3 Biotech (2021) 11:167
https://doi.org/10.1007/s13205-021-02717-8

ORIGINAL ARTICLE

Assessment of α-amylase and α-glucosidase inhibitory potential of *Citrus reticulata* peel extracts in hyperglycemic/hypoglycemic rats

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Received: 27 November 2020 / Accepted: 5 March 2021 / Published online: 15 March 2021

Abstract

Diabetes mellitus is a metabolic disorder of carbohydrate metabolism. The management of Diabetes mellitus with phytochemicals is hallmark of this research. Citrus species are known for their health benefits and are used as traditional food in South East Asia. The total phenolic content of peels was analyzed using different solvents, while Gallic acid was used as standard. Both ethanolic, aqueous extracts of *Citrus reticulata* peel showed good inhibitory activity against amylase (90.67%, 15.33%) and moderate against glucosidase (70.8%, 14.8%), respectively. Sixteen rats were randomly divided into four groups (G1, G2, G3, and G4); G1 is a negative control (water), G4 is a positive control (Acarbose), while other two are experimental groups like G2 (fed with 100 mL and 20 mg/mL in hypoglycemic and hyperglycemic trials) and G3 fed with 200 mL and 40 mg/mL in hypoglycemic and hyperglycemic trials. A significant effect of treatments and value of time was found in hyperglycemic rats. Ethanolic extract showed a significant reduction in blood glucose levels in hypoglycemic (overnight fasting) rats which was comparable to the positive control. These results suggest that *C. reticulata* peels can contribute as a useful food ingredient as a potential antihyperglycemic agent in managing type 2 diabetes mellitus. In future, *C. reticulata* peel will be a good candidate for pharmaceutical industry.

Keywords α amylase · Glucosidase · Diabetes · *Citrus reticulata* · Hyperglycemic index

Introduction

The diabetes is a major problem in population of sub-continent and it is increasing day by day. The inhibition of alpha amylase and glucosidase with natural agents (peel extracts) will be future diabetes remedy. (Mishra et al. 2018; Oboh and Ademoson 2011). An uncontrolled increase in diabetes is observed because of smoking, aging, hyperlipidemia and unhealthy diet (sweets) in the whole world (Wild et al. 2004). The synergetic action of α-amylase (E.C. 3.2.1.1) and α-glucosidase (E.C. 3.2.1.20) breaks down the dietary carbohydrate (starch) hence control and maintain blood plasma glucose level. Secretions of these enzymes are located in the mouth and secretions in brush border cells of the intestine, catalyzes the splitting of α-1–4 glycosidic bonds producing maltose and malto-triose and many units of monosaccharides. To inhibit digestive system is one of the therapeutic ways to stabilize blood glucose levels and to manage type 2 diabetes (Sekar et al. 2019; Oboh and Ademoson 2011).

Synthetic inhibitors of alpha-amylase and alpha-glucosidase are available in the market which include a group of drugs like miglitol, voglibose, and acarbose. These are

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synthetic inhibitors can cause abdominal discomfit, flatulence, and diarrhea (Harrison et al. 2019). The dried peels of *C. reticulata* have a history of conventional use in Asian cuisines that have been obsoleted over time. Peel of citrus fruits carries a good quantity of phenols, especially, acids of phenols and flavonoids. Due to the phenolic and flavonoid content of citrus fruits, they have a broad range of therapeutically important biological activities (Kalita et al. 2018).

Peels of fruits called citrus hold important quantity of phenols, especially, acids of phenols and flavonoids. Natural polyphenols have ability to eliminate unbound radicals, chelate catalysts of metals, trigger enzymes of antioxidant, lowering radicals of a-tocopherol and retard enzymes called oxidases (Aruoma et al. 2012). Epidemiological studies show that eighty percent of the diabetic population is found in low to moderate-income countries (Borg et al. 2019).

Many solvents have a capacity to extract polyphenols from complex plant matrix. Less polar solvents are frequently used like ethanol, methanol acetone, and ethyl acetate. Ethanol is considered to be good for polyphenols extraction and is safe for human consumption. Polyphenols are previously well documented for its potential to inhibit starch digestive enzymes although it varies with the source material and method of extraction (Elkhatim et al. 2018). It is, therefore, convenient to explore the possible mode of action of the phenolic components of *C. Reticulata* peels in the curing of type 2 diabetes mellitus (Proteggente et al. 2003). In the present study, Citrus reticulate dried peels extracts were used in two solvents and its inhibitory potential against carbohydrate hydrolyzing enzymes (alpha amylase and glucosidase) was reported. This inhibitory potential against carbohydrate hydrolyzing enzymes will be used as future remedy to control diabetes type I and type II.

Material and methods

Collection of *Citrus reticulata* peel

Citrus reticulata peels were purchased from the local market, washed with distilled water and processed in three different ways before extraction. (1) Oven-dried: the peels were dried in a hot air oven at 40 °C for 72 h, (2) Air-dried: the peels were dried in air for 7 days and (3) Fresh: the peels were used fresh. All the peels were then ground to mesh size of 0.2 mm and stored at 4 °C till further use (Rahman et al. 2018).

Polyphenol extraction

Two different extraction solvents were used; one is aqueous ethanol (AE) and the other is water (W). In AE, ethanol [Ethanol absolute by Anala R Normapur (VWR) case No.10h107, USA] and distilled water were mixed with a ratio of 80:20 volume by volume. The dried citrus powder, 50 g of each group was added into 500 mL of AE and incubated for 24 h in a shaking incubator at 40 °C for proper mixing and extraction of components. Extracted samples were centrifuged at 7,000 × *g* for 30 min and filtered. Both the filtrates were pooled and the volume was reduced with the help of a rotary evaporator (Menichini et al. 2011) by removing organic solvents. The water extraction was made using 5:50 ratio of dry powder to the solvent by heating at 121 °C at the pressure of 200 kPa, for 30 min. The mixture was then cool down, centrifuged at 7000 × *g* for 15 min and then filtrated for determination of polyphenolic content (Munir et al. 2018).

Estimation of total phenolic content

The concentration of the phenolic acids was determined from the linear standard calibration curve ($R^2 = 0.9890$) of Gallic acid (GA). The concentration of the GA (Gallic Acid G7384 Sigma Aldrich, USA) equivalent (GAE) was expressed in terms of mg of GA/g of dry weight (DW) of extract. Five dilutions were made using distill water to extract ratio,1:9, 2:8, 3:7, 4:6, 5:5, of both the extracts (AE and W) and each reaction mixtures were prepared in triplicate for accurate estimation. The total phenolic content of each group was calculated as GAE using $C = (c \times V)/m$ (Kumar et al. 2018). Where C = Overall Phenolic content mg/g extract, in GAE, c = the gallic acid concentration calculated from the curve of calibration (mg/mL), V = the extract volume in mL, and m = the mass of extract of *C. Reticulata* peel in g (Demiray et al. 2009).

In vitro *effect of Citrus reticulata peel extract on the a-amylase activity*

Citrus reticulata peels extract samples were prepared in four different concentrations: 100, 200, 300, and 400 µg/mL. The α-amylase (Bacterial source, AVONCHEM batch no.15594/1, USA) solution was prepared by dissolving 0.5 mL of α-amylase in 3.5 ml of 0.02 M phosphate buffer saline (pH = 6.9). Reaction mixture was prepared by mixing 1 mL of peels extract of each concentration (100, 200, 300, and 400 µg/mL), 4 mL of α-amylase solution, 3.5 mL of 1% starch (Merck, Germany) solution, 1 mL of 3,5-dinitrosalicylic acid (DNS Sigma Aldrich case no. 509-99-4, USA) and 10 mL distilled water. The mixture was incubated at 30 °C for 15 min. The reaction was stopped by 200 µL 0.1 molar sodium carbonate (Sigma Aldrich, USA). The absorbance was measured at 540 nm. The maltose (Sigma Aldrich, USA) standard curve was constructed using different concentrations of maltose in 0.1 molar sodium phosphate buffer (pH 6.9). Enzyme activity was determined by

measuring the maltose equivalents released from starch at 540 nm. The α-amylase inhibitory activity was calculated using the formula:

$$\% \text{ inhibition} = \left(A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}\right) \times 100$$

The purpose of the standard curve is to calculate the velocity of an enzyme or the release of sugar. One unit of enzyme activity was defined as the amount of enzyme that release 1 µmol reducing sugar equivalent to maltose under the assay condition (Karakaya et al. 2018).

In vitro effect of Citrus reticulata peel extract on the α-glucosidase activity

One mg of α -glucosidase (G3651 Sigma Aldrich, USA) was dissolved in 100 mL of phosphate buffer saline and mixed with 1 mL plant extract (100, 200, 300, and 400 µg/mL). The reaction mixture was composed of p-nitro-phenyl-D glucopyranoside (Sigma Aldrich, USA) as a substrate dissolved in phosphate buffer saline and varying concentration of plant extracts. The mixture was incubated for 15 min. The reaction was terminated by adding 200 µL of 0.1 molar sodium carbonate. Absorbance was taken at 400 nm (Uddin et al. 2014).

The hypoglycemic effect in overnight fasted rats

Sixteen *Sprague Dawley* rats of equal age (12 ± 2.6) and weight (80 ± 17.3 g) were randomly divided into four groups G1, G2, G3 and G4 and kept fasted overnight with free access to water. G1 received water and act as a control, G2 received ethanolic extract 100 ml/kg body weight, G3 received ethanolic extract 200 ml/kg body weight and G3 served as standard, received 5 mg acarbose. Fasting blood glucose level was estimated via puncturing the tail vein before and after 2 h of treatment by glucometer (Wilkerson et al. 1960; Habib et al. 2017).

The hypoglycemic effect in starch induced hyperglycemic rats

The hyperglycemic condition was induced by feeding rats on the high starch diet (50%) for 7 days. On 8-day 5 g glucose was fed to rats of all groups (G1, G2, G3, and G4) and then treated with water, two concentrations of extract and standard drug acarbose. A glucose tolerance test was performed after giving oral glucose (5 g). The Group 2 and 3 were given extract and G4 was given acarbose as a control. Serum glucose concentration was estimated after every 2 hours. The serum glucose was estimated as per defined criteria at 0, 2, 4, and 6 h from the tail vein using a glucometer (Vigneaud and Karr 1925; Habib et al. 2017).

Statistical analysis

The results of all the experiments are presented as the mean ± standard deviation (SD) of experiments performed in triplicate. Statistical differences among the various groups were determined by Tukey's one-way ANOVA using SPSS software. A difference was considered significant at $p < 0.05$ (Oboh and Ademoson 2011).

Results

Estimation of total phenolic content

The total phenolic content of all the groups was estimated by utilizing Folin-Ciocalteu reagent and standard Gallic acid calibration curve. Results indicated significant results of polyphenolic compounds with oven drying, 15.2 (ethanolic) and 14.5 (aqueous) mg of GAE/ gram of dry weight (DW), with air-drying method 15.9 (ethanolic) and 14.8 (aqueous) mg of GAE/ gram of DW and with fresh 14.65 (ethanolic) and 13.5 (aqueous) mg of GAE/ gram of DW (Fig. 1).

In vitro effect of Citrus reticulata peel extract on the α-amylase activity and α-glucosidase

The α-amylase and α-glucosidase inhibitory potential of *C. reticulata* were determined at different concentrations (100, 200, 300, and 400 µg/mL). From these results, it was concluded that 400 µg/mL of both extracts, ethanolic, and aqueous exhibited maximum amylase and glucosidase inhibitory activity (Figs. 2a and 3a). The percentage inhibition for amylase was (90.7% and 15.3%) and moderate against glucosidase 70.8%, 14.8% by ethanolic and aqueous extract, respectively, as shown in Table 1. The IC_{50} for amylase was 9.3 µg/mL and 14.3 µg/mL of ethanolic and aqueous preparation. This high inhibitory effect of the ethanolic extract was due to large phenolic contents in it as compared to aqueous extract.

As one unit of porcine alpha amylase produced one mg of maltose from starch in 3 min so the decrease in production of maltose per mg of the enzyme (inhibition) were 0.20 U/mL, 0.22 U/mL, 0.27 U/mL and 0.31 U/mL of amylase and 0.22 U/mL, 0.28 U/mL, 0.31 U/mL and 0.38 U/mL of glucosidase, as shown in Figures. 2a and 3a. The Km for amylase is 17.43 µg and glucosidase is 20.6 µg indicating less affinity of glucosidase than amylase for the inhibitor calculated from Lineweaver–Burk plot shown in Figs. 2b and 3b.

Hypoglycemic effect in overnight fasted rats

Fasting blood glucose level was decreased significantly by ethanolic extract (Fig. 4) and decreased as compared to the

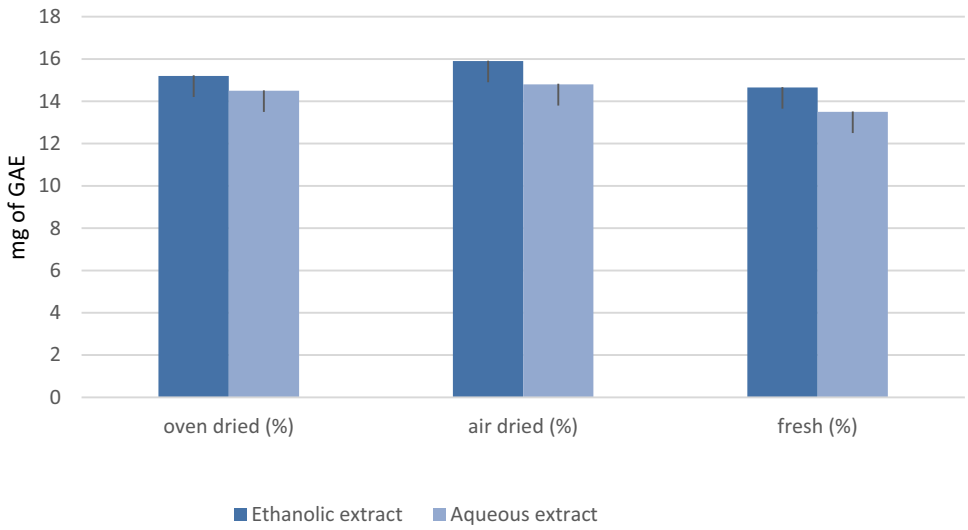

Fig. 1 Total phenolic contents as mg of GAE/ gram of DW of *Citrus reticulata* peels using different methods of drying. *value differ significantly $p < 0.05$

standard. However, a two-fold increase in the extract amount did not translate into two times decrease in the serum glucose level.

The hypoglycemic effect in starch induced hyperglycemic rats

The serum glucose levels of all the rats were 122 ± 12.5 mg/dL after 7 days of the high starch diet. A significant decrease was observed in blood glucose concentration in group 3 from 120 ± 0.288 to 83 ± 0.453 mg/dL from zero to 6 h of starvation. A significant decrease was observed in blood glucose concentration in group 2 from 118 ± 0.288 to 100 ± 0.453 mg/dL from zero to 6 h starvation but the difference was not so big in group 2 as compared to group 3 (Table 2). The in vivo study both in fasted rats and diet-induced hypoglycemic rats showed a good impact. In overnight fasting rats, a significant decrease from 92.00 mg/dL to 54 mg/dL was observed, although it was found that an increase in extract dose did not translate the impact in the same manner. This was probably due to enzyme saturation as described by Mettupalayam 2020. In glucose-induced hyperglycemia, the group receiving a high dose of the extract showed comparable pattern of control inhibitor acarbose, as shown in Table 2 (Camberos et al. 2014).

Discussion

The prevalence of Diabetes mellitus is becoming extremely alarming in developing countries. Various factors contribute including family history, urban stress and lifestyle including dietary habits and lack of exercise. Treating Diabetes is not rewarding, generally patients require lifelong management. Common targets of diabetes controlling strategy is either the enzymes or hormone's receptors (Atlas 2014). The study was focused on controlling alpha-amylase and glucosidase which were the two key enzymes of starch metabolism (Sales and Ballesteros 2012).

The dried peels of *C. reticulata* have a history of conventional use to serve and reduce the manifestation of digestive problems related to acute or chronic inflammation (Asencio et al. 2018). The significant impact of drying method was found on total phenolic content. It is found that traditional slow air drying is the most effective method showing the highest phenolic content. This is also reported by Sun et al. 2015 and the phenomena is attributed to the fact that the moderate temperatures, the activity of polyphenol oxidases are not inhibited so it can retain the phenolic compounds, whereas in case of oven-dry temperature above 50 °C the enzyme is inhibited. This finding was also reported by Yujing et al. although freeze-drying as the best option for inhibition (Sun et al. 2015). The % inhibition shown by the *C. reticulate* peel's ethanol extracts were comparable to shown by the acarbose (EC_{50} 00.0000143 ± 6.32 mg/mL) which were oral α-glucosidase and α-amylase inhibitors used to manage Type-2 Diabetes mellitus (Poovitha et al. 2016). The IC_{50} of ethanol and aqueous extract was 9.2 µg/mL and 14.5 µg/mL for α-amylase. Acarbose is a complex oligosaccharide known to reduce glucose absorption but it has many side effects like diarrhea and flatulence. Various plants and their extracts are under scrutiny as alternate medicines. Potential of *C. reticulata* peels extracts showed good potential as it showed 87% dose-dependent inhibition. Bitter gourd potential showed inhibition for both the enzymes, α-amylase, and α-glucosidase, with IC50 0.267 and 0.261 mg/mL (Poovitha et al. 2016).

When a range of plants were studied by Sindhu et al. (2013), variations were appreciated and IC50 was found from 118.88 to 70.58 µg/mL (Mourya 2018). Studies conducted

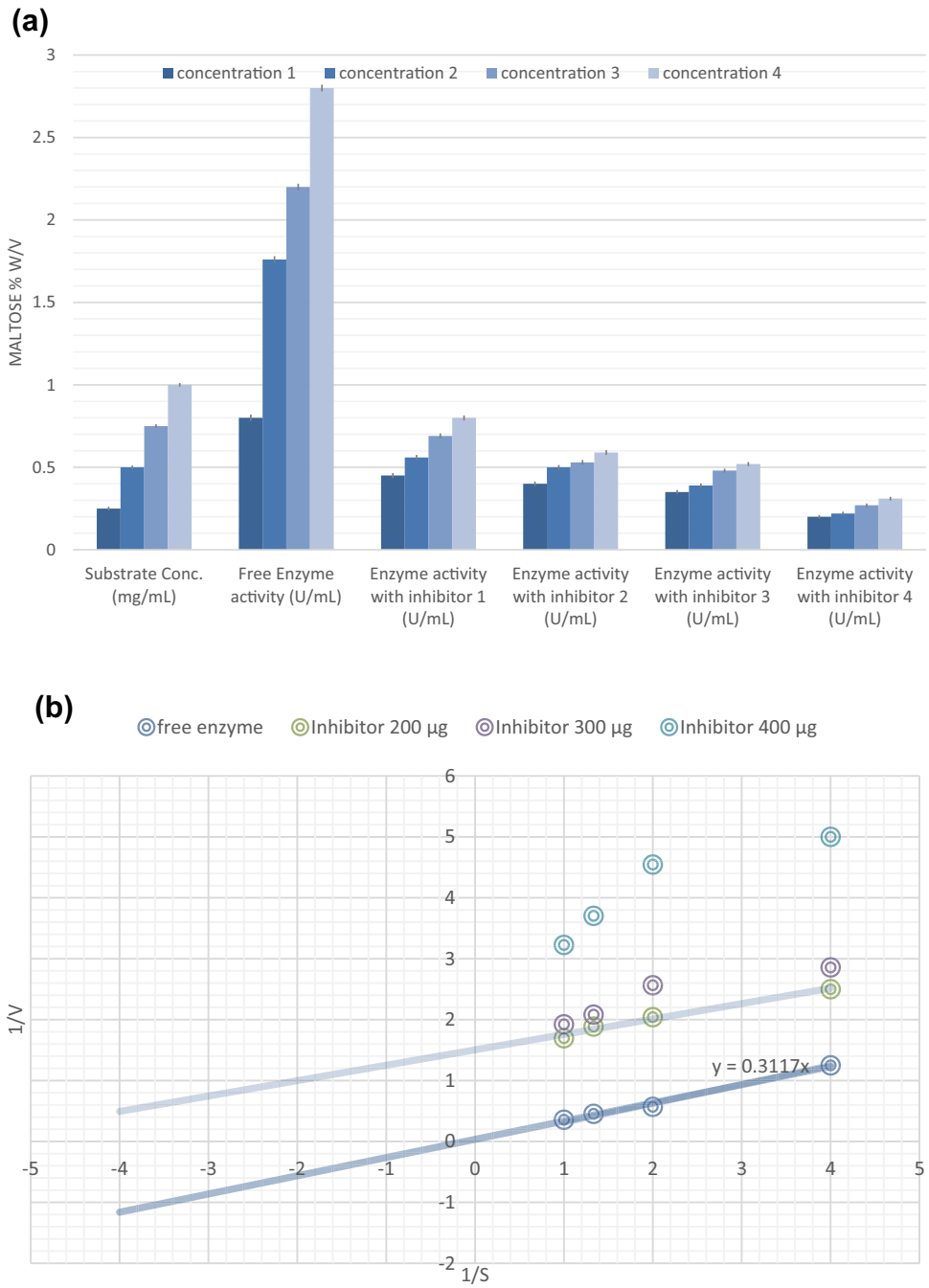

Fig. 2 **a** Alpha Amylase activity calculated from maltose standard curve. Substrate is starch (mg/mL). Inhibitor conc. 1 = 0.430 mg, Inhibitor conc. 2 = 0.8616 mg, Inhibitor conc. 3 = 1.2924 mg and Inhibitor conc. 4 = 1.7232 mg of Phenolic compounds. Lineweaver Burk plot showing kinetics of α-amylase with variable starch concentration in the absence or presence of inhibition

on *Melia azedarach* (IC_{50} 3444.11 µg/mL) (Fathima et al. 2018) *Alternanthera pungus kunth* (IC_{50} 6.96 µg/mL) (Bouabid et al. 2018), *Cassia fistula* (IC_{50} 72.44 µg/mL) (Mumtaz et al. 2018) *Atractylis gummifera* (IC_{50} 0.577 mg/mL) (Karakaya et al. 2018), *Chick pea* (85.41 µg/mL) (Bhagyawant et al. 2019) and *Ficus benjamina* (IC_{50} 19.62 µg/mL) (Yang et al. 2019) showed that *C. reticulata* peel extracts both aqueous and ethanolic had a promising effect on α-amylase inhibition when compared with other plant extracts. However, as far as α-glucosidase inhibition was concerned *C. reticulata* ethanolic and aqueous extracts were found less effective (IC_{50} 29.2 and 517 µg/mL). This inhibition is also dose-dependent. Comparatively less efficiency was also observed in other studies. It was reported by Songul (2017) that *Ferulago bracteata* roots (IC_{50} 0.42 mg/mL) only showed inhibitory potential for α-glucosidase and zero potential for α-amylase. In the case of Bitter Gourd, it was reported that both the enzymes are inhibited equally. The kinetics studies for amylase elucidated non- competitive inhibition. The V_{max} was reduced in the presence of inhibitor, while Km remained unchanged. The behavior of glucosidase was different as a mode of inhibition was mixed

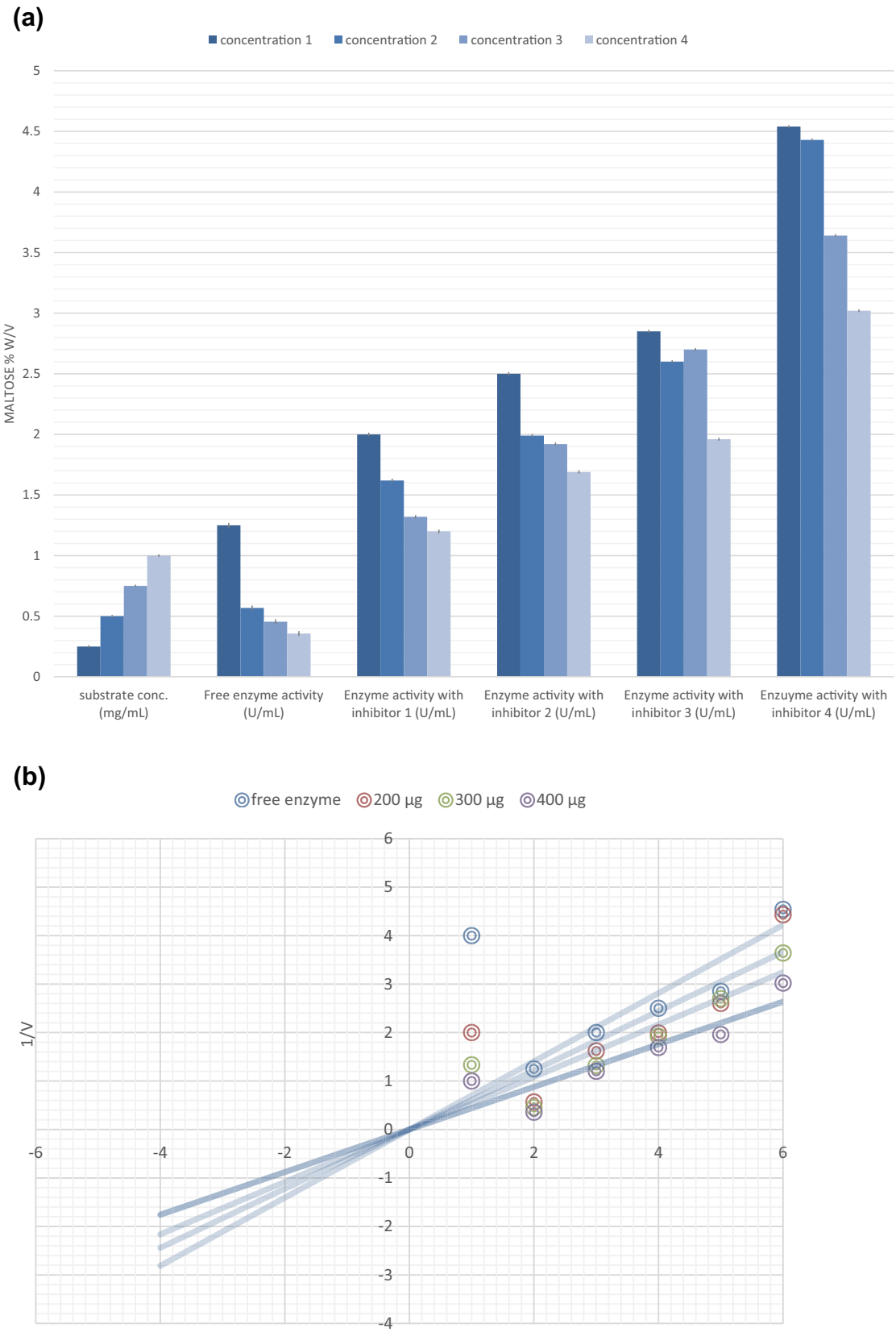

Fig. 3 **a** Alpha glucosidase activity calculated from maltose standard curve. Substrate is starch (mg/mL). Inhibitor conc. 1 = 0.430 mg, Inhibitor conc. 2 = 0.8616 mg, Inhibitor conc. 3 = 1.2924 mg and Inhibitor conc. 4 = 1.7232 mg of Phenolic compounds. **b** Lineweaver Burk plot showing the kinetics of α-glucosidase with a variable starch concentration in the absence or presence of inhibition

Table1 *a*-amylase and *a*- Glucosidase inhibitory potential of *Citrus Reticulata* dried peel was determined at different concentrations (100 µg, 200 µg, 300 µg, and 400 µg/kg of BW)

	% Inhibition against amylase				% Inhibition against glucosidase			
Volume µg/ml	Ethanol extract	IC_{50} (µg/mL)	Aqueous extract	IC_{50} µg/ml	Ethanol extract	IC_{50} µg/ml	Aqueous extract	IC_{50} µg/ml
100	85.54 ± 0.32	**9.30**	4.90 ± 0.34	**14.5**	56.7 ± 0.31	**29.2**	3.99 ± 0.34	**517.7**
200	86.20 ± 0.43		9.80 ± 0.12		58.9 ± 0.43		8.72 ± 0.12	
300	87.12 ± 0.44		14.11 ± 0.64		65.7 ± 0.44		12.32 ± 0.64	
400	90.67 ± 0.70		15.33 ± 0.1		70.8 ± 0.7		14.8 ± 0.11	

The data are expressed as mean ± S.E ($n = 5$) in each group

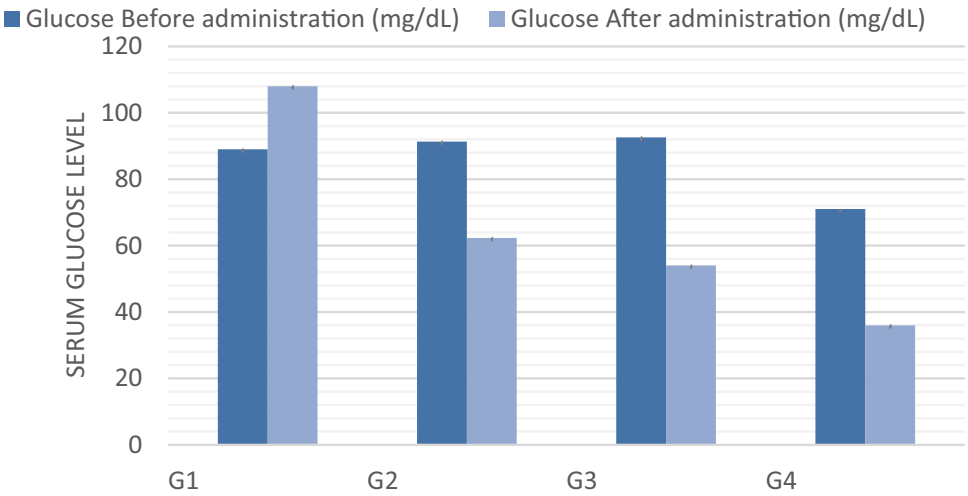

Fig. 4 Effect of *Citrus reticulata* peel ethanolic extract on fasting blood glucose level (mg/dL) in normal rats G1 = water, G2 = 930 µg ethanolic extract, G3 = 1860 µg ethanolic extract, G4 = Acarbose 5

Table 2 Effect of citrus reticulata ethanolic peel extract on glucose induced hyperglycemia (mg/dL) in normal rats (all the rats were fed with starch loaded diet for 7 days

Treatment groups	0 h glucose (mg/dL)	2 h glucose (mg/dL)	4 h glucose (mg/dL)	6 h glucose (mg/dL)
G 1	122 ± 0.577	142 ± 0.866	134 ± 0.272	120 ± 0.176
G-2	118 ± 1.154	123 ± 0.967	107 ± 0.303	100 ± 0.474
G-3	120 ± 0.288	122 ± 0.28	102 ± 0.470	83 ± 0.453
G-4	121 ± 0.12	120 ± 0.23	102 ± 0.01	82 ± 0.260

G 1 = Water, G-2 = 20.0 mg/ml/kg ethanolic extract G-3 = 40 mg/ml/kg ethanolic extract, G-4 = 5 mg/kg of BW of Acarbose significant effect of treatment and significant value for time effect ($p = 0.05$)

competitive as V_{max} was unchanged but with a decreasing value of 1/Km, the higher value of Km in the presence of inhibitor was detected. In the case of glucosidase, the mode of inhibition mixed and uncompetitive was reported by *Pisum sativum* and other plants (Ibrahim et al. 2017).

These phenolic contents of *C. retuculata* correlated with pheolic content findings in vegetables, fruits and some hot peppers and had benificial effect on diabetes control (Oboh and Ademouson 2011; Camberos et al. 2014). These *C. retucata* results correlated with inhibition of alpha amylase as revealed by *C. limetta* extracts (Camberos et al. 2014). Some other research activities revealed somehow same inhibitory effects of polyphenolic compounds against alpha amylase and glucosidase (McDougall et al. 2005; Tadera et al. 2006). Morinda lucida extracts revealed similar inhibitory action against alpha amylase due to its high polyphenolic content (Kazeem et al. 2013). These polyphenolic compound extracts showed good inhibitory action against alpha amylase and low against alpha glucosidase. The differences of results were due to different inhibitory action of polyphenolic extracts against alpha amylase and glucosidase (Camberos et al. 2014). High polyphenolic content did not mean that it revealed high inhibitory action against starch digestive enzymes. The inhibitory action of polyphenolic extractx depended on position, extent of hydroxylation and conjugation (Pulido et al. 2000).

Conclusion

This article results showed that Pakistani species of *C. reticulata* peel were rich in phenolic constituents. This study found that aqueous extract had much less phenolic contents as compared to ethanolic extract so in this way ethanolic extract had higher inhibitory effect on alpha amylase and alpha glucosidase activity as compared to aqueous extract. The peels of citrus fruits were mostly discarded in Pakistan but this research indicated that ethanolic extracts of citrus peels of *C. reticulata* were effective against alpha amylase inhibition so it could be used in the treatment of hyperglycemia and type 2 diabetes management in near future.

Acknowledgements We are thankful to Director IBBT, Prof. Dr. Wasim Shahzad for providing great support to conduct this research project.

Declarations

Conflict of interest Authors declare that manuscript has not any conflict of interest.

Ethical approval The research was conducted after approval from independent ethical committee UVAS vide letter no. 118/BeST center 18.

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