EVALUATION OF ALPHA AMYLASE INHIBITOR ACTIVITY FROM COMMON MEDICINAL PLANTS AND ITS PURIFICATION

A DISSERTATION

SUBMITTED TO THE

SCHOOL OF BIOENGINEERING & BIOSCIENCES

IN PARTIAL FULFILMENT FOR THE REQUIREMENT OF THE DEGREE OF

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

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Under the guidance of

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MAY, 2017

LOVELY PROFESSIONAL UNIVERSITY

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DECLARATION

I hereby declare that the project work entitled as "Evaluation of α-amylase inhibitor activity from common medicinal plants and its purification" submitted to Lovely Professional University, is a record of an authentic work done by me, under the guidance of Dr. Rattandeep Singh, Lovely Professional University, in order to fulfill the requirement of project. This work has not be copied from any source and whatever decoration and connection made in the circuit is a total dedicated work of mine.

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Sandeep Kaur (11210291)

CERTIFICATE

This is to certify that the Dissertation entitled "**Evaluation of α-amylase inhibitor activity from common medicinal plants and its purification.**" submitted by Sandeep Kaur (11210291) in partial fulfilment of the requirement for the award of degree of M.sc in Biotechnology to Lovely Professional University, Phagwara Punjab. It is a record of the candidates own work carried out by her under my supervision. The matter embodied in this thesis is original and has not been submitted for the award of any other degree.

Approved as to style and content by:

Dr Rattandeep Singh,
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ACKNOWLEDGEMENT

It is my honor to present my project on "Evaluation of α-amylase inhibitor activity from common medicinal plants and its purification. I wish to express my sincere gratitude to my project supervisor **Dr. Rattandeep Singh**, **Assistant Professor**, who gave me the motivation to take the project and inspired me each point of time. I deeply thank him for his dedication and patience provided for my project work. I owe a deep sense of gratitude **to Dr. Umesh Gautam, Head of Department of Biotechnology** for his continuous encouragement and guidance whenever needed.

Also, I sincerely thank all the faculty members who helped me at various stages of our project. My thanks also go out to **Mr. Chirag Chopra, Head of Laboratory of Biotechnology** for encouraging and supporting me during my project.

Last but not the least I would like to thanks my parents who always supported and encouraged me, that always helped me to move forward. I would like to thank all my friends for their kind support and stimulating thoughtful discussions that helped me so much in my project.

Sandeep Kaur (11210291)

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Abbrevations :

DMSO- Dimethyl sulfoxide

DNS- 3,5-dinitrosalycyclic acid

PPA- Porcrine pancreatic α-amylase

BSA-Bovine Serum Albumin

O.D. – Optical density

SDS-PAGE- Soduim dodecyl sulfate Polyacrylamide gel electrophoresis

SDS-Soduim dodecyl sufate

Kd-Kilodalton

Abstract: Diabetes is related with impaired insulin secretion which leads to hyperglycemia. Hyperglycemia can be lowered using the alpha-amylase inhibitor from plants, alpha amylase inhibitor act as starch blocker and prevent alpha-amylase from binding with alpha amylase. Use of medicinal plants for the treatment of diabetes is novel approach. Use of palnts is cost effective and less harmfull as compared to use of drugs available in market. In this study alpha amylase inhibitor activity of five plants was checked, these five plants are *Murraya Koenigii, Psidium guajava, Ocimum Tenuiflorum, Zingiber officinale* and *Piper nigrum.* Extarction of crude extract using soxhlet apparatus was done, alpha amylase inhibitory activity was checked using DNS reagent. Putification of plant extract done which shows maximum alpha-amylase inhibitor activity, purification steps include ammonium sulfate precipitation, dialysis, gel filteration chromatography and SDS-PAGE. After each step of purification alpha amlylase inhibitor activity and protein content was cheked to find out the specific activity and fold purification. *Piper nigrum* has shown maximum alpha amylase inhibitor activity so its purification was done. After purification *Piper nigrum* has shown 90% activity and fold purification of 8.73 was obtained. *Zingiber officinale* has shown minimum alpha amylase inhibitor activity. In furure *Piper nigrum* can be used for the treatment of diabetes and drugs can also be abtained from plant extract of *Piper nigrum.*

Chapter 1

Introduction

Diabetes Mellitus is an insulin metabolism related disease, due to the impaired secretion of the insulin it leads to the chronic hyperglycemia. Hyperglycemia is related to the carbohydrate, fat and also to protein metabolism which is caused by defects in insulin secretion or insulin action or both of them (WHO, 2006). It is shown by epidemiological studies and clinical trials that hyperglycemia is the main reason for causing the complications related to coronary artery disease, cerebrovascular disease, renel failure, blindness, limb amputation, neurological complications and pre-mature death. In the treatment of the all type of diabetes the control of hyperglycemia is very important because in the long term when the glucose concentration is not kept in the normal range, acute and chronic complications can happen (Funke I. et al,2006; Ye F. et al 2002). Various drugs are available that are used for treatment of the diabetes, widely used drugs are biguanide, sulfonylurea as ,glycosidase inhibitors, carboyl methyl benzoic acid.

Along with the hyperglycemia reduction in antioxidant defense mechanism is also a cause of diabetes mellitus. Reactive oxygen species induced oxidative stress which can develop many chronic and degenerative diseases as it cause cell membrane disintegration, protein, lipid, and deoxyribose nucleic acid damage (Styskal J. et al., 2012; Finkel T. et al., 2000). Cellular disinfection is cause by imbalance between reactive oxygen species generation and antioxidant protection mechanism which leads to various disease inducing diabetes mellitus (Johansen J.S.et al., 2005; Gutteridge J.M.C. et al., 1994). Immediate oxidative stress is induced by exaggerated postprandial spikes in blood glucose and lipids due to the highly processed, calorie-dense, nutrient depleted diet. Direct increase in postprandial blood glucose level has been observed due to induction of oxidative stress (Sangeetha R. et al., 2012). Type II diabetes mellitus is culminated and formation of advanced glycation end products is ensues by postprandial hyperglycemia. Diabetic complications and aging are promoted by these glycated products (Ashok K.T. et al., 201).

In type I diabetes insulin-producing beta cells in pancreas are destroyed by the attack of immune system so it is a autoimmune disease. The exact cause of body's immune system attack is not yet

known but possibly it is induced by autoimmune, genetic and environmental factors like viruses. It can appear at any age but mostly develop in children and young adults.

347 million people worldwide are suffering from diabetes currently (Danie G. et al., 2011). This disorder with high frequency globally is like to hit 300 million people by 2025 with India estimated to have the largest number of diabetic cases (Gupta O.P. 2003). An estimated 3.4 million people died as a result of high fasting blood sugar in 2004 (Global Health Risks, 2009). In low and middle income countries or developing countries more then 80% deaths occur (Mathers C.D., 2006). In 2030 diabetes will be the 7th leading cause of death projected by WHO (WHO,2011).

Treatment of type II diabetes consist of different therapies like endogenous insulin secretion, increase of activity at target tissues as well as inhibition of alpha-amylase enzyme to reduce the degradation of starch to decreasing glucose (Traling et al, 2008; Barley C.J., 2003).

Therapeutic approach to reduce the postprandial hyperglycemia is to slow down the dietary carbohydrate digestion through inhibition of the activity of the enzymes such as α-amylase and α- glycosidase. Alpha amylase belongs to the family of glycoside hydrolase enzymes which acts on alpha 1,4 glycosidic bonds and break down polysaccharides into monosaccharides. Alpha amylase breakdown the complex polysaccharide into oligosaccharides and disaccharides which are then converted to monosaccharide by the alpha glycosidase, monosaccharide are absorbed through intestine into hepatic portal vein. By inhibiting the alpha-amylase and alpha-glycosidase digestion, absorption of the carbohydrates can be delayed which results in lowering postprandial glucose levels. Alpha amylase inhibitor belongs to one of the anti-diabetic drug families, of which Acarbose is the most well known. These drugs are capable of healing noninsulin dependent diabetes mellitus and have very strong advantage (Cheng A.Y.Y. et al, 2005; Upadhyay R.K. et al, 2011). But use of these drugs in preventive approach is reduced because they induce gastrointestinal side effects (Sales D. et al, 2012). Research is going on to develop a nutritional strategies to completely control postprandial glycaemia and there should be no negative effect of developed drug on the digestive system (Marles R., 1994; Buyukbalci A. et al, 2008).

For the treatment of the diabetes mellitus, it can be done by inhibiting any one of the carbohydrate-hydrolyzing enzymes, α-glycosidase, and α-amylase which results into delay of absorption of glucose as well reduction of oxidative stress (Van de Laar F.A. et al.,2008; Cheng AY.Y. et al.).

Natural antioxidants are responsible for alleviating the oxidative stress condition in diabetes as well as helpful in managing the postprandial hyperglycemia as natural antioxidants has ability to neutralize the effects of reactive oxygen species (Arshya H. et al., 2013). Development of green medicines due to their higher stability, higher antioxidant potential, low cost, and low cytotoxicity is result of growing interest to combat the side effect of the drugs available for diabetes. Plants have antioxidant and antidiabetic potential both in vitro and in vivo because they are rich source of phytochemicals which posses these biological activities (Cheng A.Y.Y. et al., 2005; Sales D. et al., 2012).

Medicinal plants and herbs can be used in place of medicine to treat a number of diseases (Marles R. et al, 1994). Alpha amylase inhibitors can be obtained from the medicinal plants. Some plants have antioxidant activity as they are able to produce great amount of the phenolic substances (Cicero, 2013). In the developing countries, most of the people are unable to effort a modern treatment of various diseases so in developing countries medicinal plants can be used for the treatment of the diabetes to reduce the cost of the treatment. Extensive research has been conducted on the properties of the alpha amylase and glucosidase inhibitors because of their importance in the human nutrition and in treatment of the diabetes (Tarling A., 2008). Medicinal plants are being investigated for their antdiabetic potential as they are very promising and traditional (Babu V. et al., 2002; Parthasarathy R. et al., 2009). For the treatment of the diabetes more than 800 plants has been used throughout the world, this information is given by Ethno botanical (Bailey C.J., 2003) but still for their antidiabetic activity enough proof is not there (Rhabasa-Lhoret R., 2004). Post-prandial hyperglycemia is a major concern in type-2 diabetes and for managing the post prandial hyperglycemia alpha-amylase inhibitors obtained from medicinal plants can be used (Rothe Sagar M. et al,2014).

Inhibition of carbohydrate hydrolyzing enzymes in mammals by phenolic fractions of plants has long been recognized. It has been reported by Mai & Chuyen., (2007) that in the regulation of

carbohydrate hydrolyzing enzymes polyphenols play an important role. In garden and houses large number of exotic oranamental plants are cultivated. They are famous only for their beauty and aroma but they also carry unique medicinal potentials (S. et al, 2012).

Traditional herbal medicine re-emerging faith for the treatment of various lifestyle disorders without any side effects (Zhang P. (2010). Still 25% drugs derived from plants are part of modern pharmacopeia and they similarity with compounds originally available in plants. In India, there many plants available which possess high medicinal value. But due to the lack of the scientific investigation and characterization of the bioactive components of these medicinal plants they are used less for the treatment of diseases. And this is becoming major research area today (Bhutya R.K., (2011); Lucy H., (1999).

The aim of our study is phytochemical screening of alpha amylase inhibitors from few Indian medicinal plants and screening of their antidiabetic properties. We intend use the medicinal plants used as alternative to chemical drugs as a source of treatment of diabetes.

Chapter 2

Review of literature

Alpha amylase: Human saliva, pancreatic juice, human breast milk, serum and certain tissues like liver are the source of alpha amylase (α-1,4-glucan 4- glucanohydrolase EC 3:2:1:1). Alpha amylase hydrolyse the α (1-4) linkages present in starch and break down it into maltose and glucose. First main step of the process of digestion is performed by salivary alpha amylase. Salivary alpha amylase present in the mouth convert the starch in food into sugar and with this the process of digestion begins with the chewing of the food (Maureen et al, 2000). Because of the action of alpha amylase foods like potatoes and bread will taste slightly sweet when chewed as they contain starch. Pancreas also secrete enzyme amylase which is known as pancreatic amylase ,for the body to store and use food molecules alpha amylase that is present in gastrointestinal tract break down these food molecules (Rosenblum et al., 1998). Glycogen contain α-(1-4) linkages and α-(1-6) linkages, alpha amylase can hydrolyse the α-(1-4) linkages but it cant hydrolyse the α-(1-6) linkages, α-(1-6) linkages is responsible for the branched structure of glycogen (Maureen et al, 2000). Amylase act as digestive enzyme other then this it is also useful outside the human body.

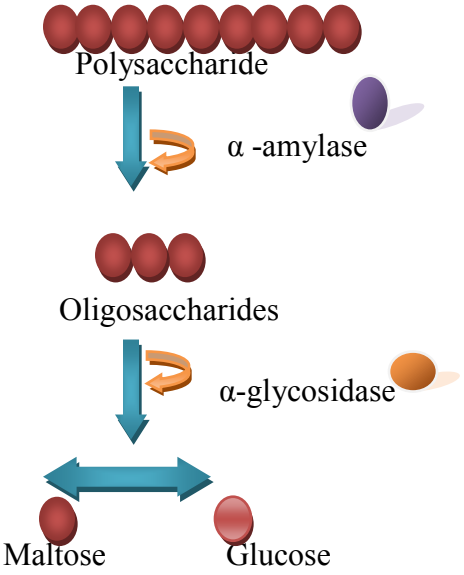


Fig 1: Action of α-amylase on polysaccharide and α-glycosidase converting polysaccharide to monosaccharides

Action of alpha amylase inhibitor : Postprandial glucose level can be lowered by inhibiting the action of α-amylase and α-glycosidase which result in delay digestion and absorption of carbohydrates. Amylase break down the starch present in flour into simple sugar during the the process of bread making. On which yeast can feed and cause bread to rise as well as impart flavour (Robyt et al, 1973). Diabetes mellitus is a metabolic disease, cause of this disease either is pancrease is not able to produce enough insulin or cells do not respond to the produced insulin (West I.C., 2000). Postprandial hyperglycemia has to be decreased to treat diabetes, during hyperglycemia blood sugar levels are elevated (Chakrabarti R. et al, 2002). By inhibiting the activity of alpha amylase and alpha glycosidase the level of sugar in blood can be decreased (Bhosale U.P. et al, 2011).

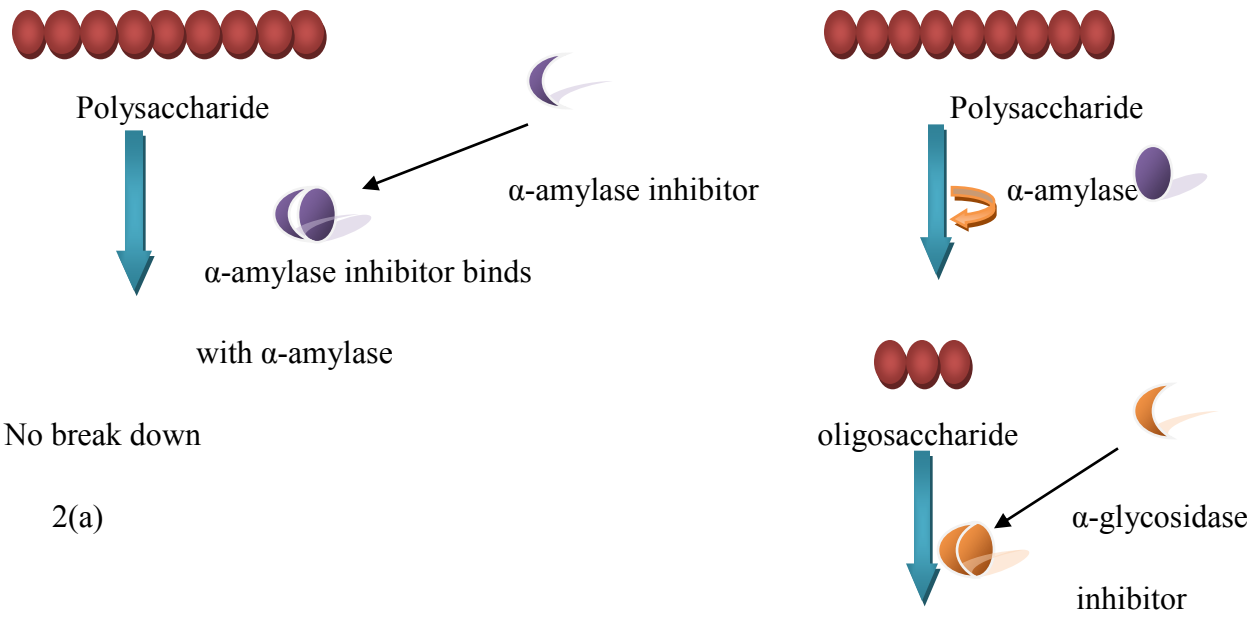


Fig 2: 2(a) Showing action of α-amylase inhibitor, as α-amylase inhibitor binds with α-amylase no break down of polyysacharide takes place, 2(b) showing action of α-glycosidase inhibitor ,it stop the break down of oligosaccharide into monosaccharide by inhibiting the action of α-glycosidase.

Carbohydrates are source of energy and are major part of human diet. To make carbohydrates absorbable from intestinal lumen so that they can be transported into blood circulation , they are converted into monosacchrides by alpha amylase, monosacchrides are absorbed from intestinal lumen (Dewai R.T. et al, 2007). By inhibiting the action of alpha amylase, carbohydrate digestion get retarded which leads to reduction in blood glucose level and for treatment of diabetes this can be used as therapeutic approach. Drawback of using inhibitors is that they are not very specific in targeting different glycosides (Cheng A.Y.Y. et al, 2005). Therefore use of traditional medicinal plants for treatment of diabetes provide an alternative and investigation of natural extracts of medicinal plants for potential of discovery of antidiabetic drugs is an important area of investigation.

Antidiabetic properties of plants: For the prevention and cure of obesity and diabetes physiological effects can be modulated by using plant based medicines and functional foods. This is new emerging interest to treat diabetes using plant based medicines. Natural effective oral hypoglycemic agents obtained from plants have slight or no side effects on human health. For the search of natural effective oral hypoglycemic agents plant kingdom is a wide field. To control the hypoglycemic activity more than 1200 plants have been used. Thus an attractive strategy has been offered by natural alpha-amylase and alpha glycosidase inhibitors to control hypoglycemia (Tundis R. et al, 2010). Alpha-amylase and alpha-glycosidase both are the major digestive enzymes and play role in intestinal absorption. So for the treatment of the diabetes these both are the major target which can done by using alpha-amylase inhibitor and alpha-glycosidase inhibitors to inhibit the activity of alpha-amylase and alpha-glycosidase respectively (Nair S.Sindhu et al, 2013).

From long time treatment of diabetes has been done by using the natural products from plants, this is mostly used in developing countries as the resources in developing countries are limited and are not affordable by everyone (Nickavar B. et al, 2009). It is reported that in the utilization of glucose, polyphenols have caused the similar effects as insulin and they inhibit the alpha amylase which is key enzyme associated with diabetes type II and plant foods are rich source of polyphenols (Reddy N.V.L.S. et al, 2010).

Hypoglycaemic activity has been shown by dietary fibers, flavonoids, alkaloids, saponins, amino acid and peptides isolated from medicinal plants either by release of insulin from pancreatic β-cells, in gut increased absorption of glucose or the body increase the glucose utilization (Li et al., 2004; S.et al, 2006, Modak et al., 2007). However most of the low molecular weight anti-diabetic compounds fall under categories of flavones and triterpenoids (Kathirvel A. et al., 2012). Number of plants have been used for treatment of diabetes as medicine from medicinal plants as they are cost effective and has less side effects.

Table 1: List of some plants that are used for extraction of alpha-amylase inhibitors

Sr. no.	Plants used	Reference	Year
1.	*Artocarpus heterophyllus*	Nair S. Sindhu et al	2013
2.	*Piper betel*	Nair S. Sindhu et al	2013
3.	*Cinnamomum zeylanicum*	Nair S. Sindhu et al	2013
4.	*Teucrium* species plants	Dastjerdi Mirzaalian Zohre et al	2015
5.	*Curcuma longa*	Kumar Prabhakar Varun et al	2013
6.	*Brassica oleracea*	Kumar Prabhakar Varun et al	2013
7.	*Adiantum caudatum Linn.*	Telagarii Madhusudhan et al	2015
8.	*Cassia fistula*	Jyothi K.S.N. et al	2013
9.	*Hibiscus rosasinensis*	Jyothi K.S.N. et al	2013
10.	Urtica dioica	Rahimzadeh Mahsa et al	2014
11.	*Syzygium aromaticum*	Salehi Peyman et al	2013
12.	Salvia species	Nickavar Bahman et al	2008
13.	Equisetum arvense	Khacheba Ihcen et al	2014
14.	Oudneya africana	Khacheba Ihcen et al	2014
15.	*Pongamia pinnata*	Jyothi K.S.N. et al	2013
16.	*Julgans regia*	Mahsa Rahimzadeh et al	2014
17.	*Catharanthus roseus et al*	Jyothi K.S.N. et al	2013
18.	*Citrus macroptera*	Nizam uddin et al	2014

19.	*Morus alba*	Slehi Peyman et al	2013
20.	*Olea europaea*	Benvaente Garcia et al	2000

The drugs are also used for the treatment of the diabtetes mellitus these drugs include sulfonylureas, biguanide, carbamoylmethyl benzoic acid etc. Better glycemic control can be used by using these drugs as monotherapy and in combination. Drugs used for the treatment of diabetes mellitus has side effects on human health like diarrhea, abdominal pain, intestinal disturbances as well as they are very costly (Mirzaalian Zohre D. et al, 2015). So altenative for this is use of medicinal plants as a source of alpha amylase inhibitors. In the introduction of new therapeutic agents, plants has played an important role. Herbal products having antidiabetic activity and minimal side effects has gained a considerable publicity and they have provides an alternative to cure these diseases, that's why plants have obtained much attention as they contain biological active substances like antioxidants and hypoglycemic agents (Marles R,J, et a). A alpha amylase inhibitors are present in plants and make plants lethal for insects as they act as starch blockers as a result dietary starch is not absorbed by body of insect and insect died, thus alpha amylase provides advantages to plants as well (Ali et al). As alpha amylase inhibitors play an important and beneficial role in plant physiology as well as in animal and human nutrition, their biological properties and effects are being studied (Garcia Olmedo et al, 1987).

In this study we have selected few Indian medicinal plants namely *Murraya Koenigii, Psidium guajava, Ocimum Tenuiflorum, Zingiber officinale* and *Piper nigrum.*

Murraya Koenigii: Although *Murraya Koenigii plant* is cultivated and distributed throughout India but it is commonly found in areas of Himachal Pradesh. It is also available in moist forests of Asian regions Nepal, Bhutan, Laos, Pakistan etc. Its common name is curry tree or kari patta, it has characteristic aroma properties. It belongs to family Rutaceae. It is aromatic shrub or small tree, its stem is woody, slender but strong, its branches are covered with dark grey bark. It is a rich source of carbazole alkaloids. For the cure of diarrhea, vomting green leaves of *Murraya Koenigii* are eaten raw. Leaves of *Murraya Koenigii* also used traditionally for flavouring soups, curries etc. It also has antifungal, anti-inflammatory, anti-oxidative, cytotoxic, anti ulcer activity (Vandana J. et al, 2012). For treatment of diabetes mellitus curry leaf is used traditionally

(Vijayanand S.V. et al, 2015). Leves of *Murraya Koenigii* has shown a hypoglycemic activity (Arulsevan P. et al, 2005).

Fig 3: ***Murraya Koenigii***

Psidium guajava: *Psidium guajava* is also known as guava, yellow guava tree.It is evergreen tree, its branches are wide spread, it is native of tropical America. Fruits of *Psidium guajava* are the source of vitamin C, vitamin A, iron, calcium and phosphorous. Leaves of *Psidium guajava* are the source of essential oil which are rich in cineol, flavonoids, quercetin. Leaves are used for sore throats, vomiting and stomach upsets (Dwek C. Anthony et al). As well as leaves also have antibacterial activity, used for treatment of diarrhea. leaves are also used for the diabetes, they are good for thirst in diabetes (Wyk et al).

During glucose tolerance test on diabetic rats induced by alloxan, it was reported that increase of plasma sugar level is inhibited by the leaves of *Psidium guajava* (Mukhtar H.M. et al, 2004). For the treatment of diabetes other then leaves other parts of *Psidium guajava* have also been used (Romila Y. et al, 2010).

Fig 4: ***Psidium guajava***

Ocimum Tenuiflorum: is a sub shrub having opposite green and purple leaves. It is native plant throughout the world, it is culvated on large scale and used as an escaped weed, for religious purposes. It belongs to Lamiales family. It contains many nutrients and biologically active compounds. *Ocimum Tenuiflorum* can also be used for treatment of diabetes as it decreases the blood glucose level (Priyabrata P. et al, 2010). It is reported that leaves of *Ocimum Tenuiflorum* has shown hypolopidaemic and antioxidant activity. Hypoglycemic affect and antidibetic activity is also reported in different reports (Shweta Gupta et al, 2005). Significant antidiabetic, anti-hyperlipidemic activity and presence of number of pharmacological actions is shown by hydroalcoholic extract of *O. tenuiflorum.*

Fig 5: *Ocimum tenuiflorum*

Zingiber officinale*:* Commomn name of *Zingiber officinale* is Ginger. It belongs to the Zingiberaceae family. In India Ginger has been cultivated in most of the states including Karnataka, Orissa, Assam, Meghalaya, Arunachal Pradesh and Gujarat, production from these states together contribute 65 per cent to the country's total production. It is used as spice in many countries, used in food for adding flavour .In traditional herbal medicine, the rhizome of ginger has also been used. Ginger is very beneficial for health as it contains number of phytochemicals. Treatment of number of ailments including degenerative disorders (arthritis and rheumatism), digestive health (indigestion, constipation and ulcer), cardiovascular disorders (atherosclerosis and hypertension), vomiting, diabetes mellitus, and cancer can be done using the ginger as ginger

has staring potential for treatment of them. The process of aging can be controlled with the help of ginger as it has anti-inflammatory and anti-oxidative properties.

In rats hypoglycemic effect of ginger (*Zingiber officinale*) has been studied (Al-Amin et al, 2006). Significant blood glucose lowering effect has been shown by ginger Juice (Sharma M. et al, 1977). As it was shown by different investigator that different parts of ginger are responsible for lowering the blood glucose level but mechanism behind same has not been yet disclosed (Shabah Shadli et al, 2014). So scope is still there to study the mechanism behind these beneficial effects.

Fig: 6 *Zingiber officinale*

***Piper nigrum*:** Black pepper is a common name of Piper nigrum (Family Piperaceae),its Indian name is kali mirch. Among various spices black pepper is considered as "The King of Spices". alkaloid piperine is present in black pepper and it smells pungent, it possess many pharmacological properties like antihypertensive, antiplatelet, antioxidant, antitumor, anti-asthmatics, analgesic, etc. black pepper possess antimicrobial, antioxidant, anti-cancer, anti-inflammatory, hepatoprotactive aswellas anti-diarrheal activity (Zohair A. Damanhouri et al, 2014). For the treatment of diabetes and hypertension essential oils from black pepper has been used as it possess alpha-amylase inhibitory activity (Ganiyu Oboh et al, 2013). It was reported that seeds of *Piper nigrum* play a role in reducing glucose and lipid levels in blood, means it

possess ant-diabetic properties. Other then *Piper nigrum* anti-diabetic activity has also been reported in other species of *Piper* (Dipali Thakur et al, 2017).

Figure 7: *Piper nigrum*

Chapter 3

Scope of study

- This study is related with inhibition of alpha amylase activity by alpha amylase inhibitors.
- In this study different medicinal plants are used which could be used as source of alpha amylase inhibitor, advantage of use of medicinal plant as source of alpha amylase inhibitor is that they does not cause any harm to human health and they are cheeper so they are cost effective as well.
- Alpha amylase inhibitor from plants can be used as an alternative for the treatment of diabetes in place of drugs, as drugs cause harm to human health and they are costly.
- Treatment of diabetes using medicinal plant as source of alpha amylase inhibitor can be proved to person who can't effort the costly medicines and it will be very beneficial for the developing countries.

Chapter 4

Objectives of study

- Extraction of alpha amylase inhibitors from common medicinal plants.
- Assessment of alpha amylase inhibitory activity of extracts obtained from selected plants using enzyme assay.
- Purification and partial characterization of alpha amylase inhibitors from the extract.

Chapter 5

Materials and methods

5.1 Collection of Plant Materials: Leaves of the plants that are mentioned in table no. 1 were collected. Leaves of each plant were washed with water and dried in sunlight for 2 days. Then crushed them into fine powder.

Table 2: Showing plant material collected and location from it is collected.

S.No.	Medicinal Plant	Plants part used	Collected from
1.	*Murraya Koenigii*	Leaves	Ludhiana,Punjab in August 2016
2.	*Psidium guajava*	Leaves	Pharala, Punjab in August 2016
3.	*Ocimum Tenuiflorum*	Leaves	Nawashahar, Punjab in September 2016
4.	*Zingiber officinale*	Rhizome	Phagwara, Punjab in January 2017
5.	*Piper nigrum*	Seeds	Phagwara market, Ounjab in January 2017

5.2 Preparation of plant extract:

15 to 20 grams of dried powder was weighed and the extraction was done with different solvents by Soxhlet extraction technique.

- The extract was then condensed by evaporating the solvent in water bath at 60°C to 70°C temperature.
- The crude extract was dissolved in 10% of DMSO and stored for further use.

Figure 8: Soxhlet apparatus for preparation of plant extract

5.3 Preparation of Maltose standard curve:

- 1% of maltose solution was prepared by dissolving 0.5gm of maltose in 50ml of distilled water.
- Standard maltose solution was pipette out in the range of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1ml in 10 separate test tubes.
- A test tube containing a blank solution was also prepared.
- By using the distilled water volume in each test tube including blank was brought to 2ml.
- 1ml of DNS regeant was added to each test tube and test tubes were covered with aluminum foil.
- Test tubes were kept in boiling water bath for 5 minutes.
- Test tubes were cooled to room temperature after taking them out of the water bath.
- 9ml of distilled water was added to each test tube and mixed well.
- Spectrophotometer reading was taken at 540nm.

5.4 Assessment of α-amylase inhibitor activity:

The method given by Le Berre-Anton et al (1997) was used but with some alterations to determine the activity of alpha amylase inhibitor.

- 500 µl the crude extract and 500 µl of porcrine pancreatic α-amylase (PPA) which was dissolved in 20mM acetate buffer (4.5 containing 20mM $CaCl_2$ and 10 mM NaCl) were incubated at 37^0C for 15 min.
- 500 µl of 1% starch (dissolved in 80mM phosphate buffer having pH 6.9) was added to each test tube and incubation at 37^0C for 15 min was done.
- Stoppage of reaction was done by addition of 1ml of 3.4 Dinitrosalicylic acid reagent.
- The contents were boiled in a water bath for about 5 minutes and then after cooling it is diluted with 4ml of water.
- Absorbance of solution was measured at 540 nm.
- Blank solution without PPA was also prepared. The amount of maltose produced was estimated from standard curve of maltose.
- α-amylase inhibitory activity was calculated according to equation shown below:

  Inhibitory activity (%) = $[M_o - M_i / M_o] \times 100$

  Where, M_1 and M_o are amount of maltose (mg/ml) produced in presence and absence of inhibitor respectively, under the same conditions.

5.5 Preparation of BSA standard curve:

- 0.1% BSA (Bovine serum albumin) was prepared by dissolving 0.1gm of BSA in 0.1N NaOH.
- Standard BSA solution was pipette out in the range of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1ml in 10 separate test tubes.
- A test tube containing a blank solution was also prepared.

- Volume make up was done with distilled water.
- 5ml of Commassive brilliant blue reagent was added in each test tube
- Vortexing was done
- O.D. was taken at 595nm.

5.6 Bradford method for protein content determination:

- 0.1ml of protein was taken in test tubes at three ratios 1:1,1:2,1:4.
- Volume make up was done using distilled water.
- 5ml of Commassie brilliant blue reagent was added in each test tubes.
- Vortexing was done.
- O.D. was taken at 595nm.

5.7 Purification strategies

5.7.1 Ammonium salt precipitation: Crude extract of protein was subjected to ammonium salt precipitation with different concentration of ammonium salt at saturation of 30, 40, 50, 60, 70, 80, and 90 % and protein content and activity towards protein of interest was studied. The cultivar showing highest activity was than subjected to dialysis. Ammonium salts precipitation was carried out by adding ammonium salt gradually while stirring the crude extract with the help of magnetic stirrer. The extract was then centrifuged at 10000 rpm for 5 minutes and pellet so obtained was re-dissolved in appropriate amount of buffer and then repeating the same for other percent saturations levels (Se-Re Park et al, 2015).

5.7.2 Dialysis: Dialysis was performed with the help of dialysis membrane at 4^0C. Ammonium salt precipitated sample was taken in a dialysis membrane and subjected to dialysis by rotating the membrane slowly on a magnetic stirrer. The membrane was immersed within the buffer and dialysis was carried out for 24 to 48 hrs. Subsequent change of buffer was done after every 4-5 hrs (Manisha N. Chalse et al, 2016).

5.7.4 Gel filtration chromatography: After the dialysis, the sample was subjected for further purification through Sephadex G-50 column (26 × 1.2 cm) The column was equilibrated with the help of Tris-HCl buffer (10mM). Different aliquots were taken in eppendorf tubes at a flow rate of 0.10 ml/min and subjected to enzyme activity, the aliquot showing maximum activity was subjected to SDS-PAGE analysis (Benny E. Knuckles et al, 1982).

5.7.5 SDS-PAGE: The purity of sample and molecular weight of protein was determined by SDS-PAGE analysis using vertical gel electrophoresis apparatus. In this technique two glass plates was taken together and three spacers were kept on the boundaries in between the glass plates and was sealed with the help of silicon grease. Various components of resolving gel was mixed and the gel was poured in between the glass plates in vertical position standing with the help of clumps. After the solidification of resolving gel, various components of stacking gel was mixed and the gel was poured in between the glass plates above the resolving gel with a 13 well comb immersing in it. The gel was allowed to solidify to form wells. After formation of wells the apparatus was connected through a power pack to carry out electrophoresis at a constant voltage. Reservoir buffer was filled up in both lower and upper tank of vertical gel electrophoretic apparatus. The sample was introduced into the wells with the help of syringe and was allowed to run for 6-7 hrs at a constant applied voltage. After completion of electrophoresis the gel was immersed into the staining solution and then was destained overnight to resolve the bands (Deepika Saini et al, 2012).

Chapter 6

Results and discussion

6.1 Preparation of standard curve of maltose:

Standard curve of maltose was prepared by using DNS.

Table 3: Various concentrations of Maltose and O.D. at 540nm

Sr. No.	Concentration (mg/ml)	O.D. at 540 nm
	Blank	0.00
1.	0.1	0.01
2.	0.2	0.078
3.	0.3	0.148
4.	0.4	0.212
5.	0.5	0.282
6.	0.6	0.304
7.	0.7	0.331
8.	0.8	0.368
9.	0.9	0.411
10.	1.0	0.545

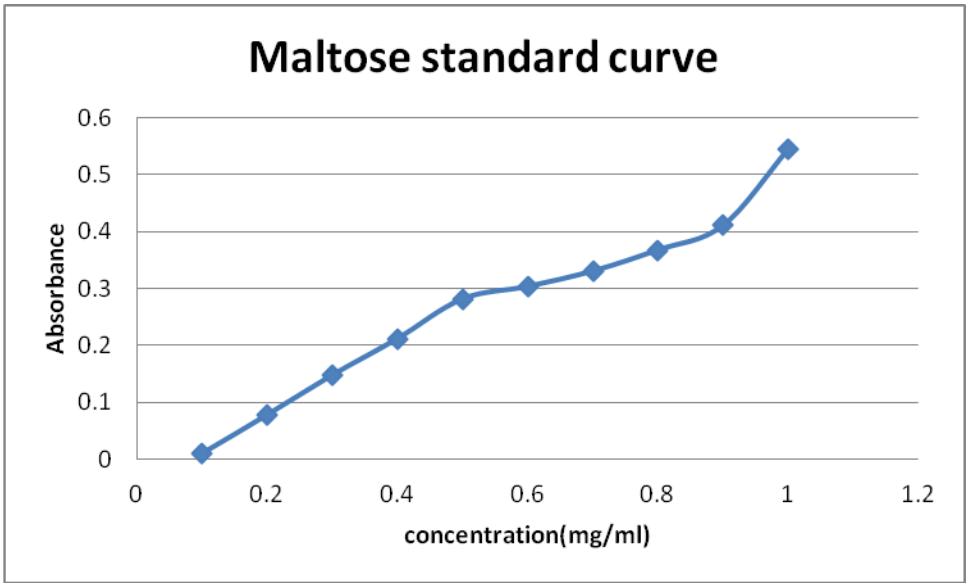


Figure 9: Standard curve of maltose

Figure 10: DNS assay of plant extracts

6.2 Assessment of Alpha-amylase inhibitory activity:

The plant leaves after Soxhlet extraction was subjected to amylase inhibitor activity against porcine pancreatic amylase (PPA). The results have shown that maximum amylase inhibitor activity of ethyl acetate extract was shown by *Piper nigrum* (48± 0.20) and minimum by *Zingiber officinale* (25±0.60). Similarly, Ethyl Acetate extract of guava was also subjected to amylase inhibitor activity and percent inhibition was found to be 40± 0.40. The results obtained in present study is in correlated with those obtained by Sudha *et al*. 2011, where the amylase inhibitor activity of ethyl acetate extract was found to be 29.2 % in *Casia Fistula*.

Protein inhibitors of α-amylase occur widely in plants. The inhibitors are believed to make plants less palatable, even lethal to insects, thus contributing some selective advantage to the plants (Sasikiran et al., 2002). Amylase inhibitors are known as starch blockers because they prevent dietary starches from being digested and absorbed by the body (Mc Evan et al. 2011). Moreover α-amylase inhibitors inhibit the action of *α*-amylase enzyme leading to a reduction in starch hydrolysis which shows beneficial effects on glycemic index control in diabetic patients (Bhat et al. 2001). During inhibition, several components of the inhibitor molecule, amylase molecule and the whole system have been reported to play important roles in the mechanism. The main components that participate in the mechanism include two loops of the inhibitor (L1 and L2) made up of residues 29–46 and 171–189 respectively (Santimone et al. 2004), the amylase domains A and B plus the active site surface loop (residues 303–312) , the active site non-loop residues (Cl binding site and Asp197, Glu233; Asp300 and Arg74 in human pancreatic amylase only (Koukiekolo et al.2001), the active site lining and gate aromatic residues (Bompard-Giles et al. 1990), the chlorine ion of the amylase and system aspects such as the inhibitor: enzyme ratio and pH (Maurus et al. 2005). Based on the effects of chemical modifications on activity of the inhibitor, Ho & Whitaker (1993) proposed that His, Trp, Tyr and Arg residues were important in the mechanism of the inhibitor

Table 4: % Alpha amylase inhibitor activity of plant extracts

Sr. No.	Plant Samples	% inhibition of alpha amylase inhibitor
1.	*Murraya Koienge*	42 ± 0.25
2.	*Ocimum Tenuiflorum*	35 ± 0.35
3.	*Psidium guajava*	40± 0.40
4.	*Zingiber officinale*	25±0.60
5.	*Piper nigrum*	48± 0.20

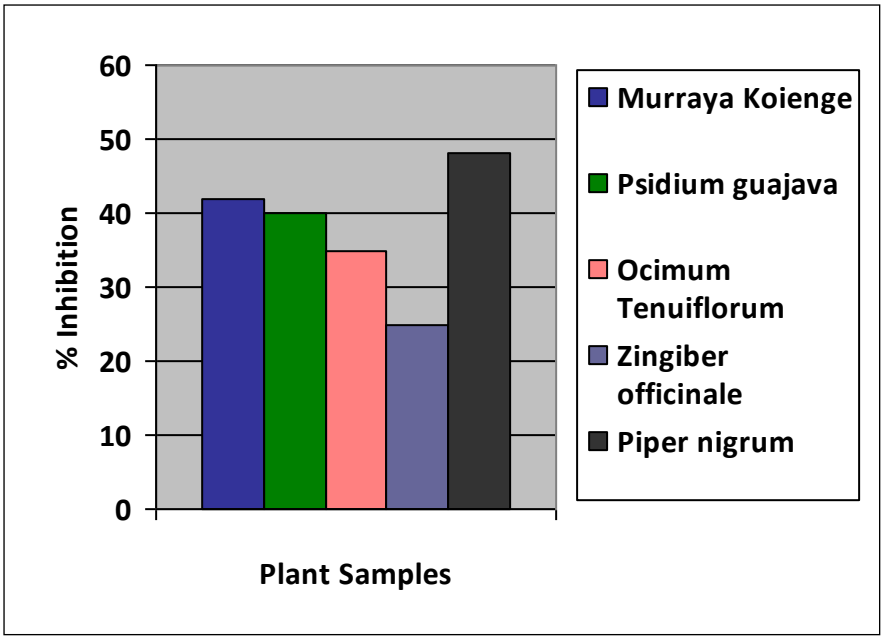


Figure 11: Amylase inhibitor activity

6.3 Purification of alpha-amylase inhibitor

On the basis of inhibitory activity *Piper nigrum* was selected for isolation and purification of alpha-amylase inhibitor. Purification was carried out with 70-90% ammonium salt precipitation followed by dialysis. The dialysed fraction was subjected to Gel- filtration chromatography using Sephadex G-50 column.

The fractions were collected at a constant flow rate and were analysed for inhibitory activity and protein concentration. The value of inhibitory activity and protein concentration was used to calculate the specific activity of the sample which is in turn used to determine fold purification.

The inhibitory activity of crude extract of *Piper nigrum* was found to be 48%. Inhibitory activity increases after ammonium salt precipitation to 55% at 70% saturation and 45% at 90% saturation.

Protein content decreases in the sample fractions, similarly it was found that specific activity increases from crude extract to salt precipitated samples. Further the inhibitory activity, protein content and specific activity was measured in purified fractions from Sephadex G-50 column. Protein content in the purified fraction was found to decrease in comparison with salt precipitated sample, where the value of inhibitory activity and specific activity was found to increase. Similarly, there is increase in fold purification as well during the purification procedures.

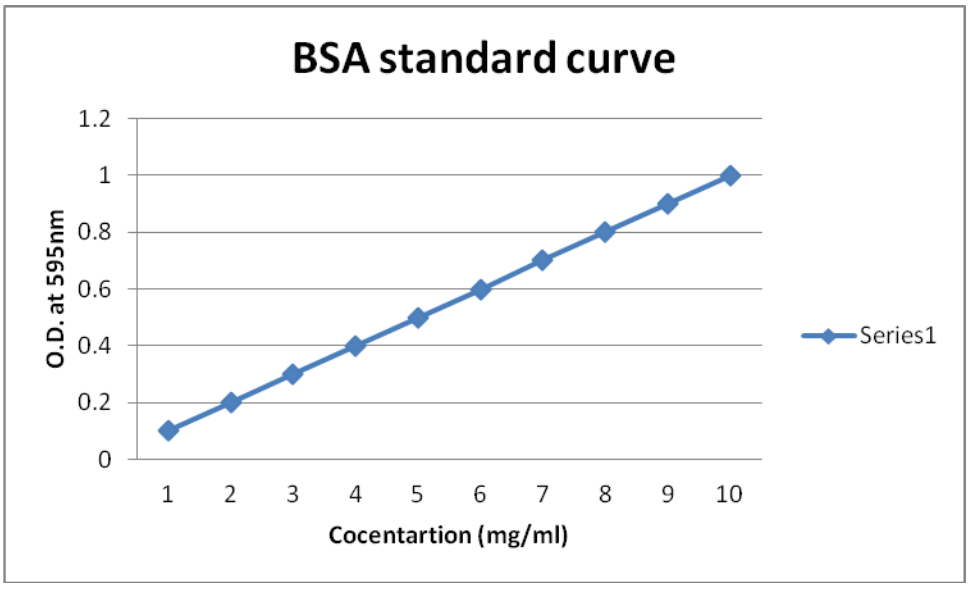


Figure 12: BSA standard curve

Table 5: Purification of alpha-amylase inhibitor from *Piper nigrum*

Sample	Inhibitory activity (%)	Protein content (%)	Specific activity (%)	Fold Purification
Crude extract	48%	69.79%	0.68	1
Amm. ppt. (70-90%)	70%	39.5%	1.77	2.62
Sephadex G-50	90%	15.14	5.94	8.73

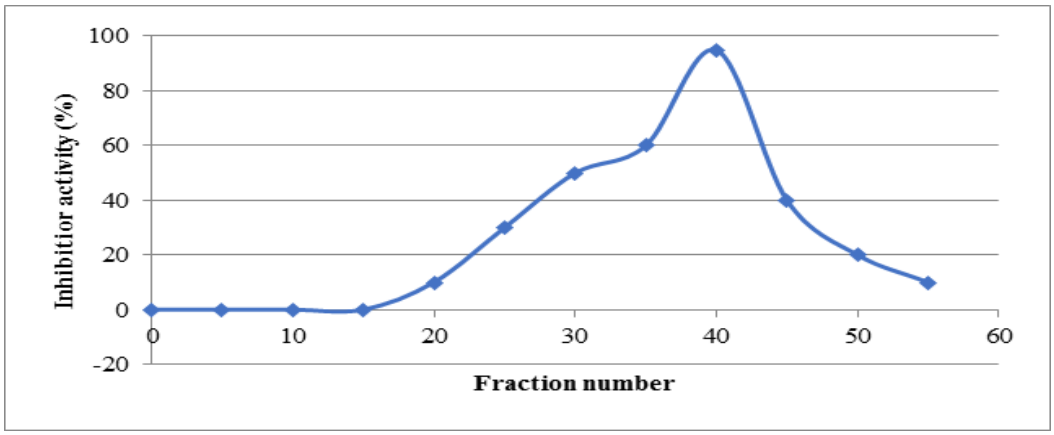


Figure 13. Inhibitory activity of alpha-amylase inhibitor from the fractions eluted from the column. Each fraction (3ml) were pooled and measured for amylase inhibitor activity against PPA.

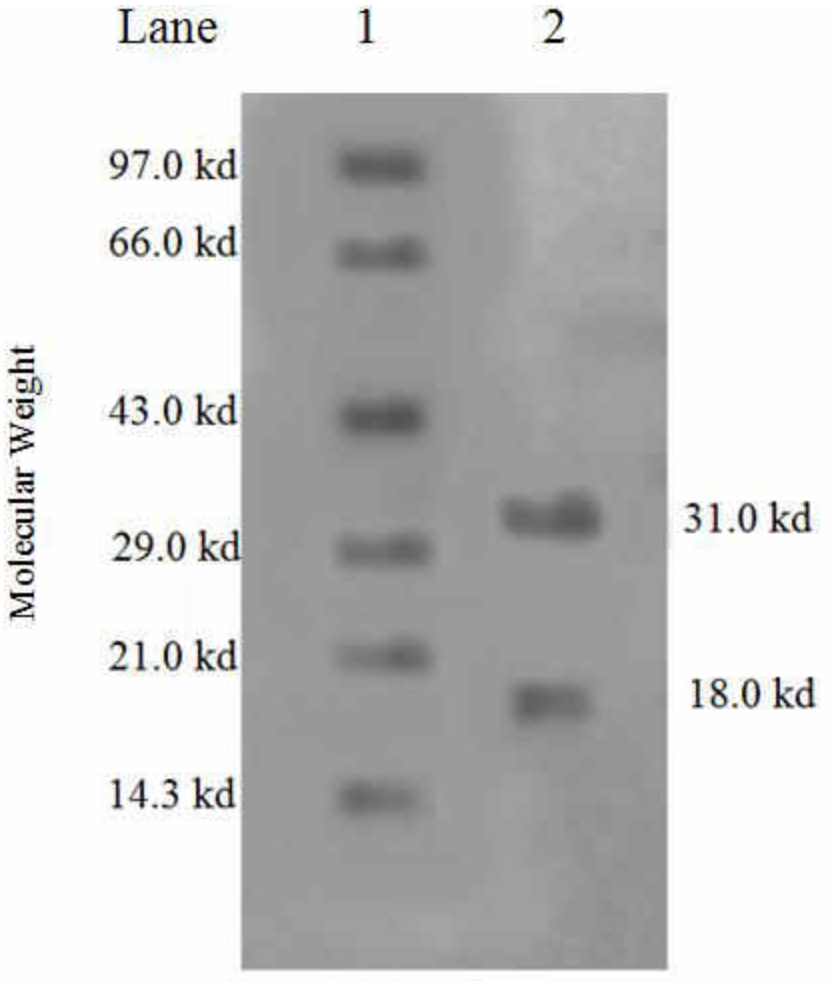
6.4 Molecular weight determination of Alpha-amylase inhibitor purified from *Piper nigrum*

Purified alpha-amylase inhibitor from Josh cultivar was also subjected to SDS-PAGE analysis on 15% gel. The polypeptide fraction was found to resolve into two bands of molecular weight in the range of 14.4-21 kd (corresponding to 18kd) and 29.0 kd-43.0 kd (corresponding to 31.0 kd).

The results obtained in present study were similar with those obtained by Kokiladevi et al (2005), where the molecular weight of α-amylase inhibitor from *Vigna sublobata* was found to be 14kd on SDS-PAGE. Similarly, Ewan et al. 2010 also observed the molecular weight of two purified amylase inhibitors from *Colocasia esculenta* to be 17 and 19 kd.

Two proteinaceous α-amylase inhibitors termed αAI-Pa1 and αAI-Pa2 were purified from seeds of a cultivated tepary bean (*Phaseolus acutifolius*) by Yamada et al. 2001. αAI-Pa1 is composed of a single glycopolypeptide with a molecular mass of 35 kDa. The αAI-Pa2 was found to resolve into three bands of molecular weight in the range of 14-18kd.

Alpha amylase inhibitor from Palo Fierro seeds (αAI-PF) was purified using affinity chromatography on a fetuin-fractogel column followed by anionic exchange chromatography. αAI-PF has a molecular mass of 77 kDa with two subunits (15.8 and 17.4 kDa). Analysis of αAI-PF peptides showed a high homology to αAI-1 from *Phaseolus vulgaris* that also inhibits PPA (Guzman-partida et al. 2007).



Lane 1- Medium Range Protein Marker (14.3-97.0 kd)
Lane 2- Purified α-amylase inhibitor from *Piper nigrum*

Chapter 7

Conclusion and future scope

In the present study, five medicinal plants have been taken to evaluate alpha-amylase inhibitor activity. The medicinal plants were subjected to Soxhlet extraction and amylase inhibitor activity was evaluated in extracts of ethyl acetate and Methanol. Both the extracts have shown alpha-amylase inhibitor activity against PPA (Porcine pancreatic amylase). The maximum amylase inhibitor activity was found in ethyl acetate extract for *Piper nigrum* (48± 0.20) and minimum amylase inhibitor activity was found in *Zingiber officinale* (25.0±0.60). The present study was actually based on standardization of protocols for the alpha-amylase inhibitor activity and in future amylase inhibitor activity of other medicinal plants will be evaluated and purification and partial characterization of alpha-amylase inhibitor will be done. Alpha amylase inhibitory activity was observed maximum in *Piper nigrum* as cpmpared to other plants that were selected that's why purification of *Piper nigrum* was done. Plant extract can be used in purify form for production of drugs for treatment of diabetes as use of these plant extract is cost effective and less harmful as compared to other drugs available in market.

Chapter 8

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Appendix:

Reagents preparation

- Phosphate buffer (0.2 M and 0.1 M)
- Sodium carbonate (2% w/v in 0.1N NaOH)
- Sodium –potassium tartarate (1% w/v)
- Copper sulphate (0.5% w/v).

DNS reagent: Dissolve 1 gm of 3, 5 dinitrosalicylic acid, 200 mg of phenol and 50 mg of sodium sulphite in 100 ml of 1% (w/v) NaOH .

- Phosphate buffer saline (PBS) : 0.01 M,
- Chilled acetone.
- Methanol (95% v/v).
- Folin-ciocalteau reagent.

SDS-PAGE

- Acrylamide –Bisacrylamide (30:0.8)
- Resolving gel buffer (1.875 M Tris-HCl, pH-8.8)
- Stacking gel buffer (0.6 M Tris HCl, pH-6.8)
- 10%SDS
- 10%APS
- TEMED

Electrophoretic buffer (Tris-glycine-pH, 8.3)

- Tris buffer: 3.0 gm
- Glycine: 14.4 gm
- SDS (10%) - 200µl
- Double distilled water- 1000 ml

Sample buffer:

- 1M Tris HCl (pH-6.8): 12.5 ml
- SDS-: 4 gm

- β-mercaptoethanol: 10 ml
- Glycerol: 20 ml

1% Bromophenol blue: 4 ml

- Staining solution:

Commassie brilliant blue dye-R 250: 1.25 gm

Methanol: 200 ml

Glacial acetic acid: 35 ml

Final volume was made by adding 500ml of double distilled water and filtered to remove any impurity

Destaining solution:

Glacial acetic acid: 75 ml

Methanol: 50 ml.

Final volume was made by adding 1000ml of double distilled water.

Composition of Stacking gel:

Acrylamide-Bisacrylamide: 25 ml

Stacking gel buffer: 3.5 ml

10% SDS: 70 µl

10% APS: 70 µl

TEMED: 10 µl

Water: 2 ml

Composition of Resolving gel:

Acrylamide-Bisacrylamide: 12 ml

Resolving gel buffer: 7.5 ml

10% SDS: 70 µl

10% APS: 0.7ml

TEMED: 70 µl

Water: 9 ml