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Research Article

In vitro screening of proteinaceous amylase inhibitors (*T. castaneum* α-amylase inhibitors) in different seed extracts

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Abstract

Most of the plant defense strategies are paying attention on selection and application of the natural amylase inhibitors (AIs) against insect pests. In addition, AIs also play a crucial role in controlling postprandial plasma glucose levels. AI activity exists mainly in seeds, leaves and flowers of plants. In search of novel α-AIs, 72 different plant seed water soluble extracts were screened for their amylase inhibitory activities by using radial diffusion assays. Most of the plant seeds screened revealed amylase inhibitory activity moderate to higher activity, few showed less activity and very few of them showed very strong activity against *T. castaneum* α-amylase. The inhibitory potency of partial inhibitors was further determined by solution assays. The lowest percent inhibition was observed in *Arachishypoganea, Azadirachta indica* and *Ricinus communis* which was less than 10% among all seed extracts. The highest percent inhibition i.e., above 90% was observed in *Zea mays, Buchanania lanzan, Echinichloa colona, Albegia saman, Avena sativa* plant seed extracts. These selected plant seed extracts inhibit the activity of *T.castaneum* amylase with varying percentage.

INTRODUCTION

Proteinaceous molecules such as α-amylase inhibitors are copious in microbes, higher plants and animals (Da silva *et al*., 2000; Toledo *et al*., 2007). These living beings create large number of diverse protein inhibitors of α-amylases in order to regulate the activity of these enzymes. Like amylase inhibitors which are also well-known as starch blockers as they avoid dietary starches from being digested and absorbed by the body. This could be helpful for treating obesity and dibetes mellitus (Ali *et al*., 2006).These inhibitors are alleged to make plants less palatable, even lethal to insects, thus contributing some discriminating advantage to the plants (Sasikiran *et al*., 2002). These are naturally used by plants as a guard instrument against insect pests (Ishimoto *et al*., 1989; kluh *et al*., 2005).

As, α-amylase inhibitors are found in numerous plant seeds and tubers, being predominantly copious in cereals and legumes (Sivakumar *et al*., 2006). The privileged theory about physiological role of enzyme inhibitors in seeds is that they act as storage or reserve proteins, as regulators of endogenous enzyme or as protective agents against the attacks of animal predators and insect or microbial pests (Abdulwahid *et al*., 2012). It appears to be likely that in specific species these proteins may satisfy a mix of these capacities (Octavio *et al*., 2000; Richardson, 1991). The inhibitor prevents insect growth through the inhibition of starch

digestion in the gut. As these insects are completely reliant on α-amylases for their survival, these enzymes are good target candidate for bioinsectisides by using α-amylase inhibitors (Franco *et al*., 2002; Svensson *et al*., 2003). Also these inhibitors show enormous potential as tool to engineer resistance of crop plant against pests (Franco *et al*., 2002). The amylase inhibitors present in seeds currently used as food in few nutritional problems for healthy people but may have some toxicological in the diets of infants who have lower production of pancreatic α-amylase than adults and for patients with impaired peptic or gastric function(Brieteneder and Radauer, 2004; Richardson, 1991; Shewari *et al*., 2001).

Among several stored product insect pests, *Tribolium castaneum* (herbst)(Coleoptera: Tenebrionidae) is most destructive pest throughout the world (Pranoto, 1991).Their presence in stored foods directly affects both the quantity and quality of the commodity (Mondal, 1994).These are considered to be the most important pests of stored foods in grocery stores and in the home (Ebeling, 2002). *Tribolium castaneum,* which is nowadays one of the most resistant insects to pesticides and insecticides (Kouninki *et al*., 2007; Assie, 2007).

It becomes important to develop alternative methods for controlling this pest. The application of biological insecticides for the protection of cereals and pulses is currently one of the major foci of research in many developing countries (Ajayi, 2007).Keeping this objective in mind we screened 72 different plant seed extracts for amylase inhibitory activity and its potential.

MATERIALS AND METHODS

Collection of materials

Different plant seeds were collected from different area of Maharashtra (Nanded, Hingoli and Osmanabad districts) and some seeds of different plants were purchased from the local market, Nanded, India. These seeds were dried and used for further study. Adult *T. castaneum,* were obtained from stored products of grocery shops from Nanded (Maharashtra) and reared in laboratory conditions. All other chemicals were of AR grade procured from SD fine, Himedia etc. All solutions were prepared in de-ionized water from Milli-Q system.

Extraction of α-amylase inhibitors from seeds

Seed powders of plants were prepared by grinding matured seeds in a mortar and pestle and or mixer-blender. For extraction of α-amylase inhibitors, with some modification Guzman- Partidas (2007) method was used. The fine powder was defatted with hexane. The solvent was removed by filtration and the seed powders were air-dried. The defatted seed powders were mixed with six volumes of distilled water and kept at 4 ^{0}C for extraction with intermittent shaking by taking care, no froth was produced. After 12-14hrs, the homogenate was centrifuged at 10,000g for 15min at 4 ^{0}C. Then the clear supernatant was used for further study. Protein concentration of α-amylase inhibitors extracts was quantified by Lowry's method (1951) using BSA as a standard.

Extraction of α-amylase from *T.castaneum* larvae

For extraction of α-amylase from *T.castaneum* Applebaum and Birk (1961) method was used. The larvae were rinsed in ice-cold distilled water, placed in a pre-cooled homogenizer. These one gram larvae were homogenized separately in six volumes of distilled water in ice-cool condition. The homogenate was centrifuged at 10,000g for 20min at 4 ^{0}C and the supernatant were pooled and stored in aliquots at 4 ^{0}C for subsequent analyses.

Preliminary Screening of α-amylase inhibitors by radial diffusion assay

For screening of α-amylase inhibitor activity, the method developed by Fossum and Whitaker (1974) with some modification was used. In the reaction mixture, enzyme (10µl) was pre-incubated with inhibitor (20µl) for 30min at room temperature. For qualitative determination of α-amylase and inhibitor activities wells 7mm in diameter were made in the starch agar gel with cork borer. A fixed volume (20µl) of reaction to be tested was introduced into the wells, and then plates were covered with a tight fitting glass plate and incubated for 10-12hrs at room temperature. At the end of incubation, the starch agar plate was flooded with Gram's iodine solution and excess solution poured off. The presence of inhibitory activity was indicated by blue color around the wells because of non-hydrolysis of starch. It was compared with control, containing insect α-amylase (10µl) solution. The presence of α-amylase activity was indicated by clear zone around the well because of hydrolysis of starch. Presence of inhibitors was indicated by a narrowing of the lysis zone at points where as absence of inhibitor was indicted by clear zone around the well compared with amylase zone.

In vitro detection of percentage inhibitory activity of plant α-amylase inhibitors by Solution assay

After screening of plant species which shows partial inhibition against amylase were further studied for

percentage inhibition activity. In this, α-amylase inhibitory activity was determined according to the method described by Ishimoto (1999).With some modifications, 20 µl of enzyme (*T. castaneum* amylase) and the plant seeds inhibitors 40 µl were mixed and incubated for 30 min at room temperature followed by addition of 1ml of 0.02 M sodium phosphate buffer and1ml of 1% starch solution in all the test tubes. After 10 min the reaction was terminated with addition of 1ml of 3, 5 dinitrosalicylic acid (DNSA) color reagent, followed by boiling in water bath for 5 min. The mixture was cooled to room temperature and diluted with 3ml of distilled water and the absorbance measured at 540 nm (Schimadzu-UV-VIS Spectrophotometer). The control samples were also prepared accordingly without any inhibitor and were compared with the test samples. The results were expressed as % inhibition calculated using the formula:

Inhibition activity (%) = Abs (control) - Abs (compound) × 100 /Abs (control) (Hao *et al*., 2009).

Determination of protein

The protein concentration of crude alpha amylase inhibitor was determined by Lowry's method (1951) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Many of the natural plant compounds and organic compounds used in the control of insect pests are known to affect digestive enzymes. Secondary organic compounds synthesized by plants have an important role in protecting plants against insect pests (Athanassiou *et al*., 2005; Pare and tumlinson, 1999). These compounds affect insects by causing a delay in larval growth and can act as antifeedant (Shekari *et al*., 2008).

Enzyme inhibitors from plant source can act as growth inhibitors of insects and hence, the genes of these inhibitors can be used for increasing the resistance of cereals to store grain pests (Pueyo *et al*., 1995). Some phytochemicals function as natural antifeedants. In integrated pest management, other than mortality of the target pest, the antifeedant and growth inhibiting activity of the insecticide is also important (Erturk *et al*., 2004). Use of enzyme inhibitors for the control of stored grain pests is a safe method as these inhibitors have been present in the human foods without causing any detrimental effect on the human beings (Hubert *et al*., 2007). The α-amylase inhibitor is not a contact poison and is involved in the impaired carbohydrate metabolism of the insect pests; it is thus of utmost importance that the inhibitor should not show repellent activity as it has to be ingested to show its effect.

In the present study 72plant seed extracts were screened for amylase inhibitory activity by radial diffusion assay. Amylase activity of *T.castaneum* was readily observed by the presence of a clear lysis zone around the well following the treatment of the starch-agar plate with Gram's iodine (Fig.1).Presence of inhibitors was indicated by a narrowing of the lysis zone around the well (Fig.1a, b).Even as, absence of inhibitors was indicated by comparing with lysis zone of amylase activity (Fig.1c). These activities were grouped under three categories as inhibitor (I), partial inhibitor (PI) and no inhibitor (NI) (Table-1).Out of 72 samples of seeds of plants, about 19.44% samples show no inhibitory activity against insect amylase (Table-1).Whereas most of the samples showed low to moderate partial inhibitory activities against tested amylase. Among these, two seed samples namely *kidney bean, Amaranthus viridis* showed complete inhibition against *T.castaneum* amylase by radial diffusion assay. Among 58 seed samples (PI), showed moderate to higher partial inhibitory activity against insect amylase (Table-1).

Usually seed extracts of plants showed more inhibitory activity than leaf and flower tissues and this may be due higher accumulation of proteins in seeds than leaves and flowers (Ambekar *et al*., 1996; Giri and Kachole, 1998). Hence, the above amylase and amylase inhibitor activities can be determined quantitatively by appropriate modifications of the Bernfeld procedure. The current result showed that all the plant seed extracts had inhibitory activity varying from nearly 4.8 to 99% inhibition by solution assay. The lowest percent inhibition was observed in *Arachishypoganea, Azadirachta indica* and *Ricinus communis* which was less than 10% among all seed extracts (Fig.-2) and 20.68% were shown negligible inhibitory activity (data not shown).The highest percent inhibition i.e., above 90% was observed in *Zea mays, Buchanania lanzan, Echinichloa colona, Albegia saman, Avena sativa* which is shown in figure-2. The extracts possess activities in the range between 51.83 to 98.78% which are considered to have strong inhibitory activity.

Many studies report the AAIs in the seeds where they are present for seed protection. Several α-amylase and proteinase inhibitors present in seeds and vegetative organs act to resist phytophagous insects (Konarev 1996; Chrispeels *et al*. 1998; Gatehouse and Gate- house, 1998;

Wisessing *et al*. 2010). Like, Pea and Azuki transgenic plants expressing α-Amylase inhibitors from common beans(α-AI) were completely resistant to the *Bruchus pisorum* and *Callosobruchus chinensis* weevils (Morton *et al*., 2000). Rye α-amylase inhibitor expressed in transgenic tobacco seeds (*Nicotiana tabacum*) caused 74% mortality in *Anthonomus grandis* first instar larvae when transgenic seed flour mixture used in artificial diet (Dias *et al*., 2010).

So, α-amylase inhibitors are attractive candidates for the control of seed weevils as these insects are highly dependent on starch as an energy source (Franco *et al*. 2000; Nagy-Gasztonyi *et al*., 2010). For weevil control, the members from α-amylase inhibitor family could be used by plant genetic engineering such as transgenic (Prescott *et al*., 2005). Many insects have several α-amylases that differ in specificity, and successful utilization of a food source is dependent on the presence of α-amylase for which there is no specific inhibitor (Silva *et al*. 2000; Bonavides *et al*. 2007).

The earlier study report only a few α-AIs from seeds against *T.castaneum* amylase (Inamdar and Nasreen, 2015; Khan, 2011, Franco *et al*., 2002, Rourke *et al*., 2001). So, the obtained results revealed that the amylase inhibitory activity from different plant seeds varied on *T.castaneum*. Further analyses are needed to determine how these extracts or their bioactive substances can be used in an integrated pest management based strategy. Besides, these outcomes could be helpful in the pursuit of fresher, more particular and biodegradable insecticidal mixes. Use of enzyme inhibitors for the control of stored grain pests is a secure method as these inhibitors have been present in the human foods without causing any harmful effect on the human beings (Hubert *et al*., 2007). In vitro, some of the proteinaceous amylase inhibitors were demonstrated to substantially reduce postprandial increases in the plasma concentration of glucose and insulin in both normal and diabetic patients (Layer *et al*., 1986, Koike *et al*., 1995, Ponnusamy *et al*., 2010).

The outcome of the present study is that all experimental seed samples show potent insect amylase inhibitors. These inhibitors block the activity of *T. castaneum* amylase with varying percentage. Based on these data, further study of the inhibitors including its purification and characterization and insecticidal properties could lead to finding of new agents for control of stored products pest. Furthermore this inhibition potential may be useful in drug design for treatment of diabetes obesity.

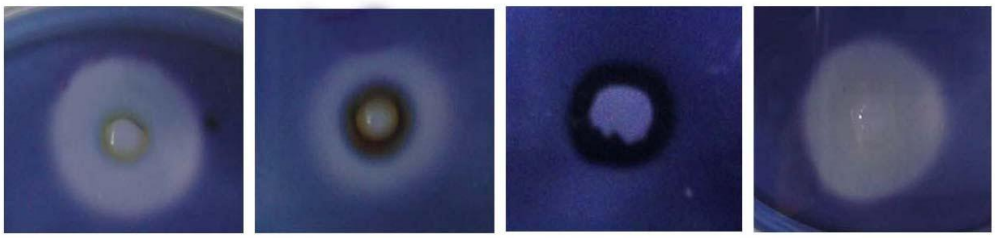

Fig.1 Fig.1 a Fig.1 b Fig.1c

Figure-1: Qualitative screening of α-AI from different seeds, - 1:*T. castaneum amylase*, 1a: Partial Inhibitor, 1b: Inhibitor, 1c: No inhibitor.

.	Sample no.	Common name	Botanical name	Inhibition
	1	Jawari	*Sorghum vulgarize*	PI
	4	Hulaga	*Dolichos lablab*	PI
	5	Kadol	*Sorghastrum nutans*	PI
	6	Bajari	*Penisehum typhoidesx*	PI
	8	YellowMaize	*Zea mays*	PI
	9	White Maize	*Zea mays*	PI
	13	Tur	*Cajanus cajan*	PI
	17	Moong	*Vigna radiate*	PI
	22	Udid	*Phaseolus mungo*	PI
	25	Masoor	*Lensculinaris syn*	PI
	27	Chawali	*Vigna ungiculata*	PI
	28	Soyabean	*Glycine max*	PI
	30	Mataki	*Vigna aconitifolia*	PI

	31	**Watana**	***Pisum sativum***	**PI**
	32	**Jawas**	***Linum usitatissium***	**PI**
	33	**Mohari**	***Brassica junesia***	**PI**
	34	**Karal**	***Helianthus annus***	**NI**
	35	**White teel**	***Sesamum tintorius***	**PI**
	36	**Black teel**	***Sesamum tintorius***	**PI**
	37	**Groundnut**	***Arachishypoganea***	**PI**
	38	**Karadi**	***Carthamus tinctorius***	**PI**
	39	**Kapalphooti**	***Cardiospermu helicacabum L.***	**NI**
	40	**Arandi**	***Ricinus communis***	**PI**
	41	**Khuskhus**	***Papaver somniferum L.***	**PI**
	42	**Karanja**	***Pongamia pinnata***	**NI**
	43	**Bibba**	***Semecarpus anacardium***	**PI**
	44	**Kaju**	***Anacardium occidantalee***	**NI**
	45	**Badam**	***Prunus amygdalis***	**NI**
	46	**Pista**	***Pistacia vera***	**PI**
	47	**Charoli**	***Buchanania lanzan***	**PI**
	48	**Rajma**	***kidney bean***	**I**
	49	**Tandool**	***Oryza sativa***	**PI**
	50	**Bhagar**	***Echinichloa colona***	**PI**
	51	**Ova**	***Trachyspermum ammi***	**PI**
	55	**Vilaichi**	***Elettaria cardamomum Maton***	**PI**
	56	**Masala vilaichi**	***Eleltaria cardamum***	**PI**
	57	**Nakeshwar**	***Mesua ferrea linn.***	**PI**
	58	**Shahajire**	***Bunium persicum***	**PI**
	59	**Karnaphool**	***Crinum brachynema***	**NI**
	60	**Lendipimpali**	***Piper iongum***	**PI**
	61	**Rajgira**	***Amaranthus viridis***	**I**
	62	**Tarbooj**	***Ctrullulus lanatus***	**PI**
	63	**Khulkhula**	***Crotalaria spectabilis***	**PI**
	64	**Ambada**	***Hibiscus cannabinus***	**PI**
	66	**Shepoo**	***Anethum graveolens***	**PI**
	67	**Bhopala**	***Cucurbita pepo***	**PI**
	69	**Bel**	***Aegle mormelos***	**PI**
	70	**Gulmohar**	***Delonix ragia***	**PI**
	71	**Raintree**	***Albegia saman***	**PI**
	72	**Kardali**	***Canna indica***	**NI**
	73	**Shirish**	***Albegiaegia lebeca***	**PI**
	76	**Chinchoke**	***Tamarindus indica***	**PI**
	77	**Chana**	***Cicer arientum***	**PI**
	79	**Dhane**	***Coriandrum sativum***	**PI**
	80	**Shewaga**	***Moringa oleifera***	**PI**
	82	**sadhi babhool**	***Acacia arabica***	**PI**
	83	**Ashoka**	***Polyalthia longifolia***	**NI**
	85	**Kadulimb**	***Azadirachta indica***	**PI**
	86	**Kadhipatta**	***Murraya koenigii***	**NI**
	87	**Cotton**	***Gossypium herbaceum***	**NI**
	88	**Agnishikha**	***Caesalpinia pulcherrima***	**NI**
	92	**Aamla**	***Emballica officinallis,Linn.***	**PI**
	93	**Seetaphal**	***Annona squamosa***	**PI**
	95	**Tumde**	***Langenaria siceraria***	**NI**
	96	**Ramphal**	***Annona reticulata***	**PI**
	97	**Mehendi**	***Lawsonia inermis***	**PI**
	98	**Vilayati chinch**	***Pithecellobium dulce***	**PI**
	99	**Gokarna**	***Clitoria ternatea***	**NI**
	100	**Kharbooj**	***Cucumis melo***	**NI**
	101	**Santri**	***Citrus aurantium***	**PI**
	102	**Mosambi**	***Citrus lemmetta***	**PI**
	104	**Oat**	***Avena sativa***	**PI**

Table-1: Radial diffusion assay for AI activity of seed samples of different plants [PI-Partial inhibitor (Narrowing hydrolysis zone), I-inhibitor (No hydrolysis zone), NI- No inhibitor (Hydrolysis zone)].

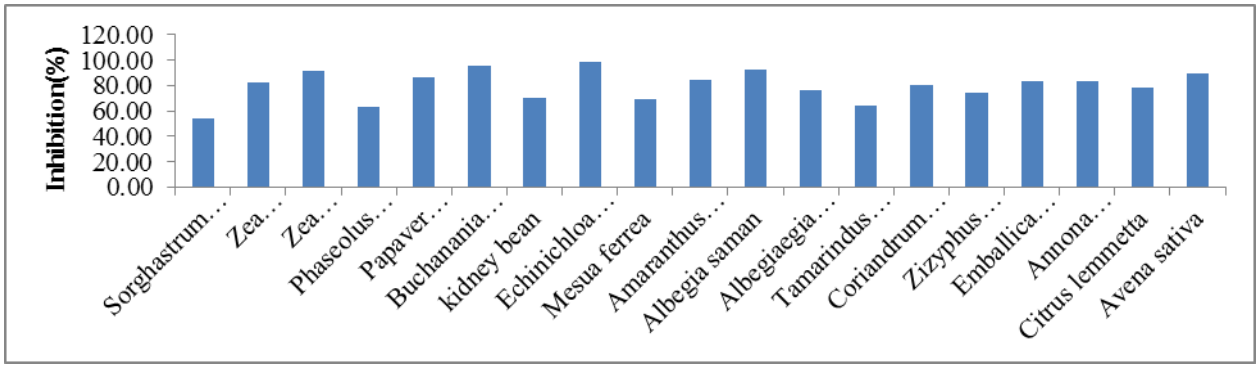

Figure-2: Inhibition of *Tribolium castaneum* amylase activity (above 50% inhibition) by different selected amylase inhibitors.

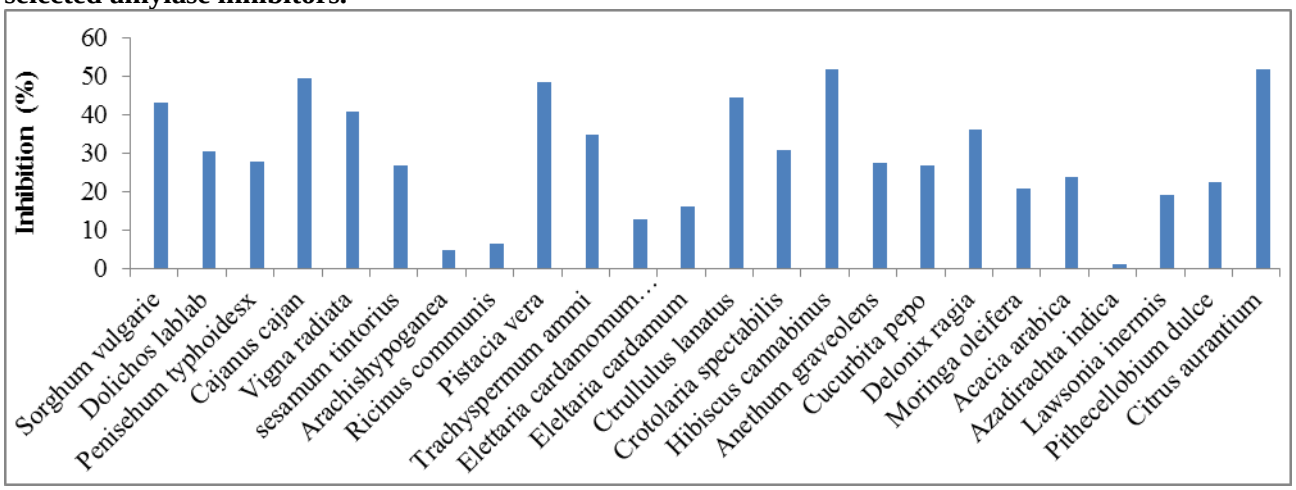

Figure-3: Inhibition of *Tribolium castaneum* amylase activity (below 50% inhibition) by different selected amylase inhibitors.

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