Antioxidant and *beta*-Galactosidase Inhibitory Studies of Methanolic Extract of *Juniperus Phoenicea* and *Calicotome Villosa* from Jordan

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Abstract

Background: We investigated *Juniperus Phoenicea* (*J. Phoenicea*) and *Calicotome Villosa* (*C. Villosa*) from Jordan for phenolic contents, antioxidant, anti β-Galactosidase activities, in an attempt to rationalize its use in lactose metabolism disorders. The kinetic parameters of leave extracts, galactose, glucose, fructose and acarbose were evaluated. Also, the thermodynamic parameters of the enzyme thermal inactivation were determined.

Methods: JP and cv crude methanolic extracts were evaluated for 1,1-diphenyl,2-picrylhydrazyl (DPPH) free radical scavenging activity and ferric reducing antioxidant power (FRAP). Further, β-Galactosidase inhibitory activities were performed using O-nitrophenyl-beta-D-galactopyranoside as substrate. Moreover, total phenolic contents, flavonoids and flavonols of plants extracts were determined and expressed in mg of gallic acid equivalent (mg GAE/g dry extract) or rutin equivalent per gram of dry extract (mg RE/g dry extract).

Results: Phytochemical screening of the crude extract of *J. Phoenicea* and *C. Villosa* leaves contained phenols, alkaloids, flavonoids, terpenoids, anthraquinones and glycosides. *J. Phoenicea* exhibited high flavonoids and flavonols contents than *C. Villosa* but both *J. Phoenicea* and *C. Villosa* contained high phenolic and showed concentration dependent DPPH scavenging activity, with *J. Phoenicea* (IC_{50} =11.1 µg/ml), *C. Villosa* (IC_{50} =15.6 µg/ml), respectively. According to FRAP assay, the antioxidant power activity of plants extracts was concentrations dependent. The β-galactosidase % inhibition was increased as the concentration of of *J. phoenicea*, *C. villosa* and rutin increased. The mode of inhibition of β-galactosidase by *J. phoenicea* (IC_{50}= 65 µg/ml) and *C. villosa* (IC_{50}= 700 µg/ml) extracts was non-competitive and mixed-inhibition, respectively. Also, rutin was affected in a competitive (IC_{50} = 75 µg/ml) inhibition.

β-galactosidase half-life was 108 min at 55°C, thermodynamic parameters revealed an activation energy of 208.88 kJ mol^{-1} and the inactivation kinetic follows a first-order reaction with k-values ranges between 0.0862 and 0.0023 min^{-1}. The enzyme showing a decreasing trend of enthalpy of denaturation ($\Delta H°$) as temperature increase but value of free energy of thermal denaturation ($\Delta G°$) for β-galactosidase was decreased with increasing in temperature. The calculated entropy of inactivation ($\Delta S°$) at each temperature showed positive values, which means there are no significant processes of aggregation.

Conclusions: *J.phoenicea* and *C.villosa* have inhibiting effect on β-galactosidase activity. Thermodynamic approach shows an enzyme stable and suggests that inactivation mechanism is based on molecular structural changes.

Introduction

Herbal medicinal plant has been used in developing countries as the primitive source of medical treatment and still used in traditional treatment of diabetes. It has special attributes as a large source of

therapeutic phytochemicals that may lead to the development of novel drugs and several pharmacological function like antioxidant, antiviral, anticancer, antimicrobial, antifungal and antiparasitic [1]. The herbal extracts have compounds that are rich in polyphenols, which interact with proteins and can inhibit enzyme activity [2].

In Jordan *J. phoenicea* and *C. villosa* species classified as a medicinal plant because it contains secondary metabolites that have beneficial effects and largely useed in Jordanian folk and traditional medicine [3, 4].

J. phoenicea (L.) belong to *Cupressaceae* family and commonly known in English term as Phoenician Juniper but in Arabic as Ar'ar.

A native of *J. phoenicea* shrub or a small tree growing to the coastal sites of the Mediterranean basin and widely distributed in Europe, northern Africa, and the Canary Islands [5].

The leaves of *J. phoenicea* are used in the form of decoction to treat diabetes, diarrhea, rheumatism, and as a diuretic against bronco-pulmonary disease[3] but their mixtures leave and berries is used as an oral hypoglycaemic agent [6]. Folk and traditional medicine in Jordan used for a treatment of variety of diseases such as diarrhea, gout, and poor appetite, eliminating gastrointestinal bacteria and parasites [7].

Calicotome villosa (or *Calicotome villosa*) described as a thorny shrub that can reach 2 m in height and has sharp terminations, trifoliate including oval leaves with yellow and grouped flowers [8]. *C. villosa* is common in the Mediterranean area and exists in an intermedia subspecies of *Calicotome villosa*, "*C. villosa* subsp. *intermedia*" grows in Morocco, Algeria and Spain. This species has been used in Sicilian folk medicine for the treatment of cutaneous abscesses, furuncles, chilblain and as an antitumor agent [4, 9]. The approach used to reduce postprandial hyperglycemia is by retarding glucose uptake through the inhibition of carbohydrate-hydrolyzing enzymes such as β-galactosidase which belong to an enzyme group called glycoside hydrolases, are a prevalent group of enzymes that hydrolyze glycosidic bonds that link the sugars in complex carbohydrates, or the linkage in glycol- conjugates [10–12].

β-galactosidase can catalyze several substrates, other than lactose, include ganglioside GM_1, lactosylceramides, and various glycoprotein. Industrially, it is an important enzyme used for the hydrolysis of lactose from milk and milk whey for several applications and lately, was enhanced by its galactosyltransferase activity, which is responsible for synthesis of transgalactosylated and oligosaccharides that act as prebiotics with several beneficial effects on the consumers[13].

Microorganisms like bacteria, fungi and yeast used to obtain β-galactosidases from plants of almonds, peaches, apricots, apples and animal sources [14–16].

Microbial β-galactosidases have a prominent position in terms of their role in the production of various industrially relevant products like biosensor, lactose hydrolyzed milk, and the production of galacto-

oligosaccharides for use in probiotic foodstuffs (), while it shown a particular importance in enzyme GOS production from *Aspergillus oryzae* which can produce trisaccharides and higher saccharides [17–18] .

Because of a deficit of the production of β-galactosidase, a condition called lactose intolerance may appear, therefore, people with lactose intolerance tend to eliminate milk and dairy products from their diet, and consequently their calcium intake may be compromised, thereby other complications like osteoporosis may occur accordingly they need to treatment of lactose intolerance can include lactose-reduced diet and enzyme replacement [19–21].

The activity and thermal stability of enzymes are influenced by diverse environmental factors (temperature, pH, reaction medium) which can strongly affect the specific three-dimensional structure or spatial conformation of the protein [22].

Different ways of biotechnological processes used to deactivate Enzymes to an inactive state, which forms a major and important constraint to analyze the estimated thermodynamic parameters, since these will lead to understanding the probable denaturation mechanism in enzymatic processes [22–23].

Materials And Methods

Chemicals

β-Galactosidase from *Aspergillus oryzae* (CAS Number: 9031-11-2), Folin-Ciocalteu phenol, 2,4,6-tripyridyl-s-triazine (TPTZ), 2, 2-diphenyl-2-picrylhydrazyl (DPPH) Rutin and Gallic acid were obtained from Sigma-Aldrich. *o*-Nitrophenyl-β-D-Galactopyranoside (CAS Number: 369-07-3) was obtained from ACROS ORGANICS. Other chemicals and solvents were used of analytical grade.

Plant material

The two plants were identified and classified by Dr. (Saleh Al-Quran) - Department of Biology Sciences at Mutah University - Jordan and specimens of the plants were preserved there.

j.phoenicea and *C. villosa* leaves were collected within spring season in March-April 2018 from western of Al-Shoubakin the southern province of Jordan.

Plant extraction and fractionation

The extracts were prepared according to [24] with some modifications, (30) grams of grinded powder from each leaves plant were treated and soaked with 300 ml of 99% methanol in 1000 ml of conical flasks at room temperature, with shaking for 48h.

Afterwards, extracts were filtered using a Buckner funnel and filter paper (WhatMan No. 4) prior to concentration to dryness under reduced pressure at 40ºC using a Mophorn 5L Rotary Evaporator RE-501 Vacuum Evaporator Digital Controller 0-90rpm Rotary Evaporator Set.

For fractionation, (4g) of the crude extract from *J. phoenicea* was subjected to column chromatography on silica gel 60 (0.06-0.2mm) and eluted with each of the following solvents: cyclohexane, ethyl acetate at different ratios (100: 0 to 0: 100), and ethyl acetate mixed with methanol at different ratios, followed by 100% methanol to yield 7 fractions, which were collected and further concentrated using Rotary Evaporator to test the biological activity of each fraction.

Phytochemical screening

Qualitative Analysis

The qualitative analysis of major groups of phytochemicals: Alkaloids, Flavonoids, Saponins, Terpenoids, Phenols, Anthraquinons, Tannins, Glycosides and Anthocyanins of *J. phoenicea* and *C. villosa* leaves extracts was done by the standard procedures of analysis [25,26]

Determination of Total Phenolic Content

Folin-Ciocalteu colorimetric method [27] used to analyze the total Phenolic content and Gallic acid was applied as a standard using 0.2 mL of (10 mg/ml) extract was mixed with Folin-Ciocalteu phenol reagent (1.5 mL). After 5 minutes, 6% sodium carbonate (1.5 mL) was added and the mixture was allowed to stand at room temperature for 90 minutes. The absorbance of the mixture was measured at 725 nm. Results were expressed as mg Gallic acid equivalent (GAE) per gram of extract.

Determination of Total Flavonoids Content

Total flavonoids content was estimated by the aluminium chloride colorimetric assay with some modifications [28] 1 ml of test extract (10 mg/ml) was added to volumetric flask containing 4 ml distilled water. To the flask 0.3 ml 5% $NaNO_2$ solution were added. After 6 min, 0.3 ml 10% $AlCl_3$ solution was added and kept for another 6 min. To this reaction mixture, 4 ml of 1M NaOH solution and 0.4 ml water was added to make up the final volume 10 ml. The reaction mixture was mixed well and allowed to stand for 15 min after which absorbance was recorded at 510 nm. Total flavonoid content was expressed as mg rutin equivalent (RE)/g plant sample. Triplicate determinations were performed.

Determination of Total Flavonols Content

Determination content of flavonols was used rutin as a standard method [29]. A 0.5 ml of methanolic plant extract (10 mg/ml) was mixed with 0.5 ml (20 mg/ml) $AlCl_3$ and 1.5 ml (50 mg/ml) sodium acetate. The absorption at 440 nm was read after 2.5 hour at 25^{o}C using spectrophotometric techniques model. Triplicate determinations were carried out.

Antioxidant Activity

DPPH Assay (Free Radical Scavenging Activity)

The antioxidant activity of *J. phoenicea*, *C. villosa* and Gallic acid were determined using 2, 2-diphenyl-2-picrylhydrazyl (DPPH) radical. It is a stable radical in solution that appears purple Colour absorbing at 517 nm in methanol. This assay method is based on the reduction of purple DPPH to a yellow colored $DPPH_2$ which was resulted from accepting a hydrogen atom from the scavenger molecule (i.e. antioxidant), concomitant with decrease in absorbance at 517 nm. The colour change is monitored by spectrophotometric according to the method described with some modifications to utilize the determination of parameters for antioxidant properties [30]. The 50 µl of each extract was added to 950 µL of DPPH in methanolic solution with final absorption of 0.747 at 517 nm. Then the mixtures were incubated for 30 minutes at room temperature in dark and the absorbance was read against the blank at 517 nm. Percent of Inhibition I (%) of free radical by DPPH was calculated as follows:

I (%) = ((A_{blank} – A_{sample})/ A_{blank}k) Î 100. Where A_{blank} is the absorbance of the control reaction (containing all reagents except the plant extract), and A_{sample} is the absorbance of the tested plant extract. Extract concentrations providing 50% inhibition (IC_{50}) are calculated from the plot of inhibition (%) against extract concentration that compared with the IC_{50} of Gallic acid value as appositive control. Samples were carried out in triplicates.

Ferric Reducing Antioxidant Power (FRAP) Assay

In the FRAP assay, the antioxidants in the sample reduce (Fe^{3+}) / tripyridyltriazine complex, which presents in stoichiometric excess, to the blue colored ferrous form (Fe^{+2}), with an increase in absorbance at 593 nm [31]

FRAP reagent was prepared by mixing 300 mM acetate buffer, pH 3.6, 20 mM ferric chloride and 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ)-dissolved in 40 mM hydrochloric acid- in 10:1:1 ratio (v:v:v). Extract of 0.1 ml was added to 0.9 ml of FRAP working reagent to have a final concentration of 5, 10, 15, 20, and 25 µg/ml, then the increase in the absorbance at 593 nm was measured after incubation at 37 ^{o}C for 10 min after calibration with $FeSO_4.7H_2O$. Absorbance change is translated into a FRAP value (µM) by relating the ratio of A593 nm of test sample to that of a standard solution of known FRAP value (100µM) using the following Equation.

FRAP = (A_{593nm}test sample/A_{593nm}test standard) Î FRAP value of standard (µM)

Enzyme Assay

Enzymatic activity of β-galactosidase was assayed using ONPG as a Substrate of Routine Assay Method which was slightly modified for assay conditions [32]. The reaction contained 20 µmoles of ONPG dissolved in 4 ml of 0.1 M citrate phosphate buffer (pH 4.5) and 40 µl of conveniently diluted enzyme. After incubation for 20 min at 30˚C, the reaction was stopped by adding 1 ml of 1M Na_2CO_3. Then the absorbance at 420 nm was measured. One unit of the activity was defined as the amount of enzyme which liberated 1µmole of o-Nitrophenol per minute under assay conditions.

Determination of Enzyme Kinetics

The double reciprocal linear plot applied to Fit the procedures for the determining V_{max} and K_M have been concerned with the development of graphical methods to estimate reaction constants [33,34.]

The dissociation constant K_i for the enzyme-inhibitor complex is particularly useful for expressing the potency of an inhibitor because, unlike IC50 and independent of substrate concentration. The precise formulas which are used to calculate Ki depends on the mode of inhibition by comparing the "apparent" values of V_{max} and K_m for an enzyme in the presence of an inhibitor to the V_{max} and K_m values in the absence of any inhibitors.

Effect of DifferentPlants Extracts and Sugars onβ-Galactosidase Activity.

Determination the percentage of relative activity (%) is compared to control of selected plants extracts and sugars on β-galactosidase activity, these effectors were incorporated in the standard assay mixture at different concentrations.

Thermal Inactivation Studies

Incubating the enzyme assays were done in duplicate at 50, 55, 60, and 65 °C for time intervals up to 3 hours was determined by thermal inactivation of β-galactosidase. Then the enzyme solution was heated in sealed tubes, which were incubated in a thermostatically controlled water bath. Tubes were withdrawn at each time interval, immediately immersed in an ice bath and enzyme activity was determined as describe earlier. The activity after 1 min of heating-up time (t = 0) was the initial activity, thereby eliminating the effects of heating-up.

Estimation of Kinetic and Thermodynamic Parameters

It is generally accepted that the thermal inactivation of several enzymes including β-galactosidase is a first-order reaction and the reaction occurs at one inactivation rate (*k*-value) in a single step, therefore kinetic and thermodynamic parameters was determined as described in previous studies [35,36].

Statistical analysis

All data analysis was performed using Microsoft Excel 2016.

Results

Phytochemical screening

The qualitative analysis of phytochemicals presents in the methanolic extract of *J. phoenicea and C. villosa* leaves were shown in table 1. Accordingly, *J. phoenicea* and *C. villosa* leaves contained Phenols, Alkaloids, Flavonoids, Terpenoids, Anthraquinones and Glycosides. Table (1) describe the screening of phytochemicals in both *J. phoenicea* and *C. villosa* leaves in methanolic extract.

Table (1): Screening of phytochemicals in *J. phoenicea* and *C. villosa* leaves methanolic extract.

No.	Phytochemicals	*J. phoenicea*	*C. villosa*
1.	Alkaloids	+	+
2.	Anthocyanins	+	+
3.	Flavonoids	+	+
4.	Phenols	+	+
5.	Saponins	+	+
6.	Tannins	+	+
7.	Terpenoids	+	+
8.	Glycosides	+	+
9.	Anthraquinons	+	+

* + Phytochemical is present.

Quantitative Analysis

Extract of *J. phoenicea* and *C. villosa* leaves were analyzed for the total phenols, flavonoids, and flavonols concentrations, as shown in table 2. The chemical analysis content showed higher concentration in *J. phoenicea* compared to *C. villosa.*

Table (2): Total phenolic, flavonoid's, and flavonols contents in *J. phoenicea* and *C. villosa* leaves in methanolic extract.

Extract	Total phenols (mg GAE/g extract)	Total flavonoids (mg RE/g extract)	Total flavonols extract)	(mg RE/g
J.phoenicea	103.6 ± 0.006	101.1 ± 0.016	30.7 ± 0.082	
C.villosa	99.1 ± 0.013	61.6 ± 0.0075	19.2 ± 0.088	

Antioxidant Activity

DPPH Free Radicals Scavenging Activity

Antioxidant activity of methanolic extracts is evaluated by using DPPH. Gallic Acid used as reference antioxidant molecule. According to DPPH assay, the scavenging effect of both extracts and reference standard at the concentration of (2 µg/ml) of extracts were in the following order: Gallic acid < *J. phoenicea* < *C. villosa*, with percentage scavenging values of 62.41%, 10 %, and 8.28%, respectively, the

IC50 values of leaves extracts compared with Gallic acid are shown in table 3. Free radical scavenging activity of these samples was also enhanced with increasing concentrations.

Table (3): DPPH antioxidant activity of *J. phoenicea* and *C. villosa* leaves methanolic extracts.

Extract	DPPH radical scavenging activity (IC50=µg/ml)
J.phoenicea	11.10 ± 0.015
C.villosa	15.6 ± 0.019
Gallic acid	1.32 ± 0.011

IC50 values are shown as the mean ± SD from three independent experiments.

Ferric Reducing Antioxidant Power (FRAP) Assay

Figure (1) shows the FRAP value (µM) of *J. phoenicea*, and *C. villosa* extracts at different concentrations and the antioxidant powers were concentration dependent.

Determination of enzyme kinetics

The kinetic parameters of β-galactosidase from *Aspergillus* oryzae were studied for the hydrolysis towards ONPG at pH 4.5 and 30˚C. Results of Km and Vmax values were obtained by a typical double reciprocal Lineweaver Burk plot (figure 2) and found to be 1.310 ± 0.091 mM and 85.344 ± 0.028 mU, respectively.

Effect of *J. Phoenicea,* and *C. villosa* on β-Galactosidase Activity

The effect of *J. phoenicea,* and *C. villosa* on the activity of β-galactosidase was determined by performing the standard assay procedure at different concentrations from 16 µg/ml to 800 µg/ml. The enzymatic activity was gradually decreased with increasing concentration. Results of the percentage of relative activity (%) for the β-galactosidase in the presence of *J. phoenicea*, and *C. villosa* were shown in detail in figure (3). The β-galactosidase % inhibition was increased as the concentration of *J. phoenicea*, and *C. villosa* increased.

J. phoenicea extract was found to inhibit β-galactosidase in a non-competitive way and *villosa* extract affected in a mixed-inhibition way with IC50 values of 65 and 700 µg/ml, respectively (figure 4). The effect of *J. phoenicea*, and *C. villosa*, on the kinetic parameters (Vmax, Km, and Ki) of β-galactosidase from *A. aryzae* are shown in table (5).

Table (5): Kinetic values of β-galactosidase from *A. oryzae* in the presence of *J. phoenicea* and *C. villosa* extracts and Galactose.

Inhibitor	Km (mM)	Vmax (mU)	Ki (mg/ml)	Mode of inhibition
Control	1.311± 0.091	85.35± 0.028	0	Normal
J. phoenicea	1.256± 0.089	35.29± 0.033	1.416± 0.058	Non-competitive
C. villosa	2.581± 0.034	58.64± 0.015	1.537± 0.039	Mixed
Galactose	2.770± 0.072	55.55± 0.024	1.866± 0.052	Mixed

Results are Mean ±SD

Thermodynamic Analysis for β-Galactosidase Thermal Inactivation

The experimental of inactivation were run at gradient raise of 50, 55, 60, and 65°C the best fit of data is obtained when Ed = 208.88 kJ mol^{-1}. Rate constants (kd) in inactivation of β-galactosidase obtained are presented in table 7, where calculated from the plot of semi-natural logarithmic of residual activity versus time (Figure 7). Similarly, the half-life (t1/2) for the enzyme to lose its 50% activity can therefore be estimated using equation: $t_{1/2}$ = 0.693/Kd. The plot of half-life in minutes expressed as a function of temperature as seen in figure 8. Also, the D value was calculated using the relationship = ln_{10}/Kd and the z value was calculated and reported to be equal to 10°C.

Table (7) summarized the thermodynamic parameters of the enthalpy of denaturation (ΔH°) for the enzyme at 50 – 65 °C, were in a range of 121.84 to 121.72 kJ/mol, which showing a decreasing trend with the increase in temperature. Other value of free energy of thermal denaturation (ΔG°) for β-galactosidase was 91.025 kJ/mol at 50 °C, which decreased with the increase in temperature. While the entropy of inactivation (ΔS°) was calculated too at each temperature, it showed positive values, which indicates that there are no significant processes of aggregation, since had this happened, the values would have been negative.

Table (7): Kinetic and thermodynamic parameters of β-galactosidase inactivation

Temp. (°C)	Kd (min-1)	R2 (%)	t1/2 (min)	D (min)	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (J/(mol . K))
50	0.0023	94.39	301.30	1001.12	121.84	91.026	95.37
55	0.0064	96.49	108.28	359.78	121.80	89.644	98.00
60	0.0123	81.88	56.34	187.20	121.76	89.201	97.74
65	0.0862	98	8.04	26.71	121.72	85.068	108.39

Discussion

In this study, the qualitative and quantitative evaluation of the phytochemical constituents of *J. phoenicea* and *C. villosa* leaves extracts approved the presence of various secondary metabolites such as Alkaloids, Glycosides, Polyphenols, Saponins, Terpenes and Anthraquinones, which confirms the medicinal importance of these plants. This finding is agreed with previous works [3,8.] The scavenging effect of the extracts against DPPH radicals and FRAP are related to the electron transfer/donating ability. Also, the evaluation of the antiradical activity of both extracts from *J. phoenicea* and *C. villosa* leaves confirmed their ability to scavenge the DPPH radicals. For example, the tested extracts exhibited a higher IC_{50} values compared to the Gallic acid, and therefore less effectiveness in DPPH radical scavenging.

With reference to FRAP assay, *J. phoenicea* which has higher antioxidants than *C. villosa* extracts, due to the higher content in total phenolic for *J. phoenicea* extract.

To understand the chemical properties of polyphenols in terms of the availability of the phenolic hydrogens which behave as hydrogen donating radical scavengers can be easily predicted their antioxidant activity [37]. Therefore, polyphenols, depending on their precise structure and the proximity or adjacency of hydroxyl groups, that have a metal-chelating potential. Thus, polyphenols may have the possibility of chelating metal ions and preventing iron- and copper-catalyzed formation to initiating radical species [38, 39].

Results of chemical analysis showed that methanolic extracts of *J. phoenicea and C. villosa* react with β-Galactosidase from *A. oryzae*, inhibiting its enzymatic activity against ONPG as a substrate in in vitro experiments with IC50 values of 65 µg/ml and 700 µg/ml respectively. As illustrated, the activity of β-Galactosidase decreased depending on the concentration of the extracts. Other studies on the inhibitory effect of medicinal plants against β-Galactosidase activity showed various IC50 values range from 50 µg/ml to 5 mg/ml more than [40,41]. Results of the degree of effect on β-Galactosidase activity in the presence of some sugars ranges from 0.08 to 4 mg/ml was tested using ONPG as a substrate, showed that galactose as a product has inhibitory effect on the hydrolysis rate of ONPG with IC50 of 600 µg/ml, but other research has also shown that galactose has inhibitory effect against β-Galactosidase activity [42, 43].

Glucose supplementation result demonstrated significant activation of hydrolysis rate at concentrations exceeded 1.5 mg/ml, which supports the hypothesis that glucose binds to the enzyme at a different site from that active one, in addition to affecting on the affinity of the enzyme for the substrate [44]. While

result agreed with other results of different concentrations in previous findings for fructose and acarbose that showed no effect at concentrations up to 4 mg/ml of β-galactosidase [45].

The present study tried to distinguish between the nature of inhibition caused by adding different plants and sugars., in addition to determining their degrees of effecting on β-galactosidase activity.

As the kinetic parameters are important for β-Galactosidase from A. aryzae the mode of inhibition were obtained by a typical double reciprocal Lineweaver–Burk plot were explained them clearly. Therefore, the results showed that the maximum velocity (Vmax) decreased in the presence of the extracts of selected plants and the galacose, but the Km increased by C. villosa and galactose or slightly decreased by J. phoenicea extract which indicating mixed inhibition and non-competitive inhibition, respectively.

The mixed competitive inhibition indicates that the inhibitor can bind to the free enzyme or to the Enzyme-Substrate complex other than catalytic site, resulting the decreasing in the Vmax proportionate to the concentration of inhibitor [46]. Otherwise, non-competitive inhibitor binds to a different site that may not the active site of the enzyme due to change in the chemical structure of the enzyme; therefore, it can block the binding or stopping of enzymatic activity and change to substrate. These results are consistent with some of previous studies which using β-galactosidase from different sources and found competitive, noncompetitive or mixed inhibition by galactose [43.]

Regarding the DPPH and FRAP values described in table 6, the fractions of J. *phoenicea* approved that the plant can be an effective source of antioxidant compounds, so, it showed inhibitory effect against β-galactosidase with different degree. Also shown, the ability of higher polarity fractions is inhibiting the enzyme higher than those with less polarity, the experimental results of highest degree of inhibition at concentration of 80 µg/ml was achieved by fraction 6 with 47.5% inhibition. The activity of β-Galactosidase did not loss after incubation at 45°C during the test period of 3 hrs. at 50°C and above while the activity decreased with increasing temperature.

Inactivation is to be a process where the secondary, tertiary or quaternary structure of a protein changes without breaking covalent bonds [47] Therefore, the plots of residual activity vs. incubation time for the enzyme were linear, with R2 > 81 %, this was indicating that the inactivation could be expressed in terms of first order kinetics in the temperature range of 50–65°C. The kinetic enzymatic modelling of thermal inactivation, and the determination of its thermodynamic parameters were analyzed at changeable temperatures and various conditions by [48].

The half-life (t1/2) determinations are more accurate and reliable especially when computing the stability properties of an enzyme at different temperatures [49]. Thus, with increasing temperature, the t1/2 and D value decreased, and the first order thermal deactivation rate constants (kd) increased. The results clearly explored that the enzyme is less thermostable at higher temperatures, that led to higher rate constant which means the enzyme process is less thermostable [50]. The z value of β -galactosidase, calculated from the slope of the graph between log D vs. temperature, was 10°C. In general, the high magnitudes of

z-values mean more sensitivity to the duration of heat treatment and lower z-values mean more sensitivity to increase in temperature [51].

The activation energy (Ed) of the thermal inactivation mechanism, which is the essential quantity to determine the thermodynamic parameters for thermal stability, is equal to 208.88 kJ mol − 1 which is comparable to the results also obtained from previous studies of the inactivation of β-galactosidases from different strains of Streptococcus thermophilus and Lactobacillus bulgaricus, and which was ranged from 200 to 215 kJ mol-1, that explained the high value for a high amount of activation energy that is needed to initiate denaturation of the enzyme[35].

Thermodynamic values of variation in activation enthalpy (ΔH), variation in activation entropy (ΔS), and variation in Gibbs free energy (ΔG) were calculated for the different temperatures as seen in Table 7. Positive ΔH values indicate that enzyme inactivation is an endothermic process [52]. The high values of ΔH° resulted from the thermal inactivation of β-galactosidase which indicates that the enzymatic activity undergoes a considerable change in conformation during denaturation [53]. The fact that ΔH° value decreases with the increase in temperature reveals that less energy is required to denature the enzyme at high temperature [54]. When entropy of inactivation (ΔS°) was calculated at each temperature, it showed positive values, which indicates that there is an increase in the molecule randomness or disorder during the exposure to high temperatures. In contrast, a negative ΔS values are expected for an aggregation process in which a few intermolecular bonds are formed [55]. And, in turn, the positive (ΔG) values indicate that the inactivation of the enzyme is not a spontaneous reaction. In the present study, there was decrease in ΔG values with increasing temperatures, which is an indication that the destabilization of the enzyme molecule is more spontaneous and faster [56] the result that the ΔG° values are lower than the ΔH° values is due to the positive entropic contribution during the inactivation process.

Conclusion

Considering the results achieved, leaf of *J. phoenicea* and *C. villosa* could inhibit key enzyme involved in the digestion of carbohydrates. Traditionally, leaf extracts could be the promising natural medicinal agents and widely used for the treatment of diabetes mellitus. In addition, *J. phoenicea* and *C. villosa* have a beneficial effect on free radical scavenging and in the prevention of oxidative stress because its extracts can reduce in vitro the radical DPPH, which triggers of several metabolic diseases such as diabetes mellitus. Furthermore, oral consumption of plants stimulate excretion, or decrease the digestion of carbohydrates by the inhibition of the enzymes responsible for metabolism of carbohydrates into glucose in the digestive tract. All these effects may be attributed to their high polyphenolic contents of *J. phoenicea* and *C. villosa* and considered as a potential source of natural antioxidants. This work demonstrates that *J. phoenicea* and *C. villosa* value as a source of metabolites with relevant pharmaceutical potential and essential to better retain enzymatic activity in food biotechnology and products supplemented with heat-sensitive enzymes.

Abbreviations

IC50
The half maximal inhibitory concentration
DPPH
1,1-diphenyl,2-picrylhydrazyl
FRAP
Ferric reducing antioxidant power
GAE
Gallic acid equivalent
RE
Rutin equivalent
ΔH
enthalpy change

Declarations

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This work was carried out at the Department of Biology, Mutah University, Al-Karak, Jordan

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

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Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

Author's Contribution

A.Al-Mustafa has designed and contributed in results analysis. M. Al-Tawarah has contributed in the major bench experiments. M. Al-Sheraideh and F. Al-Zahrany equally edit the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Figures

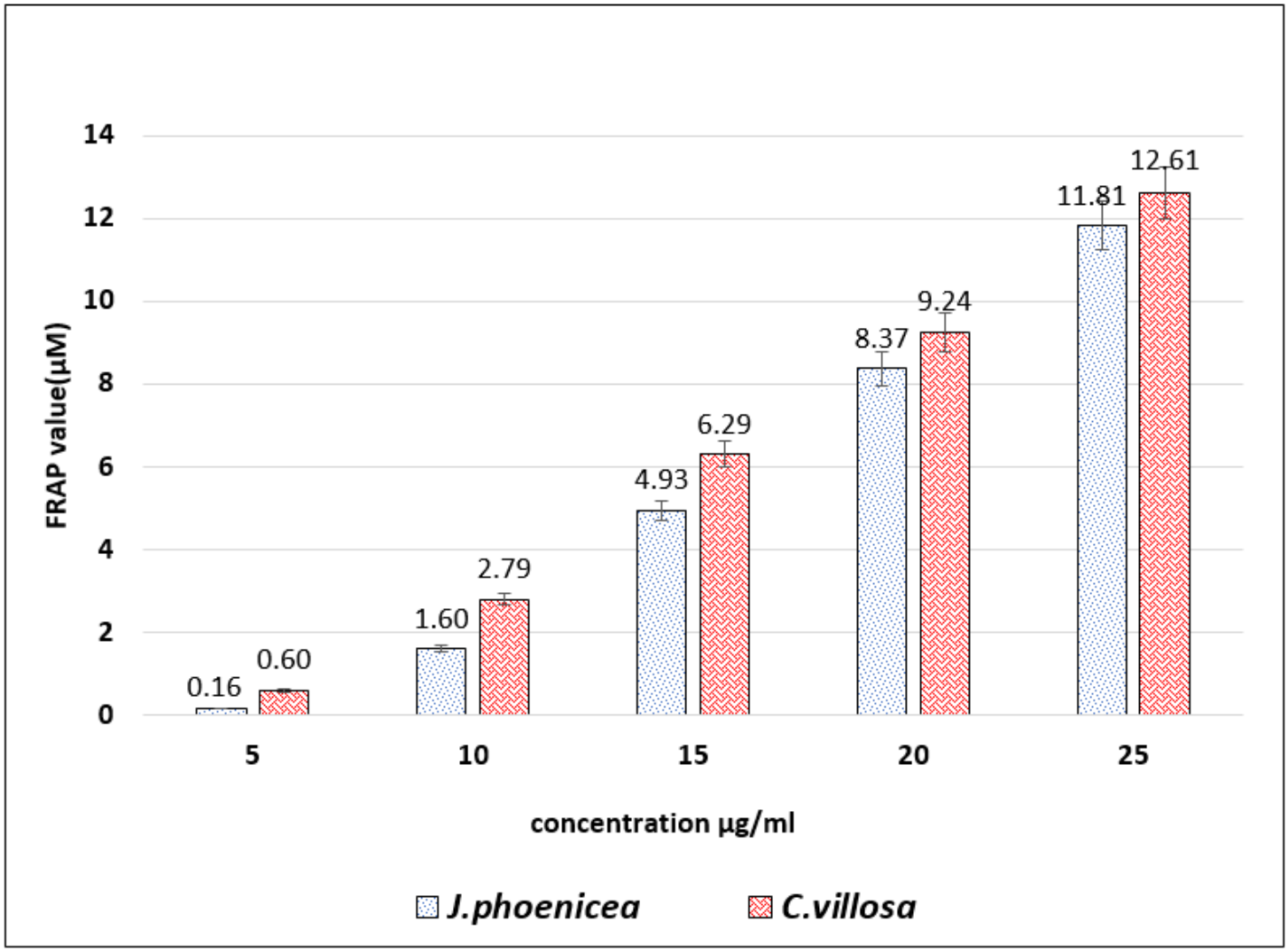

Figure 1

FRAP value of J. phoenicea and C. villosa extracts. Each value represent means ±SD (n = 3)

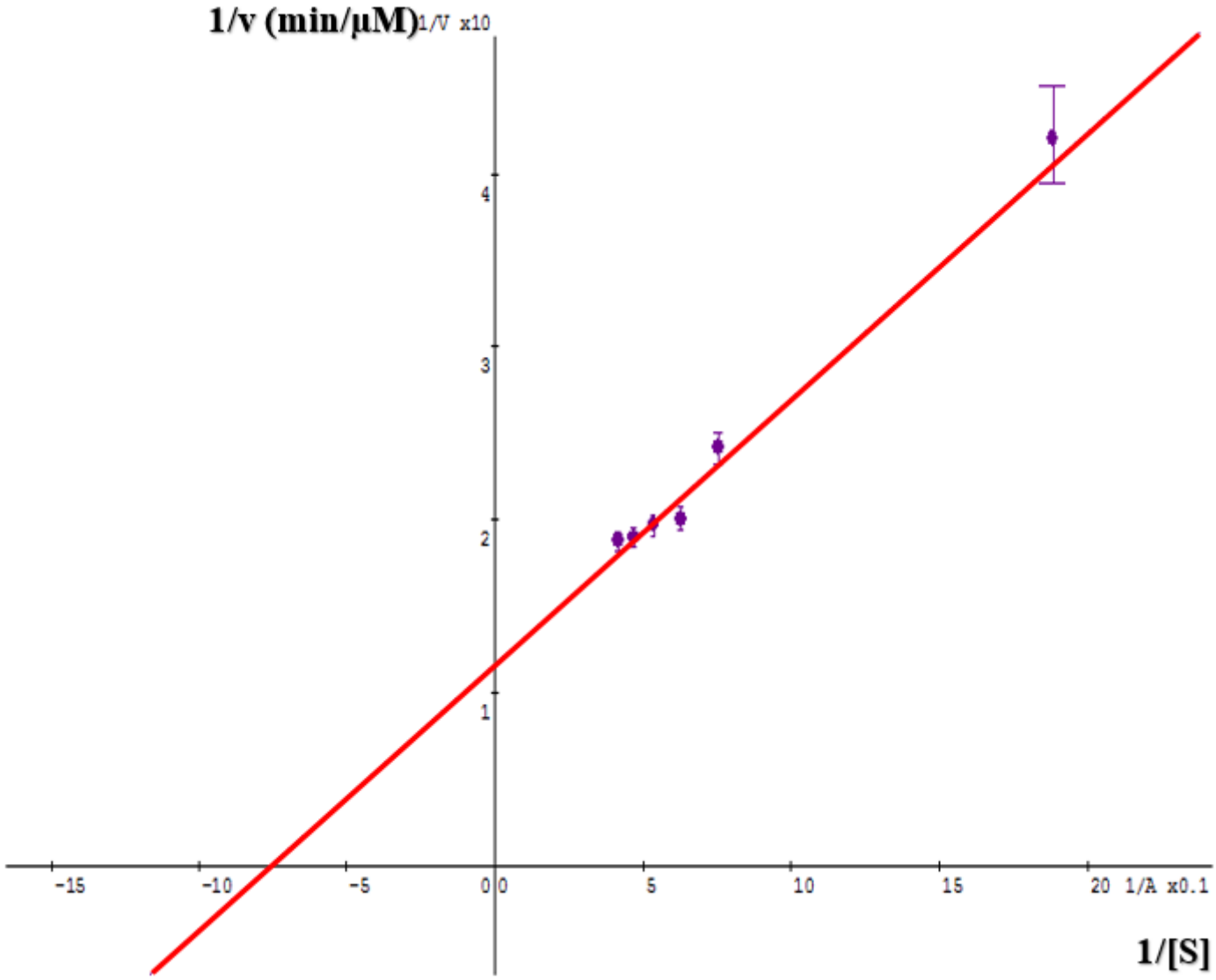

Figure 2

Lineweaver–Burk plot of (1/ v) of β-galactosidase vs. (1/ ONPG). Mean ± SD (n=3)

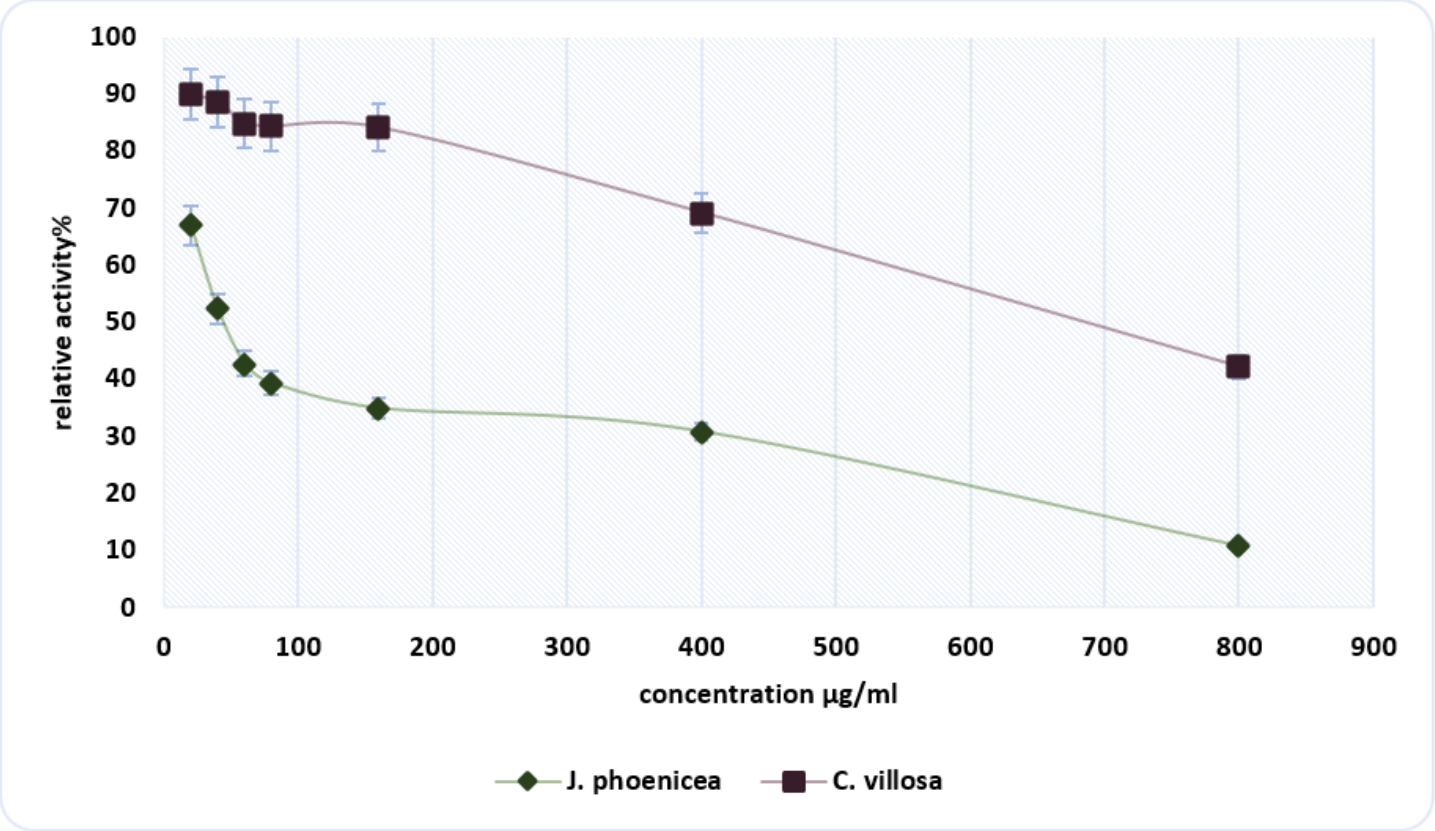

Figure 3

Relative activity (%) for β-galactosidase from A. oryzae in presence of J. phoenicea, C. villosa, and rutin, using ONPG as a substrate. Mean ± SD (n=3)

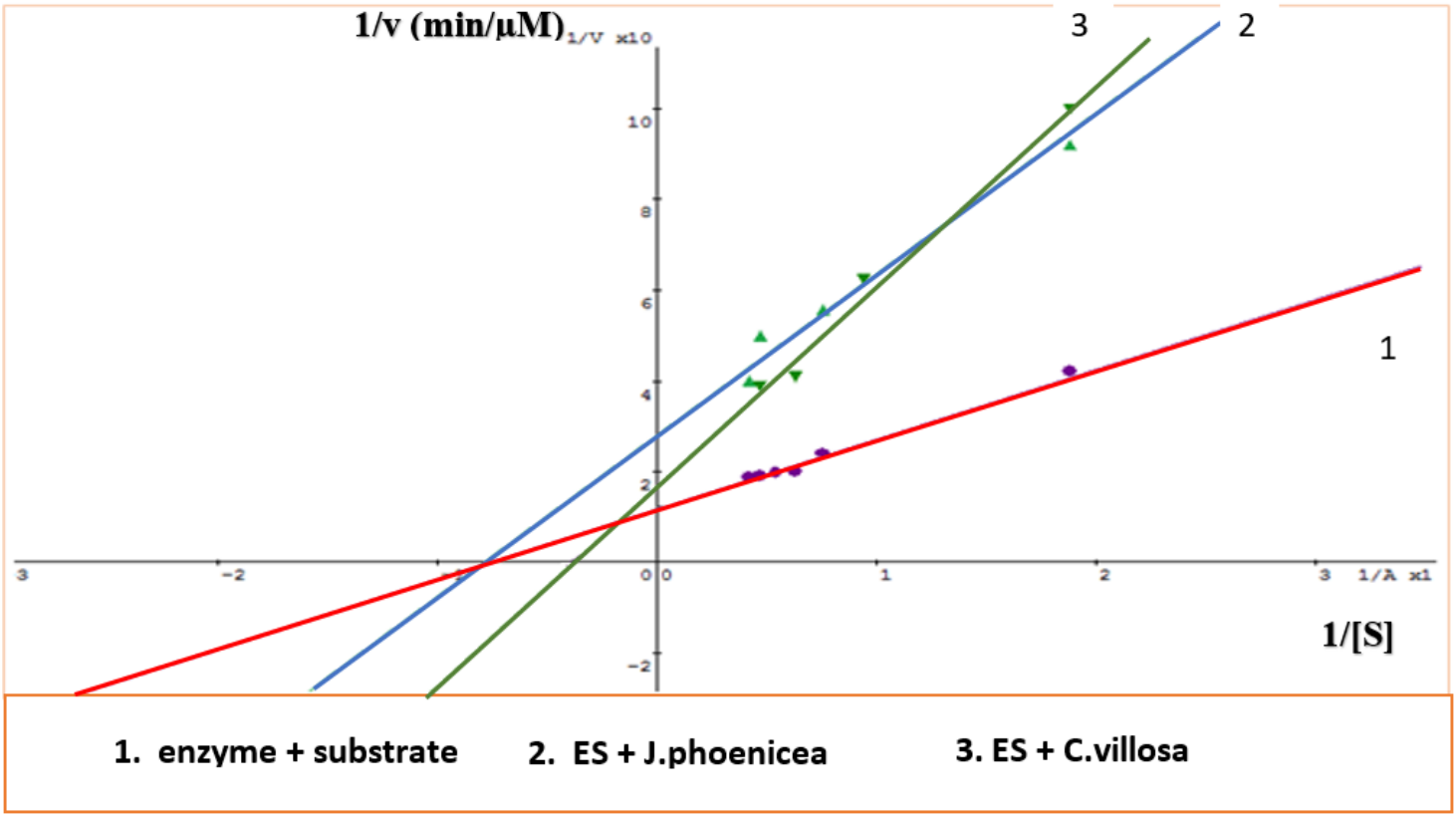

Figure 4

Inhibition of β-galactosidase by J. phoenicea and C. villosa, in the presence of various concentrations of ONPG as a substrate. Mean ±SD (n=3)

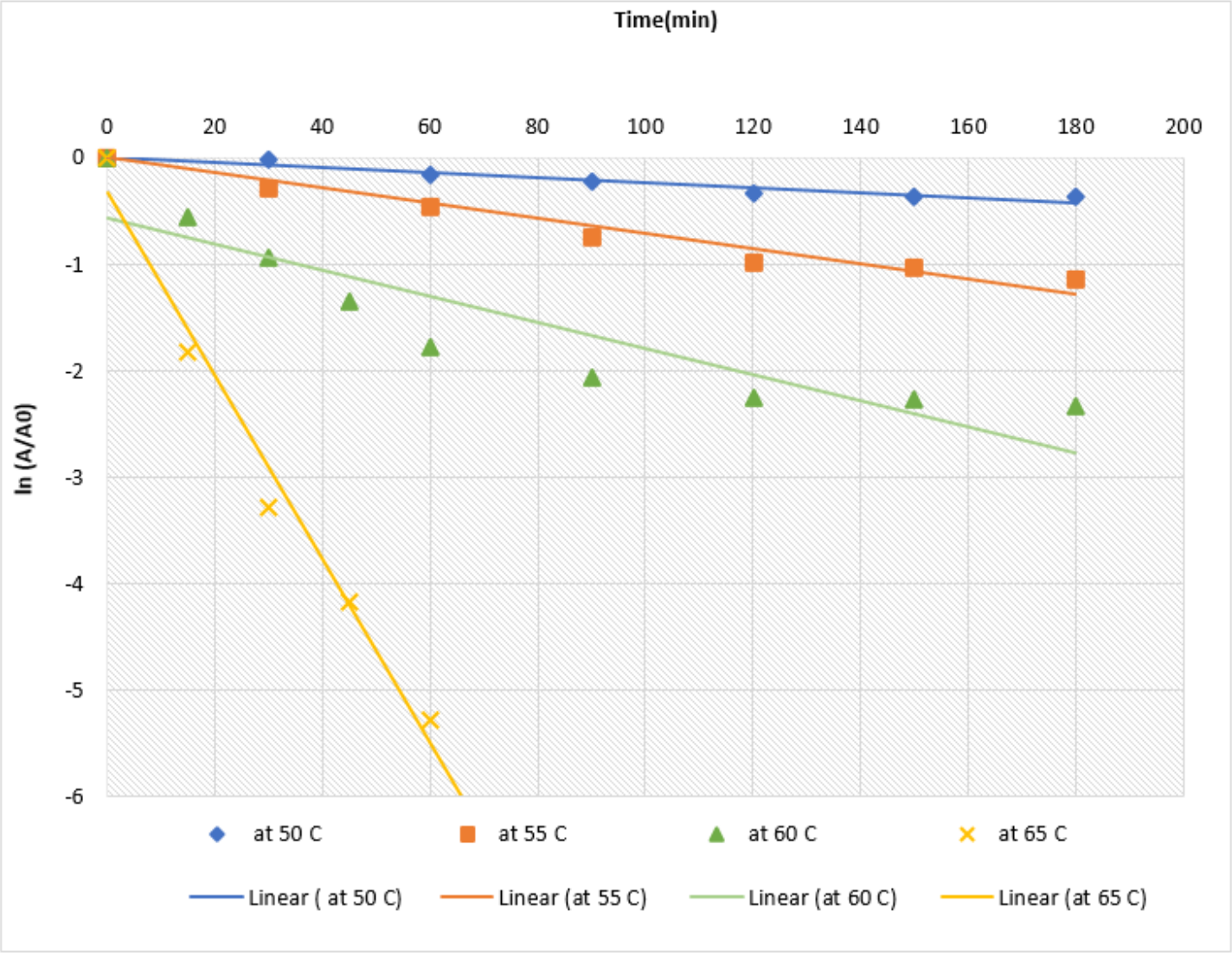

Figure 5

Thermal deactivation of β-galactosidase from A. oryzae at various temperatures using ONPG as a substrate. Mean ± SD (n=3)

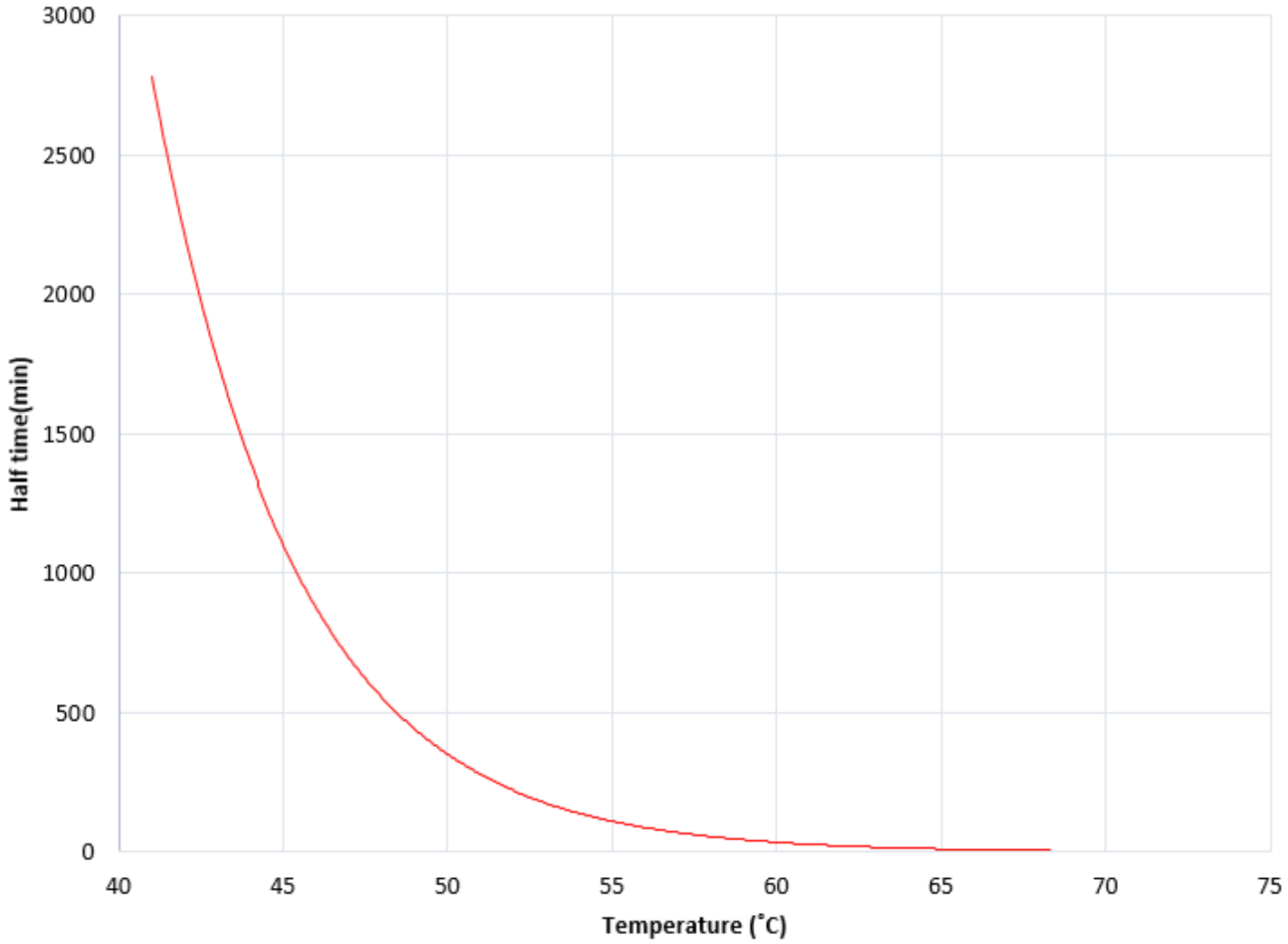

Figure 6

Half-life of β-galactosidase from A. oryzae as influenced by temperatures. Table (7) summarized the thermodynamic parameters of the enthalpy of denaturation (ΔH°) for the enzyme at 50 – 65 °C, were in a range of 121.84 to 121.72 kJ/mol, which showing a decreasing trend with the increase in temperature. Other value of free energy of thermal denaturation (ΔG°) for β-galactosidase was 91.025 kJ/mol at 50 °C, which decreased with the increase in temperature. While the entropy of inactivation (ΔS°) was calculated too at each temperature, it showed positive values, which indicates that there are no significant processes of aggregation, since had this happened, the values would have been negative.