Journal of Qassim University for Science
2023, *Vol 2, (*Issue 2), pp. 1-16
Received: 2 June 2023
Revised: 11 June 2023
Accepted: 12 June 2023

JQUS

Biochemical Characterization of α-Amylase Inhibitor Extracted from *Saussurea Costus*

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***Abstract*:** A wide variety of diseases can be treated effectively and safely with many plant-based remedies. In the current study, *Saussurea Costus* was considered due to its medicinal benefits. The main objective of the current study was enzymatic characterization of a potent proteinaceous amylase inhibitor from *Saussurea Costus*. The α-amylase inhibitor was aqueous extracted (with an inhibitory activity of 80%). The optimum activity was observed at pH 7 in 0.1 M phosphate buffer at 80°C. Thermal stability was notably significant, with inhibitory activity maintained at up to 80% after 6 h of incubation at 100°C. The fact that the *Saussurea Costus* amylase inhibitor seems to have the greatest affinity for human salivary and pancreatic -amylase is indicative of the action of the current purified inhibitor against amylase derived from various sources.

Keywords: Biochemical Characterization, α-Amylase Inhibitor, *Saussurea Costus*, Antifungal, Antidiabetics.

1. Introduction

Amylase is a type of digestive enzyme that is produced primarily by the pancreas and salivary glands and is found in trace amounts in various tissues [1]. This was described in the 1800s and was found to be among the first enzymes scientifically examined in history. The primary function of amylase is to catalyze starch hydrolysis into energy and sugars. Amylase also hydrolyzes the glycosidic bonds; thus, transforming carbohydrates into sugars in starch molecules. This type includes three main classes, which are Alpha, Beta, and Gamma, all of which function within carbohydrate molecules [2].

Salivary α-amylase is a significant element of human saliva, playing an important role in digesting starch and might be involved in colonizing bacteria in the formation of early dental plaque. Salivary amylase further includes a site for attaching to enamel surfaces and provides a potential area for bacterial binding [3]. Rosenblum et *al*., [4] showed that salivary amylase has a short contact period with starch when the food is broken down with enzymes and gastric juices [5].

An enzymatic gap of starch brings out a significant reduction in viscosity and length of glucose-polymer after comparatively few glycosidic bonds have been divided. These alterations in viscosity can have a crucial role in deciding the preference for liking food [6].

Amylase inhibitors are identified as starch blockers as they comprise substances that prevent dietary starches from getting absorbed. Starches are complex carbohydrates that are difficult to absorb until they are broken down by amylase (digestive enzyme) and other similar types of enzymes [7]. Amylase inhibitors are found to be helpful for weight loss; nevertheless, they were recognized years ago, and investigations did not show them to be effective in reducing carbohydrate absorption. Nevertheless, highly concentrated amylase inhibitor versions show a potential decline in the absorption of carbohydrates in humans [8].

The inhibitors of α-amylase play an essential part in inhibiting pancreatic activity and salivary amylase. These inhibitors have been partially described in various varieties, including black kidney beans, red and white beans.

The content of α-amylase inhibitors vary largely among legumes, with a high percentage found in dry beans [9]. They can affect the metabolism and growth of animals, which, given in high doses, may benefit diabetes or obesity treatment as well [10]. They are usually present in high numbers in *Phaseolus* species. Nevertheless, the levels in lentils and peas are low and unlikely to add considerably to the effects of legumes on metabolism. Additionally, it is noted that α-amylase inhibitors (peptides and proteins) target enzymes from several organisms feeding on plants, mammals, insects, and bacteria [10].

Amylase inhibitors reduce insulin response and post-prandial levels of blood glucose to dietary carbohydrates [11] The drop in the uptake of carbohydrates because of amylase inhibitors will result in a lower intake of calories, thereby aiding in weight loss [11] Similarly, their use has been shown to benefit the treatment of cardiovascular diseases, obesity, and diabetes; however, the long-term effects of amylase inhibition are still being researched [11]. Azzopardi et *al*. [12] add that amylase inhibitors are used and identified clinically for treating a variety of human-associated diseases and are used in surgery as well. While the physiologic pH

range of blood is between 7.35 to 7.45, the ideal pH for amylase is between 6.7 and 7.0, which would be predictable in pathophysiologic states like solid tumors and infection.

According to Bashary et *al* [13] the alpha-amylase inhibitor is an ideal target for antidiabetic activities to provide an alternative approach for type 2 diabetes mellitus treatment and drug design. Primarily, this inhibitor is divided into two, proteinaceous inhibitors and non-proteinaceous inhibitors. The latter inhibitor is recently being explored, i.e., benzothiazoles, flavones, and chalcones as potent antidiabetic agents. Furthermore, studies have shown that amylase inhibitors can be used to treat cardiovascular and obesity diseases in addition to diabetes [11].

Saussurea is a genus in the plant family of Asteraceae with about1000 genera and about 30,000 species. The *genus* itself comprises 300 species, and *Saussurea Costus* is among the most important ethnomedicinal species [13] The species is synonymous with *Saussurea lappa* and *Aucklandia Costus*. This edible plant helps in the treatment of many diseases [14]. *S. Costus* roots have been widely researched with a potential indication for medicinal importance (in Figure 1).

Figure 1: Plant and Root of S. Costus

Traditionally, *S. Costus* has been characterized for its antidiabetic, anti-inflammatory, free radical scavengers, antioxidant, antigout, antibiotics, and anti-cholinesterase potential [14-21]. It shows the significant composition of plant species with important bioactive constituents [14].

The primary objectives of this study are to aid in the scientific valorization of Saudi pharmacopoeia plants and to look for novel natural compounds with therapeutic potential for modern illnesses like diabetes. We successfully purified and characterized the -amylase inhibitor that was isolated from the roots and leaves of S. costus. Furthermore, the efficacy of the inhibitor was confirmed through the inhibition of human salivary and pancreatic amylases. as well as potential methods for their widespread use in the control of postprandial blood sugar through imit the activity of carbohydrate digestive enzymes in intestinal tract. α-Amylase is the key enzyme that degrades the polymeric substrate into shorter oligomers by catalyzing the hydrolysis of α-1,4-glucan linkages present in starch, maltodextrins, and other related carbohydrates

2. Materials and Methods

Screening of amylase inhibitor activity

There are more than 200,000 secondary metabolites, of which more than 1200 exhibit hypoglycemic activity [22]. Thus, a number of groups, such as alkaloids, saponins, flavonoids, glycosides, polysaccharides, peptidoglycans and others obtained from various plant sources, appear to have effects of particular importance in the treatment of diabetes mellitus [23].

Polypeptides and amino acids exert an excellent effect in the treatment of diabetes. Among these components are *p*-insulin isolated from ginseng glycopeptides, *Momordica charantia*, α-methylene-cyclo-propyl-glycine isolated from *Litchi chinensis,* and S-allyl-cysteine-sulfoxide isolated from *Allium sativum* [24].

Saussurea Costus is a popular plant that has various medical uses including the treatment of inflammatory diseases and stomach problems [10]. The leaves and roots of this plant are the main substance required for completing our study [19]. After these leaves have been obtained, the first step of the experiment will include the aqueous extraction of the amylase inhibitor.

In fact, the most commonly used solvent for extracting plants is water. Inexpensive, non-polluting and non-toxic are all advantages that favor the use of water over other alternatives and thus this explains why it's the most commonly used solvent for plant extraction [25].

Initially, we began our study by collecting information on antidiabetic plants used by the local population of the city of Qassim. This survey allowed us to identify ten plants. The names of the plants as well as the parts used of each plant are summarized On table 1.

Table 1: The names of plants and the different parts used for extraction.

Plant systematic name	**Part used**
Salvia officinalis	Leaves
Trigonella foenum-graecum	Seeds
Zingiber officinale	Rhizome
Curcuma longa	Rhizome
Allium sativum	Cloves
Syzygium aromaticum	Cloves
Matricaria chamomilla	Flowrs
Cinnamonum zeylanucum	Barks
Linum usitatissimum	Seed
Saussurea Costus	Roots

Assuming that the active principles are polar compounds, their extraction was done first with distilled water. The parts used were crushed manually, sifted to remove large seeds. Approximately 1 g of each powder was obtained is macerated in 10 mL of distilled water for overnight. After filtration and centrifugation (12,000 rpm, 15 min, 4°C), the crude extracts were stored at a temperature of 4°C.

Effect of temperature on the activity and the stability of the amylase inhibitor

Temperature is a primary element that influences the amylase inhibitors' activity and stability. Many inhibitors react differently at varying levels of temperature [15]. Thus, it is essential to identify the ideal levels of temperature for the inhibitors to improve their activity or inactivity areas. Therefore, this study aimed to examine the activity of amylase inhibitors at varying temperatures.

Thus, the optimum temperature for amylase inhibitor activity was determined by performing the enzyme assay at different temperatures (20–100 °C) at pH 7. In addition, the thermal stability of the inhibitor was also studied by incubating protease inhibitor at pH 7 with exposure to different temperatures ranges from 30-100 °C over different time points (15 minutes, 30 minutes, 1, 2, 3, 6, 9, 12, 15 hours). and According to the standard assay method, the residual activity over time was measured and expressed as a percentage of the control . Residual amylase inhibitor activity was expressed as a percentage of the control. For α-amylase inhibition, the unhydrolyzed isolates inhibited by 8.08% to 13.35% with the concentration increased from 2.5 to 7.5 mg/ml.

Aliquots (1.0 ml) of the protease inhibitor solution in the presence and absence of 10 mM M-ME were placed in screw-cap flasks (10 × 45 mm Pyrex with the lined lids. All flasks were placed in a boiling water bath at the same time. A study was performed at pH 7 for 250 minutes with one vial of each solution removed from the boiling water bath and subsequently cooled on ice at 30-minute intervals. This step was followed by centrifugation at 10,000 rpm for 15 min and residual protease inhibitor activity was expressed as a percentage of the control as described above.

Effect of *Saussurea Costus* amylase inhibitor on human Pancreatic and Salivary amylases

This is the focus and main objective of the study. So an experiment was conducted to test the effect of Saussurea premium on human pancreatic and salivary amylase. The Enzymes ere inactivated in the presence of a purified *Saussurea Costus* amylase inhibitor by adding 0.25 mg/mL of the inhibitor to the corresponding reaction mixture, pre-incubating for 30 minutes, and then measuring the remaining enzyme activity. The amylase inhibitory activity of each enzyme is measured in percentage of inhibition.

3. Results

Screening of amylase inhibitor activity

Saussurea Costus is a plant of Indian origin known since ancient times and used in ancient medicine, and it has many therapeutic benefits, *Saussurea Costus* roots and leaves use several medical conditions including management of diabetes patients especially type II [21].

In the present study, ten medicinal plants widely used for antidiabetic purposes in Saudi Arabia were screened for their α-amylase inhibitory potential. The herbs used in the study as well as the parts used have been listed on (table1). Several studies performed on these herbs claim them to be hypoglycemic [21], but none of these

herbs have been studied or tested for human salivary or pancreatic α-amylase in order to justify their hypoglycemic properties.

An aqueous extraction of the amylase inhibitor was carried out, according to the protocol indicated in the material and method part, on all the medicinal plants. Primary screening for α-amylase inhibition was carried out by the chromogenic DNS method The α -amylase activity is measured using a colorimetric method with 3,5-dinitrosalicylic acid (DNS) reagent. In this method, starch by α – amylase is converted into maltose. Maltose released from starch is measured by the reduction of 3,5-dinitrosalicylic acid Maltose reduces the pale yellow coloured alkaline 3, 5-Dinitro salicylic acid (DNS) to the orange- red colored. The intensity of the color is proportional to the concentration of maltose present in the sample revealed that 7 extracts exhibited weak inhibitory activity (<10%) (Figure 2), while 2 extracts exhibited significant amylase inhibitory activity (10-45%). Thus, among the extracts mentioned bove, only *Saussurea Costus* crude extract showed strong inhibition values greater than 75% toward of human salivary amylase. Thus, *Saussurea Costus* a rich source of amylase inhibitor was be retained for our studies This class of hypoglycemic agents acts mostly by reducing the absorption rate of carbohydrates in the body. Also, acarbose reversibly inhibits both pancreatic α-amylase and α-glucosidase enzymes by binding to the carbohydrate-binding region and by interfering with their hydrolysis into monosaccharides, which leads to a slower absorption together with a reduction in postprandial blood sugar levels. It's leaves and roots were subjected to amylase inhibitor purification and biochemical characterization.

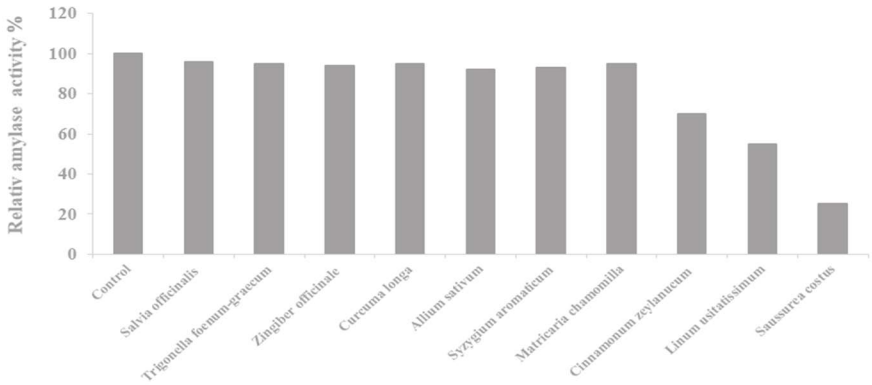

Figure 2 :Screening of Medicinal plant extract inhibition of human salivary α-amylase activity.

Alpha-amylase inhibition potential of the *Morinda lucida* extracts was determined (Figure 2). The inhibition of *α*-amylase by all the extracts at lower concentrations (0.63–1.25 mg/mL) showed no significant difference from one another but at higher concentrations (2.5–5 mg/mL), the inhibitory potential of aqueous extract was significantly different ($P < 0.05$) when compared to other extracts.

Different concentrations (10 to 150 µg/mL) of aqueous *Saussurea Costus* leaves and roots extracts were tested separately for human α-amylase inhibition activity. A dose-dependent effect was observed on increasing extract solution concentrations, suggesting a type of competitive inhibition. Plots of percent inhibition versus extract concentration showed typical dose-response curves (Figure 3).

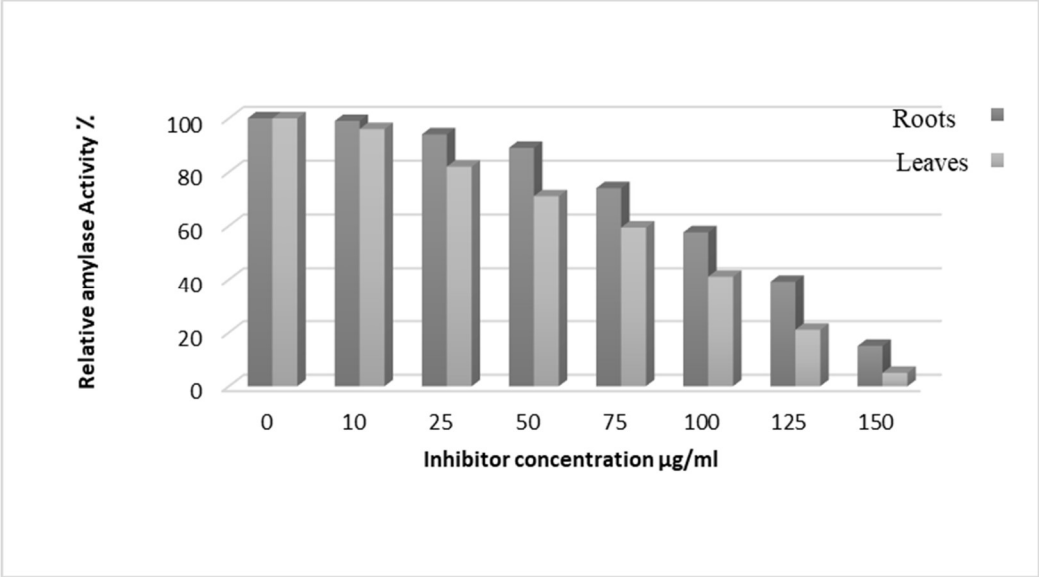

Figure 3: The percent human salivary α-amylase inhibition (%) of *Saussurea Costus* roots and leaves extracts at varying concentrations see above explanation.

The roots and leaves crude extract exhibit potent inhibitory activity exceeding 90% against human salivary amylase, suggesting their higher antidiabetic potency.

Effect of temperature on the amylase inhibitor activity and stability

Temperature values are essential parameters for the inhibitory activity of α-amylase inhibitors and their temperature tolerance determine their scope of application [25].

The effect of different temperatures on the activity and stability of purified α-amylase inhibitors from *S. Costus* roots and leaves was studied using the standard short time test (30 mn). The results in (Fig. 4) confirm that the two wedge forms were active at temperatures ranging from 4 to 100 °C, but at different rates.

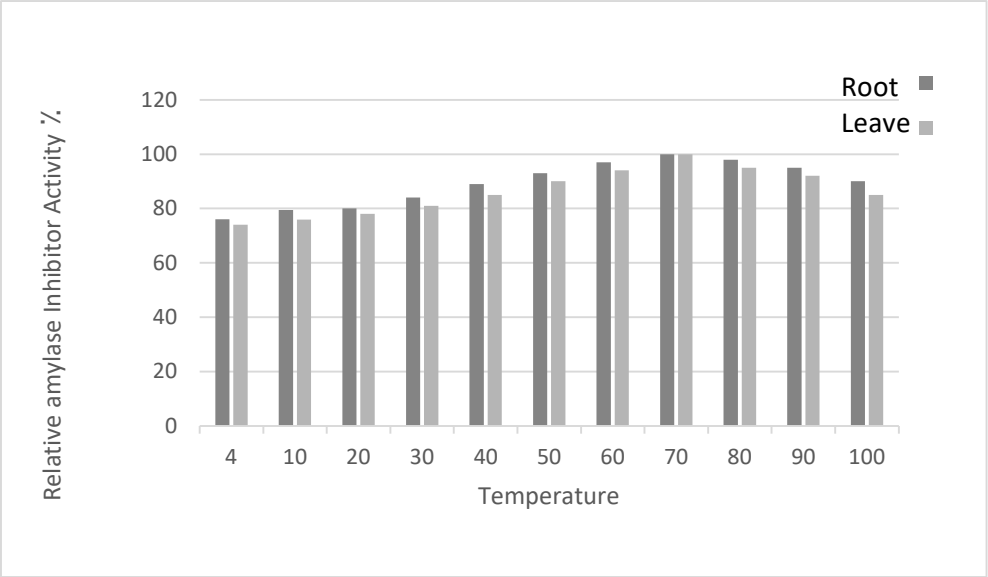

Figure 4: Temperature Effect on the Amylase Inhibitors Activity.

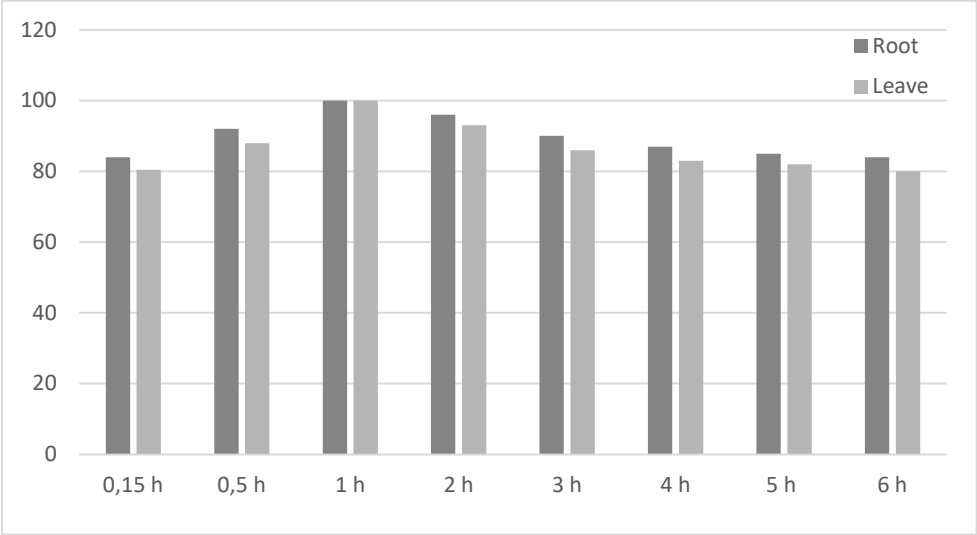

Figure 5: Stability Effect on the Amylase Inhibitors Activity.

Inhibitory activity was evaluated at different temperatures (4 °C - 100 °C) against human salivary amylase. For stability studies, the amylase inhibitor was incubated at different temperatures and withdrawn at different time intervals and the residual inhibitor activity assayed under optimal temperature conditions.

Maximum amylase inhibition activity was observed at 70 °C for the roots and leaves inhibitor. Interestingly, the two isoforms maintained over than 85% of their inhibitory activity at 100 °C, indicating the upkeep of the 3D structure active conformation at higher temperature. Sasikiran et *al* (26) and Hivrale et *al* [27] found this same important physicochemical characteristic of heat tolerence in α-amylase inhibitor from lesser yam bean (*D. esculenta*) and *Achyranthes aspera*, respectively.

As shown in the graph (Figure 5), the inhibitory activity against human amylase was maintained even after 6 hours of incubation at 100°C, indicating the incredibly high thermostability of root and leaf amylase inhibitors. The thermostability of some amylase inhibitors has been observed, such as LDI, which has a half-life of 53 and 33 min at 90 and 93 °C, respectively [28], and wheat amylase inhibitor retained 50% of its Activity 88.1 °C [29], RBI, i.e. stable and not thermally denatured up to 90 °C [2], small yam amylase inhibitor retains about 40% even after heating at 70 °C for 4 hours active. Only 18% of the activity remained after 4 h at 80°C. LYAI was rapidly inactivated at 90 °C and complete inactivation occurred only after 4 h[26], while the barley amylase inhibitor showed the highest activity and maintained about 50% inhibition after heating to 100 °C[30].

The reasons for the high heat resistance of protein α-amylase inhibitors at altitude may be due to their protein structure, which is hardly affected by temperature [31].

For example, the active sites of α-amylase inhibitors may be temperature insensitive and could react with α-amylase after high temperature processing; Or the protein formation cannot be affected by high temperatures. The high thermal tolerance of highland malt extract would enable them to withstand high processing or cooking temperature, promoting their use in developing low GI staple foods or diabetic-friendly foods.

Effect of *Saussurea Costus* amylase inhibitor on human Pancreatic and Salivary amylases

The two α-amylase inhibitors purified from *Saussurea Costus* were tested against six available amylases from human salivary and pancreatic. Results shown in (Figure 6) evince that the two isoforms possess highest affinity toward human salivary and pancreatic α-amylase (around 90-95% inhibitory activity).

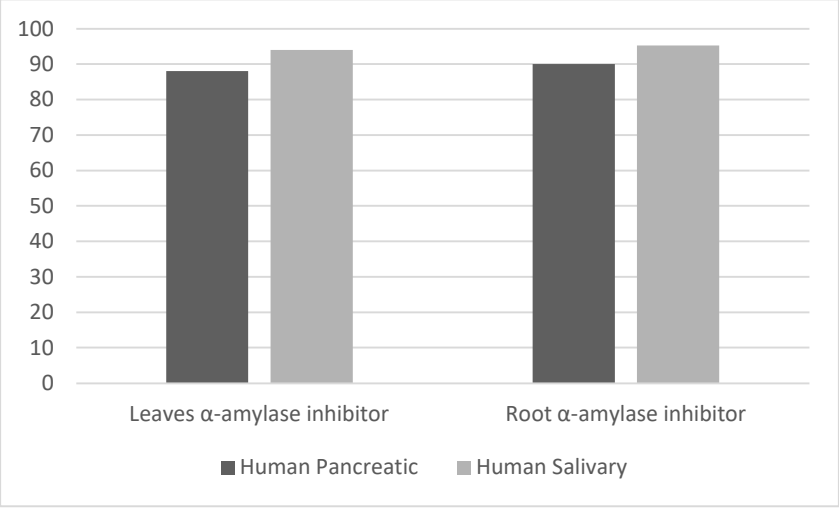

Figure 6 : Activity of roots and leaves α-amylase inhibitors from *Saussurea Costus* against human α-amylases see above explanation.

Similarly previous works on purified inhibitor from *P. vulgaris* (KR-9) [22], *Moringa olifera* [32] and Buckwheat [33] was found to be highly effective against human salivary and pancreatic α-amylase. However, the activity of human saliva α-amylase was not inhibited by proteinaceous α-amylase inhibitors extracted from Chickpea, Kidney bean, Maize, Wheat, and Millet seeds [34-38].

4. Discussion

Diabetes is a serious metabolic disorder that affects many people in the 21st century and is known as the fifth leading cause of death [39]. Various treatments, such as insulin therapy, drug therapy and diet therapy, are available to control this disease. There are several types of glucose-lowering medications that have hypoglycemic effects through different drug formulations [31]. These therapies have some drawbacks, including drug resistance, negative side effects, and even toxicity that leads to conflict [40]. On the other hand, conventional therapies make safe, effective and affordable drug candidates. Today, many therapies involving the use of medicinal plants are recommended for the management of diabetes [41].

In 2021, *Saussurea Costus* was examined by Gomaa et *al* [42] for its anti-diabetic potency. In order to evaluate the biological activities of plant extracts in relation to traditional use as above. Results found by Gomaa et al [42] suggests that the *Saussurea Costus* ethanolic extracts exhibited Anti-diabetic effect by inhibiting α-amylase activity, which confirms its ability to mitigate postprandial hyperglycemia and may be a natural source of alpha-amylase inhibitory factor in combating diabetes complications.

The inhibitory activity against human amylase was maintained even after 6h of incubation at 100°C, indicating an incredibly high thermostability of the roots and leaves amylase inhibitor. The thermostability of some amylase inhibitors has been observed, such as LDI, which has a half-life of 53 and 33 min at 90 and 93 °C, respectively [28], wheat amylase inhibitor, which retains its 50% active at pH 6.9 and 88.1 °C [29] incubated for min, RBI, i.e. stable up to 90 °C [2] Retains about 40% to 70°C after heating for 4 hours. After 4 hours at 80°C, only 18% of the activity remained. LYAI was rapidly inactivated at 90 °C and was completely inactivated only after 4 hours [26], while barley amylase inhibitors showed the highest activity, maintaining about 50% inhibition after heating to 100 °C[30].

The high thermal tolerance of highland malt extract will enable them to withstand higher processing or cooking temperature, promoting their use in the development of low-GI staple foods or diabetic-friendly foods. It is important to mention that this property is very interesting for most biotechnological applications, giving added value to the species *Saussurea Costus* for a large commercial exploitation, equally, Its high thermal stability indicates its potential for use in the management of obesity and diabetes.

The both inhibitory isoforms appear to have the highest affinity towards human salivary and pancreatic α-amylase. (around 90-95% inhibitory activity). Similarly previous works on purified inhibitor from *P. vulgaris* (KR-9) [43], *Moringa olifera* [32] and Buckwheat [33] was found to be highly effective against human salivary and pancreatic α-amylase.

Conclusion

Taking amylase inhibitors with meals can reduce the rise in blood sugar levels that is common in people with diabetes. Substances that inhibit amylase, the digestive enzyme needed to break down dietary starch into absorbable glucose units, can reduce the usual post-meal spikes in blood sugar levels in both healthy people and those with diabetes. Amylase inhibitors prevent and treat type 2 diabetes by lowering postprandial blood sugar levels and may promote weight loss.

*Saussurea Costu*s have significant alpha-amylase inhibitor activity with high potent inhibition on the saliva and pancreatic amylase enzyme So, the use of *Saussurea Costus* have significant role in management and prophylaxis against diabetic disease as well as the obesity disorder.

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التوصيف البيوكيميائي والجزيئي لمثبط الالفا اميليز المستخلص من القسط الهندي

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ملخص الرسالة

أثبتت العديد من المستخلصات النباتية أنها علاجات فعالة وآمنة لمجموعة واسعة من الحالات المرضية و نخص بالذكر منها داء السكري. من خلال دراستنا هذه، تم فحص القسط الهندي لمعرفة مدى قدرته العلاجية لمرض السكري من النوع الثاني. كان الهدف الأساسي من هذه الدراسة هو عزل وتنقية وتوصيف الخصائص البيوكيميائية و الجزيئية لمثبط الأميليز المستخلص من جذور وأوراق القسط الهندي. تم استخلاص مثبط الألفا أميليز مائيا (مع نشاط تثبيط يصل الى 80٪). بعد ذلك تمت تنقيته عن طريق المعالجة الحرارية عند درجة حرارة 100 درجة مئوية لمدة 30 دقيقة متبوعة بخطوة من تقنية الكروماتوجرافي. أظهرت النتائج أن الوزن جزيئي يقدر ب16 كيلو دالتون كما ان التسلسل الطرفي تم تحديده واثبت نسبة تشابه تقدر ب (81٪) مع مثبطي الألفا أميليز الأحادي المستخلصان من *Kengyilia melanthera* و من *Triticum dicoccoides*. وجد ان النظيرين يتحملان درجة حموضة تتراوح بين 2,0 و 12.0 واقصى درجة الفعالية هي 7,0. كما ان الاستقرار الحراري كان عليا، حيث تم الحفاظ على نشاط المثبط بنسبة تصل إلى 80 ٪ بعد 6 ساعات عند درجة حرارة 100 درجة مئوية. تشير فاعلية الانزيم المستخلص أنه يمتلك أعلى فعالية تجاه انزيمات الأميليز الموجودة بالغدد اللعابية والبنكرياسيه للأنسان على حد سواء (نشاط تثبيط يصل إلى 90٪) ، يليه الانزيم المستخلص من كلا من *Aspergillus oryzae* و *Bacillus s* p ب78٪ و 85٪ على التوالي. تم حساب المؤشرات الحركية أيضًا وأظهرت أن الإنزيم لم يتأثر بشكل واضح وكشفت أن نمط التثبيط للإنزيم المستخلص كان من النوع غير التنافسي. يمتلك المستخلص النباتي ايضا تأثير مضاد قوي للفطريات خاصة ضد *Aspergillusoryzae* و *Penicillium*.

الكلمات المفتاحية: أنزيم الألفا أميليز؛ مثبطات الألفا أميليز؛ القسط الهندي؛ علاج السكري؛ مضاد للفطريات.