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Kinetic and inhibitory effect of *Silene arenosa* extracts against pancreatic α amylase

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Abstract
Background: This study examines the inhibition of alpha amylase (α-amylase) via using *Silene arenosa* water extract. To the best of our knowledge, there are no previous studies on the α-amylase inhibition activities of *S. arenosa* extracts. For this aim, *S. arenosa* extracted with water by maceration method and their α-amylase enzyme inhibition activity was performed.
Methodology/Principal Findings: A maximum inhibition 74 and 88% by *S. arenosa* and acarbose of α-amylase activity was observed at fixed substrate concentration (20 mg). Kinetic studies indicate that the inhibition caused by *S. arenosa* was mixed type, i.e. *Km* increased from 18.7 to 22.5 mg (108.4-150.8%) while *Vmax* decreased from 0.022 to 0.004 mg (55.1-91.8%) in a concentration dependent manner. In α-amylase *K*iapp was found to increase from 5.4 to 5.05 mg (14.2–19.8%) while *Vmaxiapp* increase from 0.01 to 0.016 µg (25–100%) with increase of substrate from (10–30 mg). K_i was estimated to be 5.4 mg while the *Km* (dissociation constant of α-amylase-potato starch-*S. arenosa* complex α-amylase-*S. arenosa* complex and potato starch) was 12.5mg. The calculated IC_{50} for *S. arenosa* and standard drug acarbose against α-amylase were 55 and 44 mg, respectively at constant substrate concentration (20 mg).
Conclusions/Significance: The present results suggest that *S. arenosa* inhibit α-amylase enzyme activity and can be used as therapeutic agent for the treatment of diabetes-II.

Keywords: *Silene arenosa*, diabetes-II; α-amylase, kinetics, mixed inhibition

1. Introduction
Diabetes mellitus is a major endocrine disorder characterized by chronic hyperglycemia. It mainly affects the humans due to defects in insulin secretion or resistance [1]. In diabetes mellitus, homeostasis of carbohydrate and lipid metabolism is altered due to defects in insulin production or action. It is a major non-communicable metabolic disease involving huge healthcare cost and high mortality rate. The number of adults with diabetes was estimated to be 387 million, and diabetes alone caused 4.9 million deaths in the year 2014 [2]. In diabetic patients, postprandial hyperglycemia occurs after a meal due to glucose absorption from the gastrointestinal tract. Preventing glucose uptake in the intestines and promoting glucose uptake in tissues can control the level of blood glucose in the case of postprandial hyperglycemia, which is common for people with diabetes [3]. Pancreatic α-amylase is a key enzyme in the small intestine. These enzymes play a major role in the digestion of starch yielding glucose and maltose, leads to increased postprandial glucose levels [4]. Glycosidic linkages of α-D-(1, 4) in carbohydrates are cleaved by α-amylase to produce oligosaccharides [5]. Therefore, inhibitors of this enzyme can delay the increase in blood glucose level in people who consume carbohydrate-rich food, and keep the PPHG under control [6]. Hence, reducing the starch digestion rate by inhibition of enzymes such as α-amylase is the best way for the management of diabetes [7].
Acarbose, Miglitol, and Voglibose are the enzyme inhibitors that are currently used for controlling PPHG. Acarbose inhibits α-amylase. Though effective in controlling PPHG, these inhibitors are not desirable for long-term treatment due to their gastrointestinal side effects [8, 9]. Given the fact that about 80 % of the diabetic people are living in low- and middle-income countries [2], these drugs are expensive also. Therefore, several groups have made their efforts to find α-amylase inhibitors from plants, bacteria, marine algae, and fungi [10, 11]. Majority of them have studied the crude extracts (organic or aqueous), and some have studied pure compounds also [12, 13]. Most of the plant extracts and pure compounds were effective against α-amylase with a few exceptions being effective against this enzyme [14, 15].
This name *Silene arenosa* is the genus of *Silene* (family *Caryophyllaceae*). Critically endangered species. Two small population are known, one of which is on verge of extinction. The extent of occurrence and the area of occupancy are less than 10 km^2. Area of distribution is severely fragmented. Annual plants, 5–25 cm high. Stems and pedicels thin, rigid. Petals white or pale pink. It is distributed in Nakhichevan, North-East Anatolia, Iran.
Literature studies reveals, there is a little study of enzyme inhibition of *S. arenosa* extract and particularly α-amylase. For this reason, we planned to evaluate the *S. arenosa* for potential inhibitory activities against *a*-amylase.

2. Materials and methods
2.1. Plant collection
Leaves of *Silene arenosa* were collected from the Cantonment board dispensary, supply lines Bannu. The plant was accurately pinpoint by a taxonomists. The leaves were thoroughly washed with distilled water and were dried in a shadow at room-temperature for 22 days. The dried leaves were then crushed mechanically into fine powder by using a local grinder machine. The powder was then

subjected to further process.

2.1.1. Extraction
About 180 gm of powder was weighed using an electronic weighing machine and was kept in an air tight plastic container. Before capping the container, 960 mL of distills water was poured into the container and was sealed tightly. The container was applied on an automatic shaker machine and was randomly mixed for several hrs in order to make soluble the organic and inorganic molecules. The container was allowed to stay for 6 days.
After this time period, the sealed had been broken and the material was subjected for the process of filtration. Supernatant was discarded and filtrate was taken in a beaker and its volume was noted. The filtrate was then applied to a rotavap under reduced pressure at 50ºC than the extract was lypholysed.

2.1.2. Preparation of stock solution
About 5 mg/5mL stock solution was prepared & was further diluted into different sub-solutions i.e. 20, 60 and 100 µg/mL for various pharmacological assays. *S. arenosa.*

2.3. Anti-α-amylase activity
2.3.1. Materials
S. arenosa extract, potato starch, sodium acetate buffer, sodium potassium tartrate, 3, 5 di nitro salicylic acid, standard (Acarbose).

2.3.2. Methodology
Inhibition of α-amylase activity was determined using dinitrosalicylic acid as described before[16]. To obtained starch solution (0.1% w/v) 0.1 gm potato starch was dissolved in 100 mL of 16 mM $C_2H_3NaO_2$ buffer. To obtained enzyme solution 0.5 mg/mL α-amylase from stock (250 units/mL) was dissolved in 1 mL dH_2O. Sodium potassium tartrate and 3, 5 di nitro salicylic acid (96 mM) mixture used as calorimetric reagent. The stock solution of *S. arenosa* and standard (Acarbose) was prepared at 5 mg/5mL & was further diluted into different sub-solutions i.e. 20, 60 and 100 µg/mL for inhibitory assay. Plant extract or Acarbose (250 µL of 20, 60 and 100 µg/mL) was added to 250 µl of α-amylase (250 units/mL). The samples were pre-incubated at 25 °C for 10 min and 250 µl of 1 % starch prepared in 20 mM sodium phosphate buffer (pH 6.9) was added. The reaction mixtures were incubated at 25 °C for 10 min. The reactions were stopped by incubating the mixture in a boiling water bath for 5 min after adding 250 µL from the combine mixture of 3, 5 di nitro salicylic acid and sodium potassium tartrate. The reaction mixtures were cooled to room temperature, diluted to 1:5 ratio with water, and absorbance was measured in a spectrophotometer (double beam UV-1602, BMS-spectrophotometer) at 450 nm. The standard drug (Acarbose) served as a positive control. The experiment was repeated twice.
Percentage of inhibition of enzyme activity was calculated as

$$\% \text{ Inhibition} = \frac{Ac - As}{Ac} \times 100$$

Ac is enzyme activity standard drug (Acarbose) and As is enzyme activity of *S. arenosa*

2.3.3. Mode of α-amylase inhibition assay
Mode of inhibition of α-amylase determined as described before Ali *et al.* [17] For α-amylase, the enzyme solution (250 units/mL) was pre-incubated with plant extracts (20, 60 and 100 µg/mL), The reactions were started by adding 10, 20 and 30 mg/mL potato starch and continued at 25 °C for 10 min. The reactions were stopped by adding 0.25 mL of dinitrosalicylic acid followed by boiling for 5 min. The reaction mixtures were cooled to room temperature, diluted to 1:5 ratio with water and absorbance was measured in a spectrophotometer (double beam UV-1602,BMS-spectrophotometer) at 540 nm Double reciprocal plot (1/V versus 1/[S]) where V is reaction velocity and [S] is substrate concentration was plotted. Mode of inhibition was determined by analysing Lineweaver-Burk plot using Michaelis-Menten kinetics [18]. Michaelis constants *(*Km) were determined by two different plot of 1/V vs. 1/S Lineweaverand Burk.[18] and V vs. V/S [19, 20]. The values of inhibition constant (Ki) were obtained using Cornish-Bowden plots of S/V vs. [I]. $IC_{50,}$ was estimated at fixed substrate concentration, according to the Dixon *et al.* [21] plot using 1/V vs. [I].

2.4. Statistical Analysis
Statistical analysis was performed using one-way ANOVA, which was followed by post-hoc analysis (Duncan multiple range test). The difference was considered to be significant for $P < 0.05$.

3. Results
3.1. Anti-α-amylase activity
The extracts of *S. arenosa* were tested to evaluate their starch breakdown enzyme inhibition abilities against α-amylase. Inhibition of α-amylase activity by the *S. arenosa* and acarbose was found to be dose dependent. A maximum inhibition 74 and 88% by *S. arenosa* and acarbose of α-amylase activity was observed at fixed substrate concentration (20 mg) 'Fig 1'.

3.2. Determination of IC_{50}
The concentration of *S. arenosa* and standard drug acarbose required to inhibit 50% of α-amylase activity was found to be 55 mg/mL (Fig.2A), 44 µg/mL (Fig.2B) at constant substrate concentration (20 mg). The values are presented in Table.3.

3.3. Effects of *S. arenosa* on *Km* and *Vmax*
The effect of *S. arenosa* on *Km* and *Vmax* of α-amylase was calculated. *Km* values were increased from 18.7 to 22.5 mg (108.4-150.8%) while *Vmax* decreased from 0.022 to 0.004 mg (55.1-91.8%) (Table.1) by increase of *S. arenosa* concentration (20–100 µg/mL) for α-amylase (Fig.3).

3.4. Effects of *S. arenosa* on Kiapp and Vmaxiapp
In α-amylase *Kiapp* was found to increase from 5.4 to 5.05 mg (14.2– 19.8%) while *Vmaxiapp* increase from 0.01 to 0.016 mg (25-100) with increase of substrate from (10-30 mg) (Table.2). The values were calculated from Fig.(4A,4B and 4C).

3.5. Determination of Km
Km values for the hydrolysis of substrate (potato starch) by

α-amylase, was calculated by using Lineweaver-Burk plot.[18] and was found to be 12.5 mg (Fig.3). The values are presented in Table.3.

3.6. Determination of dissociation constants (*Ki*)

Ki (constant of α-amylase–potato starch-*S.arenosa* complex into α-amylase–potato starch complex and *S.arenosa* constant was estimated to be 5.4 mg (Fig.5) for α-amylase. The values are depleted in Table.3.

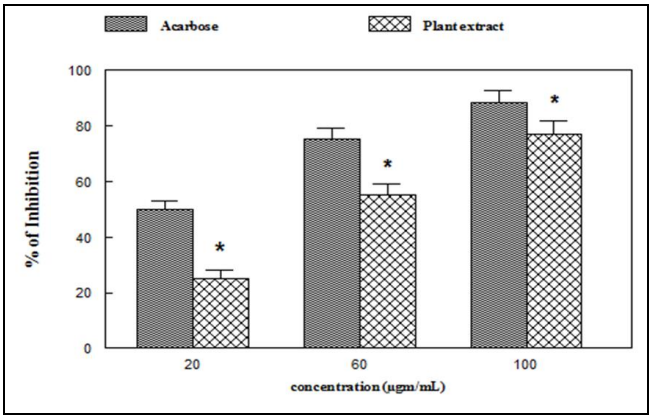
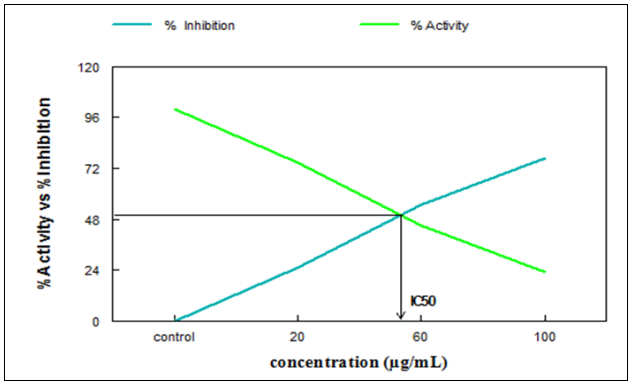


Fig 1: Concentration dependent inhibition of α-amylase in the absence and presence of water extract of *S. arenosa* were measured at 450 nm by using 20 mg substrate in 1 ml assay solutions with 20 mM sodium phosphate buffer (pH 6.9) and 96 mM combine mixture of 3,5 di nitro salicylic acid and sodium potassium tartrate was pre incubated for 10 min at 25 °C before 20 mg potato starch addition. All experiments were repeated at least two times and similar results were obtained. * P < 0.05. Significantly different from control.



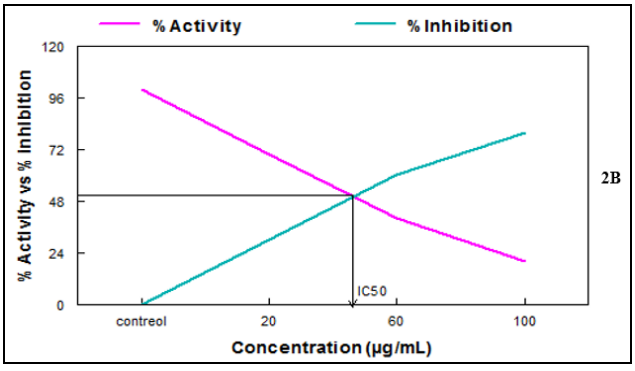


Fig 2A and 2B: A plot of the percentage residual activity in the absence and presence of water extract of *S. arenosa* and standard drug acarbose after 10 minutes incubation at 25 °C versus various concentration of *S. arenosa* extract. 30mg potato starch was used as a substrate for α-amylase. The results represent the mean of three different experiments done in duplicate.

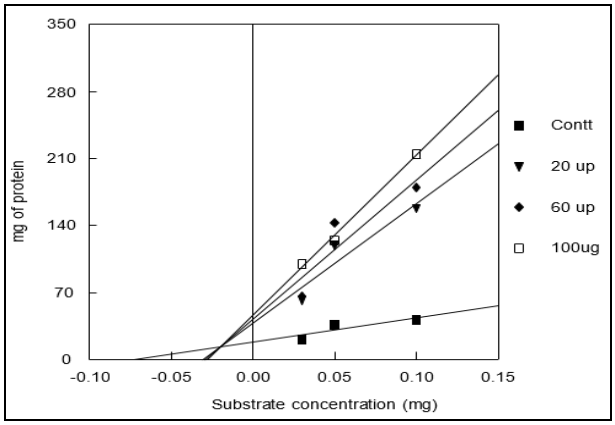
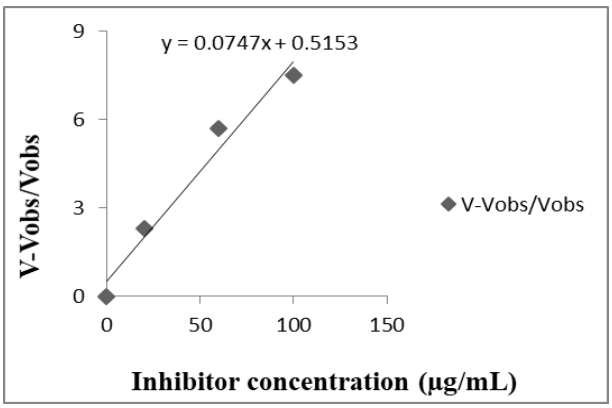
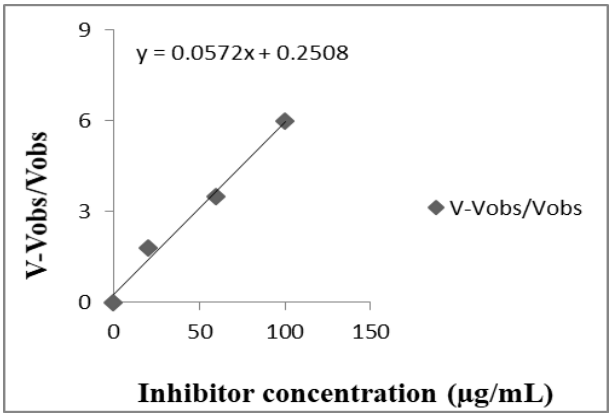


Fig 3: *S. arenosa* extract caused mixed type of inhibition of α-amylase. Data is expressed in the form of Lineweaver–Burk (reciprocal of enzyme velocity versus reciprocal of potato starch) plot. The results represent the mean of three different experiments done in duplicate by using different concentration of extract as shown in the legend boxes.





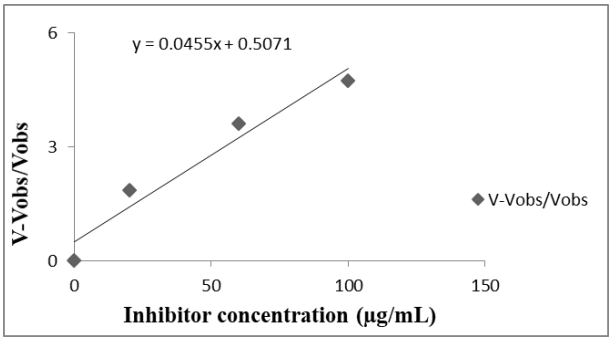


Fig 4A, 4B and 4C: Effects of *S. arenosa* on Kiapp and Vmaxiapp. Kiapp was found to increase from5.4-5.05 mg (14.2–19.8%) while Vmaxiapp increase from 0.01 to 0.016 mg (25– 100 %) with increase of substrate concentration from (10–30 mg).

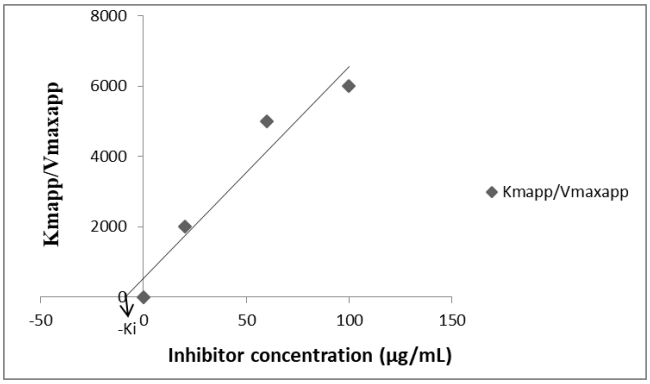


Fig 5: Determination of inhibitory constant (Ki).

Table 1: Effect of *S. arenosa* on Km and Vmax of α-amylase.

S. arenosa (µg)	*Km* (mg)	% Increase	*Vmax* (mg / min per mg protein)	% Decrease
0	8.97	0	0.049	0
20	18.7	108.4	0.022	55.1
60	20	122.9	0.005	89.7
100	22.5	150.8	0.004	91.8

Table 2: Effect of *S. arenosa* on *Kiapp* and *Vmaxiapp* of α-amylase. The *Vmaxiapp* and *Kiapp* were determined from Dixon plot of Fig.6 for α-amylase. The *Vmaxiapp* is equal to the reciprocal of y-axis intersection of each line for each potato starch concentration while *Kiapp* is equal to the x-axis intersection in Dixon plot.

Potato starch (mg)	Kiapp (mg)	% Increase	Vmaxiapp (mg / min / mg)	% Increase
10	6.3	0	0.008	0
20	5.4	14.2	0.01	25
30	5.05	19.8	0.016	100

Table 3: Comparative study of kinetic parameters of α-amylase inhibition by *S. arenosa*; K_i, inhibition constant; IC_{50}; *Km*, Michaelis–Menten constant; *Vmax* and *Vmax/Km* and of α-amylase at fixed 20 mg substrate (potato starch) concentration.

Parameters	α-amylase
K_i (mg)	5.4
IC_{50} (mg/mL)	55
Km (mg)	12.5
Vmax (mg/min)	0.0056
Vmax/Km (mg/min/mg)	0.00448

4. Discussion

4.1. Anti-α-amylase activity

Undesirable side effects and high cost of the currently available synthetic α-amylase inhibitors drive the need to explore the natural sources for new inhibitors. Being a global lifestyle diabetes that affects millions of people with diverse genetic backgrounds, the search for alternate inhibitors is also desirable from the pharmacogenetics point of view. Extracts of *S. arenosa* were reported to show anti-diabetic activities. However, α-amylase inhibiting activity was not reported for any extract from this plant. Prevention of the absorption of glucose and control of blood sugar by using the α-amylase in reducing the postprandial blood glucose level in diabetic patients is an effective treatment approach in the treatment of diabetes nowadays. A few studies have reported that α-amylase inhibitory activity of acarbose may range between 55 and 82 % depending on the experimental conditions [15, 22]. In our study 100 µg/mL acarbose and *S. arenosa* showed 47 and 88 % inhibition of α-amylase activity, respectively (Fig. 1).

The calculated IC_{50} value shows that acarbose (Fig.2A) has more inhibitory potency on α-amylase than *S. arenosa* (Fig.2A). We observed that at fixed concentration 20 mg of substrate acarbose showed 50% inhibition at concentration of 44 mg while *S. arenosa* showed same amount of inhibition at concentration of 55 mg (Table. 3).

In the present study mixed inhibition type was observed with α-amylase for *S. arenosa* (Fig.3) i.e *Km* increased from 18.7 to 22.5 mg (108.4-150.8%) while *Vmax* decreased from 0.022 to 0.004 mg (55.1-91.8%) in a concentration dependent manner (Table.1). A previous work by Poovitha and Parani [23]. has also reported the inhibition of α-amylase by *Momordica charantia* L extracts. Recent study of Eruygur and Icar [24]. Also reported the inhibition of α-amylase by *Veronica officinalis* extracts. *Km* and *Ki* (constant of α-amylase–potato starch-*S.arenosa* complex into α-amylase–potato starch complex and *S. arenosa* constant were estimated (Fig.3 and 5) and wer found to be 12.5 mg and5.4 mg (Table.3). *S. arenosa* (Fig. 3) bind other site than the active site i.e peripheral binding site or P-site. So, we propose the following scheme for the interactions between the *S. arenosa* with α-amylase.

α-amylase + *S. Arenosa* ⇔ α-amylase. *S. Arenosa* (1)

α-amylase. *S. Arenosa* + S ⇔ α-amylase. *S. arenosa*. Potato starch (2)

α-amylase. *S. Arenosa*. S ⇔ α-amylase. Potato starch + *S. Arenosa* (3)

α-amylase. S and Ch E. *S. Arenosa*. potato starch ⇔ α-amylase P (4)

α-amylase. Product ⇔α-amylase+Product (5)

Both binary (α-amylase. *S. Arenosa* or α-amylase. potato starch) and ternary (α-amylase. *S. Arenosa*. potato starch) complexes could be formed. The complex α-amylase. *S. Arenosa*. potao starch liberates the product at a lower rate, decreasing the *Vmax* in the case of the mixed inhibition type. In the present study *K iapp* and while *Vmaxiapp* were observed for α-amylase Fig. (4A,4B and 4C) i-e *Kiapp* was found to increase from 5.4 to 5.05 mg (14.2– 19.8%) while *Vmaxiapp* increase from 0.01 to 0.016 mg (25-100) (Table.2).

In conclusion, water extract of *S. arenosa* were tested for their inhibition activity against α-amylase. The plant extract exhibits higher inhibition effect. However the results demonstrated that the plant may be a good source for developing herbal formulations for diabetes-II patients. In this vein, kinetic studies with α-amylase inhibitors could be useful for novel diabetes-II purposes.

5. Acknowledgement

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