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Research Article

Phytochemical Constituents and *In Vitro* Anti-Diabetic Properties of *Ziziphus jujuba* (Rhamnaceae) Fruits

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ABSTRACT

In the current study the phytochemical profile of 70% ethanol extract of *Ziziphus jujuba* (Rhamnaceae), cultivated in Bulgaria has been investigated. The fruit extract contained numerous phytochemicals, such as triterpenes, phenolic acids and flavonoids. Five triterpenes, 11 phenolic acids and 5 flavonoids have been identified. The fruit extract had a total phenolic content of 21.62 ± 0.0265 mg/g and total flavonoid content 1.34 ± 0.017 mg/g dried extract. Among the triterpenes with the highest concentration was the betulinic acid 20943.17±527.06 µg/g dried extract, rosmarinic acid (1174.26±29.55 µg/g) among the phenolic acids, followed by myricetin (214.61±5.40 µg/g) as a representative of flavonoids and rutin 3 046.89±76.68 among the quercetin glycosides. The inhibitory effect of *Z. jujuba* fruit extract has been investigated on the key enzymes linked to diabetes - α-glucosidase and α-amylase. In order to evaluate the type of inhibition a Lineweaver-Burk plot was produced. The results obtained from the enzyme kinetic studies exhibited a mixed noncompetitive-uncompetitive type of inhibition on α-glucosidase and mixed competitive-non-competitive type of inhibition on α-amylase. Besides that the obtained results proved high inhibition of α-glucosidase (79.46% at 1.33 mg.ml^{-1} extract) and moderate inhibition of α-amylase (39.10% at 0.666 mg.ml^{-1} extract concentration). These results suggest the possible use of fruits of *Z. jujuba* in the management of diabetes mellitus.

Keywords: *Ziziphus jujuba*, phytochemical profile, α-glucosidase, α-amylase, inhibition, anti-diabetic properties.

INTRODUCTION

Diabetes mellitus is a major health challenge that concern about 220 million people over the world, estimating that this number will double till 2030[1]. Diabetes mellitus is a metabolic disease characterized by hyperglycaemia resulting from either defect in insulin secretion, insulin action, or both**.** The possibilities to predict the arising of diabetes type 2 is restricted. In spite of that abnormal glucose metabolism could be registered several years before diabetes formation. One of the therapeutic approaches for treatment of diabetes is to control the levels of blood sugar through inhibition of all enzymes responsible for degradation of starch and sugars in the food products to glucose. The inhibition of α-glucosidase and α-amylase hamper the digestion of carbohydrates and assimilation of glucose with decreasing the postprandial hyperglycemic. In humans 6 enzyme activities, 2 α-amylases and 4 α-glucosidases are involved in the degradation of carbohydrates[2]. Commercial α-glucosidase inhibitors, such as acarbose, voglibose and miglitol have a wide clinical application for controlling the blood sugar levels in patients[3]. Along with that has been reported that these substances cause numerous side effects, including flatulence, diarrhea, and abdominal pain[4]. The treatment with acarbose is not recommended to patients with cirrhosis, renal failure and gut disease[5]. Regarding these effects, the screening for more effective and safety enzyme inhibitors from natural sources are good alternative for prevention and treatment of diabetes type 2.

The medicinal properties of *Ziziphus* species result from the diverse groups of secondary metabolites identified in leaves or seeds extracts. The experimental pharmacology proves numerous activities of *Ziziphus* extracts, *e.g.* antiallergic[6,7] and analgesic[8]. The antihyperglycemic activity of leaves extracts from *Zizyphus spina*, *Z. spina-christi* (L.) and ethanol extract from seeds of *Z. jujuba* has been documented[9]. Glombitza et al.[10] reported for hypogycemic and antihyperglycemic effect of *Z. spinachristi*. The antidiabetic and antiallergic activities of water extracts from *Z. mauritiana* has been reported as well[11]. Ethanol extract from seeds *Ziziphus jujuba*[9] and extract of *Ziziphus jujuba* leaves[12] decreased the serum glucose level in diabetic rats.

The fruits of *Ziziphus* are rich sources of biologically active molecules, such as polysaccharides, phenolic compounds, terpenes, saponins etc. Each molecule possesses unique and multifactorial properties and has

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multiple mechanisms for improvement of insulin sensitivity[13].

There is insufficient information about the phenolic acids, flavonoids and quercetin glucosides contents in the fruits of *Z. jujuba*. The investigated plant species is cultivated in Bulgaria and analyses of his medicinal properties are still lacking. Although the fruits of *Z. jujuba* are used in traditional medicine for diabetes treatment[14] they are insufficient studied regarding their inhibitory activity of digestive enzymes.

In this aspect main objective of this survey is to give an insight of the biologically active compounds in 70% ethanolic extract from fruits of *Z. jujuba*, cultivated in Bulgaria, and to study the inhibitory activity of the extract, resulting from its bioactive components, against α-glucosidase and α-amylase.

MATERIALS AND METHODS

Plant material

The fruits of *Ziziphus jujuba* (Rhamnaceae), cultivated in Bulgaria were dried at 40^0C**,** afterwards grinded, frizzed and lyophilized.

Chemicals and Reagents

α-Glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), α-amylase from porcine pancreas (EC 3.2.1.1), ρ-nitrophenyl-α-D-glucopyranoside (ρNPG), acarbose, 4-hydroxybenzoic acid hydrazide (PAHBAH), starch soluble (extra pure) were obtained from Sigma-Aldrich (Darmstadt, Germany). Phenolic standards: gallic, vanillic, ferulic, p-coumaric, p-hydroxy benzoic, syringic, trans-cinnamic, chlorogenic and rosemaryin acid, myricetin, quercetin, luteolin, kaempferol, apigenin, rutin, hyperosid, carnosic acid, betulin, betulinic acid, oleanolic and ursolic acid acid were purchased from Sigma- Aldrich (Darmstadt, Germany). The other reagents were of analytical and HPLC grade and double distilled water was used for the preparation of the reagents.

Preparation of Plant Extracts

For extraction compounds the lyophilized plant material was extracted in triplicates with 70% ethanol (1:5 w/v) in an ultrasonic bath (35 kHz) at 45 °C for 45 min each. The united extracts were filtrated through a filter paper and concentrated at 45 °C on a rotary vacuum evaporator (Laborota 4002, Heidolph Instruments GmbH & Co.KG, Germany). Due to the high presence of carbohydrates the extracts were further subjected to additional cleaning step, including extract acidifying to pH 2, addition of NaCl (1g/100mL) and triplicate extraction with ethyl acetate (1:3 v/v). The collected extracts were again filtrated and vacuum concentrated. For complete dryness the extracts were frozen, freeze-dried (Christ Alpha 1-2, Germany) and stored at -20 °C prior to be used.

Phytochemical analyses by HPLC

The obtained extracts were analyzed on HPLC system consisting of Waters 1525 Binary pump (Waters, Milford, MA, USA), Waters 2487 Dual λ Absorbance Detector (Waters, Milford, MA, USA), controlled by Breeze 3.30 software and equipped with reverse-phase Supelco Discovery HS C_{18} (25 cm x 4.6 mm, 5 µm) column, thermostated at 26 °C. The extracts were dissolved in appropriate concentrations with methanol and filtrated through 0.45 µm syringe filters. The constituents were identified according to their retention time (R_T) and quantified according to calibration curve for each compound. For determination of triterpenes the method developed by Marchev et al.[15] was applied. In brief, a mobile phase consisting of methanol and 0.1% formic acid (92:8) and 0.4 ml/min flow rate was used. The detection of the substances was monitored at 210 nm. For flavonoids analysis a gradient elution was applied. The mobile phases were 2.0% acetic acid (phase A) and methanol (phase B) with wavelength of detection 308 and 380 nm. Quercetin glycosides were detected at 380 nm by applying gradient elution with 2.0% acetic acid (phase A) and acetonitrile (phase B). Rosmarinic acid was detected at 327 nm using mobile phase of methanol:H_3PO_4:H_2O = 50:0.3:49.7. The phenolic acid were quantified at 280 and 320 nm with mobile phase 2.0% acetic acid (phase A) and 0.5% acetic acid:acetonitrile (1:1). Detailed description and gradients of the mobile phase are described by Marchev et al.[16].

Assay of total phenolics content

The total phenolic content (TPC) was determined by Kerina et al.[17]. To 0.1 ml of extract (1:10 diluted) 0.5 ml Folin-Ciocalteu reagent and 2.0 ml 10% $NaCO_3$ were added. After 10 min 1.0 ml from the mix was added to 4.0 ml of distilled water. The absorbance was read at 620 nm on spectrophotometer Spectrostar Nano (BMG, Ortenberg, Germany) against a blank sample containing water instead of extract. The phenolic amount was calculated through calibration curve with 0.01% solution of gallic acid and expressed as mg gallic acid equivalents per gram of extract (mg GAE/g extract).

Assay of total flavonoids content

The total flavonoids content was determined according to Zhishen et al.[18] with minor modifications. To an appropriate diluted in DMSO extract 0.15 ml $NaNO_2$ (5%) and 0.15 ml $AlCl_3$ (10%) were added. Five minutes later 1.0 ml NaOH (1M) was added and after 6 more min the volume was adjusted to 5 ml with distilled H_2O. The reaction was conducted for 30 min at ambient temperature and afterwards the absorbance was measured at 510 nm against blank sample, which contained distilled water instead of extract. The amount of flavonoids was calculated through a calibration curve with (+) – cathecin and expressed as mg catechin equivalents per g extract (mg CE/g extract).

α-Glucosidase activity assay for inhibition tests of jujube ethanol extract

Yeast α-glucosidase has been frequently used to identify its inhibitors from medicinal plants. The method of Suresh et al.[19] was employed with some modifications. The assay uses ρNPG as substrate, which is hydrolyzed by α-glucosidase to release ρ-nitrophenol, a color agent that can be monitored at 405 nm. Briefly, 20 µl of a sample solution was mixed with 70 µl of the enzyme solution (1 unit/ml) in 0.1 M phosphate buffer (pH 6.8), since α-glucosidase is sensitive to different pH values, and incubated at 37 ºC for 6 min under shaking. After incubation, 100 µl ρNPG (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mM) solution in the above buffer was added to initiate the colorimetric reaction at 37 ºC. The

released ρ-nitrophenol was monitored at 405 nm every min for a total time of 30 min by a Spectrostar Nano (BMG, Ortenberg, Germany) 96 micro well plate reader against a blank sample without enzyme. DMSO without extracts was set up in parallel as a control and each experiment was performed in triplicates. One unit of enzyme activity was defined as the amount of enzyme that released one µmol of p-nitrophenol per minute under the assay conditions described.

α-Amylase activity assay for inhibition tests of jujube ethanol extract

The α-amylase activity was determined using the modified version of the method according to McDougall et al.[20] and Grussu et al.[21]. Briefly, 50 µL of the extracts, which were dissolved in DMSO was mixed with 200 µL of the enzyme solution (0.5 U/mL) in 0.2 M sodium phosphate buffer (pH 6.9) and incubated at 37°C for 5 min. After incubation, 250 µL of the soluble starch in concentration 0.1, 0.5, 1, 1.3 1.5 % was added to initiate the enzyme reaction. 100 mg PAHBAH substance was dissolve successively with 0.5 M HCl and 0.5 M NaOH to give the working PAHBAH reagent. Triplicate samples (250 µL) of assays were taken at fixed times and added to 1.75 mL of PAHBAH reagent in a 2 mL tube. After heating for 15 min at 100 °C and cooling, the absorbance at 410 nm was measured by a spectrophotometer SPECTROstar Nano (BMG, Offenburg, Germany). Control tubes contained only DMSO, enzyme and substrate, while in positive controls acarbose replaced the plant extracts. Mixtures without enzyme, plant extract and acarbose served as blanks. One unit of enzyme activity was defined as the amount of enzyme that released one µmol of glucose per minute under the assay conditions described.

Kinetics of inhibition of jujube ethanol extract against α-amylase and α- glucosidase activities

The kinetics of inhibition of the ethanol extract of *Z. jujuba* against α- glucosidase and α- amylase activities were measured by increasing substrate concentrations of ρNPG (0.5- 5.0 mM) and starch (0.1- 1.5 % respectively in the absence and presence of extract of *Z. jujuba* at concentrations of 0.66 and 1.33 $mg.ml^{-1}$ for both α-glucosidase and α-amylase inhibitory assay. Acarbose at concentrations 0.66 and 1.33 $mg.ml^{-1}$ was included as a possitive control. The type of inhibition was determined by Lineweaver-Burk double reciprocal plot analysis of the data, which was calculated from the result according to Michaelis-Menten kinetics[22]. Each experiment was performed in triplicates. The inhibition of α-glucosidase and α- amylase by jujube extract was calculated according to the following formula: % Inhibition = [(EAc− EAe)/EAc] × 100 where EAc and EAe are the enzyme activity of the control sample and enzyme activity of jujube extract, respectively.

RESULTS AND DISCUSSIONS

The phytochemical content of 70% ethanol extract of *Ziziphus jujuba* fruits is presented on Table 1. Among all biologically active molecules, major characteristic constituents in fructus were triterpenes, such as oleanolic, ursolic and betulinic acid which concentration reached up to 20943.17±527.06 µg/g extract. These results are in agreement with those reported by WHO monographs on selected medicinal plants[14] in fructus *Zizyphi* of *Zizyphus jujuba* Mill. or *Z. jujuba* var. *inermis* Rehd. (Rhamnaceae). The presence of the terpenoids oleanolic, betulonic, zizyberenalic and betulinic acid in fruits of Z. *jujuba* are also reported by Lee et al.[23] and Shoei et al.[24]. The investigated 70% extract had a total phenolic content of 21.62 ± 0.0265 mg/g extract. In total 11 phenolic acids were identified, among which rosmarinic (1174.26±29.55 µg/g) and sinapic acid (500.40±12.59 µg/g) were in the highest amounts. Total flavonoid content in the investigated extracts was 1.34 ± 0.017 mg/g extract and the concentration of the dominant flavonoids was as follow: myricetin (214.61±5.40 µg/g) > luteolin (69.75±1.76 µg/g) > quercetin (65.21±1.64 µg/g) > kaempferol (18.48±0.46) > apigenin (9.56±0.24) From the quercetin glycosides in high concentration could be distinguished rutin (3 046.89±76.68 µg/g) and hyperosid (94.76±2.38 µg/g). The identified phenolic compounds in our study correspond to that reported by San and Yildirim[25] in the fruits of Z. *jujuba*. In the investigated fresh fruits from four different jujube genotypes it was determined the presence of catechin, epicatechin, rutin, as well as, caffeic, ferulic, chlorogenic and 4-hydroxybenzoic acid, which concentration varied in the different selections. One of the selections had significantly higher rutin content than the other ones. Some phenolics, such as chlorogenic and caffeic acid, catechin, epicatechin and rutin, were isolated from jujube fruit by Hudina et al.[26]. The presence of terpenes, phenolic acids and flavonoids in the investigated from us 70% ethanol is a prerequisite for a potential α-glucosidase inhibitory activity of Z. *jujuba*.

Inhibition of α-glucosidase activity from 70% ethanol extract of Z. jujuba

The inhibition of α-glucosidase, which catalyzes the hydrolysis of o-nitrophenyl-α-D-glucopyranoside was increasing with the increasing concentrations of the extract (Table 2). The type of α-glucosidase inhibition of the extract was determined according to Lineweaver-Burk. The obtained graphic is presented on Figure 1A. The linear regression equitation of the control was $y = 0.0743x + 0.0151$ ($R^2 = 0.9997$). The corresponding equations for the extract concentrations 1.33 and 0.66 $mg.ml^{-1}$ were $y = 0.1916x + 0.1126$ ($R^2 = 0.9897$) and $y = 0.1625x + 0.0728$ ($R^2 = 0.9862$) respectively. In the presence of different extract concentrations K_m and V_{max} values decreased and were different from that of the control sample (Table 2). In the primary graphic the obtained correlations were crossing in third quadrant. Therefore, the inhibition was non-competitive-uncompetitive mixed. This could be also confirmed by the values of the dissociation constant: KI (0.248 $mg.ml^{-1}$) и K_i (0.843 $mg.ml^{-1}$), determined by the equations of the secondary graphics (Figure 1B, C). In the case of non-competitive-uncompetitive inhibition $KI < K_i$[22]. The lower KI (ESI) value than the (EI) is evidence for the higher affinity of the inhibitors in the extract to bind with ES- complex than the unbound enzyme. The results are summarized in Table 2.

Table 1: Content of phenolic acids, flavonoids and triterpenes in fruits of *Ziziphus jujube.*

Phenolic acids			Flavonoids and quercetin glycosides			Triterpenes		
Comp.	Conc. µg/g extract	Conc. µg/g biomass	Comp.	Conc. µg/g extract	Conc. µg/g biomass	Comp.	Conc. µg/g extract	Conc. µg/g biomass
3,4-diOH Benzoic	10.96± 0.28	0.34±0.01	Myricetin	214.61 ±5.40	6.62 ±0.17	Carnosic acid	2 642.77 ±66.51	81.50 ±2.05
2-OH Benzoic acid	144.49 ± 3.64	4.46 ±0.11	Quercetin	65.21 ±1.64	2.01 ±0.05	Betulin	3 703.66 ±93.21	114.22 ±2.87
Chlorogenic acid	202.72 ± 5.10	6.25 ±0.16	Luteolin	69.75 ±1.76	2.15 ±0.05	Betulinic acid	20943.17 ±527.06	645.87 ±16.25
Vanillic acid	221.72 ± 5.58	6.84 ±0.17	Kaempferol	18.48 ±0.46	0.57 ±0.01	Oleanolic acid	8 828.02 ±222.17	272.25 ±6.85
Caffeic acid	35.92 ± 0.90	1.11 ±0.03	Apigenin	9.56 ±0.24	0.29 ±0.01	Ursolic acid	7 669.60 ±193.01	236.52 ±5.95
Syringic acid	181.81 ±4.58	5.61 ±0.14	Rutin	3 046.89 ±76.68	93.96 ±2.36			
p-Coumaric acid	26.31 ±0.66	0.81 ±0.02	Hyperosid	94.76 ±2.38	2.92 ±0.07			
Ferulic acid	69.41 ±1.75	2.14 ±0.05						
Sinapic acid	500.40 ±12.59	15.43 ±0.39						
Rosemary acid	1174.26 ±29.55	36.21 ±0.91						
Cinnamic acid	1.02 ±0.03	0.03 ±0.00						

As a positive control in this study acarbose was used. Based on the Lineweaver-Burk primary graphic (Figure 2A), the linear regression equation of the control was $y = 0.0743x + 0.0151$ ($R^2 = 0.9997$), while at acarbose concentrations 0.66 and 1.33 mg.ml^{-1}, the respective equations were $y = 0.2572x + 0.0578$ ($R^2 = 0.9873$) and $y = 0.371x + 0.1382$ ($R^2 = 0.9751$). Both, the slopes and the vertical axis intercept increased with increasing acarbose concentration. In the primary graphic the obtained correlations were crossing in third quadrant. These results account for a noncompetitive - uncompetitive mixed. The dissociation constant determined by the equations of the secondary graphics (Figure 2B, C) for ESI-complex, KI (0.096 mg.ml^{-1}) and inhibitory constant for the EI-complex, K_i (0.333 mg.ml^{-1}), is evidence for the higher affinity of the acarbose to bind with ES- complex than the unbound enzyme. The constant of inhibition, K_i is indication for the strength of the inhibitor. K_i of acarbose (0.333 mg.ml^{-1}) had a lower value from the K_i of the extract (0.84336 mg.ml^{-1}) (Table 2). This means that the inhibitory strength of the 70% ethanol extract was lower than that of acarbose but provoked stronger inhibition of α-glucosidase (79.46%) compared to acarbose (82.98%).

There are few reports that triterpeniods have α-glucosidase inhibitory activity. Kumar et al.[27] confirmed that betulinic acid inhibited α-glucosidase activity with 52.2 ± 3.8% at concentration of 50 µg/ml. Ali et al.[28] proved that oleanolic acid inhibited α-glucosidase uncompetitive. The flavonoid structure, the position and number of the hydroxyl groups were determining factors for α-glucosidase inhibition. According to Tadera et al.[29] α-glucosidase inhibition of flavonoids increased by increasing the number of the hydroxyl groups in the B ring, while the substitution of the hydroxyl group at position 3 decreased the activity. In this regard the presence of triterpeniods and flavonoids as myricetin, quercetin, luteolin in studied extract are prerequisite for its inhibitory activity on α-glucosidase.

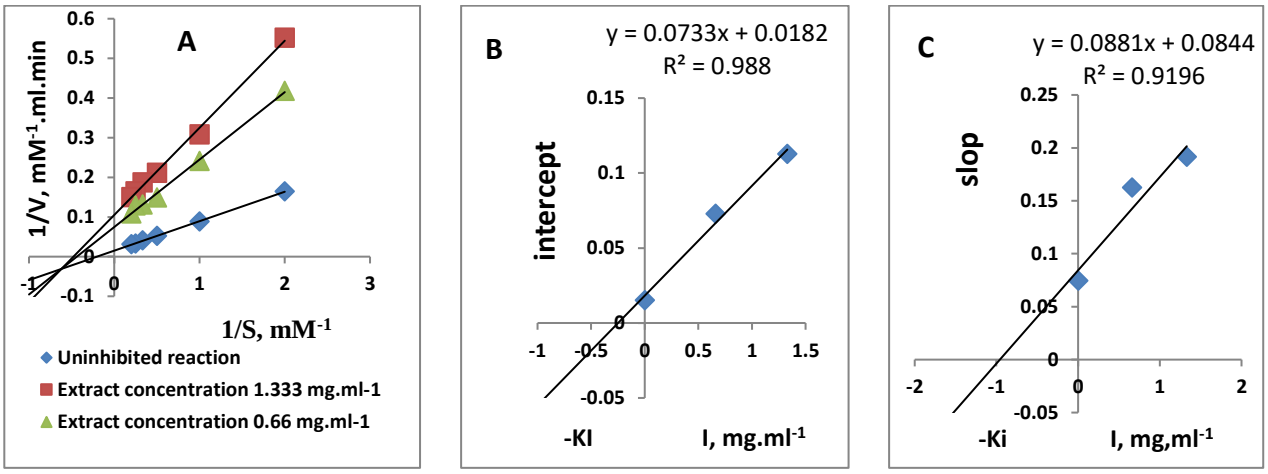

Figure 1: Lineweaver–Burk plots (A) and secondary plots (B and C) for determination of dissociation constants of ESI (KI) and EI (K_i). Reciprocal plots were obtained by variable extract concentrations.

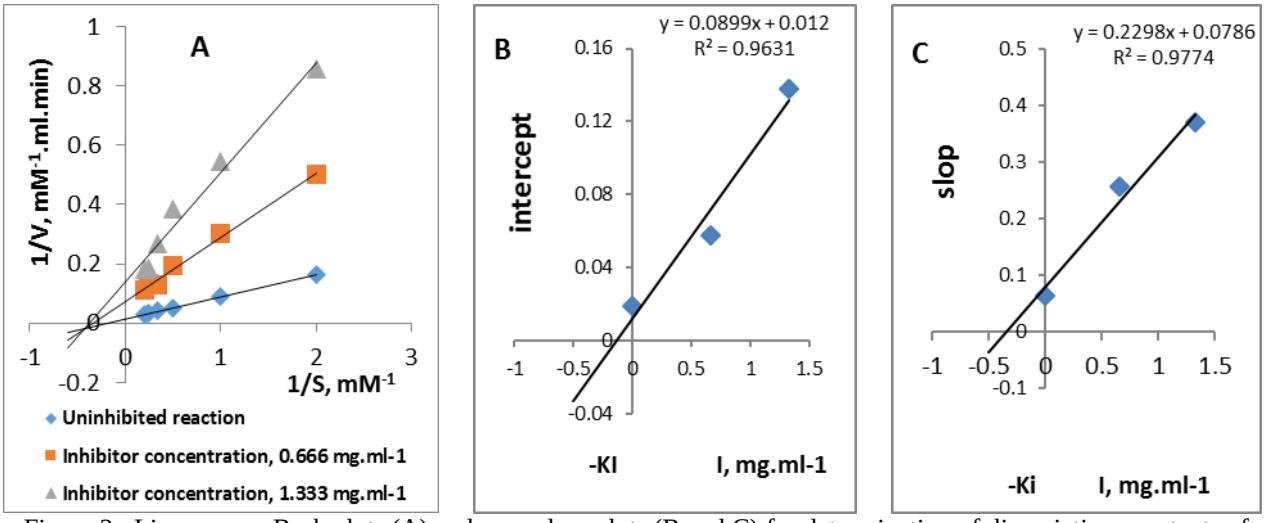

Figure 2: Lineweaver–Burk plots (A) and secondary plots (B and C) for determination of dissociation constants of ESI (KI) and EI (Ki). Reciprocal plots were obtained by variable concentrations of acarbose.

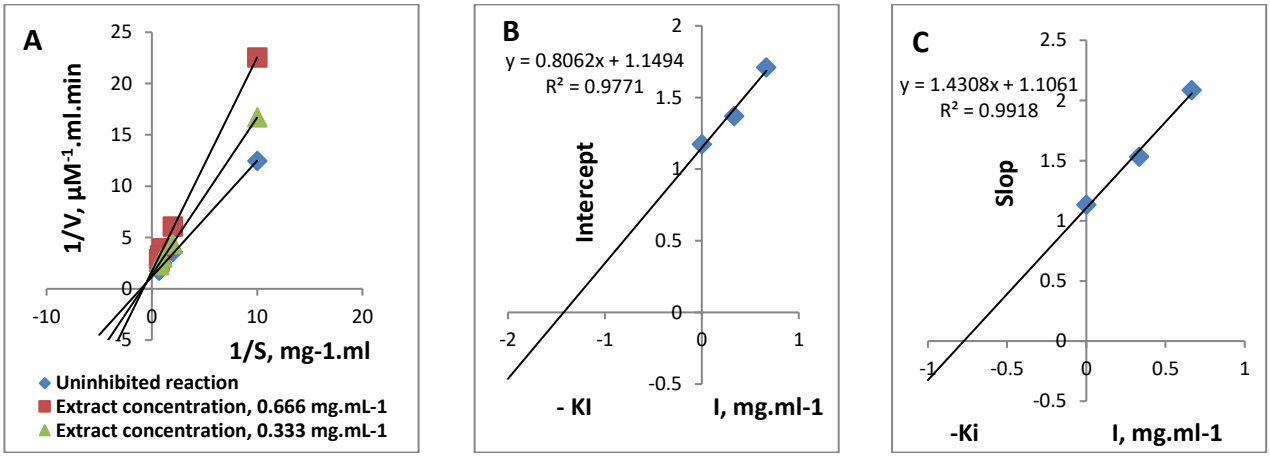

Figure 3: Lineweaver–Burk plots (A) and secondary plots (B and C) for determination of dissociation constants of ESI (KI) and EI (K_i). Reciprocal plots were obtained by variable extract concentrations.

Among the phenolic acids, with the highest amount detected was the rosmarinic acid. This acid isolated from the methanol extract of *P. madagascariensis* had α-glucosidase inhibitory activity with IC_{50} = 33.0 ± 4.6 µmol/l[30]. Molecular docking is used very often in investigation of molecular structures and functions, as well as, screening for α-glucosidase inhibitors. This method was applied by Hyun et al.[31] for investigation of flavonoids and phenolic acids, such as gallic, caffeic and ferulic acid. According to the energy of bounding of these substances

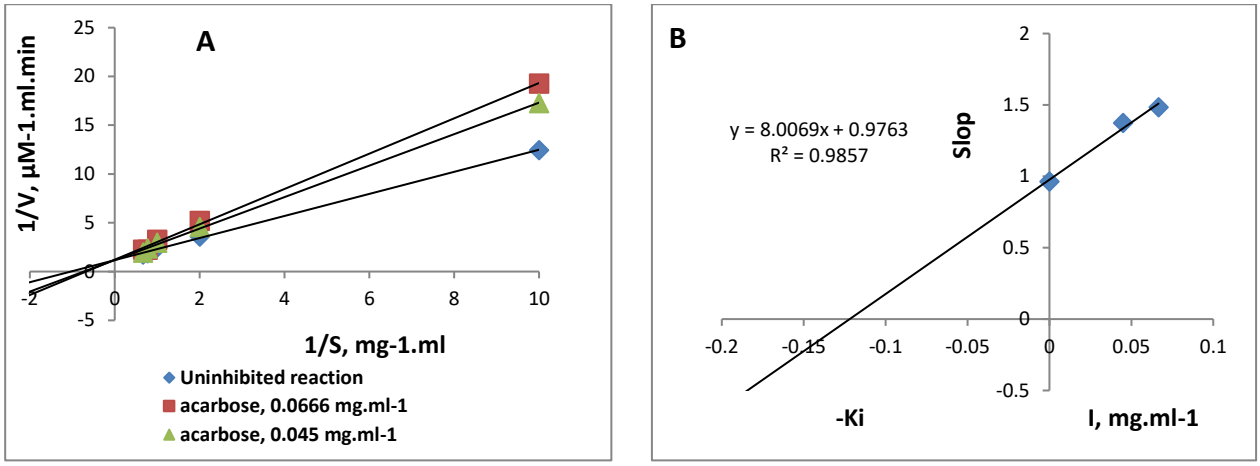

Figure 4: Lineweaver–Burk plots (A) and secondary plots (B and C) for determination of dissociation constants of ESI (KI) and EI (K_i). Reciprocal plots were obtained by variable concentrations of acarbose**.**

with α-glucosidase, they were determined as potential inhibitors of this enzyme.

Rutin was also detected in high amounts (3046.89±76.68 µg/g) in the investigated alcoholic extract. This flavonoid had mixed type of inhibition according to Hyun et al.[31], as well as myricetin, quercetin and luteolin[4].

The α-glucosidase inhibition in presence of jujube extract could be due to inhibitory activity of the reported secondary metabolites. It should be considered that the extract contains diverse groups of chemical compounds, which results in the different mode of inhibition. It could be concluded that the high concentration of rutin (with mixed type of inhibition) and oleanolic acid (with uncompetitive type of inhibition) determine the noncompetitive-uncompetitive mixed type of α-glucosidase activity in the presence of 70% ethanol extract.

Inhibition of α- amylase activity from 70% ethanol extract of Z. jujuba

Based on the Lineweaver-Burk primary graphic (фиг 3A), the linear regression equation of the control was y = 1.1312x + 1.1731 (R^2 = 0.9986), while at extract concentration of 0.333 and 0.666 mg.ml^{-1} they were y = 1.5324x + 1.3704 (R^2 = 0.9997) and y = 2.0841x + 1.71 (R^2 = 0.9996) respectively. The obtained lines were crossing in the 2nd quadrant. K_m values were increasing, while the V_{max} values were decreasing and were different from the values of the control (Table 3). It should be noted the minimum increase of K_m values with increasing the extract concentration. This fact reveals that the inhibitors have a weak impact towards the affinity to the substrate. In addition, the KI value (1.426 mg.ml^{-1}) of ESI-complex is higher from the K_i value (0.791 mg.ml^{-1}) of the EI-complex. This means that the inhibitors in the extract have higher affinity to bind with the enzyme than the ESI-complex. These results account for a mixed competitive-non-competitive inhibition[22].

The inhibitory activity of acarbose on α –amylase is showed at Figure 4A. Based on the results of Leneweaver-Burk primary graphic at acarbose concentrations of 0.045 and 0.066 mg.ml^{-1} the linear regression equations were y = 1.6136x + 1.1746 (R^2 = 0.9991) and y = 1.8108x + 1.2198 (R^2 = 0.9982). The slopes of the lines increase. The obtained lines were crossing at one point on the ordinate. The K_m values were increasing with increasing the extract concentration, while the V_{max} values did not change (Table 3). The constant of inhibition for EI-complex was K_i = 0.122 mg.ml^{-1}. These results account for a competitive inhibition, which means that acarbose and the substrate compete in one and the same point in the enzyme molecule – the active center. Such type of inhibition is possible when the competitive inhibitors are structural analogues of the substrate.

K_i of acarbose had a lower value than K_i of the extract, which means that the inhibitors in the extract were weaker than the acarbose. The extract caused moderate mixed competitive - non-competitive inhibition (Table 3).

For large number of the identified from us constituents in the ethanol extract α-amylase inhibitory activity have been reported. Pentacyclic triterpenoids, such as oleanolic acid[31], betulinic acid, oleanolic acid, ursolic acid[33] and ursolic acid[34] have been shown to inhibit α-amylase. From the flavonoids luteolin[34], myricetin and quercetin[29] inhibited α-amylase effectively. According to Ng K.[36] myricetin, exhibited inhibitory activity with 35.9% inhibition and according to Tadera et al.[29] myricetin inhibited α-amylase activity with 64%. The phenolic acids, including gallic[37], caffeic[38], ferulic acid[39], isolated from *Elusine coracana* extracts revealed inhibitory activity to α-amylase with IC_{50} value of 23.05 mg/ml[40]. Chethan et al.[41] reported strong α-amylase inhibition of gallic (67.7%), vanillic (71.9%), *trans*-cinnamic acid (79.2%) and quercetin (73.5%). Therefore, it could be assumed that the inhibitory effect of 70% ethanol extract on pancreatic α-amylase may be related to these compounds. The mechanism of activity of phenolic acids (covalent binding with reactive nucleophilic sites of α-amylase[42]) and that of flavonoids (creation of hydrogen binding with OH groups in active side chains of the enzyme[36]) is possible to explain the stronger affinity of the inhibitors from the extract to bind with the enzyme than the ES-complex. K_i has 1.8 times lower values than KI (K_i < KI)[22]. Chethan et al.[41] reported for the same type of α-amylase inhibition of millet polyphenols. The knowledge of type of inhibition reveals the precise mechanisms of action of enzyme inhibitors.

Table 2: Inhibition and kinetic parameters of o-NPG hydrolysis by α-glucosidase in the absence and presence of inhibitors in 70% extract of jujube and acarbose.

0-NPG hydrolysis by α-glucosidase	Inhibition, %	Kinetic parameters				
		Vmax, $mM.(ml.min)^{-1}$	K_m, mM	K_m, $mg.ml^{-1}$	KI, $mg.ml^{-1}$	K_i, $mg.ml^{-1}$
Uninhibited reaction	-	66.225	4.920	1.481	-	-
Extract concentration 0.66 $mg.ml^{-1}$	71.56	13.736	2.232	0.672	0.248	0.843
Extract concentration 1.33 mg,ml^{-1}	79.46	8.881	1.701	0.512		
Inhibition mod		Non-competitive - uncompetitiv mixed				
Acarbose, 0.66 $mg.ml^{-1}$	70.94	17.301	4.449	1,339	0.096	0.333
Acarbose, 1.33 $mg.ml^{-1}$	82.98	7.236	0.808	0.096		
Inhibition mod		Non-competitive - uncompetitiv mixed				

Table 3: Inhibition and kinetic parameters of starch hydrolysis by α-amylase in the absence and presence of inhibitors in 70% extract of jujube and acarbose.

Starch hydrolysis by α-amylase	Inhibition, %	Kinetic parameters			
		Vmax, $\mu M.(ml.min)^{-1}$	Km, $mg.ml^{-1}$	KI, $mg.ml^{-1}$	Ki, $mg.ml^{-1}$
Uninhibited reaction		0.852	0.964	-	-
Extract concentration 0.333 $mg.ml^{-1}$	23.55	0.729	1.118	1.426	0.791
Extract concentration 0.666 $mg.ml^{-1}$	39.10	0.584	1.219		
Inhibition mod		mixed competitive - non-competitive			
Acarbose, 0.045 $mg.ml^{-1}$	10.50	0.851	1.374	-	
Acarbose, 0.066 $mg.ml^{-1}$	21.73	0.819	1.484		0.122
Inhibition mod		competitive			

Major characteristic constituents in fructus extract are triterpenes, which makes it different from other extracts that contain mainly phenolic compounds. The variety of metabolites with different chemical structures in the extract is the possible explanation for the observed pattern of inhibition. This conclusion could be supported by the investigations of Chethan et al.[41], who reported that pure constituents, such as gallic, vanillic, trans-cinnamic acid and quercetin induced non-competitive inhibition, but the polyphenol extracts caused mixed non-competitive inhibition on α-amylase. The inhibitors from the jujube extract caused different types of inhibition towards the two enzymes, mixed non-competitive-uncompetitive towards α-glucosidase and mixed competitive-non-competitive towards α-amylase. Besides that, the obtained results prove high inhibition of α-glucosidase (79.46% at 1.33 $mg.ml^{-1}$ extract) and moderate inhibition of α-amylase (39.10% at 1.33 $mg.ml^{-1}$ extract concentration). These differences in the activity of the extracts confirm their specificity towards the inhibition of the two enzymes. Important to mention here is that starch is degraded to maltooligosaccharides from the salivary and pancreatic α-amylases. Afterwards α-glucosidases in the guts hydrolyze glycosidic bounds of maltooligosaccharides till glucose is obtained. The presence of potent α-glucosidase inhibitory activity therefore, appears more important to control the release of glucose from disaccharides in the gut than α-amylase. Therefore, moderate α-amylase inhibition with potent α-glucosidase inhibitory activity may offer better therapeutic strategy. In this aspect, α-amylase and α-glucosidase inhibitors are particularly important in the avoidance of conditions such as diabetes[29,35,43].

In the literature, there are no kinetic surveys of *Z. jujuba* fruit extract proving its anti-diabetic potential. In present research the potential of these fruit extracts as inhibitor of the enzyme α-glucosidase and α-amylase was described by K_i value not by $IC_{50.}$ The IC_{50} value depends on concentrations of the enzyme, the inhibitor and the substrate along with other experimental conditions. Ki value is thermodynamic constant that is independent of the substrate but depends on the enzyme and inhibitor[44]. Determination of the K_i value allows more easily to be compared the inhibitors determined by different surveys.

CONCLUSION

The enzyme inhibitors have an important role used as a control over the diseases and their treatment. Extract of *Ziziphus jujuba* (Rhamnaceae) possess anti-diabetic properties that could be useful in the development of herbal formulations, intended for prevention and treatment of diabetes mellitus type 2. The knowledge of type of inhibition reveals the precise mechanisms of action of enzyme inhibitors and determine their successfully application. The jujube fruit extract is a rich source of triterpenoids, with established multifactorial properties *in vivo*. The extract is rich in flavanoids and phenolic compounds that owe except enzyme and non-enzyme inhibitory mechanism acting in the digestive tract. Therefore, this extract could be used in the pharmaceutical industry for development of drug formulations.

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RFERENCES

1. WHO. Global report on diabetes. 1. Diabetes Mellitus – epidemiology. 2. Diabetes Mellitus – prevention and control. 3. Diabetes, Gestational. 4. Chronic Disease. 5. Public Health. I. World Health Organization. 20 Avenue Appia, 1211 Geneva 27, Switzerland, 2016.
2. Sim L, Willemsma C, Mohan S, Naim HY, Pinto BM, Rose DR. Structural basis for substrate selectivity in human maltase-glucoamylase and sucrase-isomaltase N-terminal domains. J Biol Chem. 2010; 285:17763-17770.
3. Van de Laar FA, Lucassen PL, Akkermans RP, van de Lisdonk EH, Rutten GE, Van Weel C. Alpha-glucosidase inhibitors for patients with type 2 diabetes: results from a Cochrane systematic review and meta-analysis. Diabetes Care. 2005; 28(1):154-63.
4. Mizuno CS, Chittiboyina AG, Kurtz TW, Pershadsingh HA, Avery MA. Type 2 diabetes and oral antihyperglycemic drugs. Curr Med Chem. 2008; 15:61-74.
5. Chakrabarti R, Rajagopalan R. Diabetes and insulin resistance associated disorders: Disease and the therapy. Current Science 2002; 83(12).
6. Yagi A, Inagaki N, Haraguchi Y, Noda K, Okamura N, Nishioka I. Studies on the constituents of *Zizyphi fructus*. IV. Isolation of an anti-allergic component. Ethyl α-d-fructofuranoside from ethanol extract of Zizyphi Fructus. Journal of the Pharmaceutical Society of Japan. 1981; 101(8):700-707.
7. Su H J, Len SC. Sonate leaf spot of Indian jujube caused by *Cristulariella manicola*. Plant Diseases 1983; 67(8):915-916.
8. Shah AH, Ai-Bekairi AM, Qureshi S, Ageel AMl. *Zizyphus sativa* fruits: evaluation of some biological activity and toxicity. *Phytotherapy Research*, 1989; 3:232–236.
9. Raju R, Murthy PS, Prabhu KM. Hypoglycemic activity of an indigenous plant material. Diabetes Research 1994; 27:89–90.
10. Glombitza KW, Mahran GH, Mirhom YW, Michel KG, Motawi TK. Hypogycemic and antihyperglycemic effect of *Ziziphus spinachristi* in rats. PlantaMed. 1994; 60:244-247.
11. Cisse A, Ndiaye A, Lopez-Sall P, Seck F, Faye B. Antidiabetic activity of *Ziziphus mauritiana* Lam (Rhamnaceae). Dakar Med 2000; 45(2): 105-107.
12. Abdel- Zaher AO, Salim SY, Assaf MH, Abdel-Hady RH. Antidiabetic activity and toxicity of *Zizyphus spina-christi* leaves. Journal of Ethnopharmacology 2005; 101(1–3): 129–138.
13. Jose M. Castellano JM, Guinda A, Delgado T, Rada M, Cayuela JA. Biochemical basis of the antidiabetic activity of oleanolic acid and related pentacyclic triterpenes. DIABETES 2013; 62(6).
14. WHO. Monographs on selected medicinal plants. 1. Plants, Medicinal. 2. Angiosperms. 3. Medicine, Traditional. Edn 3, Vol. 3, World Health Organization, Ottawa, Ont, 2001; 359-369.
15. Marchev A., Ivanov I, Georgiev V, Pavlov A. Determination of di-and triterpenes in *Salvia tomentosa* Mill. cell suspension culture by high-performance liquid chromatography. Scientific works – University of Food Technologies 2012; LIX: 229-233.
16. Marchev A, Georgiev V, Ivanov I, Badjakov I, Pavlov A. Two-phase temporary immersion system for *Agrobacterium rhizogene*s genetic transformation of sage (*Salvia tomentosa* Mill.). Biotechnology letters 2011; 33 (9):1873-1878.
17. Kerina HJ. Trudgill PW, Hopper DJ. Evidence of two pathways for the metabolism of phenol by *Aspergillus fumigatus*. Archives of Microbiology 1995; 163:176-181.
18. Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chemistry 1999; 64(4): 555–559.
19. Suresh Babu K., Tiwari A.K.,.Srinivas P.V., Ali A.Z., China Raju B., Rao J.M, 2004. Yeast and mammalian alpha-glucosidase inhibitory constituents from Himalayan rhubarb Rheum emodi Wall.ex Meisson, Bioorg. Med. Chem. Lett., 14: 3841-3845.
20. McDougall, G. J., Shpiro, F., Dobson, P., Smith, P., Blake, A.;Stewart, D., 2005. Different polyphenolic components of soft fruits inhibit α-amylase and α-glucosidase. J. Agric. Food Chem., 53: 2760–2766.
21. Grussu D., Stewart D. and McDougall J.G,. Berry polyphenols inhibit α-amylase in vitro; Identifying active components in rowanberry and raspberry, J. Agric.Food Chem. 2011; 59: 2324-2331.
22. Palmer T. Enzymes. Biochemistry, Biotechnology, Clinical Chemistry. East-West press Edition, New Delhi, 2004, 42-146.
23. Lee S, Min B, Lee C, Kim K, Kho Y. Cytotoxic triterpenoids from the fruits of *Ziziphus jujuba.* Planta Medica 2003; 69: 18-21.
24. Shoei SL, Buh, FL, Karin CL. Three triterpene esters from *Zizyphus jujuba.* Phytochemistry1996; 43(4):847-851.
25. San B, Yildirim AN. Phenolic, alpha-tocopherol, beta-carotene and fatty acid composition of four promising jujube (*Ziziphus jujuba* Miller) selections. Journal of Food Composition and Analysis 2010; 23,706–710.
26. Hudina M, Liu M, Veberic R, Stampar F, Colaric M. Phenolic compounds in the fruit of different varieties of Chinese jujube (*Ziziphus jujuba* Mill.). Journal of Horticultural Science and Biotechnology 2008; 83(3):305–308.
27. Kumar S, Kumar V, Prakash O. Enzymes Inhibition and Antidiabetic Effect of Isolated Constituents from *Dillenia indica*. BioMed Research International 2013;7.
28. Ali MS, Jahangir M, Hussan SS, Choudhary MI. Inhibition of alpha-glucosidase by oleanolic acid and its synthetic derivatives. Phytochemistry 2002; 60:295–299.
29. Tadera K, Miinami Y, Takamatsu K, Matsuoka T. Inhibition of a-Glucosidase and a-Amylase by Flavonoids. J Nutr Sci Vitaminol 2006; 52:149-153.
30. Kubínová R, Pořízková R, Navrátilová A, Farsa O, Hanáková Z, Bačinská A, Cížek A, Valentová M.

Antimicrobial and enzyme inhibitory activities of the constituents of Plectranthus madagascariensis (Pers.) Benth. J Enzyme Inhib Med Chem 2014; 29(5):749-52.
31. Hyun TK, Eom SH**,** Kim JS. Molecular docking studies for discovery of plant-derived α-glucosidase inhibitors. Plant Omics Journal 2014; 7(3):166-170.
32. Bailey CJ. New approaches to the pharmacotherapy of diabetes. In: Pickup, J.C., William, G. (Eds.), Textbook of Diabetes, Vol. 2, 3rd ed. Blackwell Science Ltd., UK, 2003:73.1-73.21.
33. Castellano JM, Guinda A, Delgado T, Rada M, Cayuela JA. Biochemical basis of the antidiabetic activity of oleanolic acid and related pentacyclic triterpenes. Diabetes 2013; 62:1791–1799.
34. Chakrabarti R, Rajagopalan R. Diabetes and insulin resistance associated disorders: Disease and the therapy. Current Science 2002; 83(12):1533-1538.
35. Kim JS, Kwon CS, Son KH. Inhibition of Alpha-glucosidase and Amylase by Luteolin, a Flavonoid. Biosci. Biotechnol. Biochem. 2000; 64 (11), 2458-2461.
36. Ng K. Evaluation of α-Amylase and α-Glucosidase Inhibitory Activity of Flavonoids. International Journal of Food and Nutritional Science 2015; 2(6): 1- 6.
37. Kimura K, Lee JH, Lee IS, Lee HS, Park KH, Chiba S. Two potent competitive inhibitors discriminating alpha-glucosidase family I from family II. Carbohydr Res 2004; 339:1035-40.
38. Skeggs LT, Khan JR, Shumway NP. The preparation and function of the hypertension – converting enzyme. J Exp. Med. 1956; 103:295-299.
39. Bowles DJ. Defense-Related Proteins in Higher Plants. Annu. Rev. Biochem. 1990; 59: 873-907.
40. Shobana S, Sreerama YN, Malleshi NG. Composition and enzyme inhibitory properties of *finger millet (Eleusine coracana L.)*seed coat phenolics: Mode of inhibition of α-glucosidase and pancreatic amylase. Food Chem 2009; 115:1268-1273.
41. Chethan S, Sreerama YN, Malleshi NG. Mode of inhibition of finger millet malt amylases by the millet phenolics. Food Chemistry 2008; 111(1): 187-191.
42. Rohn, S., Rawel., Kroll, J. Influence of plant phenols on the activity of enzymes. *Food Sci. Biotechnal*. 2002a; 11:4-9.
43. Kim SD, Nho HJ. Isolation and characterization of α-Glucosidase inhibitor from the fungus Ganoderma lucidum. Journal of Microbiology 2004; 42:223-227.
44. Cer R. Z., Mudunuri U., Stephens R. and Lebeda F. J. IC50-to-Ki: a web-based tool for converting IC50 to Ki values for inhibitors of enzyme activity and ligand binding. Nucleic Acids Research, 2009; 1–5.