In vitro Anti-Diabetic Activity of Aqueous Methanolic Extract of *Ailanthus Altissima* Bark

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Abstract

After meals a prominent factor to raise the blood glucose level is the intestinal enzyme alpha-glucosidase whose substrate is carbohydrates. In diabetics, the inhibition of this enzyme would be a therapeutic tactic to reduce postprandial glucose levels. The current study was designed to explore the inhibition of the α-glucosidase enzyme by *Ailanthus altissima* bark extract. Three different plant extracts were formulated by using distilled water, methanol, and a mixture of methanol and water (70:30) to compare the best effect of the extract, while negative and positive controls were used as distilled water and metformin respectively. Four different concentrations of plant extract in all solvents and controls were used to find the percentage inhibition of alpha-glucosidase. Enzyme inhibition was observed in a dose-dependent manner, maximum percentage inhibition was examined against 1.5 mL of all solvent extracts and controls but aqueous methanolic plant extract showed a maximum73% inhibition that was comparable to standard drug inhibition (80%). One-way ANOVA was followed for statistical analysis to find out the P-value based on which the most significant and insignificant were declared. Aqueous methanolic plant extract showed the most significant results because the value of $p<0.5$, while aqueous plant extract showed insignificant results because the P- value is > 0.05. The assay results are evident for the presence of some bioactive compounds in the bark extract of plants which confer versatile medicinal properties based. The current study proves aqueous methanolic extract of *A. altissima* bark has anti-diabetic activity in vitro and is a strong candidate for postprandial control of blood glucose levels.

Keywords: Anti-diabetic, Alpha-glucosidase, *Ailanthusaltissima,* Aqueous Methanolic

1. Introduction

Diabetes mellitus (DM) is considered to be a complex type of persistent, and severe disorder associated with multifarious etiologies with deep significance and merely identified as diabetes (Kumari et al. 2011). DM is a worse complication disease that may not only host many other physiological morbidities but also playan impedimental role in other indispositions that mostly affect the people in the developing states, prominent to the key socioeconomic contest. According to a study survey a decade before, 25% population of the world is badly affected by diabetes (Arumugam, Manjula, and Paari 2013). Broadly classified into two major categories as type-I DM (insufficient insulin production by pancreatic beta cells) mostly onsets in either childhood or adolescence (Anjana et al. 2011)and type-II DM (cells unable to

utilize sufficiently produced insulin by pancreatic beta cells; insulin resistance) mostly onset after 40years age (Spellman 2010). As DM develops, the metabolism of sugar by most of the body cells does not occur normally either due to poor action of insulin on non-pancreatic cells or pancreas insufficient production of insulin. Resultantly body starts to utilize other sources of energy and conversion of protein, lipids and glycogen into sugars, which is coupled with the production of by-products by the liver namely ketones(Buowari 2013). A notorious factor of DM is "chronic hyperglycemia" which is either insulin-dependent or not or even both which disturbs the metabolism of macromolecules. Damage or dysfunction even failure of multiple organs(e.g. heart, eyes, kidneys, nerves, and blood vessels), may result in disability and early death (Ayepola et al. 2013). The intensity and duration of hyperglycemia determine the severity of damage to the respective tissues, organs, and organ systems. Both of these factors are also important to analyze the duration of the occurrence of the disease and how to overcome or control hyperglycemia. Two or three hours after a meal, the blood glucose level rises suddenly which is known as postprandial hyperglycemia (PPHG). A Human's dietis majorly comprised of starch, sucrose, or other carbohydrates. The α-glucosidase is a small intestine localized, membrane-bound enzyme found in epithelium for the catalysis of disaccharides and oligosaccharides to glucose that is absorbed and enter into the bloodstream. Inhibition of α- glucosidase enzyme can help in delaying the digestion of carbohydrates, thereby reducing the levels of glucose in the blood (DiNicolantonio, Bhutani, and O'Keefe 2015). Several drugs are available as α-glucosidase inhibitors such as acarbose and miglitol that inhibit the absorption of carbohydrates from the gut. These inhibitors can prevent or mask the manifestation of the disease. Generalsymptoms accompanied by DM include polyuria, weight loss, thirst, and blurring of vision. Diabetes mellitus is a serious health problem with continuously increasing rates of incidence and mortality. Recovery from diabetes is expected to be associated with genetic and environmental factors. Keeping in view the high prevalence, morbidity, and chronicity of DM, it will not be objectionable to nominate it "3rd killer" for human health after cardiovascular disorders, obesity, and cancer (Freedman et al. 2012).

As mentioned above, in DM severity of damage to any tissue or organ/s is associated with PPHG intensity and duration, for which the intestinal epithelial enzymeα-glucosidase is considered to be responsible, so there is a need to find the natural, low co and human-friendly therapeutic agents that can inhibit the catalytic activity of the enzyme α-glucosidase to avoid the high cost and adverse effects of available drugs.

2. MATERIALS AND METHODS

2.1 Collection of the plant material

The collection of plant bark was done from the bio park of *Bahauddin Zakariya University (BZU), Multan.* Then it was authenticated with the cooperation of the *Department of Botany, (BZU), Multan,* byan expert taxonomist and herbarium specimens were submitted for the record.

2.2 Preparation of extract

For decontamination and removal of dust, the bark was washed up with tape water before shade drying for 10 days. Later, dried bark was grounded well in an electrical grinding machine to get it into fine powdered form. The powdered bark was soaked in a mixture of distilled water and methanol (30:70) at room temperature for seven days. A mixture of aqueous and methanol extract was prepared by adding 10g *A. altissima* powder to 100 mL of a mixture of distilled water and methanol and shaking water bath at 55℃ for 4h. The solution was filtered and the supernatant was freeze-dried at -2° C. Same procedure was

repeated with distilled water and methanol separately to find out the comparative solubility of phytochemicals responsible for enzyme inhibition as shown in

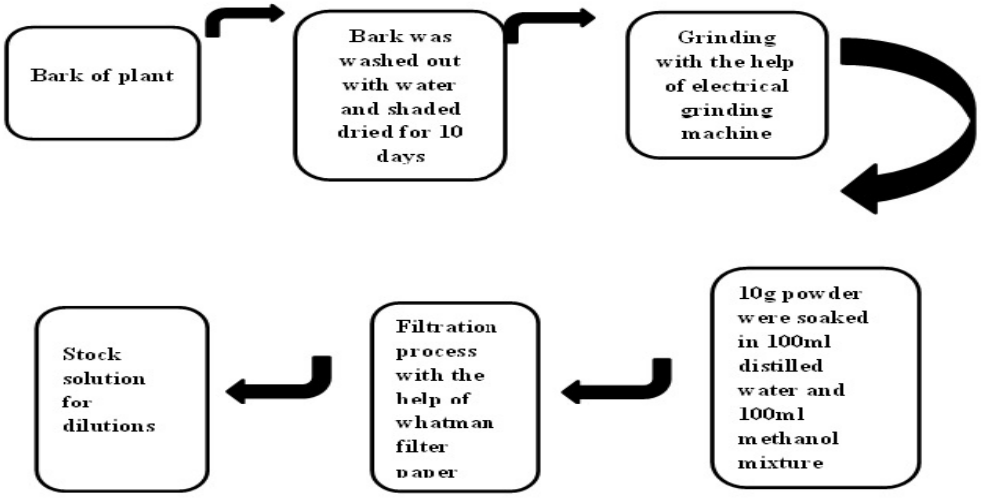

Figure 1. Schematic diagram for extract preparation.

2.3 Chemicals

Starch (2% w/v), Tri's buffer (0.2M), HCl (2mL), methanol (100mL), alpha-glucosidase enzyme, metformin (500mg/tablet). All the chemicals of analytical grade were used in the experiments.

2.4 Analytical Concentrations of Prepared Stock Solutions

Various concentrations from each stock solution (0.1, 0.5, 1 & 1.5 mL) of plant bark extract in aqueous methanol (70%), distilled water, and methanol were used to monitor comparative inhibitory activity against alpha-glucosidase by phytochemicals.

2.5 Negative Controls

Distilled water was used as a negative control in the case of four different concentrations.

2.6 Positive Control

Metformin is an effective anti-diabetic drug that is specifically involved in the inhibition of mitochondrial glycerophosphate dehydrogenase and alpha-glucosidase (Kyriachenko et al. 2019).As a positive control, **a** standard drug (Metformin) was used in the following experiment.

2.7 Aqueous Plant Extract

The mixture of various concentrations of aqueous plant bark extract (0.1-1.5 mL),1mLsolution of the starch substrate (2% w/v) with Tris buffer (pH= 8.0) of 0.2M was incubated for 5min at 37°C. Later, 1mL of alpha-glucosidase enzyme (1U/mL) was added toeach aliquot to monitor the inhibitory assay of the prepared solution that was keptincubatedat 37°C for 40 min. Termination of inhibition was ensured by adding 2mLHCl (6 N). The spectrophotometer was used to measure the color intensity at 540 nm using an according to the previously followed protocol (Krishnaveni, Balasubramanian, and Sadasivam 1984).

2.8 Methanolic Plant Extract

The same procedure was revised including all the chemicals, reagents, and enzymes in the same concentrations replacing the aqueous plant extract with methanolic plant extract as reported in a previous study to find out the enzyme inhibition using a spectrophotometer(Krishnaveni et al. 1984).

2.9 Aqueous-Methanolic Plant Extract

All the previously discussed experimental steps were revised with the same concentrations of chemicals, reagents, and enzymes for aqueous methanolic plant extract to find its inhibitory effect on alpha-glucosidase based on changes in color intensity using a spectrophotometer(Krishnaveni et al. 1984).

2.10 Negative Control and Positive Control

Mixtures of solutions containing various concentrations (0.1-1.5 mL) of distilled water (negative control),1mLstarch substrate (2% w/v), and Tris buffer (pH= 8.0) of0.2M were incubated for 5min at 37°C. Later, 1mL of alpha-glucosidase enzyme (1U/mL) was added to each aliquot to monitor the inhibitory assay of the prepared solution that was kept incubated at 37°C for 40 min. Termination of inhibition was ensured by adding 2mLHCl (6 N). The spectrophotometer was used to measure the color intensity at 540 nm using an according to the previously followed protocol (Krishnaveni et al. 1984).

Except for plant bark extract, all other chemicals, reagents, and enzyme solutions were used as in previously mentioned concentrations while various solution concentrations (0.1-1.5mL) of metformin (99% W/V) were added as positive controls. The percentage inhibition was measured thrice for each of the controls and their standard error of the mean (SEM) was also calculated statistically.

2.11 Comparative Statistical Analysis of Alpha-Glucosidase Inhibition

One-way ANOVA was applied to data to evaluate the results against all solvent extracts to find out the P-value, the results having a P-value < 0.05 were considered the most significant while results with a P-value > 0.05 were considered the insignificant

2.12 Inhibitory assay via spectrophotometer

The assay was applied for the identification of inhibition of alpha-glucosidase enzyme. After 40 min rate of absorbance through the spectrophotometer was monitored at 540nm (Farman and Hadwan 2021). Percentage inhibition (I%) was calculated by

Percentage Inhibition= $100\times[1-(X-MIN)/(MAX-MIN)]$ (Shai et al. 2010a)

3. RESULTS

Postprandial anti-diabetic activities of plant bark extract were recorded against α-glucosidase by applying prepared stock solution concentration in various solvents (distilled water, methanol, and aqueous methanol), aqueous methanol solution (negative control) and metformin (positive control). Different analytical concentrations (0.1, 0.5, 1 & 1.5 mL) from each prepared stock solution in different solvents showed different percentage inhibition of alpha-glucosidase. Maximum percentage inhibition was examined in 1.5 mL concentration of aqueous methanolic plant bark extract that was 70.1% which is comparable with standard drug metformin that showed 80.2% inhibition of alpha-glucosidase. Moreover, a linear relationship was found between analytical grades of aqueous methanol extract of plant bark and alpha-glucosidase inhibition that represents dose-dependent inhibition. Plant bark stock solutions in distilled water and methanol also showed a maximum of 20% and 68% alpha-glucosidase inhibition against 1.5mL analytical concentrations which demonstrates that the solubility of phytochemicals responsible for inhibition of enzymes is lesser in distilled water than in methanol.

Minimum percentage inhibition of alpha-glucosidase was found to be 10% and 45.6%, 25.3% by 0.1mL analytical concentrations stock solutions prepared in distilled water, methanol, and aqueous methanol (70%) respectively that also showed that phytochemicals responsible for alpha-glucosidase inhibition are more soluble in methanol and aqueous methanol.

3.1 Percentage Inhibition by Aqueous Plant Extract

Aqueous plant extract's all analytical concentrations showed the least potent inhibitory effect on alpha-glucosidase. Minimum percentage inhibition was found to be 1% and maximum 15% by 0.1mL and 1.5mL analytical grades of aqueous plant extract respectively. Percentage inhibition shown by analytical concentrations of aqueous plant extract was observed to be accelerated with the increase in extract concentrations as shown in Table.1.

Table 1. In vitro anti-diabetic activity of the alpha-glucosidase enzyme by aqueous methanolic, methanolic and aqueous plant extract in comparison with negative (distilled water) and positive controls (metformin).

Concentration (mL)	Inhibition by Negative Control (%)	Inhibition by Positive Control (%)	Inhibition by aqueous methanolic plant extract (%)	Inhibition by methanolic plant extract (%)	Inhibition by aqueous plant extract (%)
0.1	2	19	6	4	1
0.5	5	39	25	16	6
1	8	59	47	36	9
1.5	11	78	73	58	15

3.2 Percentage Inhibition by Methanolic Plant Extract

All analytical concentrations of methanolic plant extract showed a potent inhibitory effect on alpha-glucosidase. Minimum percentage inhibition was found to be 4% and maximum 58% by 0.1mL and 1.5mL analytical grades of methanolic plant extract respectively. Percentage inhibition shown by analytical concentrations of methanolic plant extract was observed to be accelerated with the increase in extract concentrations as shown in Table 1.

3.3 Percentage Inhibition by Aqueous Methanolic Plant Extract

All analytical concentrations of aqueous methanolic plant extract showed a potent inhibitory effect on alpha-glucosidase. Minimum percentage inhibition was found to be 6% and maximum 73% by 0.1mL and 1.5mL analytical grades of aqueous methanolic plant extract respectively. Percentage inhibition shown by analytical concentrations of aqueous methanolic plant extract was observed to be accelerated with the increase in extract concentrations as shown in Table 1.

3.4 Percentage Inhibition by Negative Control and Positive Control

Negative and positive controls showed the percentage inhibition as 11±2 and 80.2±2 respectively as mentioned in Table.1.

3.2 Comparative Statistical Analysis of Alpha-Glucosidase Inhibition

One-way ANOVA results were analyzed comparatively and evaluation of data based on the P-values was done easily, shown in Table 2.

Interpretation

Table 2 deliberated the one-way ANOVA of the aqueous plant, Methanolic plant, and aqueous Methanolic plant. The result described that the value of aqueous methanolic plant extract is significant because of the value of $p<0.5$ through the goodness of the model. On the other hand, the methanolic plant extract is significant because of the value of $p<0.05$. Lastly, the Aqueous plant extract is insignificant

because the value is > then the P<0.05. The graphical representation (Figure 2) showed the inhibition of the enzyme by comparative analysis of aqueous methanolic (70%), methanolic, and aqueous plant extract of *A.altissima* bark using four different concentrations, negative control, and standard drug. A proportional relation was found between the percentage inhibition of alpha-glucosidase enzyme and dose-dependent increment of bark extract.

Table 2. Analysis of variance (one way) showing the mean differences and P-value for plant extracts in different solvents

Variables	*SS*[a]	*Df*[b]	*MS*[c]	*F*[d]	*p-value*
aqueous methanolic plant extract					
Between Groups	1537.351	1	1537.351	5.477149	0.02
Within Groups	1684.108	6	280.6846		
Total	3221.459	7			
methanolic plant extract					
Between Groups	2734.301	1	2734.301	6.562697	0.04
Within Groups	2499.858	6	416.6429		
Total	5234.159	7			
Aqueous plant extract					
Between Groups	97.30125	1	97.30125	5.621236	0.055*

SS[a](static single assignment**), Df[b]**(Degrees of freedom between groups) **MS[c]**(Mean Square)**F[d](**Variations between/within samples**) P-value**(Probability)

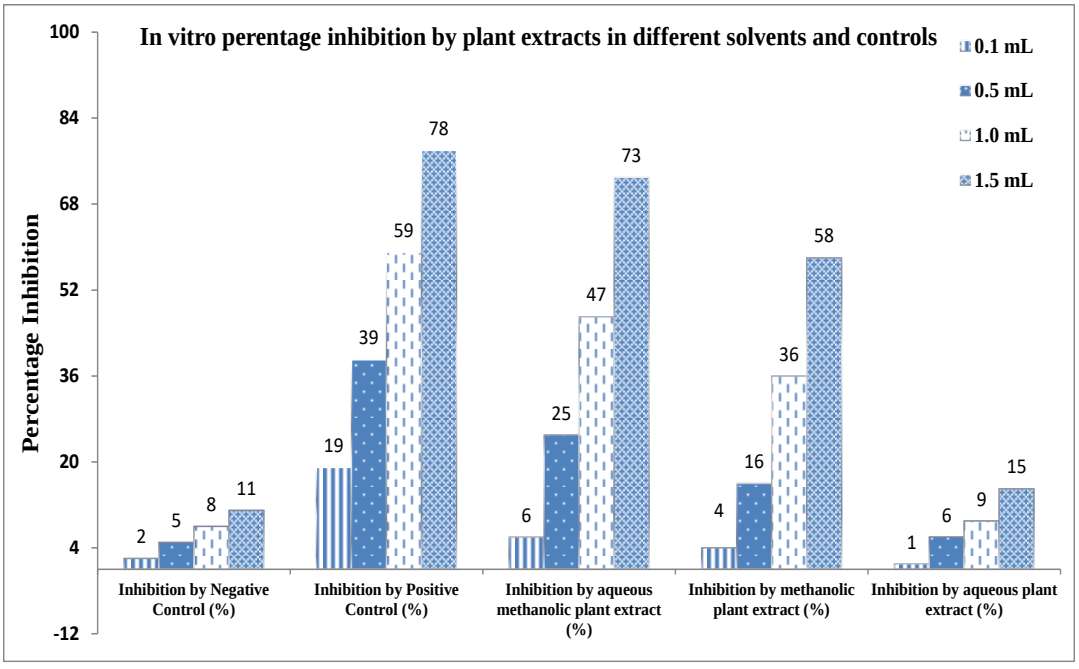

Figure 2. Graphical representation of α-glucosidase percentage inhibition.

4. DISCUSSION

Our study reveals that a 1.5mL analytical concentration of aqueous methanolic extract of *A. altissima* bark showed maximum inhibition of alpha-glucosidase up to 73% due to the solubility of polar and non-polar phytochemicals like saponins and flavones, in the crude extract of *A. altissima* bark which are richly available in aqueous methanol. The percentage inhibition of α-glucosidase by the activity of aqueous methanolic extract was found to be comparable with standard drug/positive control(metformin), which causes 80% inhibition of α-glucosidase. Minimum inhibition of alpha-glucosidase induced by 0.1mL aqueous methanolic extract was found 6% which is also greater than the negative control. It shows a linear relationship between analytical concentrations and alpha-glucosidase inhibition.

The aqueous extract of *A. altissima* bark in analytical concentration 1.5mL showed maximum 15% alpha-glucosidase inhibition which shows polar phytochemicals soluble in water can also induce the inhibition. Minimum inhibition shown by 0.1mL of *A. altissima* bark aqueous extract was found 1% which also indicates the proportional relation between extract concentrations and alpha-glucosidase inhibition. Aqueous extract of *A. altissima* bark-induced inhibition results indicate aqueous extract is the least potent inhibitor as its inhibitory potential is even lesser than the negative control showing 15% inhibition of alpha-glucosidase. The methanolic extract of *A.altissima* bark (1.5mL) as compared to the aqueous extract (1.5mL) showed maximum inhibition of up to 56% due to the presence of non-polar phytochemicals like flavones which are comparatively more soluble in methanol than distilled water. Methanolic extract analytical concentrations results also indicate that by increasing the extract concentrations, the inhibition of alpha-glucosidase enhanced gradually. Aqueous methanolic extract (majorly flavones) showed inhibition comparable to the standard drug (Metformin) and is involved in weight loss over time when combined with diet and exercise. Moreover, metformin does not treat the underlying causes of diabetes. It manages the symptoms of diabetes by lowering blood sugar or glucose (Hermann 1979).

Due to hyperglycemia, there are chances to develop many serious complications including nephropathy and cardiovascular diseases that indicate chronicity of DM. Nowadays, its prevalence in all populations of the world is increasing due to an increase in obesity and the aging populace. The rate of rapidly increasing number of diabetic patients predicted that Its incidence will increase from 2.8% to 4.4% of the world population within 30yearstillthe year 2030. Diabetics hardly bear a huge burden of high-cost treatment and management strategies. It's the PPHG that causes type-II DM which contributes to more than 90% of diabetes cases and its complications (Alongi and Anese 2018). Alpha-glucosidase inhibitors delay the PPHG through reduced absorption of glucose from the intestine, which are efficient therapeutic agents(Kadouh et al. 2016).

Flavonoids have been reported to be efficient inhibitors for α-glucosidase as these form non-covalent interaction complexes with enzymes which bring some conformational changes and energy transfer that can be detected in common analytical techniques including fluorescent spectrometry, surface plasmon resonance, isothermal titration calorimetry, circular dichroism, etc., (He et al. 2019). However, during the analysis of these changes, researchers have to face multiple barriers in experimental designs and analysis, even though most technologies have failed to quantitatively visualize the structure of the complexes formed by non-covalent interactions (Ahmed et al. 2021).

The comparative analysis of 15 different flavonoids has been evaluated to find the percentage inhibitions of these compounds, that supported the evidence that flavonoid aglycones significantly inhibited the α-glucosidase superior to that of the flavonoid glycosides. A positive relationship between the number of phenolic hydroxyl groups in the B ring of flavonoids and the activity was also determined.

Although myricetin and dihydromyricetin are similar in structure, myricetin showed significantly higher inhibition ofα-glucosidase than that dihydromyricetin (He et al. 2019b).

Fluorescence analysis revealed that myricetin also presented a greater binding capacity with α-glucosidase. To understand the real process of docking of the inhibitor's molecular mechanism of α-glucosidase inhibition, a molecular simulation of myricetin and dihydromyricetin was carried which revealed both molecules had different alignments in the active center of α-glucosidase. Immunoglobulin M (IGM) analysis represented stronger hydrogen bonding and van der Waals force interactions with α-glucosidase by myricetin. The study results declare flavonoids as quick hypoglycemic nutraceuticals (He et al. 2019b).

Phenolic group substitution reactions of sugars at different positions with Gallic acid, comeric acid, etc. also increased the inhibition of α-glucosidase. Hydroxy or methoxy substitutions on the phenyl group and geometric isomerism in the phenylpropenoyl moiety did not play an important role in enzyme inhibition. In contrast, acetylation of glucose increased the inhibition, but lower level inhibition was observed with 3,4-di-O-acetylation of rhamnopyranoside. The bioactivity of flavonoids was found to be reduced when O-glycosylation was observed while C-glycosylation of flavonoids significantly enhances it (Chen et al. 2021). However, it seems both O and C-glycosylation reduce the alpha-glycosidase inhibitory potential of flavonoids.

Previous research results demonstrate a positive correlation between the phenolic content of plants and their respective anti-diabetic activities, these analytical studies also demonstrate that phenols and flavonoids do not directly inhibit the enzyme rather the water fractions of both as a whole, were found to be better inhibitors of α-glucosidase as compared with ethyl acetate fractions and the crude aqueous extracts(Lachkar et al. 2021). Different concentrations of acarbose were tested against α-glucosidase and α-amylase but tested concentrations were found not to be involved in the inactivation of enzymes while some other studies reported a minor level of inhibition by acarbose against yeast α-glucosidase. Manypotent inhibitors may inhibit α-glucosidase of baker's yeast but show a low level of inhibition against mammalian α-glucosidase due to the difference of molecular recognition in the binding site of the enzyme (Shai et al. 2010b).

The aqueous methanolic extract of *A.altissima* bark reveals a greater significance of inhibition of alpha-glucosidase due to the presence of phytochemicals especially a large number of flavonoids like quercetin and total phenolic compounds (119.84 mg GAE/g dry extract) including gallic acid (Luís et al. 2012).Based on the previously conducted research studies, it is easy to understand our research outcomes based on available data, and depicting the experimental correlation between the extract of *A.altissima* bark and alpha-glucosidase inhibition. Keeping in view the inhibitory role of the extract of *A.altissima* bark due to the presence of flavonoids and phenols, the therapeutic significance of *A.altissima* bark extract can be expected. Paraprandial hyperglycemia can be controlled in diabetics especially, if a small quantity of this extract is used with the first bite of each meal. In Diabetes, the human body is either unable to produce or respond to the insulin hormone. The alpha-glucosidase is responsible to balance the insulin level. Thus using *A.altissima* bark extract not only reduces the hyperglycemic condition but also normalizes insulin levels in type-II DM. In addition, *A.altissima* bark extract has an advantage over other anti-diabetic drugslike metformin that it's a natural product with no adverse effects.

According to a previous research study in 2013, the psidium extract was found to be effective for the inhibition of alpha-glucosidase enzymes due to phytochemicals in psidium extract. Psidium extract contains tannins, flavones, alkaloids, and phenols which could be responsible for its versatile medicinal properties. Results of this study shows similar linear correlation between plant extract and enzyme

inhibition (Anon n.d.) but no proposed mechanism of enzyme inhibition was furnished. The anti-diabetic action of Psidiumguajava can also be attributed to intestinal alpha-glucosidase inhibitory activity (Anon n.d.).

Keeping in view, the ease of availability, least or no side effects, and low cost of treatment, and adverse effects of available drugs, the world's interest is in finding plant-based natural products that could be promising therapeutic candidates that can be used as lead molecules in the drug development. Natural plant-based products not only contain therapeutic agents but also a richer source of many other bioactive chemicals, which possess powerful pharmacological actions. Many drugs are being used nowadays whose exemplary sources directly or indirectly are plants.

Conclusion

The current study was designed to evaluate the inhibitory activity of aqueous methanolic extract of *A.altissima* bark againstα-glucosidase. The plant extract exhibit comparable inhibition of α-glucosidase with standard drugs. In the future, exact therapeutic compound isolation, quantification in extract, purification from extract, and characterization are required which is responsible for in vitro inhibition of alpha-glucosidase that will prove it a strong candidate drug to control postprandial glucose in diabetic patients.

Conflict of Interest

All authors are duly informed and have gone through this manuscript. There is no conflict of interest in this paper.

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