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Inhibitory potential of *Phaseolus lunatus* L. Seeds to Digestive Enzymes

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Abstract: Introduction: Study was taken with an objective to investigate the inhibitory effect of *Phaseolus lunatus* seeds extract on three enzymes (α-amylase, maltase and glucoamylase) which are involved in diabetes. The disadvantages related with the use of synthetic drugs make it necessary to search for alternative drugs from medicinal plants. One therapeutic approach for the treatment of postprandial hyperglycaemia is to decrease the plasma glucose level which can be attained by lowering the starch hydrolysis by digestive enzymes such as α-amylase, maltase and glucoamylase. The use of synthetic antidiabetic agents caused gastrointestinal side effects. **Methods:** Hence, the research was aimed to evaluate the potential of *Phaseolus lunatus* seeds extract to inhibit α-amylase, maltase and glucoamylase as a possible mechanism of hypoglycemia caused by this plant with less side effects. *Phaseolus lunatus,* also known as Lima bean, is native to Tropical America and are grown throughout the world. Lima bean has been prescribed traditionally to treat different diseases including diabetes. **Result:** The results of the present study showed that *Phaseolus lunatus* seeds extract have exerted both time and concentration dependent significant inhibitory effect on these digestive enzymes which might be due to cumulative effect of phytoconstituents present in the plant extract. **Conclusion:** Therefore, it can be said that the part of mechanisms by which *Phaseolus lunatus* exhibited its antidiabetic activity might be through the inhibition of these digestive enzyme.

Keywords: *Phaseolus lunatus*, α-amylase, Maltase, Glucoamylase, Diabetes

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Introduction

Dietary starch is the foremost reason of blood glucose in the body. Dietary starch hydrolysis is completed by a group of enzymes [1]. Among many digestive enzymes in the human body, α-amylase found in human saliva where mechanical digestion starts. Pancreas also makes α-amylase to degrade starch into disaccharides and trisaccharides. α-Amylase cleaved inner α-(1,4) glycosidic bond to yield glucose, maltose and maltotriose units as end products [2] . As only glucose can be absorb in the intestinal lumen [3], so further hydrolysis takes place by other enzymes such as maltase. Maltase act on the α-(1,4) glycosidic linkage of maltose and produce 2 units of α-D-glucose [4]. Maltase hydrolyze oligosaccharides more rapidly such as maltose and malto-oligosaccharides [5]. Another digestive enzyme such as glucoamylase also known as γ-amylase or amyloglucosidase (EC3.2.1.3) present in the intestinal brush border membrane [2, 6] prefers sequential cleavage of polysaccharide substrates and hydrolyze α-(1,4)-glycosidic linkage, while slowly catalyzes α-(1,6) bond to produce α-D-glucose [5]. This happens normally in a

healthy individual but when there is an increase in the activity of enzymes and insulin deficiency occur, causes elevated glucose level lead to hyperglycemia[7]. In post prandial hyperglycemia, there is an persistent increase in blood glucose level after taking meal [8] and prolonged postprandial hyperglycemia are the main casual factor of Diabetes mellitus (DM) [9]. To overcome this issue, slows down the digestion of carbohydrate by enzymes through a suitable inhibitor causing a delay in glucose absorption in the blood, thus reduce glucose level and treat hyperglycemia [10]. Miglitol, Voglibose and Acarbose are the synthetic enzyme inhibitors used for the treatment of diabetes but they have several gastrointestinal side effects as flatulence, diarrhea and abdominal pain [11]. Hence, these synthetic medicines become less attractive as therapeutic agent and researchers explore and identify enzyme inhibitors through natural source with fewer side effects.

Phaseolus lunatus L. belongs to Papilionaceae family and native to Tropical America and cultivated throughout the world. *Phaseolus lunatus* L. also known as Lima Bean, is perennial climbing plant. The leaves are trifloliate and fruit contains 3-4 seeds [12]. Its seeds and leaves are effective for its astringent property in Pakistan and India and can be taken as a meal for fever. Traditionally, powdered seeds are prescribed to apply over wounds and abscesses on skin for therapeutic purpose [13]. Pharmacological properties of *Phaseolus lunatus* describing its anti-ulcerative [14], hypolipidemic, hypoglycaemic, antifungal, anti-proliferative and antioxidant activity [13, 15]. Our current study is aimed to evaluate concentrations and time-dependent effect of *Phaseolus lunatus* on α-amalyse, glucoamylase and maltase enzyme and found possible mechanism of hypoglycaemic effect of *Phaseolus lunatus*.

Materials And Methods

Identification of plant and preparation of extract

Phaseolus lunatus seeds (2 kg) were procured from local market and the authenticated sample with voucher specimen number (PLS-04-15/16) were deposited. Seeds were coarsely crushed and soaked for three days in ethanol at room temperature and then filtered. The mixture then dried and concentrated at 40^{o}C by rotary evaporator. From this crude extract, different concentrations of plant extract (200,400,600,800 and 1000µg/ml) were prepared to use in enzyme assay.

EPLS=Ethanolic extract of *Phaseolus lunatus* seeds. This study was conducted in the Research laboratory of Department of Pharmacognosy and Department of Biochemistry, University of Karachi.

Production of α- amylase, glucoamylase and maltase enzyme

Crude ***α-*** amylase and glucoamylase enzymes were prepared by inoculating the fungal strain of *Aspergillus* specie, obtained from Department of Biochemistry, University of Karachi and incubated for 4 days at 30^{o}C in the medium [16] with some modifications containing (gdl^{-1}): peptone, 1.00; yeast extract, 1.00; K_2HPO_4 , 0.10; starch, 1.50; $MgSO_4$, 0.05; (pH 6.0). Cell free filtrate were obtained by centrifugation at 4^{o}C, for 15min at 10,000 rpm.

For producing crude maltase enzyme, bacterial strain of *Bacillus fermis* was obtained from Department of Biochemistry, University of Karachi. Cell culture were grown for 48 hours at 37^{o}C in modified medium [17] containing (gdl^{-1}): $CaCl_2$, 1.0; starch, 1.5; peptone 0.5; maltose, 1.0; $NaNO_2$, 1.0 (pH 7.0). Cells were centrifuged (15mint, 1000rpm, 4^{o}C) to obtain the cell free filtrate (CFF) and evaluated the effect of EPLS on these crude enzymes.

Concentration and preincubation time-dependent effect of EPLS on α-amylase

Crude α-amylase with *Phaseolus lunatus* was quantitatively analysed by modified DNS method [18]. Solutions of different concentrations (200, 400, 600, 800 and 1000µg/ml) of extract were preincubated with α-amylase for 15minutes at 37^{o}C. 1% starch solution as substrate (phosphate buffer 0.05M, pH 7) was used and maltose released was detected by adding DNS under standard

assay conditions. Absorbance was measured at 546 nm by spectrophotometer and compared with the control. Similarly, α-amylase with EPLS (200µg/ml) was preincubated at 37°C for 10, 15, 20, 25 and 30 min prior to enzyme assay. Acarbose was used as standard. The protocol was repeated thrice. α-Amylase unit is stated as ***"amount of enzyme which generate 1.0µmol of maltose per minute from the starch hydrolysis as substrate in potassium phosphate buffer of pH 7.0 at 40°C."*** One inhibitory activity of EPLS is defined as ***"the amount of inhibitor which reduced the α-amylase enzyme activity by one unit."*** Percentage inhibition of α-amylase is appended below:

$$\text{α-Amylase inhibition by } \textit{Phaseolus lunatus}(\%) = \left(\frac{EA_c - EA_t}{EA_c}\right)x100$$

Where EA_c is enzyme activity units of control and EA_t are enzyme activity units of test.

Concentration and pre-incubation time-dependent effect of EPLS on maltase

The effect of concentrations of EPLS on maltase activity was analysed by using modified method of glucose-oxidase diagnostic kit . Plant extract from each concentrations (200, 400, 600, 800 and 1000µg/ml) and maltase was preincubated at 37°C for 15min. 1% maltose solution (phosphate buffer 0.05M, pH 6.5) was added and measured the release of glucose under standard assay conditions using glucose oxidase diagnostic kit reagent. Absorbance was measured at 546 nm by spectrophotometer and compared with the control. Similary, extract (200 µg/ml) with maltase was preincubated for 10, 15, 20, 25 and 30 min at 37°C prior to enzyme assay. Acarbose was used as standard. The whole procedure was repeated thrice. Maltase unit is stated as ***"amount of enzyme that liberate 1.0µmol of glucose per minute from the maltose as substrate in potassium phosphate buffer of pH 6.5 at 40°C."*** One unit of inhibitory activity of EPLS is stated as ***"the amount of inhibitor which reduced the maltase enzyme activity by one unit."*** Percentage inhibition of maltase is appended below,

$$\text{Maltase inhibition by } \textit{Phaseolus lunatus}\ (\%) = \left(\frac{EA_c - EA_t}{EA_c}\right)x100$$

Where EA_c is enzyme activity units of control and EA_t is enzyme activity units of sample test.

Concentration and preincubation time-dependent effect of EPLS on glucoamylase

Glucose oxidase diagnostic kit procedure was applied for estimation of glucoamylase activity with EPLS [16]. Different concentrations 200, 400, 600, 800 and 1000 µg/ml of EPLS with glucoamylase were preincubated at 37°C for 15minutes. Substrate (1% starch solution) was used and prepared in 0.05M phosphate buffer (pH 7.0). Glucose oxidase diagnostic kit method was used to measure glucose which was released after starch digestion under standard assay condition and absorbance was noted at 546 nm by spectrophotometer and compared against control. Similarly, extract (200 µg/ml) with enzyme were preincubated for 10, 15, 20, 25 and 30 min at 37°C prior to enzyme assay. The whole protocol was repeated thrice. Standard drug Acarbose was used. One glucoamylase unit is described as ***"the amount of enzyme required to generate 1.0 µmol of glucose per minute from the starch hydrolysis as substrate in potassium phosphate buffer of pH 7.0 at 40°C".*** One inhibitory unit of EPLS is described as ***"the amount of inhibitor which reduced the glucoamylase enzyme activity by one unit".*** Percentage inhibition of glucoamylase was calculated by formula,

$$\text{Glucoamylase inhibition (\%)by } \textit{Phaseolus lunatus} = \left(\frac{EA_c - EA_t}{EA_c}\right)\times 100$$

Whereas EA_c is enzymatic activity of control in units; EA_t is the enzymatic activity of sample test in units.

Statistical Analysis

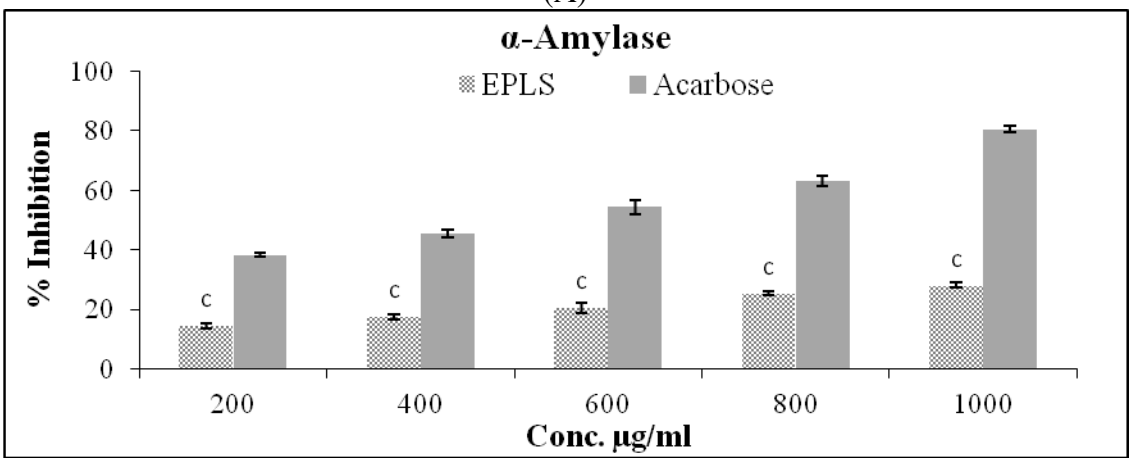
Data were repeated thrice and presented by mean ± standard error. Resultant were evaluated statistically by one-way ANOVA followed by tukey's HSD test . Values are presented as $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ (significant, more significant and highly significant respectively) using SPSS version 16.0. IC_{50} values were calculated from graph.

Results

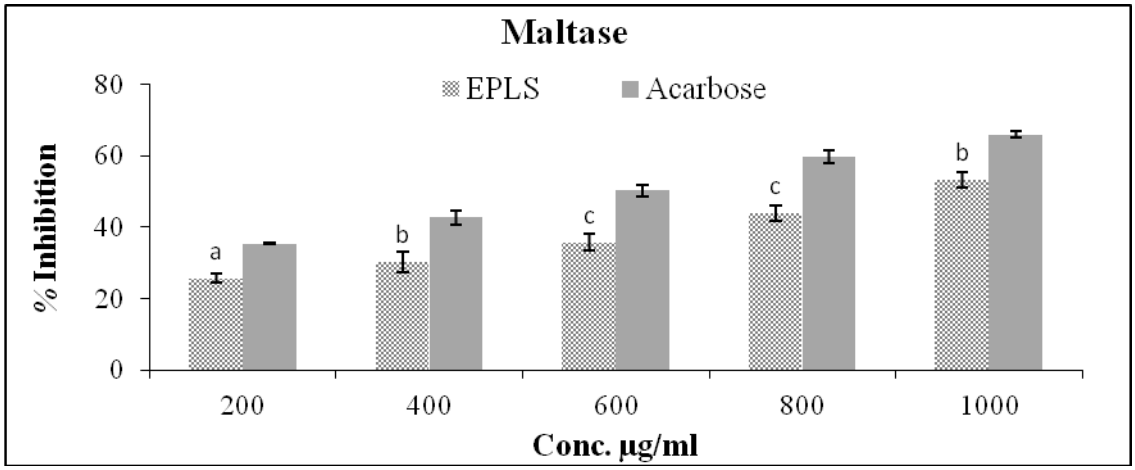
α-Amylase, maltase and glucoamylase inhibition suggested that the ethanolic extract of *Phaseolus lunatus* seeds had inhibitory activity. In this experiment, a control of all three digestive enzymes were prepared and compared the enzymatic activity with test sample (EPLS /Acarbose-standard drug) having different concentration and time-dependent effect on these enzymes.
The dose and time-dependent α-amylase, maltase and glucoamylase activities by EPLS were studied and inhibition of α-amylase, maltase and glucoamylase were increased when concentration and preincubation time of extract with these enzymes were increased.

Concentration-dependent effect: At concentration of 1000µg/ml, α-amylase, maltase and glucoamylase gave highest percentage inhibition of 28.1%, 53.23% and 51.67% respectively by extract as shown in Fig-1(a,b,c). In all concentrations of EPLS, highly significant difference ($p \leq 0.001$) in percentage inhibition of α-amylase was observed when compared by concentrations of Acarbose statistically while percentage inhibition of maltase was increased significantly at 200µg/ml ($p \leq 0.05$), at 400µg/ml ($p \leq 0.01$) more significantly and highly significant at 600 and 800µg/ml ($p \leq 0.001$). There is significant increase in percentage inhibition of glucoamylase at initial three concentrations of extract ($p \leq 0.05$) when compared with respective concentrations of Acarbose as shown in Fig-1(a,b).

(A)



(B)



(C)

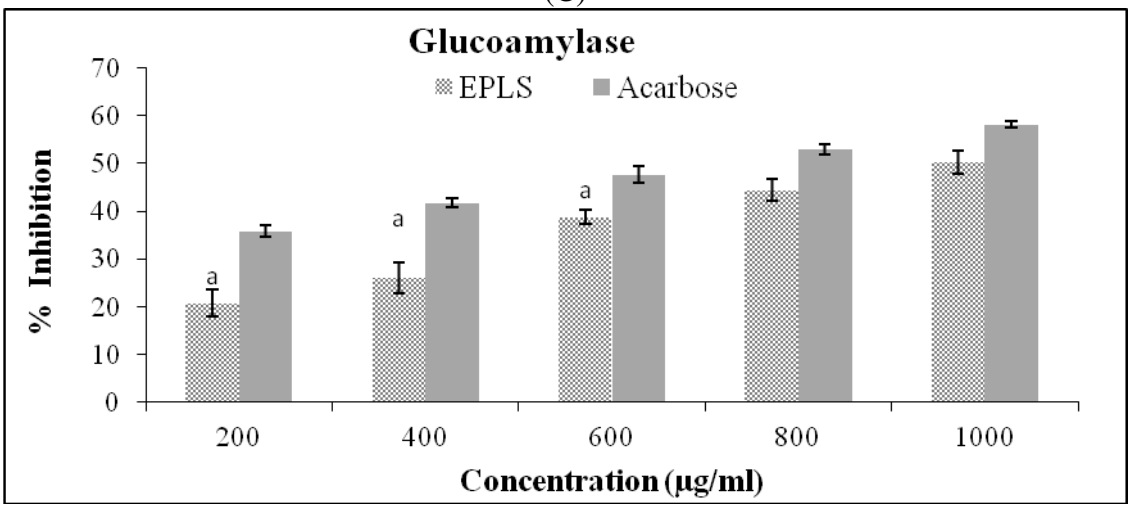


Fig-1: α-Amylase (A), maltase (B) and glucoamylase (C) percentage inhibition by different concentration of EPLS and Acarbose; carrying error bars with a = p≤*0.05* significant, b=*p≤0.01* very significant and c=*p≤0.001* highly significant difference between the concentration of extract and Acarbose. Values represent the mean ± SE (n=3).

Table-1 showed no significant decrease in units of α-amylase except at only 1000µg/ml of extract (p≤0.05), maltase enzyme units in table-2 were decreased significantly at 600 (p≤0.05), 800 (p≤0.01) and 1000µg/ml (p≤0.001) and enzyme activity units of glucoamylase were decreased at all concentration significantly but highly significant at 600, 800 and 1000µg/ml (P≤0.001) as shown in table-3 while Acarbose significantly decreased all three digestive enzymes activity units at all concentrations when compared against control.

Table-1: Units of α-amylase inhibited by concentrations of EPLS and Acarbose

Conc. (µg/ml)	**EPLS**		**Acarbose**	
	α-Amylase (Unit/ml/min)	**IC_{50} µg/ml**	**α-Amylase (Unit/ml/min)**	**IC_{50} µg/ml**
Control	343.24 ± 26.63	2232.39	-	475.26
200	293.03 ± 21.82		211.73 ± 17.12^{b}	
400	282.60 ± 21.73		186.71 ± 10.24^{c}	
600	272.54 ± 21.55		156.4 ± 14.35^{c}	
800	255.56 ± 19.21		126.4 ± 10.99^{c}	

1000	246.50 ± 19.0[a]		66.64 ± 5.37[c]	

Data presented as mean ± SE; [a] =P≤0.05 significant, [b] =P≤0.01 more significant, [c] =P≤0.001 highly significant between control and concentration of sample (extract/Acarbose).

Table-2 Units of maltase inhibited by concentrations of EPLS and Acarbose

Conc. (µg/ml)	**EPLS**		**Acarbose**	
	Maltase (Unit/ml/min)	**IC_{50} µg/ml**	**Maltase (Unit/ml/min)**	**IC_{50} µg/ml**
Control	318.47 ± 33.18	956.96	-	578.08
200	235.83 ± 20.94		205.59 ± 20.94[a]	
400	221.72 ± 23.24		183.42 ± 23.24[b]	
600	203.58±17.22[a]		159.2 ± 21.04[c]	
800	177.3 ± 13.21[b]		129.00 ± 19.22[c]	
1000	149.16 ±17.22[c]		108.84 ± 13.96[c]	

Data presented as mean ± SE; [a] =P≤0.05 significant, [b] =P≤0.01 more significant, [c] =P≤0.001 highly significant between control and concentration of sample (extract/Acarbose).

Table-3 Units of glucoamylase inhibited by concentrations of EPLS and Acarbose

Concentrations µg/ml	**EPLS**		**Acarbose**	
	Glucoamylase (Unit/ml/min)	**IC_{50} µg/ml**	**Glucoamylase (Unit/ml/min)**	**IC_{50} µg/ml**
Control	2136.2 ± 107.35	972.69	-	708.85
200	1638.7± 123.25[a]		1395.4± 82.10[c]	
400	1497.1±130.58[b]		1276.2± 62.63[c]	
600	1364.2± 93.11[c]		1145.1± 61.17[c]	
800	1210.0± 103.52[c]		1000.8±68.22[c]	
1000	1036.0± 92.51[c]		880.17± 53.18[c]	

Data presented as ±SEM; [a] =p≤ 0.05significant, [b] =p≤ 0.01more significant, [c] = p≤0.001 highly significant difference between control and concentration of sample (extract/Acarbose).

Time-dependent effect: At different time parameter, increased in percentage inhibition by extract on α-amylase was not as high as Acarbose (Fig-2a) but when compared statistically with respective time of Acarbose (10-30min), increase in percentage inhibition was highly significant (p≤0.001) at all preincubation time while percentage inhibition of maltase was significantly increased at only 10 and 15min (p≤0.05) and very significant (p≤0.01) at 25 min against Acarbose (Fig-2b). Fig-2c showed percentage inhibition of glucoamylase at 10, 15 and 20 min by extract was significant (P≤0.05) when compared against preincubation time of Acarbose.

(A)

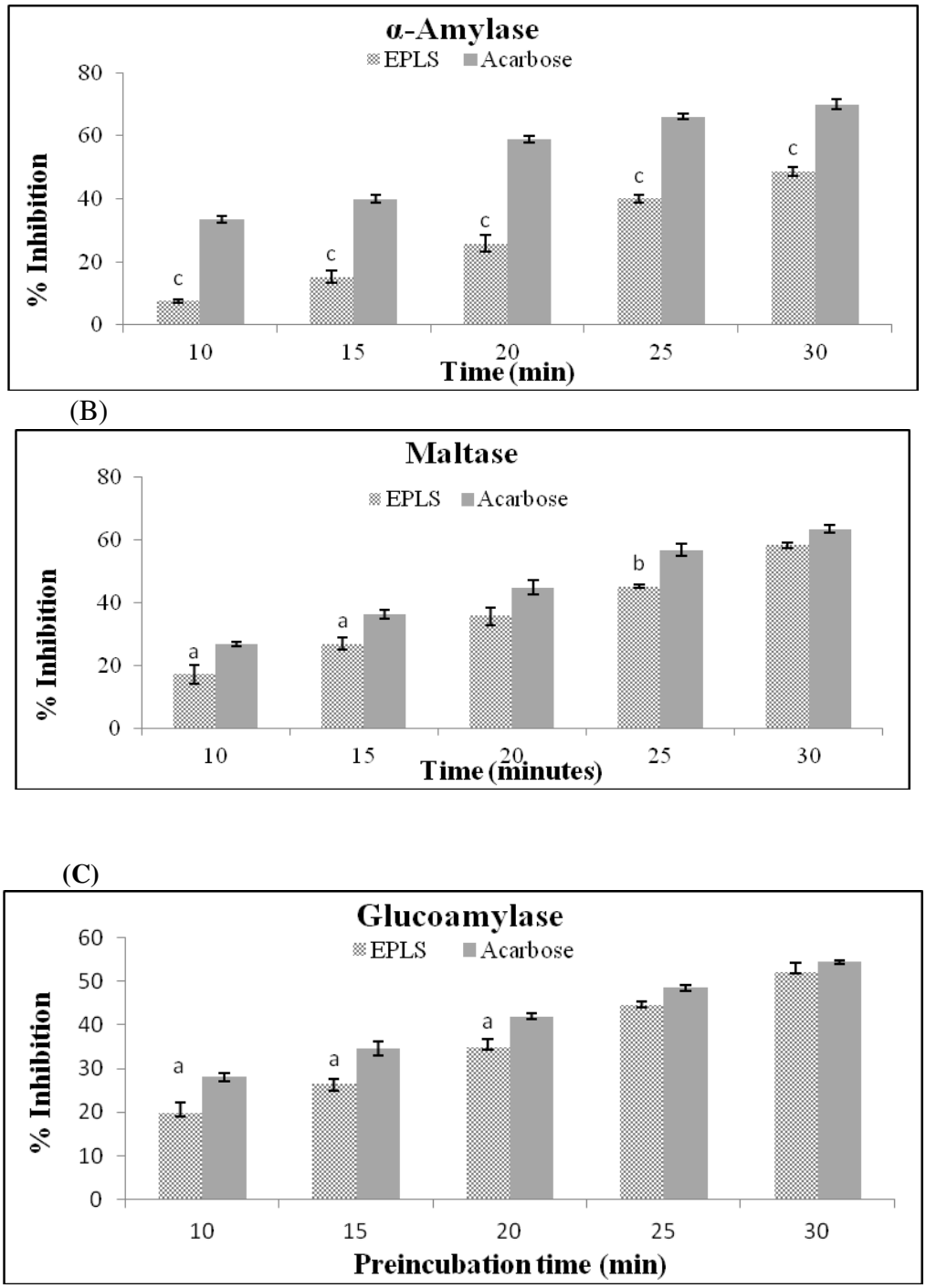


Fig-2: α-Amylase (A), maltase (B) and glucoamylase (C) percentage inhibition by different preincubation time of EPLS and Acarbose (200µg/ml each); carrying error bars with a = p≤*0.05* significant, b=*p≤0.01* very significant and c=*p≤0.001* highly significant difference between preincubation time of extract and Acarbose. Values represent the mean ± SE (n=3).

Table-4 showed that decrease in enzyme units of α-amylase was highly significant at 25 and 30min (p≤0.001) while maltase and glucoamylase units were significantly reduced by extract at all preincubation time when compared against control while Acarbose reduced all three digestive enzymes units significantly at all preincubation time.

Table-4 Units of α-amylase, maltase and glucoamylase inhibited by preincubation time parameter of EPLS and Acarbose

Time (min)	Enzymatic activities (Unit/ml/min)					
	EPLS (200µg/ml)			Acarbose (200µg/ml)		
	α-Amylase	Maltase	Glucoamylase	α-Amylase	Maltase	Glucoamylase
Control	350.87 ± 26.61	487.78 ± 17.57	2130.7± 59.32	-	-	
10	324.07 ± 24.15	403.12 ± 15.74[a]	1707.1± 78.75[c]	233.36 ± 19.95[b]	356.76 ± 15.99[c]	1533.3± 37.94[c]
15	296.99 ± 19.25	356.76 ± 21.23[c]	1564.7± 44.38[c]	211.29 ± 20.30[c]	310.41 ± 17.91[c]	1396.4± 56.61[c]
20	261.1 ± 26.05	312.42 ± 5.33[c]	1382.0± 41.68[c]	145.06 ± 14.68[c]	270.09 ± 21.04[c]	1236.2± 29.29[c]
25	210.10 ± 13.98[c]	268.08 ± 12.26[c]	1177.9± 20.99[c]	119.41 ± 11.69[c]	211.64 ± 17.45[c]	1096.0± 16.18[c]
30	179.52 ± 10.75[c]	203.58 ± 7.26[c]	1015.8± 32.94[c]	106.35 ± 13.31[c]	179.39 ± 12.26[c]	970.81± 16.62[c]

Data presented as mean ± SE; [a] =P≤0.05 significant, [b] =P≤0.01 more significant, [c] =P≤0.001 highly significant between control and concentration of sample (extract/Acarbose).

IC_{50} values

Concentration of EPLS required for 50% inhibition (IC_{50}) was 2232.39 µg/ml for α-amylase (table-1), 956.96 µg/ml for maltase (table-2) and 972.69 µg/ml for glucoamylase (table-3). IC_{50} values of EPLS for these enzymes were higher than IC_{50} values acquired by Acarbose which is 475.26 µg/ml for α-amylase, 578.08 µg/ml for maltase and 708.85 µg/ml for glucoamylase.

Discussion

Hyperglycemia is a condition in which blood glucose level is increased rapidly and is caused by the persistent carbohydrate hydrolysis through digestive enzymes. To control the postprandial hyperglycemia, the therapeutic way is to reduce the digestion of starch by inhibiting the carbohydrate hydrolyzing enzymes in the digestive tract [17].

From early times, natural compounds have been considered as the vital source of providing medicines and nowadays most of the medicinal agents are originated from natural substances and because of the accessibity of the natural compounds, low cost and less toxic medicine are prepared [18]. Research on non-conventional medicines, especially with natural medicinal products, has been acquired more importance and more space in pharmaceutical industries for investigating novel phytotherapeutics and phytomedicines as there are large number of untapped plant species, which are still needed to be explored [19]. This research has been executed in search for identifying natural medicines with minimum adverse effect, which become beneficial in the treatment or relief of hyperglyceamia, as their bioactive constituents have good antihyperglycemic activity [18].

In the present study, EPLS exhibited significant increase in α-amylase, maltase and glucoamylase inhibition with increasing concentration and time as compared against Acarbose and control so that less glucose is produced, ultimately less absorption of glucose take place thus treating increased postprandial glycaemia in blood [10] and the presence of phytoconstituents in EPLS play their role individually or synergistically as suitable inhibitor which might be accountable for antidiabetic activity. There was a significant increase in inhibition of α-amylase, maltase and glucoamylase enzyme with increasing concentration of EPLS, suggested a dose dependent effect. Similarly, when extract and digestive enzymes such as α-amylase, maltase and glucoamylase preincubation time was increased, inhibition of α-amylase, maltase and glucoamylase enzyme activity increases significantly suggested a time-dependent effect of EPLS. Extract in minimum

concentration (200 µg/ml) efficiently inhibited the enzyme when interaction time of EPLS with glucoamylase increases, lead to more inhibition with less concentration of extract. Lesser amount of glucose absorbed thus helpful in the treatment and management of hyperglycaemia and this inhibition of glucoamylase may be due to the presence of suitable inhibitor as phytochemical present in the extract. For example, researchers mentioned that the incidence of hyperglycemia can be reduced by taking phytochemicals such as plant sterols and dietary fibers in meal [20]. Polyphenols and flavonoids bind with proteins thus entirely or partly decreasing α-amylase and maltase contributing its hypoglycaemic activity [21]. Alkaloids and terpenoids have antidiabetic property [22]. Johnson *et al*., 2013 mentioned the *in vivo* hypoglycaemic activity of *Phaseolus lunatus* thus confirmed the antidiabetic activity of *Phaseolus lunatus.* It has been reported previously that 1mg/ml of total saponins extract from *Phaseolus lunatus* revealed < 40% inhibition of maltase [23] and our study showed > 50% inhibition of maltase at 1mg/ml EPLS which might be due to the synergistic effect of phytoconstituents such as alkaloids, tannins, phenols, flavonoids, saponins, sterols etc. present in the EPLS. Talukdar, 2013 reported that 1mg/ml of total phenolic content from *Phaseolus lunatus* showed 21.34% α-amylase inhibition and our EPLS crude extract showed 28.1% inhibition suggested that lima bean mildly inhibit α-amylase. In recent research, an increase in gastric emptying also observed by phenols and tannins obtained from red fruit extract which inhibited α-amylase and maltase lead to fullness and ultimately loss of weight [24] as well as binding of tannins isolated from cocoa, pomegranate, grapes and cranberries to glucoamylase, thus delaying carbohydrate hydrolysis and therefore aid in control of diabetes [25]. As these bioactive components are present in the *Phaseolus lunatus* as a suitable inhibitor of enzymes therefore EPLS may be effective in treating obesity as well as hyperglycaemia.

By calculating IC_{50} values, effectiveness of α-amylase, maltase and glucoamylase inhibition by EPLS were evaluated and showed that EPLS mildly inhibited α-amylase (2232.39µg/ml) and moderately inhibited maltase (956.96 µg/ml) and glucoamylase (972.6939µg/ml) whereas concentration of Acarbose required to inhibit 50% α-amylase, maltase and glucoamylase were 475.26, 578.08 and 708.85 µg/ml respectively. Acarbose presented strong potential of inhibition towards these digestive enzymes. Moderate potential of EPLS to inhibit enzymes have the capability to integrate into diet of patients having type II diabetes as a natural alternative therapeutic agent. Early study revealed that the phytoconstituents should mildly inhibit α-amylase and strongly inhibit maltase which are effective as antidiabetic agents because Acarbose inhibit these enzymes strongly lead to undigested dietary starch in the intestine and unusual fermentation by bacteria causing gastrointestinal disorders such as flatulence, abdominal distention, meteorism and diarrhoea. Hence, mild inhibition of α-amylase is beneficial by EPLS [26, 27]. Therefore, enzyme inhibitors from EPLS have effective hypoglyceamic activity as their bioactive compounds act individually or synergistically to inhibit α-amylase mildly and moderately inhibit maltase and glucoamylase. The inhibition shown by EPLS was less than Acarbose standard drug; though it was more than control thus, justifies its effect. More research work is required to confirm the precise mechanism of inhibition and to reveal the structure of bioactive constituents for the claimed antidiabetic activity.

Conclusion

From the results of ethanolic extract of *Phaseolus lunatus* seeds against α-amylase, maltase and glucoamylase activity, it may be concluded that compound(s) present in the extract significantly inhibited *in vitro* digestive enzyme α-amylase, maltase and glucoamylase with respect to concentration and pre-incubation time parameters and this beneficial activity can be utilize in the prophylaxis and supportive treatment of hyperglyceamia. Further work is needed to identify the exact mechanisms of inhibition by EPLS and to isolate the bioactive compounds accountable for inhibition of these digestive enzymes.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
Not applicable.

HUMAN AND ANIMAL RIGHTS
No animals were used in this study. The study on humans was conducted in accordance with the ethical rules of the Helsinki Declaration and Good Clinical Practice.

CONSENT FOR PUBLICATION
Not applicable.

AVAILABILITY OF DATA AND MATERIALS
None.

FUNDING
None.

CONFLICT OF INTEREST
The authors declare no conflict of interest, financial or otherwise.

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