INDO AMERICAN JOURNAL OF PHARMACEUTICAL RESEARCH

STUDIES ON A-AMYLASE INHIBITOR ISOLATED FROM *ADIANTUM AETHIOPICUM* L.

Abhijit Surendra Limaye*, Shankar L. Laware
Department of Botany, Nowrosjee Wadia College, Pune, S. P. Pune University, M. S. India.
ACS College, Sonai, Ahmednagar, S. P. Pune University, M. S. India.

ARTICLE INFO

Article history
Received 14/06/2017
Available online
30/06/2017

Keywords
Adiantum,
Alpha Amylase Inhibitor (AAI),
Proteolytic Enzymes,
Hypoglycemic Agent.

ABSTRACT

Fresh fronds *of Adiantum aethiopicum* L were collected from Fergusson College campus at various growth stages like young fronds, mature fronds without sori, mature fronds with immature sori (pale green colour) and mature fronds with mature sori stage (brown to black colour). They were extracted with 0.15 M NaCl. The clear supernatant obtained after centrifugation was subjected to protein fractionation with ammonium sulphate. The protein fractions were dialyzed against 0.15M NaCl and tested for salivary alpha amylase inhibitor assay. Fractions between 31 and 60% saturation (F_{31-60}) proved to have maximum salivary amylase inhibitory activity; hence it was applied to a Sephadex G-100 column and eluted at a flow rate of 3.0 ml / 10 min. Each eluted fraction was measured spectrophotometrically at 280 nm and tested for salivary alpha amylase inhibitor assay. The elution number 6, 7, 8, 9, 10, 18, 19 and 20 exhibited maximum salivary AAI activity. Maximum inhibitory activity (68%) was observed in eluted fraction no. 8. The fraction is moderately thermo-stable as it is stable to the temperature ranging from 30^0C to 60^0 C. The Maximum activity was recorded at 30^0 and retained about 60% activity up to 50^0 C. It was also proved to be stable to different pH ranging from 3 to 9, but at pH 7.0 it showed maximum inhibitory activity. The fraction was tested for its proteolytic actions against proteolytic enzymes such as trypsin and chymotrypsin. It was found to be more effective amylase inhibitor than the commercial acarbose tablet (Glucobay). Thus, the results of present investigation could suggest the possible use of AAI extracted from *Adiantum aethiopicum* L. can be used as hypoglycemic agent in non-insulin dependent diabetes mellitus and obesity patients.

Corresponding author
Dr. Abhijit Surendra Limaye
Assistant Professor, Department of Botany,
Nowrosjee Wadia College,
Late Prin. V. K. Joag Path,
Pune-411 001; Maharashtra, India
09011902969/07588322493
abhijitlimaye123@gmail.com

*Please cite this article in press as **Dr. Abhijit Surendra Limaye** et al. Studies on α-Amylase Inhibitor Isolated from Adiantum aethiopicum L. Indo American Journal of Pharmaceutical Research.2017:7(06).*

Copy right © 2017 This is an Open Access article distributed under the terms of the Indo American journal of Pharmaceutical Research, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

INTRODUCTION

The use of starch blockers has recently gained in popularity with the success and growth of carbohydrate restricted diets. There are many enzymes in the human digestive system, which help digest the variety of foods we consume.[1] The alpha amylase enzyme is responsible for breaking down starchy foods, such as breads, potatoes, rice, and pasta into sugars that can be absorbed through the intestinal wall. Alpha amylase inhibitors (AAI) are molecules, which interfere with the action of the alpha amylase enzyme. [2] Commercial starch blockers available in market contain protein extracted from wheat or white kidney beans, both of which naturally contain alpha amylase inhibitors (AAI). An alpha amylase inhibitor inhibits the digestion of starch thereby potentially improving postprandial carbohydrate tolerance in people with low glucose tolerance. As excess dietary carbohydrate is metabolized to fat; inhibition of carbohydrate digestion may help in weight management as well. Recent studies documented that the AAI was found to exert a starch blockade effect in normal people and in patients with diabetes mellitus by reducing the post-prandial increase in plasma concentrations of glucose and insulin.[3] Plant alpha amylase inhibitors, particularly abundant in cereals [4, 5] and leguminous plants have been extensively studied, because they play a role in plant resistance to insect and microbial pests. Teixeira [6] studied the effect of tea prepared from leaves of *Syzygium jambolana* on glucose tolerance in non-diabetic subjects and found that tea prepared from leaves enhances the glucose tolerance. Arai [7] studied the effect of *Eugenia uniflora* extract on hyperglycemia and hypertriglyceridemia in mice and observed that the extract is beneficial in hyperglycemic mice. Kedar and Chakrabarti [8] studied the effect of Jamun seed extract on blood sugar; lipids and urea in streptozotocin induced diabetes in rabbits and reported that seed extract treatment is effective in maintaining the blood sugar level. Achrekar[9] studied the hypoglycemic activity of *Eugenia jambolana* and *Ficus bengalensis* and reported their beneficial effects. Similarly Pepato[10] assessed antdiabetic activity of *Myrcia uniflora* extracts in streptozotocin-diabetic rats and reported that the extracts are beneficial in management of diabetes in rat. Melo[11] studied alpha amylase inhibitor from cowpea seeds. However there are no reports on characterization of alpha amylase inhibitor proteins in pteridophytes. *Adiantum* is one of the important medicinal plants mentioned in Ayurveda. Ethnomedicinally, the genus is important and popularly known as *Hansraj* or *Hanspadi* in Ayurvedic system of medicine. It has been used in cold, tumors of spleen, liver and other viscera, skin diseases, bronchitis and inflammatory diseases. However, it has not been characterize for their active chemicals and there were no reports based on amylase inhibitory activity on human salivary amylase. Hence, the present investigation was based on extraction, isolation and characterization of AAI obtained from *Adiantum aethiopicum* L. fronds at various growth stages.

MATERIALS AND METHODS

Preparation of plant extract

Adiantum aethiopicum L. fronds were collected from the Fergusson College campus and local area of Pune. They were separated at various growth stages like young fronds (YF), mature fronds without sori (MF), mature fronds with immature sori (IM), mature fronds with mature sori (MS). Fronds were extracted with 0.15 M NaCl (1:5, w/v) with continuous stirring for 4 h at 4^0 C. The material was then centrifuged at 10,000 ×*g*, 4^0 for 30 min. This supernatant was saved and further used for ammonium sulphate precipitation for different fractions like 0-30%, 31-60% and 61-90%.

Dialysis of ammonium sulphate fractions and Gel filtration by Sephadex G-100

The proteins fractions obtained were dialyzed in a cellophane casing dialysis sacks against 0.15 M NaCl solution and tested for salivary alpha amylase inhibitor (AAI) assay on human saliva [12]. The fraction between 31 and 60% saturation (F_{31-60}) was centrifuged and applied to a Sephadex G-100 column at a flow rate of 3.0 ml/10 min. Each fraction was then measured spectrophotometrically at 280 nm. The proteins in individual peaks were tested for salivary alpha amylase inhibitor assay. The protein inhibitors were determined [13] and tested at a standard concentration of 100 µg/ml. The elution number 6, 7, 8, 9, 10, 18, 19 and 20 exhibited maximum salivary AAI activity. These elutions were pooled to get composite sample, which was stored at 0^0C to 4^0C and used for further studies.

Assay of alpha amylase inhibitory activity

The alpha amylase inhibition was assayed by quantifying the reducing sugars liberated during assay condition. Activity was measured where, 1 unit is defined as the amount of enzyme required to liberate 1µmol of maltose per minute under assay conditions. A modified DNSA method of Bernfeld's (1955) was used to determine the reduction in human salivary alpha amylase (EC 3.2.1.1) inhibitory activity, when the individual eluted fractions collected from the column and added to reaction mixture. Starch (1%) was used as a substrate and sample (with three replicates) including:

1. Blank: Distilled water was used as a blank for all the reactions.
2. Control: Human salivary alpha amylase (1unit) incubated with distilled water without any inhibitor.
3. Ip: Proteins (alpha amylase inhibitor) isolated from *Adiantum aethiopicum* L. (eluted fractions).
4. Test: Human salivary alpha amylase incubated with protein fractions (alpha amylase inhibitors).

All the reactions were incubated at 30^0C temperature for 20 minutes and then they were terminated by addition of 3,5-Dinitrosalicylic Acid (DNSA). Reaction mixtures were boiled for 10 minutes in water bath, cooled to room temperature. Final volume of reaction mixture was adjusted to 10 ml with distilled water and the absorbance was measured at 540 nm on UV-visible spectrophotometer (Shimadzu-1700) against water blank as a reference.

The percent alpha amylase inhibitory activity was calculated as:

% activity = [Control + Ip] – [Test] x 100

Characterization and electrophoretic analysis

The AAI was characterized for temperature stability, optimum pH range, and proteolysis with proteolytic enzymes like trypsin, chymotrypsin. Finally the activity was compared with that of standard commercial drug (Glucobay). The inhibitor protein (eluted fraction no.8) was tested for its purity by native PAGE [14]. About 80 µg of protein sample was loaded on 10% polyacrylamide gel. SDS-PAGE was carried out for molecular weight determination. The same inhibitor protein (fraction-8) was redissloved in Laemmli buffer. The same protein fraction (80 µg) was loaded on 12% polyacrylamide gel. Silver staining was used to stain the electrophoresed proteins. The standard protein molecular markers were used to get exact molecular weight of proteins (range 14.4 kDa to 116.0 kDa).

Statistical analyses

The experiments were conducted in ten replicates and experimental results were mean of ± SD of ten parallel measurements. The data with respect to various parameters were processed using student *t*-test. The values for $p < 0.05$ were regarded as significant.

RESULT AND DISCUSSION

The present investigation is based on purification and characterization of alpha amylase inhibitors from *Adiantum aethiopicum* L. As the properties of plant depend on the amount and type of metabolites produced at different growth stages of life cycle, hence, fronds of *A. aethiopicum* L. were harvested at different growth stages *viz.* i) young fronds (YF), ii) mature fronds (MF), iii) mature fronds with immature sori (IM) and iv) mature fronds with mature sori (MS). The results of the crude fractions were tabulated in the table 1; where the maximum activity was recorded at IM stage.

Table 1: Crude activity of AAI at different growth stages.

Growth stage	AAI (%)
Young fronds (YF)	21.23 ± 0.27
Mature fronds (MF)	33.00 ± 0.16
Mature fronds with immature sori (IM)	45.34 ± 0.30
Mature fronds with mature sori (MS)	19.08 ± 0.19

The protein fraction showing maximum activity was dialyzed overnight against 0.15 M NaCl at 4^0C. The inhibitory activity of dialysed protein was measured in order to check effect of dialysis on activity. Further the dialysed protein sample (2ml) was applied on Sephadex G-100 column for purification. The fractions (1 to 24) at the volume of 3 ml were collected with a flow rate of 3.0 ml min^{-10}. The absorbance of each eluted fraction was measured at 280 nm on UV-VIS spectrophotometer and their protein contents were calculated. All the eluted fractions were tested for their inhibitory effect on human salivary alpha amylase

Table 2: alpha amylase inhibitory activity of the elutions from fraction 31-60%.

Elution number	Amount of proteins (µg/3ml)	AAI (%) at 100 µg
06	231.44 ± 0.11	20.93 ± 0.24
07	385.74 ± 0.23	11.26 ± 0.34
08	1276.56 ± 0.21	64.10 ± 0.15
09	257.64 ± 0.12	49.86 ± 0.14
10	200.87 ± 0.10	28.41 ± 0.23
18	181.95 ± 0.16	23.50 ± 0.19
19	202.33 ± 0.14	25.00 ± 0.13
20	181.95 ± 0.21	32.53 ± 0.32

All the elutions obtained by gel filtration were tested for their alpha amylase inhibitory activity. Among these eluted fractions, fractions 6, 7, 8, 16, 17, 18, 19 and 20 showed higher AAI activities (Table 2). Maximum inhibitory activity (55%) was observed in elution 8.

Table 3: Effect of temperature and ph on stability of elution no. 8 isolated from *Adiantum aethiopicum* L. (IM stage).

Temperature (^{0}) (at pH 7.00)	Inhibition of salivary amylase (in %)	pH (at 30 ^{0})	Inhibition of salivary amylase (in %)
30	54.76 ± 0.01	3.00	32.68 ± 0.01
40	51.59 ± 0.02	4.00	28.00 ± 0.03
50	44.71 ± 0.01	5.00	43.30 ± 0.04
60	13.76 ± 0.01	6.00	21.00 ± 0.01
70	6.88 ± 0.02	7.00	68.24 ± 0.01
80	0.00	8.00	38.44 ± 0.02
90	0.00	9.00	19.57 ± 0.06

The data tabulated in Table 3 clearly show that AAI is stable to the temperature ranging from 30^0C to 50^0C indicating that it is moderately thermo-stable. Maximum activity was recorded at 30^0C and retained about 80% activity up to 50^0C. The data pertaining effect of pH on inhibitory activity given in same table, reveal that AAI is stable under pH range 3 to 9. However, maximum activity was recorded at pH 7.

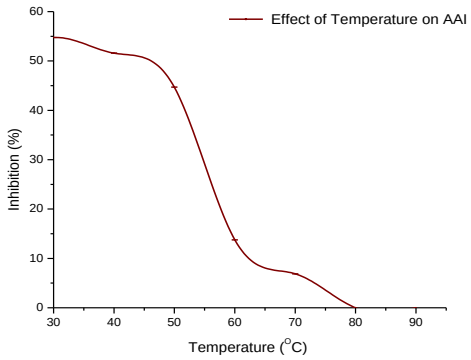


Fig. 1

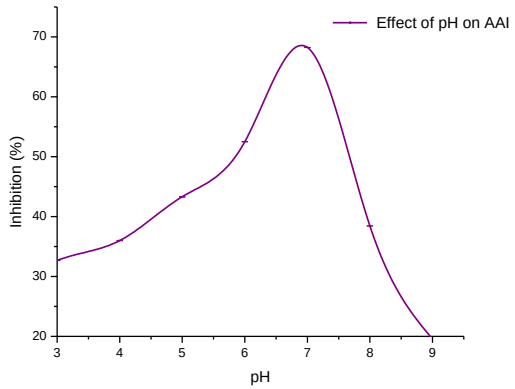


Fig. 2

Fig. 1 and 2: Effect of Temperature and pH on Human salivary amylase inhibitor.

Salivary amylase catalyses hydrolysis of 1-4 glycosidic linkage of starch and convert it into sugar before food enters in intestine. AAI are currently most common use oral agent to control sugar level in diabetic patient[3]. The results of present investigation reveal that AAI isolated from *A. aethiopicum* L. are inhibiting salivary amylase by 68.24% at pH 7.0. This is important because salivary amylase digest a portion of ingested starch in stomach before it enters the intestine and is exposed to the pancreatic amylase. The findings of present investigation are in line with the findings of Baldwa[15], Sarkar[16] and Grover[17]. They reported that bitter melon fruit contains insulin like polypeptide called polypeptide-p, plant insulin or p-insulin; which has hypoglycemic activity in humans and animal models of diabetes.

Similarly Petrucci[18] isolated AAI wheat flour and reported its inhibitory activity on human salivary amylase. Correspondingly AAI is also identified in the protein fraction obtained from *Phaseolus vulgaris* seeds. This protein binds with the active sites of alpha amylase and prevents the starch metabolizing activity; hence it is also referred as starch blocker. The alpha-amylase inhibitor from *Phaseolus vulgaris* is a protein composed of two glycosylated protomers of 30 kDa, which are cleaved into two polypeptides of 16 and 14 kDa, respectively. The protein fraction no. 8 elucidates the dimeric nature of inhibitor protein. The purified AAI protein fraction with two polypeptides that have relative mobility values 0.63 and 0.66 with molecular weights of 15.5 kDa and 15 kDa respectively as confirmed on 12 % SDS-PAGE. This result is in close agreement to that reported by Heidari[19] in seeds of *Triticum aestivum* Var. *Zarrin*.

The *Trigonella foenum-graceum* L. (fenugreek seeds), has been reported to possess hypoglycemic and hypolipidemic properties in animal experiments, as well as in human and clinical cases. The holy basil (*Tulasi* in Hindi), *Ocimum sanctum*, and *O. album* have been observed to decrease the fasting and postprandial blood and urinary glucose levels in Type II diabetic patients. The dried powder of these leaves also mildly reduced cholesterol level [3].

The results tabulated in Table 3 indicate that this protein elution is stable. This indicates that AAI isolated from *A. aethiopicum* L. is stable at lower pH similar to pH of gastric juice.

Table 4: Effect of proteolytic enzymes on stability of elution no 8 isolated from *Adiantum aethiopicum* L. (IM stage).

Incubation time	Stability of AAI to inhibit salivary amylase activity (% Inhibition)			
	Trypsin	%	Chymotrypsin	%
00.0 min	64.20	100.0	61.08	100.0
15.0 min.	64.20	100.0	61.08	100.0
30.0 min	64.20	100.0	61.08	97.0
45.0 min	53.24	82.0	53.75	90.0
60.0 min	50.92	80.0	17.58	30.0
75.0 min	42.11	70.0	0.00	00.0
90.0 min	0.00	00.0	0.00	00.0

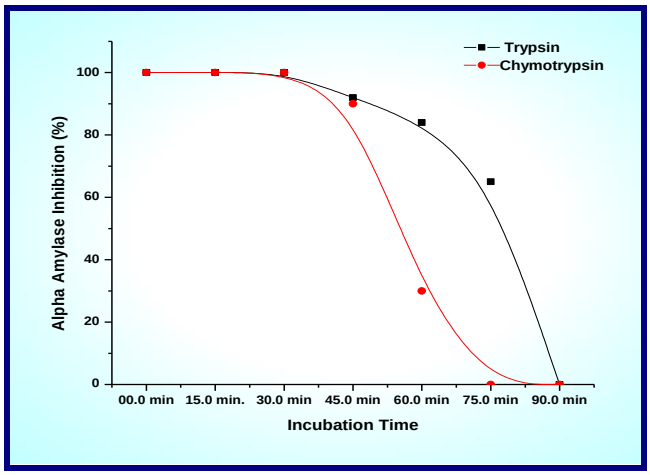


Fig. 3 Effect of proteolytic enzymes on AAI activity of protein fraction no. 8.

The AAI pre-incubated with trypsin, chymotrypsin for 15, 30, 60, and 90 min. and checked for its inhibitory activity showed that it was stable to proteolytic actions up to 75 min. and 60 min. respectively for trypsin, and chymotrypsin (Table 4). This would suggest that the disulphide bonds are present in the inhibitor protein and are important for its activity. The comparative study of extracted protein elutions offers better results than the commercial tablet Glucobay (Acarbose) manufactured by Bayer Pharmaceuticals.

Table 5: Comparative AAI activity of protein inhibitor (eluted fraction 8) isolated from *A. aethiopicum* L. with commercial drug Glucobay (Acarbose).

Product	AAI (%)
Glucobay (Acarbose)	61.40 ± 1.18
Protein inhibitor (eluted fraction 8)	64.10 ± 0.16
P (T <= t) at 5%	6.22836×10^{-6}

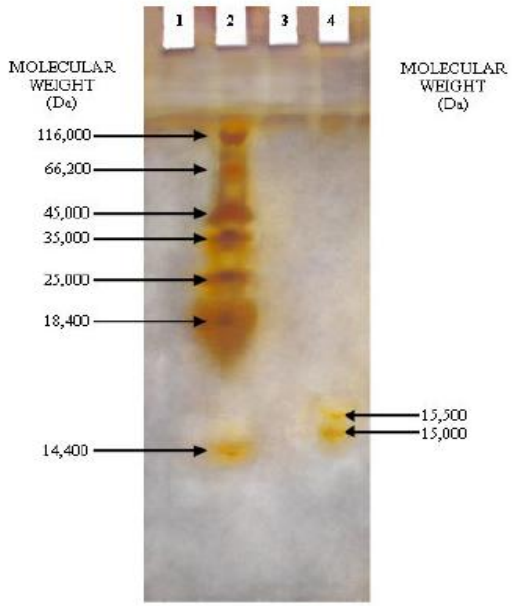


Fig. 4 SDS-PAGE analysis of eluted fraction no. 8.

Lane no. 2: Marker proteins (14.4 kDa to 116.0 kDa)
Lane no. 4: Eluted fraction no. 8

The inhibitor protein fraction was further subjected to native PAGE to check its purity. The protein appeared as single distinct band. However, when subjected to SDS-PAGE it resolved into two different bands. This indicates that the inhibitor protein is dimeric in nature i.e. made up of two polypeptides. The molecular weights of the polypeptides are found to be ≈ 15 kDa and 15.5 kDa.

CONCLUSION

The results of present investigation could suggest the use of AAI isolated from fresh fronds (IM stage) of *Adiantum aethiopicum* L. as hypoglycemic agent in non-insulin dependent diabetes mellitus and for obesity patients. It can also be an alternative drug as it possesses AAI activity more than commercial drug (Glucobay). However, the clinical value needs to be further evaluated by characterizing the toxic or undesirable effects of the inhibitor protein.

List of abbreviations

Molar : M
Sodium Chloride : NaCl
Fraction of 31 to 60 % saturation with ammonium sulphate: F(31-60)
Nanometer : nm
Alpha Amylase Inhibitors : AAI
Micro-gram : ug
Milliliter : ml
3,5 Dinitro Salicylic Acid : DNSA
Inhibitor protein : Ip
Polyacrylamide Gel Electrophoresis: PAGE
Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis: SDS-PAGE
Kilo Dalton : kDa
Standard deviation : SD
Young fronds : YF
Mature fronds without sori: MF
Mature fronds with immature sori: IM
Mature fronds with mature sori: MS

ACKNOWLEDGEMENT

We are grateful to Principal, Dr. Venkatraghvan, N. Wadia College and Principal, Dr. Ravindrasinh Pardeshi, Fergusson College for providing all necessary laboratory facilities for current investigation. We are thankful to Dr. S. S. Gadekar, Head, Department of Botany, N. Wadia College for his encouragement and support.

Conflict of interest:

Nil

REFERENCES

1. Maton A, Hopkins J, McLaughlin CW, Johnson S, Warner MQ, LaHart D, et al. Human Biology and Health. Englewood Cliffs, New Jersey, USA: Prentice Hall; 1993.
2. Ho MF, Whitaker JR. Purification and partial characterization of white kidney bean (Phaseolus vulgaris) alpha-amylase inhibitors from two experimental cultivars. J Food Biochem 1993; 17:15-33.
3. Tiwari AK, Madhusudan Rao J. Diabetes mellitus and multiple therapeutic approaches of phytochemicals: present status and future prospects. Current Science 2002; 83:30-8.
4. Garcia-Maroto F, Carbonero P, Garcia-Olmedo F. Site-directed mutagenesis and expression in Escherichia coli of WMAI-1, a wheat monomeric inhibitor of insect α-Amylase. Plant Mol Biol 1991; 17:1005–11.
5. Marshall JJ, Lauda CM. Purification and properties of phaseolamin, an inhibitor α-Amylase, from the kidney bean, Phaseolus vulgaris. J Biol Chem 1975; 250: 8030–33.
6. Teixeira CC, Fuchs FD, Blotta RM, Knijnik J, Delgado IC, Netto MS, et al. Effect of tea prepared from leaves of Syzygium jambos on glucose tolerance in nondiabetic subjects. Diabetes Care 1990;13: 907-8.
7. Arai I, Amagaya S, Komatsu Y, Okada M, Hayashi T, Kasai M, et al. Improving effects of the extracts from Eugenia uniflora on hyperglycemia and hypertriglyceridemia in mice. J. Ethnopharmacology 1999;68:307-14.
8. Kedar P, Chakrabarti CH. Effects of jambolan seed treatment on blood sugar, lipids and urea in streptozotocin induced diabetes in rabbits. Indian J Physiol Pharmacol. 1983; 27:135-40.
9. Achrekar S, Kaklij GS, Pote MS, Kelkar SM. Hypoglycemic activity of Eugenia jambolana and Ficus bengalensis: mechanism of action. In Vivo 1991; 5:143-47
10. Pepato MT, Oliveira JR, Kettelhut IC, Migliorini RH. Assessment of the antidiabetic activity of Myrcia uniflora extracts in streptozotocin-diabetic rats. Diabetes Res. 1993; 22:49-57.
11. Melo FR, Sales MP, Pereira LS, Bloch C Jr, Franco OL, Ary MB. α-Amylase inhibitors from cowpea seeds. Prot Pept Lett 1999;6:385–90.
12. Bernfeld, P. Amylase, α and β. Methods Enzymol 1955; 1:149-58.
13. Lowry OH, Rosebroough NJ, Farr AL, Randall RJ. Protein measurement with folin phenol reagent. Biochemistry 1951; 15:529-36.
14. Laemmli H. Cleavage of structural protein during the assembly of the head of Bacteriophage T_4. Nature 1970; 227:680-85.

15. Baldwa VS, Bhandari CM, Pangaria A, Goyal RK. Clinical trial in patients with diabetes mellitus of an insulin-like compound obtained from plant sources. Upsala J Med Sci 1977; 82:39-41.
16. Sarkar S, Pranava M, Marita R. Demonstration of the hypoglycemic action of Momordica charantia in a validated animal model of diabetes. Pharmacol Res 1996; 33:1-4.
17. Grover JK, Vats V, Rathi SS, Dawar R. Traditional Indian anti-diabetic plants attenuate progression of renal damage in streptozotocin induced diabetic mice. J Ethnopharmacol 2001; 76:233-38.
18. Petrucci T, Tomasi M, Cantagalli P, Silano V. Comparison of wheat albumin inhibitors of α- amylase and trypsin. Phytochemistry 1974; 13:2487-95.
19. Heidari R, Zareae S, Heidarizadeh M. Extraction, Purification and inhibitory effect of alpha amylase inhibitor from Wheat (Triticum aestivum Var. Zarrin). Pak J Nutrition 2005; 4:101-05.



