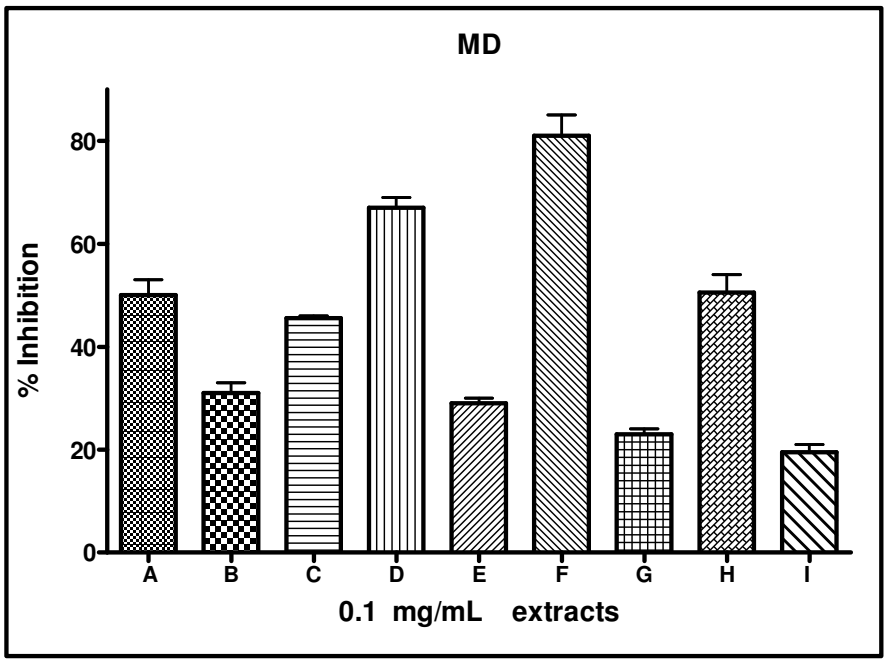


Figure 31: Aldose reductase inhibitory activity of ***Malus domestica*** (apple). **A:** Methanol extract, **B:** Petroleum ether, **D:** Diethyl ether, **F:** Ethyl acetate, **H:** n-Butanol, **C, E, G, I:** Water fractions

Specific activity of control (without extract): 1.50 ± 0.02 nmole/min/mg protein

Important bioactive compounds contained in ***Malus domestica*** (apple) were listed in Table 5. In fractionation the following list of compounds were considered to be soluble in the related solvents given in Figure 31. Aglycones such as quercetin, myricetin, (+) catechin, and (-) epicatechin are soluble in diethyl ether fractions. Quercetin and myricetin are same type of flavonoids (flavonols). Quercetin is one of the most potent inhibitors among all flavonoids, however myricetin having less number of OH groups, is not so potent as quercetin. (+)Catechin, and (-)epicatechin are 3-flavanols which do not show high inhibition on aldose reductase [Varma., S. et al. (1976)]. Therefore, quercetin should be the responsible compound for aldose redutase inhibition in diethyl ether fraction of ***Malus domestica*** among the other phenolics.

Quercetin 3-glycosides such as quercitrin, isoquercitrin, hyperoside, rutin, and phenolic acids like chlorogenic acids, ferrulic acid, and phloridzin are also soluble in ethyl acetate fraction. In the literature quercetin is one of the most abundant compounds found in the contents of apple [Schiweber, A., Keller, P. (2000)]. Quercetin glycosides are found in high amounts in ***Malus domestica*** (apple). Among the glycosides, dissolved in ethyl acetate fraction, quercitrin must be responsible for the high inhibition potency due to the rhamnose moiety at C-3 position. Another compound probably important as an inhibitor could be the chlorogenic acid soluble in ethyl acetate fraction. Whereas, chlorogenic acid, quercetin glycosides like quercitrin and quercetin were reported to have strong inhibitory activity at the previous studies [Varma, S., and Kinoshita, J. H. (1976)].

3.5.4 Inhibitory Effects of *Malus floribunda* (japaneese apple) Fractions on Lens Aldose Reductase

120.0 g Dry powdered ***Malus floribunda*** was extracted in methanol, and dried to 105.9 g crude extract, then it was dissolved in 50 mL of distilled water and fractionated in the organic solvents with increasing polarities such as, petroleum ether, diethyl ether, ethyl acetate, n-butanol respectively. Each fraction was completely dried and redissolved in water to obtain approximately 1mg/mL solution before its additon to the assay mixtures. Aldose reductase inhibition study was carried out by using 0.1 mg/mL of extracts in the final assay solutions, results are given in Figure 32.

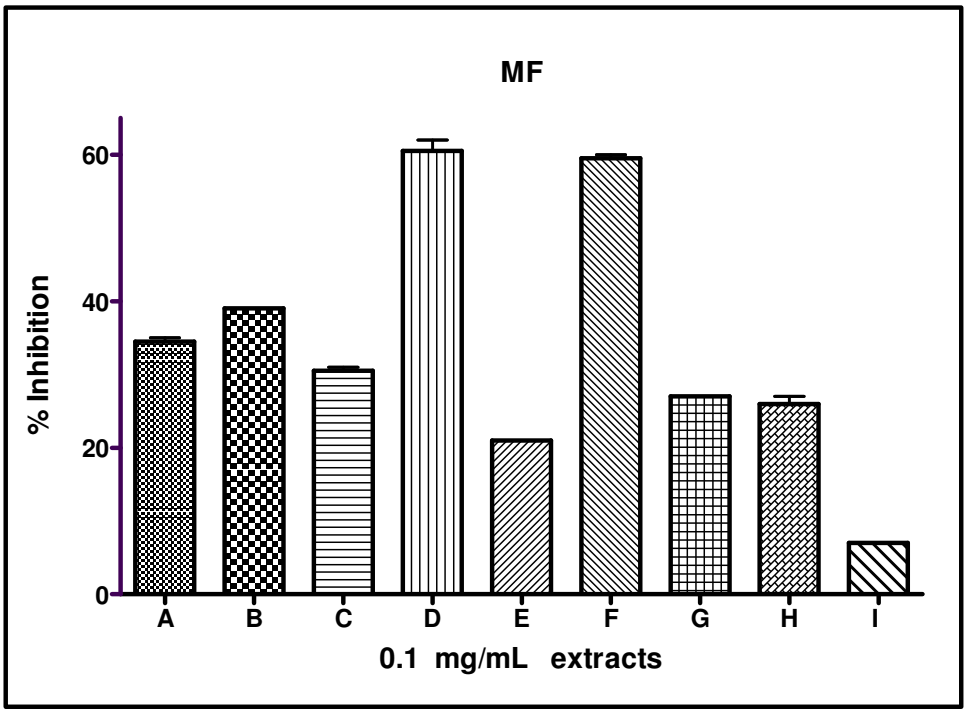


Figure 32: Aldose reductase inhibitory activity of ***Malus floribunda*** - (japaneese apple) **A:** Methanol extract , **B:** Petroleum ether, **D:** Diethyl ether, **F:** Ethyl acetate, **H:** n-Butanol, **C, E, G, I :** Water fractions.
Specific activity of control (without extract): 1.50 ± 0.02 nmole/min/mg protein

As illustrated in Figure 32, diethyl ether and ethyl acetate fractions have shown quite high inhibition on the aldose reductase activity which was quite similar to the inhibition from the diethyl ether fraction of ***Malus domestica***. Even though, both of them belong to the ***Rosaceae*** family, ***Malus domestica*** appears to have much higher inhibition in the ethyl acetate fraction than that of ***Malus floribunda***.

Literature on the phenolic content of ***Malus floribunda*** is unavailable, as a result, our discussion about the phenolic contents of ethyl acetate fraction from ***Malus floribunda*** will be related to the TLC data from Figures 20 and 21. In Figure 20 the intense yellow band must be from the quercetin monoglycosides as shown in the standard bands, and pink bands were due to the other phenolic compounds in ***Malus floribunda*** with 1% vanilin sulfuric acid spray reagent visualization.

In Figure 21 spray reagent used was $FeCl_3$ which visualizes the phenolics in dark olive green spots. From the TLC results shown in Figure 20 responsible groups, for potency of ethyl acetate fraction, might be from flavones, flavonol monoglycosides, and phenolic acids of ***Malus floribunda.***

3.5.5 Inhibitory Effects of *Punica granatum* (pomegranate) Fractions on Lens Aldose Reductase

142 mL of ***Punica granatum*** fruit juice from one pomegranate was concentrated under vacuum to dryness, and 25.3 g crude extract was obtained**.** Then the crude extract was dissolved in 10 mL of distilled water and fractionated in solvents with increasing polarities such as, diethyl ether, ethyl acetate, n-butanol respectively. Each fraction was completely dried and redissolved in water to obtain approximately 1mg/mL solution before its additon to the assay mixtures. Aldose reductase inhibition study was carried out by using 0.1 mg/mL of extracts in the final assay solutions, results are given in Figure 33.

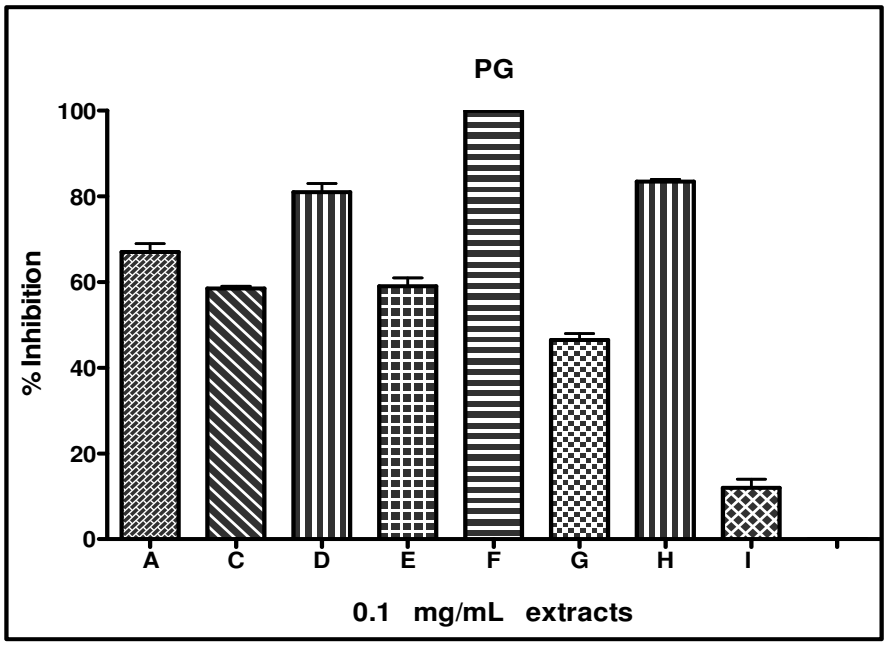


Figure 33: Aldose reductase inhibitory activity of ***Punica granatum*** (Pomegranate) **A:** Fruit juice, **D:** Diethyl ether, **F:** Ethyl acetate, **H:** n-Butanol , **C, E, G, I :** Water fractions
Specific activity of control (without extract): 1.50 ± 0.02 nmole/min/mg protein

The ***Punica granatum*** was the most effective plant even as an untreated fruit juice. Among all of the organic fractions tested, the ethyl acetate was found to be the most effective. Protocatechuic acid, gallic acid, chlorogenic acid, ferulic acid, phloridzin, quercetin, catechin were the phenolic compounds found in pomegranate as shown in Table 6. Gallic acid, quercetin and catechin were soluble in diethyl ether fractions which could be the reason for a high inhibitory effect of the fruit juice. Chlorogenic acid, ferrulic acid, phloridzin on the other hand were soluble in ethyl acetate fractions.

From the Figure 33 at above, one can easily see that ethyl acetate seems to be the most effective inhibitory solvent in fractionation. This result may be related to its content, rich in phenolics, especially chlorogenic acid and gallic acid.

Gallic acid, chlorogenic acid, ferulic acid, ascorbic acid and phloridzin as the phenolic acids of pomegranate are all freely soluble in ethyl acetate fractions (Table 6).

Results of this study may indicate the importance of flavone and flavonol monoglycosides and phenolic acids of pomegranate especially the chlorogenic acid, caffeic acid, catechin, which have been reported as inhibitors of aldose reductase. Also among the phenolic acids mentioned only the chlorogenic acid is known as a strong inhibitor of aldose reductase [Varma, S., and Kinoshita, J. H. (1976)]. In addition to chlorogenic acid, ascorbic acid was also reported for its aldose reductase inhibitory effect. [Vinson, J., Marienne, E., Staretz, et al., (1989), Cunningham, J. J., et al. (1994), Vincent, T., Mendiratta, S., May, J. M. (1998)].
The overall high inhibitory activity of pomegranate may be due to the phenolic acids which were found in high amounts, and other phenolics like flavonoids even though, they are not found in high concentrations as phenolic acids [Poyrazoğlu, E., et al. (2002)].

3.5.6 Inhibitory Effects of *Spinacia oleraceae* (spinach) Fractions on Lens Aldose Reductase

194.7 g Dry powdered *Spinacia oleraceae* was extracted in methanol, dried to 34.5 g crude extract which was dissolved in 50 mL of distilled water and fractionated in solvents with increasing polarities such as, petroleum ether diethyl ether, ethyl acetate, n-butanol respectively. Each fraction was completely dried and redissolved in water to obtain approximately 1mg/mL solution before its additon to the assay mixtures. Aldose reductase inhibition study was carried out by using 0.1 mg/mL of extracts in the final assay solutions results are given in Figure 34.

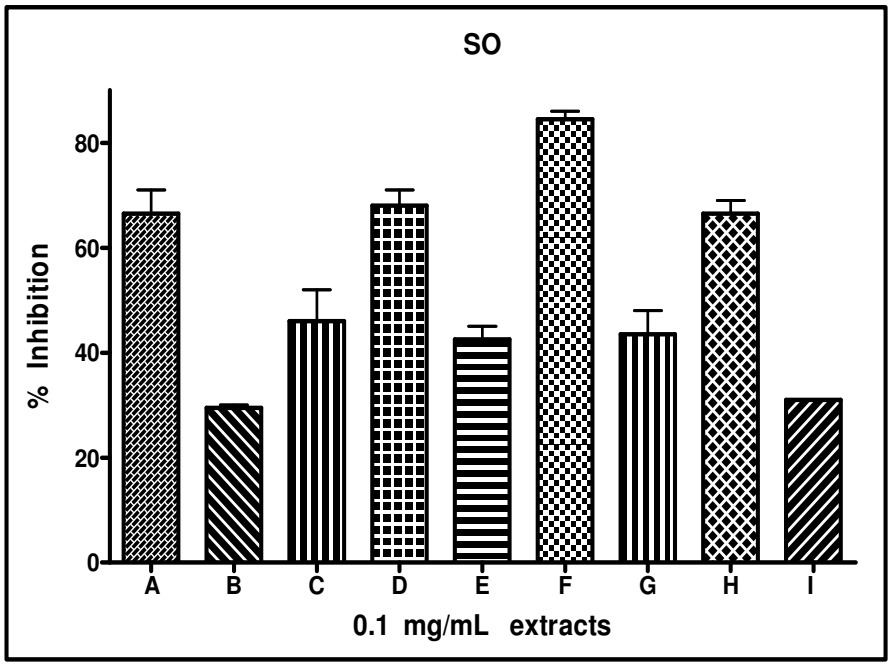


Figure 34: Aldose reductase inhibitory activity of ***Spinacia oleraceae*** (spinach) **A:** Methanol extract, **B:** Petroleum ether, **D:** Diethyl ether, **F:** Ethyl acetate, **H:** n-Butanol, **C, E, G, I :** Water fractions
Specific activity of control (without extract): 1.50 ± 0.02 nmole/min/mg protein

Petroleum ether fractionation was most useful for ***Spinacia oleraceae*** because it was necessary to get rid of the high content of wax and green color of chlorophylls.

Crude methanol extract, diethyl ether, and n-butanol fractions seems to have similar effects on aldose reductase activity. Nevertheless, ethyl acetate fraction was much more effective as an inhibitor. The spinach extract contain high amounts of flavonol monoglycosides in ethyl acetate fraction that provides main requirements of the flavonoids for their inhibitory activity. The compounds listed in Table 4, have also the methoxyl groups and acidic moieties attached to the sugar in the structure which made them less polar, results in a decreasing solubility in polar solvents.

The most active fraction of ***Spinacia oleraceae*** is ethyl acetate but its methanolic extract was also active, this may be explained by synergy. All of the polar groups were soluble in methanol, but only the specific groups like flavonoid monoglycosides and some phenolic acids can be soluble in ethyl acetate. The p-coumaric acid and ferulic acid were the only phenolic acids reported, but nothing found about their aldose reductase inhibitory properties [Ferreres, F., et al., (1996), Nuutila, A. M., et al., (2001)].

3.5.7 Comparison of Aldose Reductase Inhibitiory Effects of Selected Fruits and Vegetables

Ethyl acetate fractions of all the vegatables and fruits were found to be most effective comparing with the other fractions. Reason for this may be the high content of quercetin-3-O-glycosides of those plants which dissolve in ethyl acetate freely. Consequently, IC_{50} calculations were only compared for the ethyl acetate fractions of each plant material. The results were given on the Figure 35. Different concentrations of each individual ethyl acetate fractions were obtained by serial dilutions to reach a useful % inhibition curves. Fifty percent of the total inhibition was calculated as the IC_{50} values (Table 9). From the data available on IC_{50} values we can organize the decreasing potency for inhibition as following: ***Punica granatum > Allium cepa > Malus japonica > Spinacia oleraceae > Malus domestica > Allium porrum***.

From the results given, pomegranate and onion are chosen the most potent plants with their low IC_{50} values. ***Punica granatum*** (pomegranate) showed the highest inhibitory effect on enzyme with IC_{50} value of 5.4 µg/ml among the other plants tested.

Table 9 : IC_{50} values of ethyl acetate fractions of selected fruits and vegetables

Latin Name	Common Name	IC_{50} Value (µg/ml)
Allium cepa	Onion	6.45 ± 0.38
Allium porrum	Leek	25.5 ±1.8
Malus domestica	Apple	20.5 ± 2.8
Malus floribunda	Japaneese Apple	12.3 ± 1.8
Spinacia oleraceae	Spinach	18.5 ± 2.8
Punica granatum	Pomegranate	5.4 ± 0.07

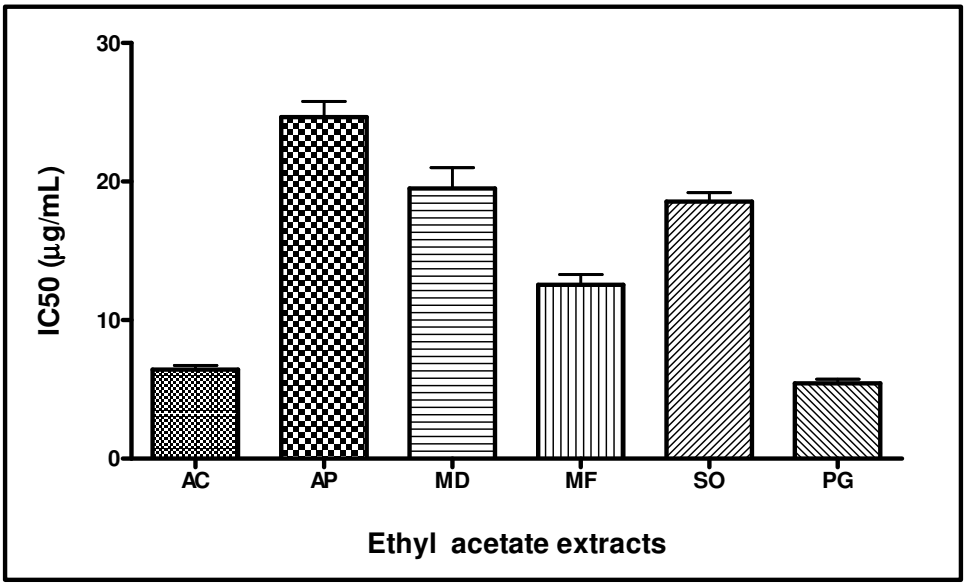


Figure 35: IC_{50} values of ethyl acetate fractions of selected fruits and vegetables

CHAPTER 4

CONCLUSION

The observation of van Heynengen show that in rat lens large quantities of polyol production was induced sugar cataractogenesis. Accumulation of high concentration of polyol in the lens leads to an increase in the intracellular ionic strength that is resulting in excessive hydration, eventuallly a loss of membrane integrity and leakage of free aminoacids, glutathione and myoinositol [(Heynengen, F. (1959)]. The hyperosmotic theory of sugar cataract identifies aldose reductase as the primary factor that responsible for this pathological condition. Recent human genetic and biochemical data suggested a strong link between raised aldose reductase activity and strongly altered risk of diabetic complications such as cataract, nephropathy, retinopathy and neuropathy [Collier and Small (1991)]. These results together with recent clinical, experimental and pharmacological data, provide powerful new support for the rationale on research and development of aldose reductase inhibitors. There are reports of aldose reductase inhibiting activity of few natural products such as ***Salacia oblonga*** [Matsuda, K., et. al. (1999)], and ***Salvia multiorrhizae***, ***Glycyrrhiza uralensis*** [Zhang and Zhou, (1989)]. These plants are rich in flavonoids, which are reported to reduce the aldose reductase activity. Flavonoids take part in a wide range of biochemical reactions such as, inhibition and inducement of a large variety of mammalian enzyme systems. In the plant kingdom, flavonoids are ubiquitiously distrubuted and they are common components of our diets, generally found as O-glycosides bound at C-3 positions. Since flavonoids which are present commonly in food are relatively non-toxic and used as folk medicines in many preparations.

In this study, six diffrent daily consumed vegatables and fruits that are rich in flavonoids were investigated for their aldose reductase inhibitory activity. Fruits and vegatables chosen for their unlimited consumption in diabetic patients' diets. Crude extracts were prepared with methanol to obtain all polyphenols. Methanol was chosen as polar solvent to get all the compounds dissolving in water. The methanolic extracts were fractionated with different solvents with varying polarities in order to get different types of phenolic acids and flavonoid groups. All fractions (petroleum ether, diethyl ether, ethyl acetate and n-butanol) were tested for their aldose reductase inhibition potency. The results of the study suggest that the flavonoid glycosides which we guess to be found mostly in the ethyl acetate fraction showed the most potent inhibitory effect supported by the literature [Okuda, J., et al. (1982) and Shimizu, M., et al. (1984)].

The IC_{50} values of the most potent ethyl acetate fractions of these vegetables and fruits were calculated. Findings of this study suggested that onion and pomegranate should be investigated in vivo studies for prevention of different diabetic complications through the pharmacologic action of aldose reduction inhibition.

In conclusion, vegetables and fruits are very important sources for structures of high chemical diversity, many of them possesing interesting biological activities and medicinal properties. In the context of world-wide spread of diabetic complications, an intensive search for the new natural lead compounds for the development of novel pharmocological therapeutics is extremely important.

REFERENCES

1. Arauz, S. (1992) **Journal of Diabetic Complications**, Vol 6, 131-137

2. Akagi, Y., Yagıma, Y., ,Kador, P. F., Kuwabara, T., and Kinoshita J.H (1984) ***Diabetes***, Vol 33, 562-566

3. Aritomi, M., and Kawasaki, T. (1984) ***Phytochemistry***, Vol 23, 2043

4. Bagnasco, M. S., Uchida, S., Balaban, R. S., Kador, P. F. (1987) ***Proc. Natl. Acad. Sci.USA*** , Vol 84 , 1718-1720

5. Bergman, M., Varshavsky, L., Gottlieb, H., Grossman, S., (2001), ***Phytochemistry*** , Vol 58, 143-152

6. Bergman, M., Perelman, A., Dubinsky , Z., Grossman, S., (2003) ***Phytochemistry***, Vol 62, 753-762

7. Bhatnagar, A., Liu, S., Das, B., Srivastava, K. (1989) ***Molecular Pharmacology*** , Vol 36, 825-830

8. Bilia, A. R., Catalano, S., Pistelli, L., Morelli, I. (1993) ***Phytochemistry*** Vol 33, 1449-1452

9. Bhatmagar, A., Srivastava, S. K. (1992). ***Biochem. Med. Metab. Biol***. Vol 48, 91-112

10. Bohren, K. M ., Bullock, B., Wermth, B., Gabbay, K. H. (1989) ***Journal of Biological Chemistry,*** Vol 264, 547-9551

11. Boulton, H. (1990) ***Diabetologia,*** Vol 33, 431-437

12. Boghosion, R. A., McGuiness, E. T., (1979) ***Biochim. Biophys. Acta.,*** Vol 567, 278-286

13. Brandwein, B. J., (1965), ***Journal of Food Science*** Vol 30, 360

14. Bandyukova, V. A., and Shinkarenko, G. L., (1967) ***Farmatsevtichnii Zhurnal-Kiiv,*** Vol 22, 975

15. Chaudry, P. S., Cabrera, J., Juliani, H. R., Varma, S., (1983) ***Biochemical Pharmacology,*** Vol 32, 1995-1998

16. Crabbe, M. J. C., Hoe, S. T. (1991) ***Enzyme,*** Vol 45, 188-193

17. Crabbe, M. J. C., Goode, D. (1998) ***Prog. Retin. Eye Res.,*** Vol 17, 313

18. Calış, I., Kuruüzüm, A., Demirezer, Ö., Sticher, O., Ganci, W., Rüedi, P. (1999) ***Journal of Natural Products,*** Vol 62, 1101-1105

19. Carotenuto, A., Fatturusso, E., Lanzotti, V., Magno, S., Cicala, C. (1997) ***Phytochemistry***, Vol 44, 949-957

20. Cromlish, J. A., Flynn, T. G. (1983) ***Journal of Biological Chemistry,*** Vol 258, 3583-3586

21. Cromlish, J. A., Flynn, T. G. (1983) ***Journal of Biological Chemistry,*** Vol 258, 416-3424

22. Conrad, S. D., Doughty, C. C (1982) ***Biochim. Biophys. Acta.,*** Vol 708, 48-357

23. Crabbe, J. M. C. (1991) ***Int . Opthalmol.,*** Vol 15, 25-36

24. Crabbe, M. J .C., Petchey, M., Burgess, S. E. P., Cheng, H. (1985) ***Experimental Eye Research,*** Vol 40, 95-99

25. Clarke, B. F.,Young, R. J., Martyn, C. N., Ewing, D. J., (1984) ***Diabetological Medicine,*** Vol 1, 88-90

26. Constantino, L., Rastelli, P.,Vienello, G. and Barlocco, D. (1997) ***Exp. Opin. Ther. Pat.,*** Vol 7, 843

27. Costantino, L., Rastelli G., Vianello, P., Cignarella, G., and.Barlocco D. (1999) ***Medicinal Research Reviews***, Vol 19, 3-23

28. Dias, C. P. A., Barberan, F. A. T., Ferreaira, M. F., Ferreres, F., (1998) ***Phytochemistry*** ,Vol 48, 165-1168

29. Dvornik, D., Duquesne, N. S., Krami, M. (1973) ***Science***, Vol 182, 1146-1148

30. Das, B., Srivastava, S. K., (1985) ***Diabetes*** Vol 34, 1145-1149

31. Del Corso, A., Barsacchi, D., Camici, M., Garland, D., Mura, U. (1989) ***Arch. Biochem. Biophys,*** Vol 270, 604-610

32. Friedman, E. A. (1999) ***Diabetes Care*** , Vol 22 (suppl 2), B65-B71

33. Fattorusso, E., Lanzotti, V., Scafati, O., Cicala, C. (2001) ***Phytochemistry,*** Vol 57, 565-569

34. Ferreres, F., Castaner, M., Barberan, F. A. T. (1997) ***Phytochemistry*** , Vol 45 , 1701-1705

35. Fico, G., Bilia, A. R., Morelli, I., Tome, F. (1999) ***Biochemical Systematics and Ecology*** , Vol 28, 673-678

36. Fossen, T., Pederson, A. T., Andersen, Q. M., (1998) ***Phytochemistry*** , Vol 47, 281-285

37. Flynn, T. G. (1972) ***Biochem. Pharmacol.***, Vol 31, 2705-2712

38. Futh, A., Hardings, J. J. (1989) ***New Scientist***, 44-47

39. Greene, H. (1992) ***Journal of Diabetic Complications,*** Vol 6, 35-38

40. Heyningen, V. R. (1959) ***Nature,*** Vol 184, 194-195

41. Haraguchi, H., Ohmi, I., Sakai, S., Fukuda, A. (1996) ***Journal of Natural Products,*** Vol 59, 443-445

42. Hollman, P. C. H., Katan, M. B. ,Kromhout, D. (1993) ***The Lancet***, Vol 342, 1007-1011

43. Hermann, K., (1956) ***Die Naturwissenschaften*** , Vol 7, 158

44. Halder, N., Joshi, S., Gupta, S. K. (2003) ***Journal of Etnopharmacology***, Vol 86, 113-116

45. Hotta (1990) ***Diabetes***, Vol 39 (suppl 1), 61A

46. Hayman, S., Kinoshita, J. H. (1965) ***Journal of Biological Chemistry,*** Vol 240, 877-882

47. Harborne T. J., Mabry, H. ***The Flavonoids***, Chapman & Hall ltd. London (1975)

48. Ishii, H., Jirousek, M. R., Koya, D., Takagi, C., King, H. P.X, Aubert, R.E., and Herman, W. H. (1998), ***Diabetes Care,*** Vol 21, 1414-1431

49. Iwu, M. M., Igboko, O, A., Okunji, O,C., Tempesta, S. (1990) **J. *Pharm. Pharmacol.*** Vol 42, 290-292

50. Kador, P. F., Sharpless, N. E., (1983) ***Molecular Pharmacology***, Vol 24, 521-531

51. Karakaya, S., El, S. (1999) ***Food Chemistry***, Vol 66, 289-292

52. Kador, P. F., Kinoshita, J. H., Brittain, D. R., Mirrlees, P. J., Sennitt, C. M., Stribling, D. (1986) ***Biochemical Journal***, Vol 240, 233-237

53. Kador, P. F., Kinoshita, J. H., Kuwabara, T., Yajima, Y., Akagi, Y. (1984), ***Diabetes,*** Vol 33, 562-566

54. Kador, P. F. (1990) ***Experimental Eye Research,*** Vol 50, 615-620

55. Kubiseski, T. J., Hayman, D. J., Morjana, N. A., Flynn, T.G . (1992) ***Journal of Biological Chemistry,*** Vol 267, 6510-6517

56. Koeppen, B., and Van der Spuy, J. E., (1961) ***South African Journal of Agriculturural Science***, Vol 4 , 557

57. Koya, D., King, G. L. (1998) ***Diabetes***, Vol 47, 859-866

58. Kador, P. F., ***Medicinal Research Reviews,*** (1988)

59. Kador, P. F., Robinson, G. W. Jr., Kinoshita, J. H. (1985***) Annuals of Reviews of Pharmacology***, Vol 25, 691-714

60. Kinoshita, J. H., Drovnik, D., Kraml, M., Gabbay, K. H. (1968) ***Biochem. Biophys. Acta*** . Vol 158, 472-475

61. Kinoshita, J. H., Fukushi, S., Kador, P. F., Merola, L. O. (1979) ***Metabolism***., Vol 28, 462-467

62. Kawamura and Hamanaka, N., (1997) ***J. Synth. Org. Chem., Japan,*** Vol 37 , 651-657

63. Liu, S. Q.,Bhatnagar, A., and Srivastava, S. K.(1992) ***Biochem. Biophys. Acta*** , Vol 1120, 329-336

64. Liu,S. Q., Bhatnagar, A., and Srivastava, S. K. (1993) ***The Journal of Biological Chemistry***, Vol 268, 25494-25499

65. Lee, H. S., (2002) ***J. Pharm. Pharmaceut Sci***., Vol 5 (3), 226-230

66. Lightman, S. (1993) ***Eye,*** Vol 7, 238-241

67. Lu, Y., Foo Y., (1996) ***Food Chemistry,*** Vol 59, 187-194

68. Masson, E. A., Boulton, A. J. M., (1990), ***Drugs***, Vol 39, 190-202

69. Mylari, L. B., Beyer, T. A., Siegel, T. W. (1991), ***Journal of Medicinal Chemistry***, Vol 34, 1011-1018

70. Matsuda, H., Cai, H., Kubo, M., Tosa, H., Iınuma, M., (1995), ***Biol. Pharm. Bull***., Vol 18, 463-466

71. Matsuda, H., Morikawa, T., Toguchida, I., Yoshkawa, M. (2002), ***Chemical Pharmaceutica l Bulletin,*** Vol.50, 788-795

72. Miyamoto, S. (2002) ***Chem-Bio Informatics Journal,*** Vol 2, 74-85

73. Narayanan, S. (1993) ***Annuals of Clinical Labaratory Science*** Vol 23, 148-153

74. Nakai, N., Fujii, Y., Kobashi, K., Nomura, K.(1985) ***Archieves of Biochemistry and Biophysics***, Vol 239, 491-496

75. Naeser, P., Brolin, S. E., Erikson, U. J.(1988) ***Metabolism***, Vol 37, 1143-1145

76. Naeser, P., Brolin, S. E (1991) ***Acta Ophthalmologica,*** Vol 69, 591-595

77. Ohta, M.,Tanimoto, T., Tanaka, A., Hayakawa, T. (1993) ***Int . J. Biochem,*** Vol 25, 1165-1174

78. Okuda, J., Miwa, I., Inagaki, K., Horie, T., Nakayama, M. (1982), ***Biochemical Pharmacology,*** Vol 31, 3807-3822

79. Okuda, J., Inagak, K., Nakayama, M., Horie, T. (1984) ***Chemical and Pharmaceutical Bulletin,*** Vol 32, 767-772

80. Preifer, M. A., Schumer, M. P., and Gelber, D. A. (1997) ***Diabetes,*** Vol 46(suppl. 2), S82-89

81. Perkin, A. G., and Hummel, J. J. (1896) ***Chemical News,*** Vol 74, 96

82. Porte, D., and Schwartz, M. W. (1996), ***Science***, Vol 272, 699-700

83. Petrash, J. M., Delucas, L.J., Bowling, E., Egen, N. (1991) ***Electrophoressis,*** Vol 12, 84-90

84. Peterson, M. J., Sarges, R., Aldwieger, C. E., and McDonald (1979) ***Metabolism***, Vol 28, 456-461

85. Park, Y., and Lee, C. (1994) ***Polyphenols*** *,* Vol 94, 265

86. Reymann, J. M., Rondeau, J. M., Barth, P., Jaquinod, M., Dorssealer, V. A., Bielman, J. F. (1992) ***Biochim. Biophys. Acta***, Vol 1122, 1-5

87. Robinson, W. G. (1994) ***Diabetes,*** Vol 43, 337-338

88. Raskin, P., Rosenstock, J. (1987) ***The American Journal of Medicine***, Vol 83, 298-306

89. Rastelli, G., Antolini, S., Constantino, L. (2000) ***Boorganic Medicinal Chemistry***, Vol 8, 1151

90. Rondeau, J. M., Podjarney, F., Reyman, J. M., Barth, P., Biellman, J. F., Moras, D. (1992) ***Nature***, Vol 355, 469-472

91. Segelman, A. B., Varma, S. D., Wagner, H,. Seligmann, O. (1977), ***Journal of Pharmaceutıcal Science,*** Vol 66, 1358-1359

92. Shen, C., Sigman, D. S.(1991) ***Archieves of Biochemistry and Biophysics,*** Vol 286, 596-603

93. Stribling, D., Mirrlees, D.J., Harrison, E., Earl, D. C. N.(1985) ***Metabolism,*** Vol 34, 336-343

94. Sima, A. A. F., Bril, V., Nathaniel, T. A., McEwen, J.,(1988), ***N.Engl.J.Med***, Vol 319, 548-555

95. Sima, A. A. F., Sugimoto, K. (1999) ***Diabetologia***, Vol 50, 21-33

96. Sima, A. A. F., Greene, D. A.,Brown, M. B., Hohman, T. C. (1993) ***Journal of Diabetic Complications***, Vol 7, 157-169

97. Schimuzu, M., Horie, S., Arısawa, M., Hayashi, T., Suzuki, S., Berganza, L., Ferro, E., Basualdo, I. (1987) ***Chemical and Pharmaceutical Bulletin,*** Vol 35, 1234-1237

98. Schimuzu, M., Ito, T., Terashıma, S., Hayashi,T., Arisawa, M., Morita, N., Kurokawa, S., Hashmoto, Y. (1984) ***Phytochemistry***, Vol 23, 1885-1888

99. Sluis, A. A., Dekker, M., Jongen, W. M. F. (1997) ***Cancer Letters***, Vol 114, 107-108

100. Schieber, A., Keller, P., Carle, R. (2001) ***Journal of Chromatography***, Vol 910, 265-273

101. Stribling, D. (1990) ***Experimental Eye Research,*** Vol 50, 621-624

102. Srivastava, S. K., Ansari, H. N., Brown, J., Petrash, M.(1982) ***Biochem. Biophys. Acta. ,*** Vol 717, 210-214

103. Sheaff, C. M., Daughty, C. C. [1976] ***Journal of.Biological Chemisry,*** Vol 251, 2696-2702

104. Srivastava, S. K., Ansari, H. N., Hair, A. G., Das, B (1984) ***Biochem. Biophys. Acta., ,*** Vol 800, 220-227

105. Miyamoto, S. (2001) ***Expert Opin. Ther. Patents***, Vol 12, 621-631

106. Schade, S. Z., Early, S. L., Williams, T. R., Kezdy, F. J., Heinrikson, R. L., Grimshaw, C. E., Doughty, C. C.(1990) ***Journal of Biological Chemisry,*** Vol 265, 3628-3635

107. Sorbinil Retinopathy Trial Research Group (1990) ***Archieves of Ophthalmology,*** Vol 108, 1234-1244

108. Sundkvist, T. (1992) ***Journal of Diabetic Complications,*** Vol 6, 123-130

109. Spielberg, S. P,. Shear, N. H. Cannon, M., Hutson, N. J. and Gunderson, K. (1991) ***Ann. Intern. Med.,*** Vol 114, 720-724

110. Tanimoto, T., Fukuda, H., Kawamura, J. (1983) ***Chemical and Pharmaceutical Bulletin***, Vol 31, 2395-2403

111. Thorpe, S. R., and. Baynes, J. W.(1999) ***Diabetes,*** Vol 48, 1-9

112. Yamagishi, M., Yamada, Y., Ozaki, K., Asao, M., Shimizu, R., Suzuki M., Matsumoto, M., Matsuoka, Y., and Matsumoto, K. (1992) ***Journal of Medicinal Chemistry,*** Vol 35, 2085-2094

113. Yabe-Nishimura, C. (1998) ***Pharmacological Review***, Vol 50, 21-33

114. Van Heyningen, R.(1959) ***Nature,*** Vol 184, 194-195

115. Varma, S. D., Mikuni, I., Kinoshita, J. H. (1977) ***Science***, Vol 195, 205-206

116. Varma, S. D., Mikuni, I., Kinoshita, J. H. (1975) ***Science,*** Vol 188, 1215-1216

117. Varma, S. D., Kinoshita, J. H. (1976) ***Biochemical Pharmacology,*** Vol 25, 2505-2513

118. Yabe-Nishimura, C. (1998) ***Pharmacological Review,*** Vol 50, 21-33

119. Yue, D. K., Brooks, B. (1993) ***The Medical Journal of Australia,*** Vol 159, 76-78

120. Wilson, D. K., Bohren, K. M., Gabbay, K. H., Quiocho, F. A (1992), ***Science***, Vol 257, 81-84

121. Wilson, K. D., Tarle, I., Petrash, J .M. (1993) ***Biochemistry***, Vol 90, 9847-9851

122. Wood ***Academic Press,*** 1985

APPENDIX

Table 1: Aldose reductase inhibitory activity of ***Allium cepa*** (onion) **A:** Methanol extract, **B:** Petroleum ether, **D:** Diethyl ether, **F:** Ethyl acetate, **H:** n-Butanol, **C, E, G, I:** Water fractions

Specific activity of control (without extract): 1.50 ± 0.02 nmole/min/mg protein

Fractions	A	B	C	D	E	F	G	H	I
%Inhibition	**42**	**84.5**	**41**	**55**	**35**	**99**	**31**	**75**	**21**
Standard Deviation	**2.8**	**4.9**	**4.9**	**2.8**	**1.4**	**0.21**	**3**	**2.1**	**1.4**

Table 2: Aldose reductase inhibitory activity of ***Allium porrum*** (leek) **A:** Methanol extract, **B:** Petroleum ether, **D:** Diethyl ether, **F:** Ethyl acetate, **H:** n-Butanol, **C, E, G, I :** Water fractions

Specific activity of control (without extract): 1.50 ± 0.02 nmole/min/mg protein

Fractions	A	B	C	D	E	F	G	H	I
%Inhibition	**26**	**40**	**8.7**	**60**	**21**	**59**	**28**	**26**	**7**
Standard Deviation	**1.4**	**1.7**	**0.7**	**0**	**1.1**	**0.4**	**1.4**	**0.7**	**0**

Table 3: Aldose reductase inhibitory activity of ***Malus domestica*** (apple) **A:** Methanol extract, **B:** Petroleum ether, **D:** Diethyl ether, **F:** Ethyl acetate, **H:** n-Butanol, **C, E, G, I :** Water fractions

Specific activity of control (without extract): 1.50 ± 0.02 nmole/min/mg protein

Fractions	A	B	C	D	E	F	G	H	I
% Inhibition	**50**	**31**	**45**	**67**	**29**	**81**	**23**	**50**	**19.5**
Standard Deviation	**4.2**	**1.4**	**0.7**	**2.8**	**1.4**	**5.6**	**1.4**	**5.6**	**2.1**

Table 4: Aldose reductase inhibitory activity of ***Malus floribunda*** (J.apple) **A:** Methanol extract, **B:** Petroleum ether, **D:** Diethyl ether, **F:** Ethyl acetate, **H:** n-Butanol, **C, E, G, I :** Water fractions

Specific activity of control (without extract): 1.50 ± 0.02 nmole/min/mg protein

Fractions	A	B	C	D	E	F	G	H	I
%Inhibition	**35**	**39**	**31**	**60.5**	**21**	**75**	**27**	**26**	**7**
Standard Deviation	**0.7**	**0**	**0.7**	**0.7**	**1.4**	**0.7**	**0**	**1**	**0**

Table 5: Aldose reductase inhibitory activity of ***Punica granatum*** (pomegranate) **A:** Methanol extract, **B:** Petroleum ether, **D:** Diethyl ether, **F:** Ethyl acetate, **H:** n-Butanol, **C, E, G, I :** Water fractions

Specific activity of control (without extract): 1.50 ± 0.02 nmole/min/mg protein

Fractions	A	C	D	E	F	G	H	I
% Inhibition	**67**	**58**	**81**	**59**	**99.9**	**46**	**83.5**	**12**
Standard Deviation	**2.8**	**2.8**	**2.8**	**2.8**	**0.07**	**2.1**	**0.7**	**2.8**

Table 6: Aldose reductase inhibitory activity of ***Spinacia oleraceae*** (spinach) **A:** Methanol extract, **B:** Petroleum ether, **D:** Diethyl ether, **F:** Ethyl acetate, **H:** n-Butanol, **C, E, G, I :** Water fractions

Specific activity of control (without extract): 1.50 ± 0.02 nmole/min/mg protein

Fractions	A	B	C	D	E	F	G	H	I
% Inhibition	**66**	**30**	**46**	**68**	**42**	**84**	**43**	**66**	**31**
Standard Deviation	**6.3**	**0.7**	**8.4**	**4.2**	**3.5**	**2.1**	**4.9**	**3.5**	**0**

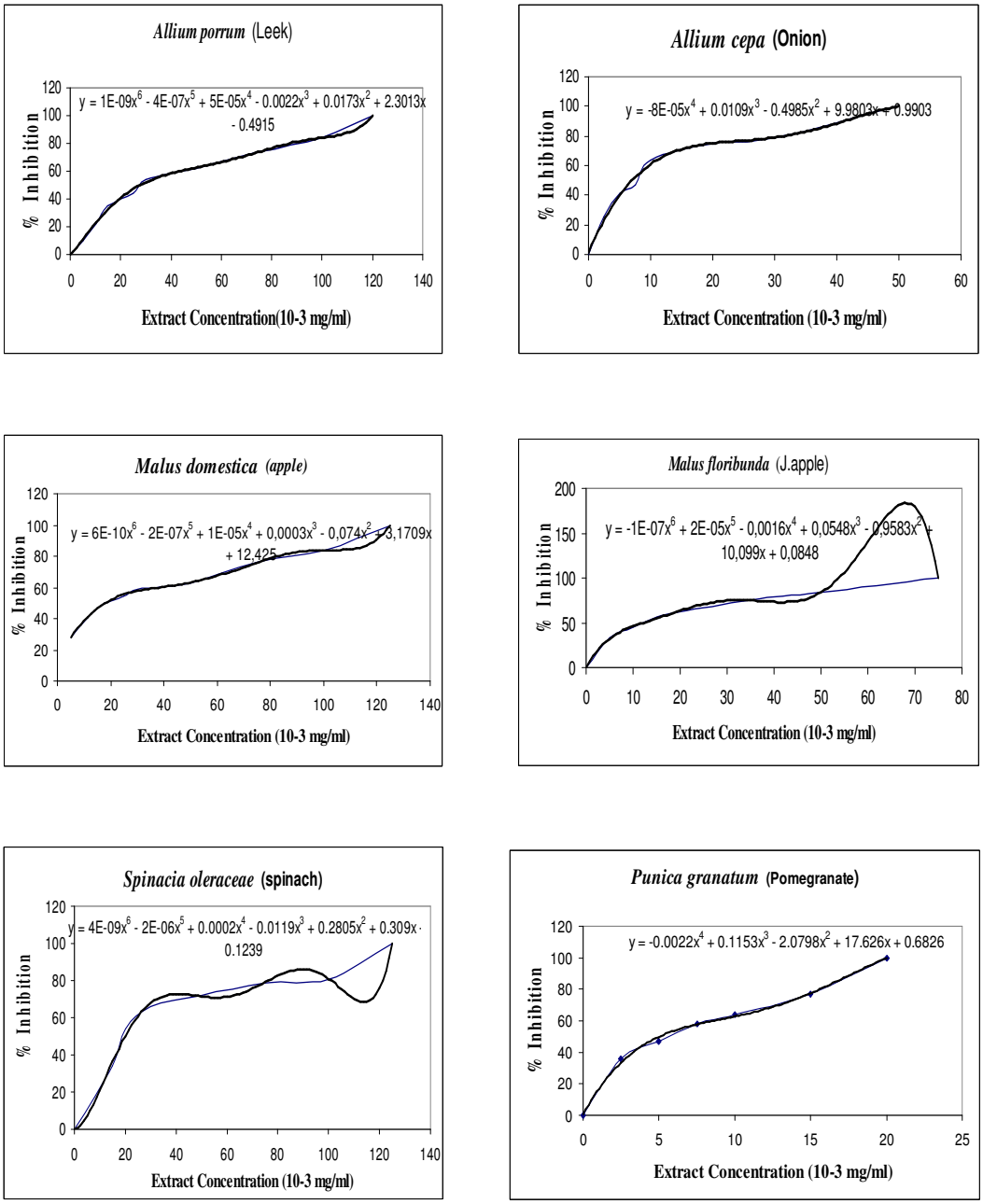


Figure 1: EtAoc extract concentration - % inhibition curves of selected fruits and vegetables