



## Anti-diabetic activity and stability study of the formulated leaf extract of *Zizyphus spina-christi* (L.) Willd with the influence of seasonal variation

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### ARTICLE INFO

#### Article history:

Received 29 May 2010

Received in revised form 19 August 2010

Accepted 1 September 2010

Available online 15 September 2010

#### Keywords:

*Zizyphus spina-christi*

Anti-diabetic activity

Seasonal variation

*In vitro* dissolution test

Arrhenius equation

Accelerated stability

Shelf life

### ABSTRACT

**Aim of the study:** The present study aimed to evaluate the anti-diabetic activity of *Zizyphus spina-christi* leaf extract (200 mg/kg b.w.), plain and formulated in STZ-diabetic rats. Percentage yield of extracts, marker yield (christinin-A) and antihyperglycemic potencies, depending on seasonal variation were investigated. The chemical stability, study of storage conditions, shelf life T90 prediction of both plain extract and formulated soft gelatin capsules by accelerated studies were studied.

**Material and methods:** Changes in all studied parameters after oral administration of *Z. spina-christi* extract for 28 days were reported. Seasonal variation affecting yield and activities was studied. Flavonoid contents were HPLC evaluated. The capsules were stored at 30, 40 and 50 °C [75% relative humidity] and their residual christinin-A content was assayed for 24 weeks. Christinin-A chemical degradation was monitored by HPLC stability indicating method previously validated. Possible physical examination was checked by dissolution test of the content of the capsules using dissolution tester USP XXIV.

**Result:** Oral administration of *Z. spina-christi* leaf extract, plain and formulated for 28 days reduced blood glucose level with significant increase in serum insulin and C-peptide levels. Marked elevation in total antioxidant capacity with normalization of percentage of glycated hemoglobin (HbA1C%) was reported. Moreover, they succeeded to reduce the elevated blood lactate level and to elevate the reduced blood pyruvate content of diabetic rats. In line with amelioration of the diabetic state, *Zizyphus* extract, plain and formulated restored liver and muscle glycogen content together with significant decrease of hepatic glucose-6-phosphatase and increase in glucose-6-phosphate dehydrogenase activities. *In vitro* experiments showed a dose-dependent inhibitory activity of *Zizyphus* extract against  $\alpha$ -amylase enzyme with (IC<sub>50</sub>) at 0.3 mg/ml. Such finding has been supported by the *in vivo* suppression of starch digestion and absorption by *Zizyphus* extract in normal rats.

The flavonoids content of the formulated leaves (collected during June 2009) were found to be 11.5  $\mu$ g/g of the dry plant material (expressed as quercetin) and 58.8  $\mu$ g/g of the dry plant material (expressed as rutin). The shelf life for the capsules stored at 30, 40 and 50 °C [75% relative humidity] for plain and formulated extract were calculated to be 66.90 and 70.74 weeks, respectively.

**Conclusion:** The current work revealed that *Z. spina-christi* leaf extract, plain and formulated, improved glucose utilization in diabetic rats by increasing insulin secretion which may be due to both saponin and polyphenols content and controlling hyperglycemia through attenuation of meal-derived glucose absorption that might be attributed to the total polyphenols. Studies showed that leaves are preferably collected from June to October. Finally, the release followed the Higuchi kinetic model, indicating diffusion dominated drug release with no drop in the dissolution values throughout the test period.

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### 1. Introduction

Traditional medicine has selected groups of plants that time and experience have shown to be beneficial. Among these plants is

*Zizyphus spina-christi* (L.) Willd which is reported to possess anti-hyperglycemic activity (Glombitza et al., 1994). Four triterpenoidal saponin glycosides, christinin-A, B, C and D, were isolated from the butanol extract of the leaves of *Z. spina-christi* (L.) Willd (Mahrn et al., 1996) and leaves were investigated for their flavonoids (Nawwar et al., 1984).

Diabetes mellitus is a complex metabolic disorder that is alarmingly increasing with more than 180 million people affected worldwide as recorded by WHO in 2008. Although currently available therapeutic modalities can control many aspects of diabetes,

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serious complications are still encountered in addition to the side effects of the commonly used anti-diabetic drugs. An alternative strategy for diabetes treatment is the use of medicinal plants as a useful source for the development of new pharmaceuticals, as well as, dietary supplements to existing therapies (Bailey and Day, 1989).

Based on previous findings concerning the formulation of the ethanol 70% leaf extract of *Z. spina-christi* (L.) Willd (Nesseem et al., 2009), the authors were stimulated to carry out the present investigation aiming to elucidate the possible mechanisms of anti-diabetic activity of the extract either plain or formulated. In this respect, the first part of the anti-diabetic study was concerned with its effect on glucose utilization in streptozotocin-diabetic rats with referring to the antioxidant status of the animals. Meanwhile, the second part dealt with examination by *in vitro* experiments, the ability of the extract to inhibit the intestinal  $\alpha$ -amylase activity in addition to the *in vivo* effect on starch digestion and absorption in normal rats.

Furthermore, from the economical standpoint the time of leaf collection should be carefully studied to provide the best yield of extractives and physiologically active metabolites, these being usually affected by seasonal variation. It is well known that the development of fleshy fruits involved considerable accumulation of organic metabolites into the succulent pericarp and associated tissues leading to consumption of the active metabolites. Taking this in consideration, the present work included an analytical profiling of the leaf extract as regards its yield and christinin-A content (bioactive marker) and an evaluation of the antihyperglycemic activity of samples collected at different time intervals from the beginning of flowering to fruiting seasons.

Finally, a study of the stability of pharmaceutical products and of stability testing techniques was performed firstly, from the point of view of patient safety. The present trend in the pharmaceutical industry is towards production of highly specific, chemically stable potent drugs. It is important therefore, that the patient receives a uniform dose of drug throughout the whole of the shelf life of the product. In addition, although a drug may have been shown to be safe for use, this is not necessarily true for the decomposition products. Secondly, considerations must be given to the relevant legal requirements concerned with the identity, strength, purity and quality of the drug, and finally such a study is important to prevent the economic repercussion of marketing an unstable product (Hung, 1990). Furthermore, stability testing is important in providing evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light. It also enables recommended storage conditions, retest periods and shelf lives to be established (ICH Harmonised Tripartite Guideline, 2003). Basically, there are two different types of stability studies: short-term and long-term studies. A typical short-term study is the accelerated stability study under stressed conditions (at higher temperature and relative humidity). The purpose of the accelerated stability study is to increase the rate of the chemical and physical degradation of the drug product, so the significant degradation can be observed in a relatively short period (Chow and Liu, 1995). According to the regulatory definition, a stability indicating method is an analytical procedure, which is capable of discriminating between the major active pharmaceutical ingredients from any degradation products formed under defined storage conditions during the stability evaluation period (El-Massik et al., 2003; Hong and Shah, 2005). In this work, we evaluated the shelf life of T90 of the soft gelatin capsules, as well as the plain extract stored at 30, 40 and 50 °C. The christinin-A (Bioactive Marker) chemical degradation was monitored by HPLC method previously validated (Nesseem et al., 2009). Possible physical modifications were checked by dissolution test of the content of the capsules.

## 2. Experimental

### 2.1. Materials and apparatus

#### 2.1.1. Plant materials

*Z. spina-christi* (L.) Willd leaves were collected during June 2008 (Bf; beginning of the flowering stage), November 2008 (Ef; end of the flowering stage) and February 2009 (Fs; fruiting stage) from the cultivated plants in the Experimental Station of Medicinal Plants of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Giza, Egypt. The identity was confirmed by Professor Dr. Nabil Hadidy, late Professor of Botany, Botany Department, Faculty of Science, Cairo University, Cairo, Egypt. Voucher specimens are kept at the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University. Air-dried leaves were coarsely powdered, packed in dark, coloured tightly closed containers and kept for extraction, formulation and biochemical studies.

#### 2.1.2. Animals

Male Sprague Dawley albino rats weighing  $200 \pm 20$  g were obtained from the animal house colony, National Research Center, Cairo, Egypt. The rats were kept under controlled environmental conditions with free access to standard laboratory diet and water throughout the experimental period. This study was conducted in accordance with ethical procedures and policies approved by Animal Care and Use Committee of Faculty of Pharmacy Cairo University, Cairo, Egypt following the 18th WMA General Assembly, Helsinki, June 1964 and updated by the 59th WMA General Assembly, Seoul, October 2008.

#### 2.1.3. Kits and chemicals

BioMerieux enzyme  $\alpha$ -amylase RTU kit. Glucose Enzymatic GOD-PAP kit, Biocon Diagnostik (Germany). Sensitive Rat Insulin RIA kit supplied from Linco Research Inc. (USA). C-peptide serum level was assessed by a double-antibody RIA kit (Double Antibody C-peptide, EURO/DPC Ltd., U.K.). Blood lactate kit, Spinreact–Spain. Blood pyruvate kit, Greiner Diagnostic GmbH–Germany. Glycated hemoglobin kit, Stanbio, San Antonio (USA). Acarbose (Bayer, Germany). All chemicals and methanol HPLC grade were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

#### 2.1.4. Material for formulation of soft gelatin capsules

Polyoxyethylene (20) cetyl ether (BC-20TX), Croda, Bx 11143.U.K., Meglyol 810 (Huls AG, Witten, Ruhr, Germany), ascorbic acid, PEG 6000, and PEG 400 (Prolabo, France), Soft gelatin capsules were gift samples supplied from Glaxo Co., Egypt. Polyethylene glycol 400 and 4000 (Prolabo, France).

#### 2.1.5. Apparatus

USP release tester (Hanson SR6, Hanson Research Corporation, California, USA), sieves of mesh size 250  $\mu$ m and 100  $\mu$ m (VEB, Metal-Weberei, DDR, Italy) were used. UV-Spectrophotometer (Shimadzu, Kyoto, Japan), electrical balance (Sartorius GMBH, Göttingen, Germany), electrical blender (Philips, Holland), magnetic stirrer (Thermolyne Corporation, Dubuque, IA, USA), thermostatically controlled electric water bath (Julabo Labortec Hink GMBH, Seelbach, Germany). HPLC determination of flavonoids was conducted on a LC-10 ADVP Shimadzu liquid chromatograph equipped with DGU-12A Shimadzu Degasser, SLC-10 AVP Shimadzu system controller and a SPD-10 AVP Shimadzu UV–Vis detector. Column: Extra column “Waters” RP<sub>18</sub> (4.6 mm  $\times$  250 mm, 5  $\mu$ m, Ireland). Column temperature: ambient temperature; flow rate: 1 ml/min; Detector range: 200–400 nm; injection volume: 20  $\mu$ l; Mobile phase: methanol:water (80:20 v/v); Run time: 15 min. HPLC determination of the marker compound (christinin-A) as previously mentioned in Nesseem et al. (2009). Teflon homogenizer

(Potter-Elvehjem type). Dupont Sorvall combiplus ultracentrifuge (USA). Gama Counter Mini, Model 3600 (U.K.).

### 3. Methods

#### 3.1. Calculation of the percentage yield of the different extracts

Powdered leaves, 20 g collected during the three studied stages (Bf, Ef and Fs) were repeatedly refluxed with ethanol 70% till exhaustion (4 times, 400 ml). The ethanolic extracts were evaporated to dryness under reduced pressure at 40 °C. The obtained residues were kept in desiccators till constant weight, weighed and the percentage yields were recorded as a mean of three experiments.

#### 3.2. Preparation of the standard solutions and samples

A stock solution (0.25 mg/ml) of standard quercetin and rutin in methanol 80% were prepared. Standard calibration curves were prepared using serial dilutions of standards viz, 20, 40, 80, 120 and 160 µg from the stock solution. Typical calibration curves were prepared by plotting peak area ( $y$ ) against injected amount ( $x$ , µg). Residues (Bf, Ef and Fs, 10 mg each) were extracted separately by ultrasonication with methanol 80% at room temperature for 20 min. Each extract was filtered, clarified by centrifugation, transferred into a 25 ml volumetric flask and the volume adjusted with methanol 80%. Flavonoids were assayed using standards rutin and quercetin by HPLC. HPLC and Spectrophotometric determination of the marker compound "christinin-A", [3-O-( $\alpha$ -L-fucopyranosyl) (1 → 2)- $\beta$ -D-glucopyranosyl (1 → 3)- $\alpha$ -L-arabinopyranosyl] jujubogenin, was detected at  $\lambda = 200$  nm following the same condition stated by [Nesseem et al. \(2009\)](#).

#### 3.3. Acute antihyperglycemic activity

Diabetic rats were prepared according to the method described by [Eliasson and Samet \(1969\)](#).

#### 3.4. Preparation of soft gelatin capsules

The selected formula was prepared by encapsulating 450 mg of the ingredient in a soft gelatin capsule prepared in our laboratory which was then sealed with gelatin solution. The control formulation was prepared similarly by separately encapsulating 200 mg of each plain extract in a soft gelatin capsule as mentioned before in a previous publication ([Nesseem et al., 2009](#)).

#### 3.5. Biochemical studies

##### 3.5.1. Effect of *Z. spina-christi* extract, plain and formulated on glucose utilization in STZ-diabetic rats

Rats were divided into normal ( $n = 10$ ) and diabetic ( $n = 50$ ) groups. Diabetes was induced by i.p. injection of Streptozotocin (STZ) (50 mg/kg b.w.) freshly prepared in 0.1 M citrate buffer pH 4.5 ([Wohaieb and Godin, 1987](#)). Forty eight hours after STZ-administration, diabetic rats with non fasting plasma glucose >300 mg/dl were randomly divided into three groups, diabetic and diabetic treated with either plain *Zizyphus* leaf extract or formulated leaf extract in soft gelatin capsules. The first group was left without treatment and served as a control diabetic group. The second group received oral daily dose of 200 mg/kg of plain *Zizyphus* extract while the third group received the content of one soft gelatin capsule equivalent to 200 mg/kg *Zizyphus* extract for 28 days. At the end of the experimental period, all animals were deprived from food for 3 h, and then sacrificed. Blood samples were collected and serum was separated for measurement of levels of blood glucose,

insulin, C-peptide and total antioxidant capacity. A second portion of blood samples were collected on EDTA and used for the estimation of HbA1C%. A third portion of blood samples were collected on 0.6 M perchloric acid for immediate deproteinization and consequent estimation of lactate and pyruvate concentrations. Liver and skeletal muscles were rapidly isolated, where portions of the tissues were immediately digested in 30% KOH solution for subsequent determination of glycogen content. Meanwhile, the liver was quickly divided into two portions; the first one was homogenized in 0.1 M citrate buffer pH 6.5, ultracentrifuged for 15 min at  $1000 \times g$  at 4 °C. The supernatant was used for determination of hepatic glucose-6-phosphatase activity. The second portion of the liver was homogenized in 1.15% KCl solution and centrifuged for 30 min at  $20,000 \times g$  at 4 °C. The supernatant was recentrifuged for 60 min at  $100,000 \times g$  at 4 °C and the resulting supernatant was used for assay of glucose-6-phosphate dehydrogenase activity.

##### 3.5.2. In vitro effect of different extract concentrations on the activity of $\alpha$ -amylase enzyme

Determination of  $\alpha$ -amylase activity was done kinetically using bioMerieux enzymeline  $\alpha$ -amylase RTU kit. The method is based on hydrolysis of the substrate 2-chloro-4-nitrophenyl maltotrioid by  $\alpha$ -amylase. The rate of production of the hydrolysis product 2-chloro-4-nitrophenol (measured by the change in absorbance at 405 nm per min) is proportional to  $\alpha$ -amylase activity. The  $\alpha$ -amylase enzyme was incubated with either phosphate buffer (control experiments) or the buffer containing different concentrations of the extract. At the end of the incubation time, the substrate was added and the increase in absorbance was measured for 3 min. The percentage inhibition of  $\alpha$ -amylase enzyme activity by different plain and formulated extract concentrations was calculated by the following formula: % inhibition =  $[\Delta AC - \Delta AS / \Delta AC] \times 100$ , where  $\Delta AC$  is the increase in absorbance of the control and  $\Delta AS$  is the increase in absorbance in presence of different extract concentrations. The % inhibition of  $\alpha$ -amylase enzyme activity was plotted against different extract concentrations. The final concentration of an inhibitor (extract) in the reaction mixture required to inhibit 50% of enzyme activity is defined as the  $IC_{50}$  value.

##### 3.5.3. Effect of *Z. spina-christi* extract, plain and formulated on starch digestion and absorption in normal rats

This experiment is based on measuring blood glucose level in rats after 18 h overnight fast and at different time intervals following starch ingestion either alone or in combination with either acarbose or *Zizyphus* extract. Rats were fasted for 18 h, blood samples were taken from the tail vein for assessment of blood glucose at zero time, and the animals were divided into normal and two treated groups. The rats in the normal group were given starch load (3 g/kg b.w.) via oral gavage and blood samples were withdrawn at 30, 60, 90, 120 and 150 min after starch ingestion. Blood glucose levels were measured enzymatically using Accu-check Roche diagnostic method. In the treated groups, the same previous regimen was followed except that 200 mg/kg b.w. of *Zizyphus* extract or 25 mg/kg b.w. of acarbose was given orally just before the starch load.

##### 3.5.4. Assessment of biochemical parameters

Blood glucose was assessed in the serum according to the method of [Schmidt \(1961\)](#). Serum level of insulin and C-peptide were estimated using radioimmunoassay kits. Blood pyruvate and lactate were measured using commercially available kits. The percentage of HbA1C was assessed following the method of [Abraham et al. \(1978\)](#). Total antioxidant capacity was measured in serum by the ferric reducing antioxidant power (FRAP) assay ([Benzie and Strain, 1996](#)) which measures the ferric reducing ability of antioxidants in serum. Glycogen content in the digested hepatic and skeletal mus-

cle tissues was determined according to the method of Hassid and Abraham (1957) where the liberated glucose in the hydrolyzate was estimated. Glucose-6-phosphatase activity was assayed by the method of Baginsky et al. (1974) and the inorganic phosphate liberated was measured by the method of Fiske and Subbarow (1925). One unit of glucose-6-phosphatase is defined as n mol of Pi liberated/mg protein.min at 37 °C. Glucose-6-phosphate dehydrogenase activity was determined by the method of Leveille (1972). Protein content of different hepatic fractions was measured using the method of Lowry et al. (1951).

### 3.5.5. Statistical analysis

Values are expressed as mean  $\pm$  S.E.M. The level of statistical significance was taken at  $p < 0.05$  using one-way analysis of variance (ANOVA) followed by Tukey–Kramer's test to judge the difference between various groups.

### 3.6. Accelerated stability testing of soft gelatin capsules and extract of *Z. spina-christi*

Soft gelatin capsules containing 450 mg equivalent to 200 mg extract as well as the capsules containing 200 mg of plain extract as a control were packed into well capped vials protected from light and stored at a temperature of 30, 40 and 50 °C [75% relative humidity] for 24 weeks. The samples were withdrawn at intervals 0, 1, 2, 3, 4, 6, 8, 12, 16 and 24 weeks and allowed to stand at room temperature. The concentration of christinin-A in the extract at different time points was determined by HPLC using a preconstructed calibration curve of the extract in methanol 80%.

#### 3.6.1. Current statistical methodology

Thermal induced instability is generally assumed to follow the Arrhenius relationship (Patrick, 2006):

$$K = Ae^{[-(E_a/RT)]}$$

where  $K$  is the reaction constant,  $A$  the Arrhenius constant,  $E_a$  the activation energy,  $R$  the molar gas constant, and  $T$  is the absolute temperature ( $K_0$ ).

The logarithmic form of the Arrhenius equation (Patrick, 2006) indicates that there is a linear relationship between the logarithm of the rate constant and the inverse of absolute temperature. By determining the rate constants for degradation at elevated temperatures, one can estimate  $K$  at a lower temperature by extrapolation. It is necessary to see if the potency of a formulation is above 90% its original or of its labeled concentration after storage at various temperatures for certain periods of times. According to the ICH guideline on evaluation for stability data (ICH Harmonised Tripartite Guideline, 2003), for an attribute known to decrease with time (e.g., the active ingredient content), the shelf life is calculated as the time point at which the lower one-sided 95% confidence limit for the mean degradation curve intersects the acceptance criterion. In terms of an attribute known to increase with time (e.g., the concentration of an impurity), a similar definition can be given but for the upper limit. In the present work, we deal with active ingredient herbal extract data and assume that the strength of the product decreases linearly with time, which is the commonly employed approach for describing the degradation of active ingredients. In case of a linear degradation relationship, the mean parameter that can be estimated is the slope of the degradation line.

#### 3.6.2. In vitro release study

In vitro release of christinin-A from the selected formula, as well as, the plain extract was performed using the USP XXIV dissolution tester apparatus-I (rotating basket). An accurately weighed amount of soft gelatin capsules equivalent to 200 mg of extract

relative to control containing 200 mg of plain extract was placed in USP dissolution basket. Two capsules of the formulated extract stored at 30, 40 and 50 °C [75% relative humidity] were simultaneously assessed for a period of 120 min. The basket was rotated at  $100 \pm 5$  rpm in 1000 ml distilled water and thermo statistically adjusted to a temperature of  $37 \pm 0.5$  °C. At each time interval, 3 ml sample were withdrawn, meanwhile an equal volume of distilled water was added to maintain the volume constant. Christinin-A was determined spectrophotometrically at  $\lambda$  200 nm at each specified time intervals from the previously constructed calibration curve.

#### 3.6.3. Kinetic analysis of the release data

The data obtained from the release studies were kinetically analyzed to determine the mechanism and the order of drug release from different formulations (Basak et al., 2007). Linear regression analysis was done to test the goodness of fit of the data to the following models:

$$C_t = C_0 - Kt \quad \text{for zero-order kinetics}$$

$$\log C_t = -\frac{Kt}{2.303} + \log C_0 \quad \text{for first-order kinetics}$$

where  $C_t$  is the amount of drug released in time  $t$ ,  $C_0$  is the initial amount of the drug, and  $K$  is the first-order rate constant.

$$Q = Kt^{1/2} \quad \text{for Higuchi Diffusion model}$$

where  $Q$  is the amount of the drug released in time  $t$  and  $K$  is the Higuchi dissolution constant (Higuchi, 1962).

## 4. Results and discussion

### 4.1. Effect of *Z. spina-christi* extract on general characteristics of diabetes

Rats in the untreated diabetic group, as shown in Table 1, exhibited marked hyperglycemia with significant reduction in serum insulin, C-peptide levels and total antioxidant capacity (TAC), whereas percentage of glycosylated hemoglobin (HbA1C%) was significantly elevated when compared to the normal group. Oral administration of 200 mg/kg b.w. of *Z. spina-christi* leaf extract either plain or formulated in STZ-diabetic rats for 28 days resulted in significant reduction in blood glucose level together with significant rise in serum insulin, C-peptide levels and TAC with significant lowering in HbA1C%. The current results are in harmony with those obtained by Glombitza et al. (1994) who demonstrated that the butanol extract (saponin fraction) of *Zizyphus* leaves or its main saponin glycoside, christinin-A, improved glucose utilization in diabetic rats. In fact, plasma C-peptide is considered an indicator of insulin secretion (Polonsky et al., 1984). The parallel increase in plasma insulin and C-peptide produced by the extract seems to rule out the inhibition of insulin turnover. Thus, the plasma glucose-lowering action is undoubtedly mediated by increased insulin secretion.

Determination of the amounts of flavonoids in the formula contributing to the anti-diabetic activity were found to be 11.5  $\mu\text{g/g}$  of the dry plant material (expressed as quercetin) and 58.8  $\mu\text{g/g}$  of the dry plant material (expressed as rutin) in *Zizyphus* leaves, as determined by HPLC. Nawwar et al. (1984) reported the presence of quercetin, hyperoside and quercetrin as flavonols in *Z. spina-christi* leaves. Hii and Howell (1984,1985) reported that exposure of isolated rat islets of pancreas to certain flavonoids such as (–)-epicatechin or quercetin enhances insulin release. They argue that such flavonoids may act on islet function, at least in part, via alteration in  $\text{Ca}^{++}$  fluxes and in cyclic nucleotide metabolism. Vessal et al. (2003) suggested that quercetin has proven to be beneficial

**Table 1**Blood glucose, lactate and pyruvate levels, serum insulin and C-peptide concentration, TAC and HbA1C% in *Zizyphus spina-christi* extract-treated groups.

Parameters	Groups			
	Normal	Control diabetic	Plain extract (200 mg extract/kg b.w.)	Formulated extract (200 mg extract/kg b.w.)
Glucose (mg/dl)	93.4 ± 3.2	541.1 ± 15.4 <sup>a</sup>	285.6 ± 21.4 <sup>a,b</sup>	289.7 ± 20.5 <sup>a,b</sup>
Insulin (ng/ml)	1.4 ± 0.14	0.44 ± 0.05 <sup>a</sup>	0.87 ± 0.08 <sup>a,b</sup>	0.89 ± 0.1 <sup>a,b</sup>
C-peptide (ng/ml)	0.9 ± 0.08	0.35 ± 0.04 <sup>a</sup>	0.58 ± 0.04 <sup>a,b</sup>	0.57 ± 0.03 <sup>a,b</sup>
TAC (μmol/ml)	711.3 ± 20.1	526.8 ± 15.8 <sup>a</sup>	626.6 ± 24.2 <sup>a,b</sup>	624.1 ± 24.6 <sup>a,b</sup>
HbA1C%	6.2 ± 0.76	15 ± 1.9 <sup>a</sup>	10 ± 0.99 <sup>b</sup>	9.8 ± 1.0 <sup>b</sup>
Lactate (mg/dl)	13.6 ± 1.0	24.4 ± 1.8 <sup>a</sup>	18.9 ± 1.1 <sup>a,b</sup>	18.7 ± 1.0 <sup>a,b</sup>
Pyruvate (mg/dl)	1.2 ± 0.13	0.7 ± 0.1 <sup>a</sup>	1.0 ± 0.1 <sup>a,b</sup>	0.98 ± 0.1 <sup>a,b</sup>

Values are means ± S.E.M. (*n* = 8 in each group), values in the same row are significantly different. TAC: total antioxidant activity, HbA1C: glycated hemoglobin.<sup>a</sup> *P* < 0.05 compared to normal group.<sup>b</sup> *P* < 0.05 compared to control diabetic group.

in decreasing blood glucose concentration, promoting regeneration of the pancreatic islets, as shown by increased number of islet cells, and increasing insulin release in STZ-induced diabetic rats. Compounds that facilitate glucose transporter-4 (GLUT-4) translocation can be potentially beneficial for the treatment of diabetes. Flavonoids-rich fraction from *Cephalotaxus sinensis* leaves extract (Wei et al., 2007), showed a potent antihyperglycemic effect on STZ-induced diabetic rats by demonstrating that GLUT-4 is translocated to the cell membrane of mice adipocytes after incubation with flavonoids-rich fraction.

Although the β-cell cytotoxic action of STZ is not fully understood, it is thought to be mediated by the inhibition of free radical scavenger-enzymes thereby enhancing the generation of reactive oxygen species (ROS) leading to oxidative damage (Evans et al., 1965). From the typical characteristics of diabetes is the increase of serum glycated protein such as glycated hemoglobin (HbA1C), which is a parameter for glycemic control where glucose or other reducing sugars react with the amino residues of proteins to form Amadori products, for instance, glycated hemoglobin (Thorpe and Baynes, 1996). Due to the presence of aromatic hydroxyl groups, flavonoids have strong antioxidant properties, as they are scavengers of reactive oxygen species and, therefore, inhibit peroxidation reactions (Duthie and Crozier, 2000). Antioxidants can inhibit oxidative glycation (glycoxidation) of tissue proteins with reducing sugars as reported by Yamaguchi et al. (2000). These findings could explain the observed enhancement of the total antioxidant capacity with the concomitant lowering of HbA1C% in rats treated with *Zizyphus* extract, plain and formulated.

In the present results, hyperglycemia was accompanied by marked elevation in blood lactate whereas blood pyruvate was significantly reduced. Increased lactate production may result from increased glucose conversion to lactate in adipocytes as shown by Newby et al. (1989). In line with amelioration of the diabetic state, *Z. spina-christi* extract, plain and formulated succeeded to reduce significantly the blood lactate levels and to elevate the reduced blood pyruvate contents. The increase in plasma lactate concentration is also in accordance with the study of Mondon et al. (1992)

who found that STZ-induced diabetes in rats was characterized by reduced pyruvate dehydrogenase (PDH) activity and increased lactate production in skeletal muscle and adipose tissue. These changes, in the presence of insulin deficiency, could contribute to enhanced hepatic glucose production. The ability of the *Zizyphus* extract to modulate the insulin secretion might help in ameliorating the condition. It should be mentioned that there is no significant difference between the effects of the plain *Zizyphus* extract and the formulated one on the above parameters.

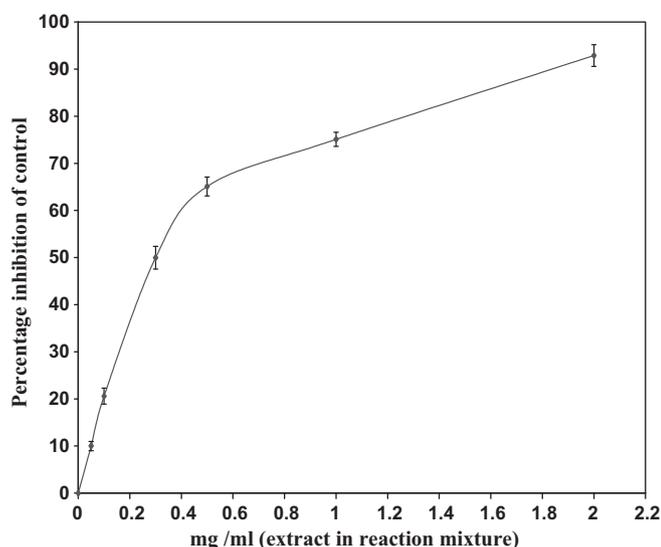
#### 4.2. Effect of *Z. spina-christi* extract on liver and muscle glycogen content, hepatic glucose-6-phosphatase and glucose-6-phosphate dehydrogenase activities in STZ-diabetic rats

Data presented in Table 2 revealed that STZ-induced diabetes in rats caused significant reduction in liver and muscle glycogen content when compared to normal group. Glycogen is the primary intracellular storable form of glucose and its level in various tissues especially in liver and skeletal muscles indicates direct reflection of insulin activity since it regulates glycogen deposition by stimulating glycogen synthase and inhibiting glycogen phosphorylase. Since STZ causes selective destruction of β-cells of islets of Langerhans resulting in marked decrease in insulin levels, it could be predicted that glycogen levels in tissues (muscle and liver) decrease as the influx of glucose is inhibited in the absence of insulin (Golden et al., 1979). As shown in the present study, STZ-diabetic rats exhibited significant increase in the hepatic gluconeogenic enzyme, glucose-6-phosphatase activity together with significant decline in the activity of hexose monophosphate shunt enzyme, glucose-6-phosphate dehydrogenase. This could be referred to increased blood glucagon/insulin ratio reflecting under-utilization of glucose in the liver. Oral administration of *Zizyphus* extract either plain or formulated succeeded to correct significantly the defective glycogen storage of diabetic muscle and liver and caused significant decrease of hepatic glucose-6-phosphatase and increase in glucose-6-phosphate dehydrogenase activities. These results could be explained on the basis that the observed better glucose utiliza-

**Table 2**Liver and muscle glycogen content, hepatic glucose-6-phosphatase and glucose-6-phosphate dehydrogenase activities in *Zizyphus spina-christi* extract-treated groups.

Parameters	Groups			
	Normal	Control diabetic	Plain extract (200 mg extract/kg b.w.)	Formulated extract (200 mg extract/kg b.w.)
Muscle glycogen (mg/g tissue)	3 ± 0.32	1.5 ± 0.2 <sup>a</sup>	2.5 ± 0.25 <sup>b</sup>	2.6 ± 0.27 <sup>b</sup>
Liver glycogen (mg/g tissue)	40.7 ± 2.9	19.8 ± 1.2 <sup>a</sup>	31.9 ± 2.6 <sup>b</sup>	32.7 ± 2.3 <sup>b</sup>
Liver glucose-6-phosphatase (U/mg protein)	60.1 ± 3.6	122 ± 5.5 <sup>a</sup>	81.4 ± 4.2 <sup>a,b</sup>	88.1 ± 6 <sup>a,b</sup>
Liver glucose-6-phosphate dehydrogenase (U/mg protein)	42.7 ± 3	16.9 ± 1.4 <sup>a</sup>	28.3 ± 2.2 <sup>a,b</sup>	25.9 ± 1.6 <sup>a,b</sup>

Values are means ± S.E.M. (*n* = 8 in each group), values in the same row are significantly different.<sup>a</sup> *P* < 0.05 compared to normal group.<sup>b</sup> *P* < 0.05 compared to control diabetic group.



**Fig. 1.** *In vitro* effect of plain *Zizyphus spina-christi* extract at different concentrations on the activity of  $\alpha$ -amylase enzyme. Each point represents the mean of 5 experiments  $\pm$  S.E.M. Y-axis is the percentage inhibition of  $\alpha$ -amylase enzyme as related to the control. X-axis is the concentration of the extract in the final reaction mixture (mg/ml).

tion may be due to an increase in insulin release which is supported by the concomitant rise in serum C-peptide level demonstrated in the current work. Glombitza et al. (1994) reported significant rise in serum insulin and pancreatic cAMP levels as a result of administration of the saponin fraction in the butanol extract of *Zizyphus* leaves to diabetic rats. It has been stated that potentiators of insulin release include agents, which elevate cAMP levels (Hedekov, 1980). Available evidence suggests that flavonoids also inhibit cAMP phosphodiesterase (Ferrel et al., 1979).

#### 4.3. *In vitro* effect of *Z. spina-christi* extract, plain and formulated on the activity of $\alpha$ -amylase enzyme

*Zizyphus* extract, exerted dose-dependent inhibitory activity against  $\alpha$ -amylase enzyme, after incubating different concentrations of the extract with the enzyme where 50% inhibition ( $IC_{50}$ ) occurs at 0.3 mg/ml. Moreover, almost complete inhibition of  $\alpha$ -amylase activity was reached at concentrations higher than 2 mg/ml (Fig. 1).

**Table 3**

Influence of seasonal variation on the yield of extracts, marker christinin-A and antihyperglycemic potencies.

Parameters	Seasons		
	Spring <sup>a</sup> (June 2008, Bf)	Autumn (November 2008, Ef)	Winter (February 2009, Fs)
Yield (% w/w)	31.7	32.2	30.3
Marker yield (mg/g)	65.6	61.4	16.6
Antihyperglycemic potencies	86	82	51

<sup>a</sup> Flowering stage starts from May (late spring) to November.

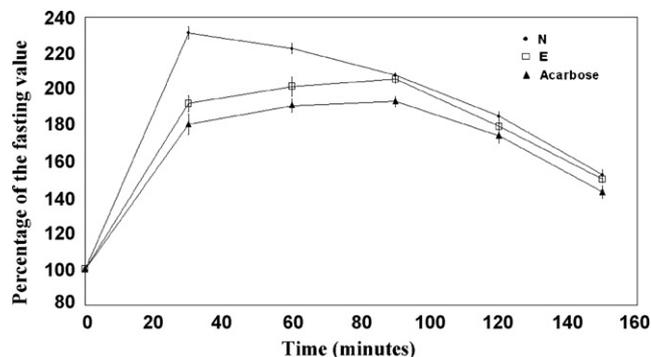
**Table 4**

Effect of the *Zizyphus spina-christi* extracts (200 mg/kg b.w.) and metformin reference (150 mg/kg) on blood glucose level in diabetic rats ( $n = 10$ ).

Groups/doses	Zero time (mg/dl, mean $\pm$ S.E.)	4 h (mg/dl, mean $\pm$ S.E.)	8 h (mg/dl, mean $\pm$ S.E.)
Control (10 ml saline) diabetic untreated	243.7 $\pm$ 9.2	242.8 $\pm$ 10.3	245.9 $\pm$ 11.2
Beginning of flowering stage extract	251.6 $\pm$ 8.1	181.9 $\pm$ 8.4*	115.2 $\pm$ 6.1*
End of flowering stage extract	247.1 $\pm$ 7.9	175.4 $\pm$ 6.5*	119.3 $\pm$ 5.1*
Fruiting stage extract	253.8 $\pm$ 9.2	189.8 $\pm$ 6.2*	171.8 $\pm$ 6.4*
Metformin	249.5 $\pm$ 8.9	161.7 $\pm$ 5.4*	92.4 $\pm$ 3.2*

Values in the same row are significantly different.

\*  $P < 0.01$ .



**Fig. 2.** Effect of plain *Zizyphus spina-christi* extract on starch digestion and absorption in normal rats. Blood glucose values were presented as percentage of fasting value taken at zero time for each group before starch ingestion (3 g/kg b.w.) and plotted against different time intervals. Each point represents the mean of 5 experiments  $\pm$  S.E.M. N = Rats received starch load only. E = Rats received plain *Zizyphus* extract (200 mg/kg b.w.) just before the starch load. Acarbose = Rats received acarbose (25 mg/kg b.w.) just before the starch load.

#### 4.4. Effect of *Z. spina-christi* extract, plain and formulated on starch digestion and absorption in normal rats

The *in vitro* results prompted us to examine the inhibitory effect of *Zizyphus* extract, on starch digestion and absorption in normal rats. Acarbose (25 mg/kg b.w.) was used as positive control in this experiment. Fig. 2, demonstrated that oral administration of starch load of 3 g/kg b.w. to normal rats, after 18 h fast, caused an increase in blood glucose level reaching a maximum of 231.2% of the fasting level at 30 min after starch ingestion. A gradual decline in blood glucose was observed reaching to about 152.4% of the fasting level after 150 min from the oral starch loading. Oral administration of *Zizyphus* extract, plain and formulated (200 mg/kg b.w.) just before the starch load elicited an *in vivo* decline in starch digestion and absorption. *Zizyphus* extract reduced the area under the blood glucose curve, as compared to the normal curve, and tended to delay the absorption as the maximum blood glucose level was attained between 60 and 90 min. The inhibitory effect of *Zizyphus* extract on starch digestion and absorption was lower than that of acarbose regarding both the peak of blood glucose level and the area under the blood glucose curve. To the best of our knowledge, there is no report on the inhibitory activity of *Z. spina-christi* extract against carbohydrate-hydrolyzing enzymes. Starch in meals is first decomposed into oligosaccharides by the enzyme  $\alpha$ -amylase in saliva and  $\alpha$ -glucosidase in pancreatic juice, which is a membrane bound

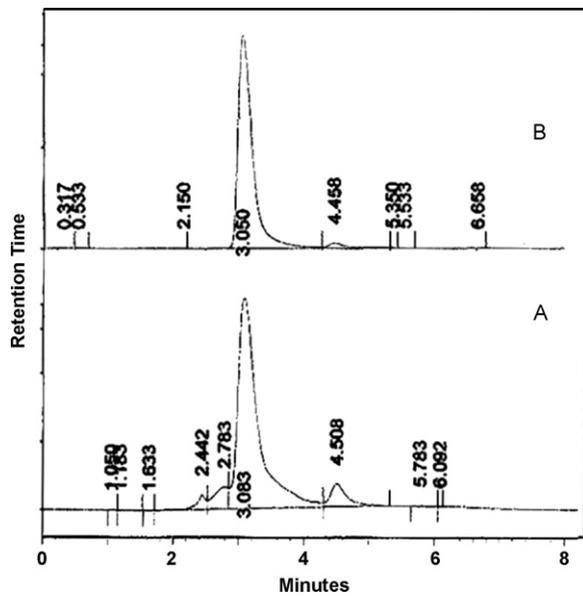


Fig. 3. HPLC trace of *Zizyphus spina-christi* plain extract A and the marker rutin B; detection: 374 nm; retention time 3.083 min.

enzyme located at the epithelium of the small intestine, catalyzing the cleavage of glucose from disaccharides and oligosaccharides. Dietary  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibitors have been identified as a potentially natural and safe approach for controlling hyperglycemia (Bischoff, 1994). Polyphenolic extracts from a number of plants were found to be effective inhibitors of intestinal  $\alpha$ -glucosidase activity (Matsui et al., 2001) that is comparable to synthetic inhibitors (acarbose and voglibose) already being used therapeutically to control hyperglycemia (Toeller, 1994). These extracts also inhibited  $\alpha$ -amylase activity, which could prove to be synergistic to their potential therapeutic effect on post-meal blood glucose levels (Matsui et al., 2001).

The current work revealed that the *Zizyphus* leaf extract, plain or formulated, improved glucose utilization in diabetic rats by increasing insulin secretion which may be due to both saponin and polyphenol contents and controlling hyperglycemia through attenuation of meal-derived glucose absorption that might be attributed to the total polyphenols.

#### 4.5. Influence of seasonal variation on yield of extracts, marker content (christinin-A) and antihyperglycemic activity

The yield of extracts were slightly influenced by seasonal variation reaching the maximum 32.2% in November 2008 (Ef, End of the flowering stage) being lower, 31.7% in June 2008 (Bf, Beginning of the flowering stage) and lowest, 30.3% in February 2009 (Fs, Fruiting stage). HPLC determination of the christinin-A content at 200 nm showed a slightly different pattern, the highest yield was 65.6 mg/g (Bf) followed by 61.4 mg/g (Ef) and the least 17.3 mg/g of the dry plant material at the (Fs). Evaluation of the antihyperglycemic activity of the different samples (Table 3) at a dose of 200 mg/kgb.w. was found to be 86% (Bf), 82% (Ef) and 51% (Fs), as potent as Metformin (150 mg/kgb.w.,  $P < 0.01$ ) after 8 h of administration (Table 4). The highest yield of extract was thus recorded for the leaves collected at the end of flowering stage (Ef) while the highest content of marker and antihyperglycemic potency were recorded for samples gathered at June 2008 (Bf) suggesting that this time should be recommended for collection up to October.

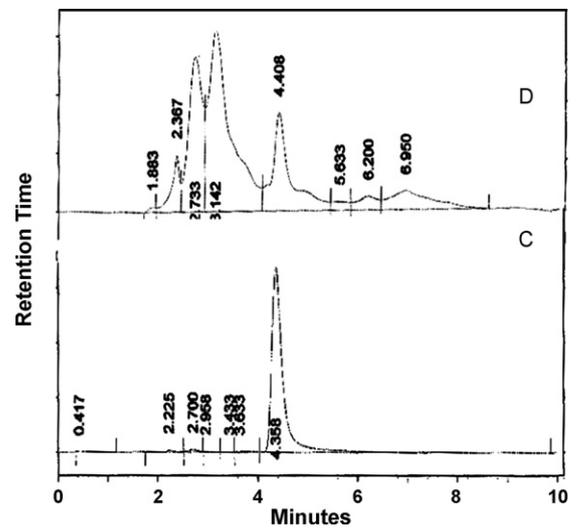


Fig. 4. HPLC trace of *Zizyphus spina-christi* plain extract D and the marker quercetin C; detection: 254 nm; retention time 4.408.

#### 4.6. Determination of the flavonoid content in the formulated leaves extract contributing in the antihyperglycemic activity

HPLC determination of the flavonoid content of the formulated leaf extract collected at the beginning of the flowering stage (June 2008) were 11.5  $\mu\text{g/g}$  (expressed as quercetin) and 58.8  $\mu\text{g/g}$  of the dry plant material (expressed as rutin – Figs. 3 and 4). The calibration curves have correlation coefficient close to 1. The resulting data and the mean regression equations were computed and found to be  $y = 73,877x$  for quercetin with a correlation coefficient  $R = 0.9969$  and  $y = 45,814x$  for rutin with  $R = 0.9951$ , where  $y$  is the absorbance and  $x$  is the concentration in  $\mu\text{g/ml}$ .

#### 4.7. Accelerated stability testing of soft gelatin capsules and leaf extract of *Z. spina-christi*

The chemical stability of the tested formula in comparison with the plain extract was evaluated according to Kenneth et al. (1979).

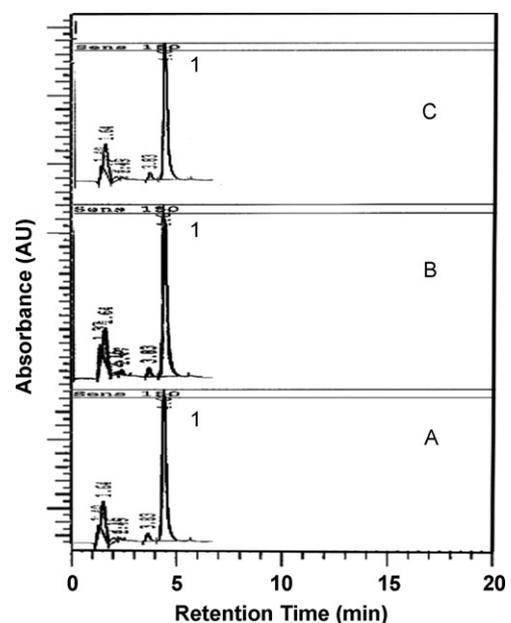


Fig. 5. HPLC trace of *Zizyphus spina-christi* plain extract (detection: 200 nm) after 4 weeks (A), 12 weeks (B) and 24 weeks (C) at 50 °C.

The formulation is meeting the requirement of 1 year shelf life for herbal formulations. The use of HPLC method in this stability study revealed that the degradation products did not show any interference with absorbance at 200 nm after 4, 12 and 24 weeks (Figs. 5 and 6). Kinetic analysis of the stability data obtained for the tested capsules revealed that the degradation followed first order kinetics. Figs. 7 and 8 illustrated graphically the first order degradation of the tested capsules at 30, 40 and 50 °C [75% relative humidity] in comparison to plain extract. Figs. 9 and 10 illustrated the logarithmic plot of  $k$  (degradation rate constant) of the plain extract and formulated extract respectively at different temperatures. From these figures, the degradation rate constant at 25 °C ( $K_{25}$ ) and the expiration date were calculated according to the Garret and Carper equation (Garrett and Carper, 1955).  $t_{0.9} = 0.105/K_{25}$ , where  $t_{0.9}$  is the time at which the percent drug remaining is 90%. The results are shown in Table 5 and the expiration dates of the prepared formula relative to control of plain extract were calculated to be 70.74 and 66.90 weeks, respectively.

#### 4.8. In vitro release study

The dissolution test was performed using apparatus-I Dissolution tester USP XXIV. The percentage of christinin-A release from plain and formulated extract packed in soft gelatin capsules were

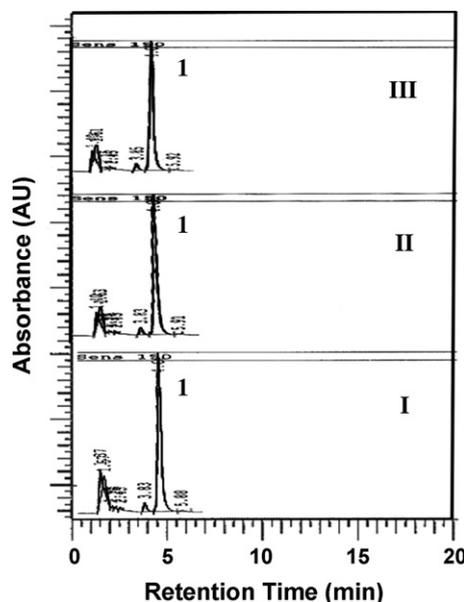


Fig. 6. HPLC trace of *Zizyphus spina-christi* formulated extract (detection: 200 nm) after 4 weeks (I), 12 weeks (II) and 24 weeks (III) at 50 °C.

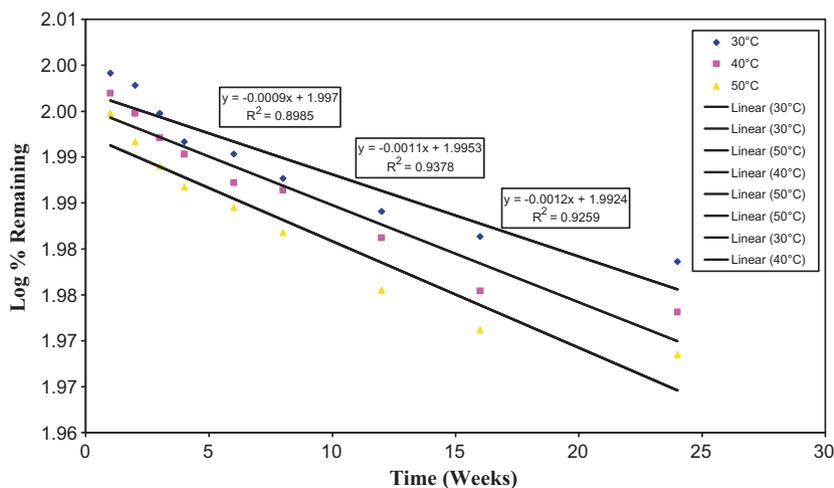


Fig. 7. First-order degradation of *Zizyphus spina-christi* plain extract at 30, 40 and 50 °C.

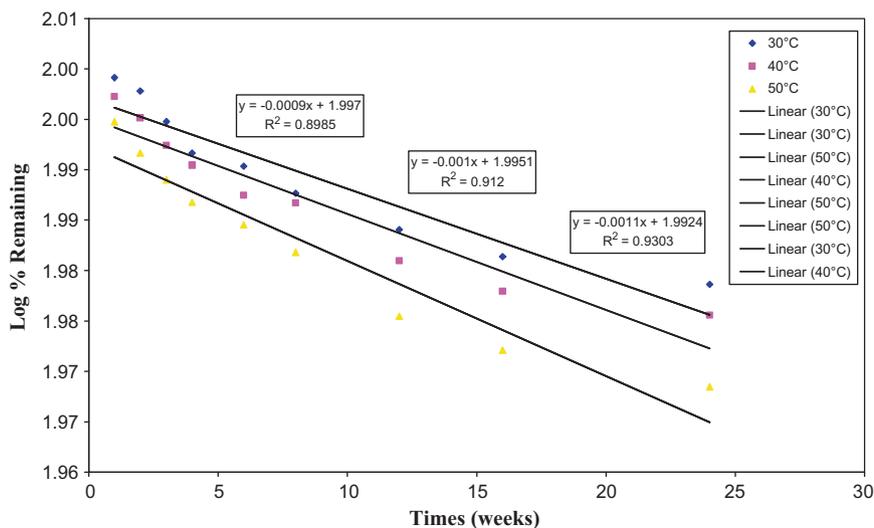


Fig. 8. First-order degradation of *Zizyphus spina-christi* formulated extract at 30, 40 and 50 °C.

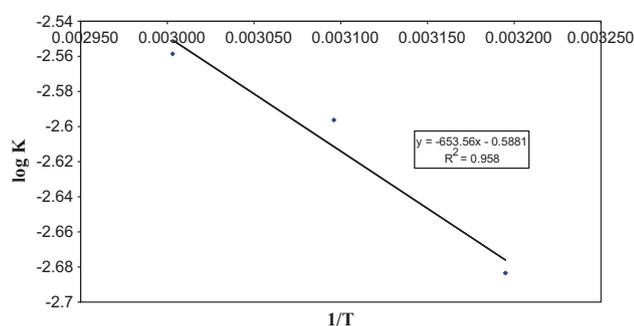
**Table 5**Logarithmic  $K$  values of the prepared formula relative to control of plain extract at 30, 40 and 50 °C [75% relative humidity].

Extracts	Logarithmic $K$ values at different temperatures			Calculation of expiration time (weeks)
	303 °K	313 °K	323 °K	
Plain extract	-2.683	-2.559	-2.524	66.90
Formulated extract	-2.683	-2.638	-2.524	70.74

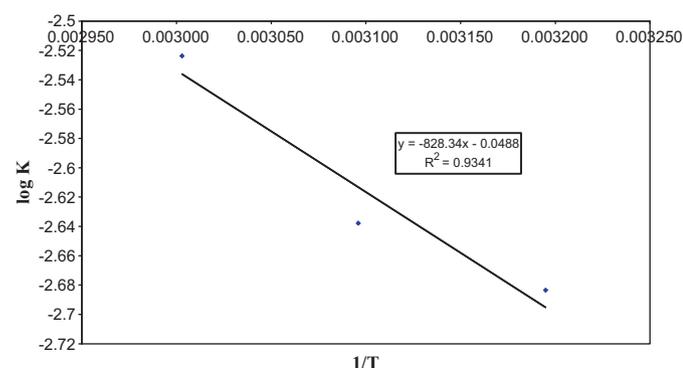
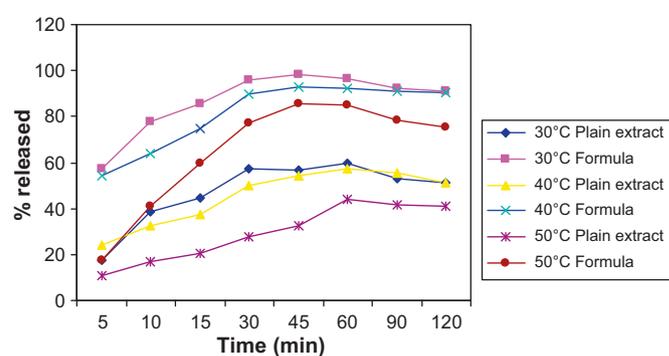
I = Plain extract (P-Ext), II = Prepared formula.

**Table 6**Release of christinin-A from plain and formulated extract of *Zizyphus spina-christi* after storage at 40 and 50 °C [75% relative humidity] according to zero, first order kinetics and diffusion model.

Formulations	Correlation coefficient			Mechanism of release
	Zero order	First order	Diffusion	
Plain extract stored at 30 °C	0.7	0.6	0.8	Diffusion
Formulated extract stored at 30 °C	0.4	0.4	0.9	Diffusion
Plain extract stored at 40 °C	0.7	0.7	0.9	Diffusion
Formulated extract stored at 40 °C	0.6	0.6	0.8	Diffusion
Plain extract stored at 50 °C	0.9	0.8	0.9	Diffusion
Formulated extract stored at 50 °C	0.5	0.5	0.7	Diffusion

**Fig. 9.** Logarithmic plot of  $K$  value of plain extract.

calculated spectrophotometrically at 200 nm. Fig. 11 displayed the *in vitro* release of the extract after 120 min. The release rate of christinin-A from the formulated extract stored at 30, 40 and 50 °C [75% relative humidity] was found to be 98.4, 93.0 and 85.6%, respectively after 45 min. While the plain extract stored at 30, 40 and 50 °C [75% relative humidity] gave 59.4, 57.4 and 44.3%, respectively after 60 min. The kinetic analysis of the extract release data from different preparations was calculated by linear regression according to zero, first order kinetics and simplified Higuchi model. The release followed the Higuchi kinetic model, indicating diffusion dominated extract release with little or no drop in the dissolution values throughout the test period as shown in Fig. 11 and Table 6.

**Fig. 10.** Logarithmic plot of  $K$  value of prepared formula.**Fig. 11.** Release of christinin-A from soft gelatin capsule, relative to plain extract, containing *Zizyphus spina-christi* extract after storage at 30, 40 and 50 °C [75% relative humidity].

#### 4.9. Conclusion

The current work revealed that the *Z. spina-christi* leaf extract, plain or formulated, improved glucose utilization in diabetic rats by increasing insulin secretion which may be due to both saponin and polyphenol content and controlling hyperglycemia through attenuation of meal-derived glucose absorption that might be attributed to the total polyphenol content.

The highest yield of extract was recorded for the leaves collected at the end of flowering stage (Ef) while the highest content of marker and antihyperglycemic potency were recorded for samples gathered at June 2008 (Bf) suggesting the time for collection from June to October from the economical standpoint.

The formulation is meeting the requirement of 1 year shelf life for herbal formulations. The degradation products, measured by HPLC method did not show any interference with absorbance at 200 nm. Kinetic analysis of the stability data obtained for the tested capsules followed first order kinetics. The expiration dates of the prepared formula relative to control of plain extract were 70.7 and 66.9 weeks, respectively. The release rate of christinin-A from the formulated extract stored at 30, 40 and 50 °C [75% relative humidity] was 98.4, 93 and 85.6% respectively after 45 min. The plain extract stored at the same conditions gave 59.4, 57.4 and 44.3% respectively after 60 min. The release followed the Higuchi kinetic model, indicating diffusion dominated extract release with little or no drop in the dissolution values throughout the test period.

However, clinical trials are still needed to evaluate the efficacy of the formulated *Zizyphus* leaf extract in diabetic patients.

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