Indian Journal of Traditional Knowledge
Vol 18(4), October 2019, pp 662-669

# A modern formulation of traditional medicine: Jujube (*Ziziphus jujuba* Mill.) fruit syrup

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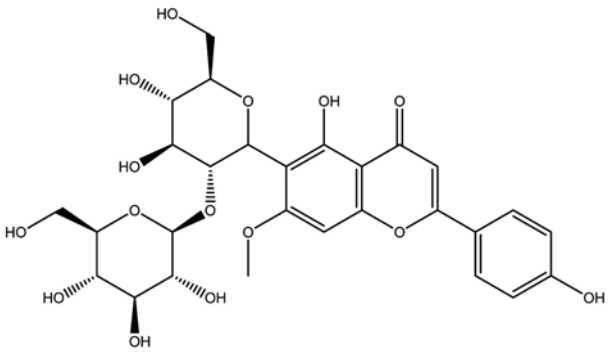
*Received 03 December 2018; revised 14 May 2019*

*Ziziphus jujuba* Mill.,commonly called jujube (Rhamnaceae) has been known for its health benefits and used to cure different diseases such as asthma, cough and anxiety. The aim of this study is a formulation of its syrup and evaluation of physicochemical properties in accelerated stability conditions. The isosbestic point of spinosin (as marker) and its stability kinetic was studied in different buffers (pH range: 3-8). The isosbestic point was 259 nm that was different from $\gamma_{max}$ (334 nm). It was found that spinosin was more stable at pH of 7. So, the pH of the syrup was adjusted to 7. The syrup was a brown viscous liquid with jujube fruit flavor. Dry residue, pH, density and viscosity of the syrup were found to be 0.8 g/mL, 7, 1.29 g/mL and 0.14 Pa/s, respectively. During accelerated stability studies no significant changes were observed in physical properties and 3.2% decrease in spinosin content was seen that is acceptable. The preservative effectiveness test showed that the free preservative formulation met the USP criteria. In conclusion, *Z. jujuba* fruit syrup has a suitable potential to be manufactured on the mass production for traditional herbal medicine markets.

**Keywords**: HPTLC, Rhamnaceae, Spinosin, Stability, Syrup, *Ziziphus jujuba*

**IPC Code**: Int. Cl.[19]: A61K 38/00, A61K 36/72, A01N 25/30, B63B 43/04, C13K 1/00, A61K 8/97

Traditional herbal medicine, as a natural source, plays an important role in treatment of various diseases and maintenance of health[1,2]. Traditional treatments that were used in various traditional medical schools have been repeatedly examined by a number of great physicians through experiments and clinical trials over thousands of years. Even now, hundreds of years later, traditional medicine has not lost its value[3]. *Z. jujuba*, a small deciduous tree or shrub, is widely cultivated around the world and its ripe fruit is used as food and medicine since ancient times[4,5]. The various parts of this plant such as fruits, leaves, roots and seeds have medicinal value. *Z. jujuba* is beneficial for the treatment of different diseases, such as asthma, constipation, cough, inflammation, insomnia, anxiety, laryngitis as well as heart, liver and kidney diseases[6,7,8]. Various chemical constituents from different phytochemical classes such as alkaloids, polysaccharides, flavonoids are found in *Z. jujuba* fruit. For example, spinosin (Scheme 1), flavones C-glycoside, was isolated from *Z. jujuba* seed. Spinosin at 5 mg/kg exerts its anxiolytic-like effects by modulation of γ-aminobutyric acid-A ($GABA_A$) and 5-hydroxytryptamine-1A ($5\text{-}HT_{1A}$) receptors compared with diazepam as a control group in mice[9]. In an *in vivo* study the sub chronic administration of spinosin (5 mg/kg), exhibited significant increase in the proliferation and survival of neuronal cells and the number of immature neurons in the hippocampus




Scheme 1 — Molecular structures of spinosin

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dentate gyrus region[10]. Spinosin in dose dependent manner enhance phenobarbital induced sleep by increasing sleep time and reducing sleep latency via a serotonergic mechanism[11]. In addition, due to the presence of carbohydrates, oral administration of aqueous extract of *Z. jujuba* fruit is a beneficial treatment for constipation[12,13].

The aim of this study is to formulate *Z. jujuba* fruit syrup and to investigate the quality control and stability under accelerated storage condition (40±2°C/75±5% RH (relative humidity), 6 months) as specified in drugs regulatory agencies guidelines[14-17]. Each sample was evaluated for physicochemical stability parameters as appearance, pH, viscosity, organoleptic properties and content of the marker (spinosin). In addition, the isosbestic point, preservative effectiveness test and the effect of pH on the stability kinetic of spinosin were studied at different buffers (pH range 3-8).

## Materials and methods

### Plant material

The fruits of *Z. jujuba* were bought from a local market in Birjand city, South Khorasan province, Iran in September 2017. The voucher specimen was authenticated and verified by Ms M Souzani from herbarium of Pharmacy school, Mashhad University of Medical Sciences, Iran. A voucher specimen was also deposited (No 13246).

### Chemicals

The standard spinosin was purchased from Sigma-Aldrich, (Steinheim, Germany) and all solvents (dichloromethane, ethyl acetate, methanol and water) which were used in this study were HPLC grade and purchased from Merck (Darmstadt, Germany). The other chemicals and reagents were of pure analytical grade and were supplied by Merck.

### Instruments

Chemical stability studies were evaluated by using HPTLC instrument (CAMAG, Muttenz, Switzerland) on pre-coated glass plate with a 200 µm layer of silica gel $60F_{254}$ (Merck, Darmstadt, Germany). A freeze dryer (Operon Co. Ltd., Korea) was used to dry the extracts. The pH was measured using digital pH meter (Five Easy plus FEP 20-ATC kit, Mettler Toledo AG, Analytical, Schwerzenbach, Switzerland). Brookfield viscometer (LVDV-1 Prime viscometer, Brookfield Engineering Laboratories, New York, USA) was used to measure viscosity of each stability sample. UV spectrophotometer (UV-1206 UV–Vis spectrophotometer, Shimadzu, Japan) was used for spectrophotometry analysis.

Stability studies were performed by using a stability chamber (programmable environmental test chamber, Remi) capable of controlling temperature and humidity within range of 40±2°C and 75±5% RH, respectively.

### Quality control of plant material

*Determination of moisture content (loss on drying)*

Ten grams of herbal powder (without initial drying) was placed in a cruiser vessel and dried at 105°C for 5 h and then weighed. Drying and weighing continued at 1 h interval. Whenever the difference between 2 seriate weights after drying for 30 min and cooling for 30 min in a desiccator, was not more than 0.01 g, the constant weight was recorded[18].

*Total ash value*

Five grams of dried and pulverized *Z. jujuba* fruit were weighed accurately and ignited in a muffle furnace at 500℃ in a tared crucible until the sample turned into white ash with the constant weight. The percentage of total ash value was calculated[18].

*Acid-insoluble ash value*

Twenty-five milliliters of HCl (2 M) was added to the total ash. The mixture was heated on a water bath for 10 min. The insoluble matter was collected on a Whatman ash-less filter paper (Whatman No. 41) and was washed with hot distilled water until the filtrate neutralization. The filter paper with residue was ignited in the furnace, cooled in a desiccator until constant weight was achieved. The acid-insoluble ash was weighed and the percentage of acid-insoluble ash was calculated[18].

*Water–soluble ash value*

The total ash was boiled with 25 mL water for 5 min. It was filtered by an ash-less filter paper (Whatman No. 41) and the residue was ignited in the furnace until constant weight was achieved. The water-soluble ash was weighed and the percentage of water-soluble ash was calculated[18].

*Determination of pectin value*

Twenty grams of *Z. jujuba* fruit was boiled in absolute ethanol for 5 min. The residue was extracted with 200 mL of boiling water. The pH of the extract was adjusted to 6.5 with ammonia solution (1%). By addition of an equal volume of ethanol (95%), pectin

precipitated. The precipitates were separated, washed successively with ethanol (70%) and dried at room temperature. The percentage of pectin was calculated[19].

***Extraction methods***

*Z. jujuba* fruits were placed in an oven for removing the moisture. Then the dried fruits were grinded into a coarse powder. 100 g of powder was extracted with ethanol (70%), by a Soxhlet apparatus and percolation methods, separately. The equal amount of fruit powder was extracted with distilled water by decoction method. The extracts were filtrated with filter paper and concentrated with rotary evaporator at a reduced pressure at a temperature 45℃ prior to freeze-drying[20].

**Determination of spinosin in different extracts by HPTLC method**

The content of spinosin in different extracts of *Z. jujuba* fruit, which gained by Soxhlet, percolation, and decoction methods, was determined by HPTLC methods. In this method, prior to analysis, all samples were filtered with 0.45 μm membrane syringe filters.

*HPTLC conditions*

A CAMAG HPTLC system equipped with an automatic TLC Sampler 4 (ATS 4) was used to analyze the sample on (20 cm×10 cm) glass plates that were pre-coated with a 200 μm layer of silica gel $60F_{254}$, a CAMAG twin trough development chamber was used in which the vertical development of the plate was performed using ethyl acetate-dichloromethane- methanol- water (18:10:15:5, *v/v/v/v*) as the mobile phase. Visualization of the plates was performed by treatment with aluminum chloride - ethanol solution (10%) as a reagent that was followed by heating at 110°C for 20 min. Detection was carried out at 334 nm using a CAMAG TLC scanner III and associated integration software (win CATS 1.4.2 version).

TLC visualizer was used for taking a photo from chromatograms with a digital camera (Power Shot G5 with Neck Strap NS-DC2, Canon, Japan).

**Isosbestic point determination**

The absorption spectra of spinosin in acidic, neutral and basic solution with equimolar concentration, was recorded from 200 to 600 nm, separately. The wave lengths that the spectra cross each other are the isosbestic point which is independent of the solution pH.

**Buffers preparation**

Citrate buffers (pH 3, 4, 5, 6; 0.1 M) and phosphate buffers (pH 7, 8; 0.1 M) were prepared by mixing two stock solutions as described in Table 1. The final solutions were diluted with water to volume 100 and 200 mL for citrate and phosphate buffers, respectively.

**Effect of pH on spinosin stability (pH profile)**

In order to obtain the most stable pH, the stability kinetic of spinosin was studied in the pH range 3–8 (citrate buffer: 3-6, phosphate buffer: 7–8) at room temperature.

To investigate the effects of pH on spinosin stability, the samples obtained by the Soxhlet method at a constant concentration were used. The pH of samples was adjusted using citric acid (0.1 M) − sodium citrate (0.1 M) solutions with different ratios to achieve the pH values (3, 4, 5, 6) and monobasic sodium phosphate (0.1 M) − dibasic sodium phosphate (0.1 M) solutions with different ratios to achieve the pH values 7 and 8.

All samples were stored for 30 days at room temperatures in dark place. At least 12 time points were chosen to obtain the reaction kinetics.

**Reaction kinetics**

To investigate the effects of different pHs on stability kinetics of spinosin, zero and first-order models were applied to evaluate the amount of spinosin remaining in the solutions over times. Spinosin content can be described by $C=-Kt+C_0$ in zero-order kinetics and by $\ln C_= -K\ t+\ln C_0$ in first-order kinetics. Where C is the concentration of spinosin at time t (day), $C_0$ is the initial concentration and k is the reaction rate constant.

**Preparation of *Z. jujuba* fruit syrup**

For preparation of simple syrup, 850 g of sucrose was added to 450 mL of purified water and the syrup

Table 1 — Buffer preparation methods

| pH | Buffers | stock solution |
|---|---|---|
| 3 | Citrate | 46.5 mL of Citric acid 0.1 M |
| | | 3.5 mL of sodium citrate 0.1 M |
| 4 | Citrate | 33 mL of Citric acid 0.1 M |
| | | 17 mL of sodium citrate 0.1 M |
| 5 | Citrate | 20.5 mL of Citric acid 0.1 M |
| | | 29.5 mL of sodium citrate 0.1 M |
| 6 | Citrate | 9.5 mL of Citric acid 0.1 M |
| | | 41.5 mL of sodium citrate 0.1 M |
| 7 | Phosphate | 39 mL of monobasic sodium phosphate 0.1 M |
| | | 61 mL of dibasic sodium phosphate 0.1 M |
| 8 | Phosphate | 5.3 mL of monobasic sodium phosphate 0.1 M |
| | | 94.7 mL of dibasic sodium phosphate 0.1 M |

was heated until sugar was completely dissolved. To prepare *Z. jujuba* fruit syrup, 187.5 g of extract powder (based on the Iranian traditional medicine)[21] obtained through Soxhlet extraction method diluted with simple syrup, pH was adjusted to 7 by adding phosphate buffer (pH 7) and the volume made up to 1000 mL. The solubility was examined by observing the clarity of solution by visual inspection. Minimum concentration of methyl paraben (0.015%) and propyl paraben (0.01%) was added as preservative[22].

**Stability studies**

The formulated syrup was subjected to accelerated stability conditions and samples were withdrawn at 0, 1, 3 and 6 months[23].

*Chemical stability*

In order to determine the spinosin content, all samples were analyzed using HPTLC method.

*Physical stability*

All samples were studied for its physical and organoleptic properties such as dried residue, color, clarity, odor, taste, pH, density and viscosity.

**Preservative effectiveness test**

For the preservative effectiveness test, in addition to syrup sample containing preservative, two controls (formulated syrup and simple syrup without preservative) were used. The test organisms were bacteria (*Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739), fungi (*Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404). From each culture a $10^8$ cfu/mL suspension was prepared. Twenty milliliters of each sample was inoculated with 0.5–1 mL of the prepared suspension to reach final count of approximately $10^5$-$10^6$ cfu/mL of organisms on day 0. The inoculated samples were incubated at 22.5±2.5°C and investigated for microbial survivors after 14 and 28 days.

To assess the number of survivors, 1 mL of each sample after sufficient dilution was transferred to plate and mixed with soybean-casein digest agar (Hi Media, Mumbai, India) by gentle swirling (pour-plate method). The plates containing bacteria were incubated at 32.5±2.5°C for 1-3 days, and the fungal plates were incubated at 22.5±2.5°C for 3–7 days. Following incubation, the cfus were counted and recorded. The log reduction (log [final count] /log [initial count]) was calculated for each sample at different time intervals. The criteria of the USP preservative effectiveness test for bacteria require a one log reduction by day 14, no increase in survivors from days 14 to 28 and no increase in survivors for the fungi from initial today 28 of the test[24,25].

**Statistical analysis**

All data was presented as the mean±standard deviation. Data normality was verified using the Shapiro-Wilk test and homogeneity of variance was checked by Brown–Forsythe test. If the data failed to pass the test, a logarithmic transformation of the data was performed and retested.

Significant differences were assessed between treatments and the control using repeated measures analysis of variance (ANOVA), followed by, Dunnett's test for stability studies and one-way ANOVA, and followed by Tukey's multiple comparisons test was used for selection of the extract with the highest amount of spinosin. $p<0.05$ was considered to be statistically significantly different. All statistical tests were conducted using GraphPad PRISM (Version 6.00) software.

## Results and discussion

Traditional medicine is "the sum total of the knowledge, skill and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness"[26]. Majority of the world population is still using traditional medicine to support their health [27]. *Z. jujuba* fruit syrup is one of the most popular traditional medicines in different parts of the world. Syrup has advantages like ease of swallowing in patients with dysphagia, children and the elderly people and above all, the dosage can be adjusted easily[28].

**Quality control of plant material**

The ash values (total ash, acid-insoluble, and water-soluble), moisture content, and pectin value were determined and the results are presented in Table 2. The herbal ingredients were in compliance with the quality control standards. Therefore, they were used for formulation studies.

*Extractive value*

The percentage yields of different extraction methods are showed in Table 3.

Table 2 — Quality control of plant material

| Parameters | mean±SD (n=6) |
|---|---|
| Moisture content (%) | 13.95± 0.05 |
| Total ash (%) | 2.98± 0.03 |
| Acid-insoluble ash (%) | 0.31± 0.03 |
| Water-soluble ash (%) | 1.52± 0.04 |
| Pectin content (%) | 2.12± 0.05 |

Table3 — Extractive values of different extraction methods

| Extraction method | Solvent | Extractive value (w/w %) |
|---|---|---|
| Soxhlet | ethanol 70% | 25.98±3.2 |
| Percolation | ethanol 70% | 25.23±2.02 |
| Decoction | distilled water | 26.31±4 |

***Determination of spinosin content in different extracts from Z. jujuba fruit***

The content of spinosin in different extracts of *Z. jujuba* fruit which gained by Soxhlet, percolation and decoction procedure that determined by HPTLC methods were 2.81±0.04, 2.03±0.03, 1.36±0.03 µg/g, respectively.

The quantitative results showed that there is a significant difference between the amount of spinosin in different extracts and the highest amount of spinosin was extracted by Soxhlet method. Therefore, this extract is used for preparation of oral liquid dosage form.

**Isosbestic point**

The isosbestic point occurred at a wavelength 259 nm. At this isosbestic wavelength, the absorptivities are independent of the solution pH. Indeed, there are three strong absorption wavelengths for spinosin (215, 272 and 334 nm). However, spinosin at 334 nm was easily separated from other compounds. Hence, all studies were done at 334 nm, which is different from the isosbestic point.

**Effect of pH on spinosin stability (pH profile)**

Hydrolysis is one of the most important chemical instability reactions in liquid dosage forms, which can be prevented by using appropriate buffers[29,30]. In order to find more stable pH for spinosin, its stability kinetic was studied in different buffers and pHs at room temperature and the results of reaction kinetics are illustrated in Fig. 1. A series of k values (reaction rate constants) were obtained through linear regression with using first-order kinetic equation and the time at which 10% spinosin degraded was calculated and listed in the Table 4. The log-linear relationship between K values and pH is shown in Fig. 2. The pH of samples before and after pH treatments was monitored and no significant difference was found. The results showed that spinosin was more stable at pH 6 and 7, which can be explained by the sensitivity of the glyosidic bonds to hydrolysis[31,32]. The proposed mechanisms for degradation of spinosin are depicted in Scheme 2. The acid-catalyzed hydrolysis of glyosidic bonds involves the protonation of the glycosidic oxygen atom to form intermediate 1, then the cleavage of this etheric bond result the intermediates 2 and 3. The intermediate 3 converts to the compound 4 in the presence of $H_2O$[31]. According to the results in Table 4 and hydrolysis of spinosin in acidic conditions, the final liquid dosage form was formulated at pH 7.

Table 4 — pH effect on rate constant and $t_{90}$ values for spinosin

| pH | Curve equation | $R^2$ | K | $t_{90}$ (day) |
|---|---|---|---|---|
| 3 | Y= -0.0422 X+0.0183 | 0.8587 | 0.0422 | 2.4 |
| 4 | Y= -0.0216 X+0.1773 | 0.9638 | 0.0216 | 4.8 |
| 5 | Y= -0.0091X+0.2616 | 0.9828 | 0.0091 | 11.5 |
| 6 | Y= -0.0008 X+0. 2945 | 0.9895 | 0.0008 | 131.7 |
| 7 | Y= -0.0004 X+0.2962 | 0.9941 | 0.0004 | 263.4 |
| 8 | Y= -0.0136X+0.2201 | 0.9535 | 0.0136 | 7.7 |

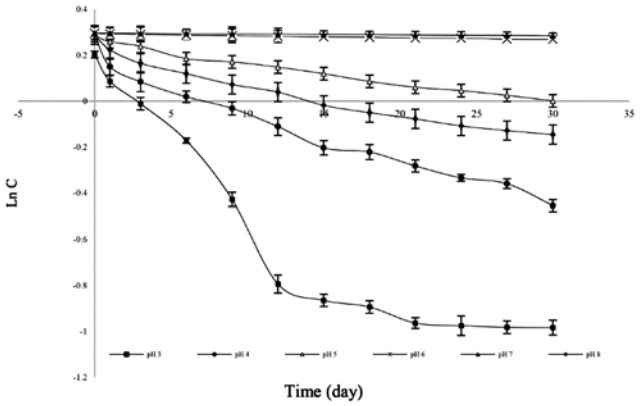



Fig. 1 — First-order stability kinetic regression lines of spinosin at different pHs at room temperature.

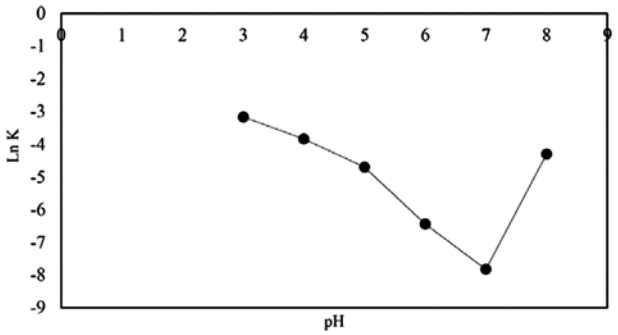
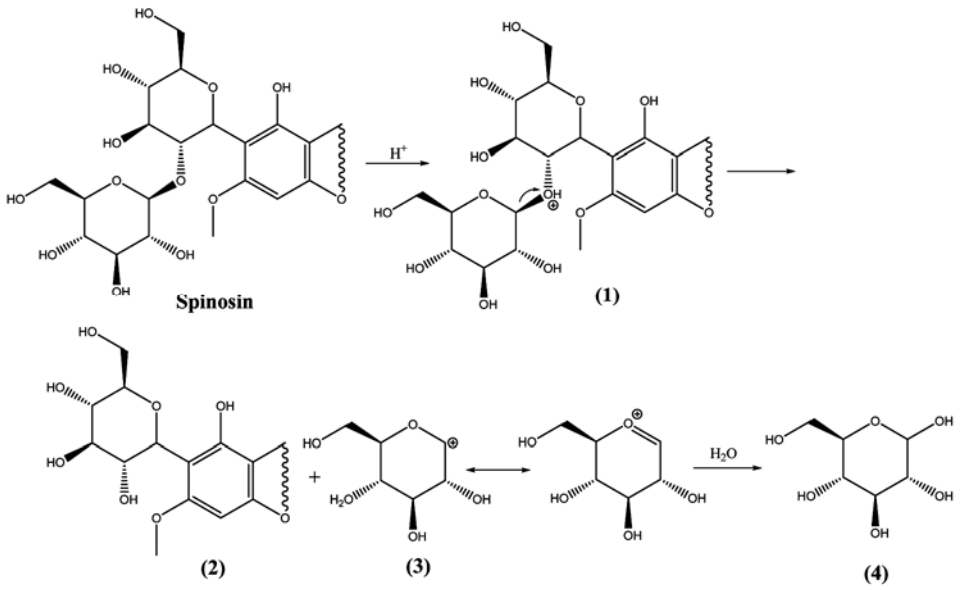



Fig. 2 — Log-linear relationship between the rate constant k and pH at 25°C.




Scheme 2 — The proposed mechanism for acid hydrolysis of spinosin

**Preparation of Z. jujuba fruit syrup**

According to the obtained results, for the preparation of the final *Z. jujuba* fruit syrup, 187.5 g of extract powder obtained through Soxhlet extraction method diluted with simple syrup, pH was adjusted to 7 by adding phosphate buffer (pH 7) and the volume made up to 1000 mL.

**Stability studies**

*Chemical stability*

Chemical stability of the syrup was investigated in terms of spinosin content as the marker. Content of spinosin was determined by HPTLC method. Spinosin was separated at $R_f$ 0.38±0.02. Presence of spinosin in each stability sample was confirmed through matching of $R_f$ values and comparing UV absorption spectra at the start, middle and the end position of the spectrum. The results showed that spinosin content decreased by 3.2% after six months at the accelerated storage condition, which is acceptable with a maximum allowable reduction of 5% (Fig. 3)[33].

*Physical stability*

The results of the physical evaluation and organoleptic properties demonstrated that all stability samples were clear, viscose, dark brown with specified odor and taste of *Z. jujuba* fruit. Crystallization and precipitation were not observed in the samples.

These results revealed that there was no significant change in physical stability of any samples at the accelerated storage condition (Table 5). The results of stability studies showed that the syrup had acceptable stability after 6 months of storage, which confirms the stability of product.

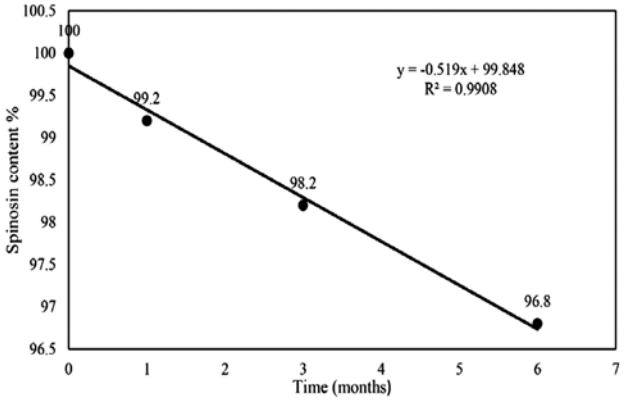



Fig. 3 — Percentage of spinosin content of *Z. jujuba* fruit syrup during 06 months at accelerated conditions.

**Preservative effectiveness tests**

All groups were tested by preservative effectiveness test. The results showed that the *Z. jujuba* fruit syrup is effectively preserved, even without preservative. The results (Table 6) demonstrated that the syrup formulations exceeded the USP criteria. In accordance with the present results, previous studies have explained that the *Z. jujuba* fruit also has antimicrobial effects[34]. In the same way, ethanolic extract of *Z. jujuba* fruits showed a wide antimicrobial activity against *E. coli*, *S. aureus*, *C. albicans* and *Aspergillus fumigatus*[35].

Table 5 — Results of physical stability studies of *Z. jujuba* fruit syrup under the accelerated condition

| Test | Time (month) | | | |
|---|---|---|---|---|
| | 0 | 1 | 3 | 6 |
| Physical appearance | viscous liquid | viscous liquid | viscous liquid | viscous liquid |
| Color | dark brown | dark brown | dark brown | dark brown |
| Odor | sweet aromatic | sweet aromatic | sweet aromatic | sweet aromatic |
| Taste | *Z. jujuba* fruit | *Z. jujuba* fruit | *Z. jujuba* fruit | *Z. jujuba* fruit |
| Specific gravity (g/mL) | 1.29±0.03 | 1.29±0.01 | 1.29±0.02 | 1.29±0.01 |
| Viscosity(pa/s) | 0.14±0.02 | 0.14±0.04 | 0.14±0.02 | 0.14±0.03 |
| pH value | 7 ± 0.01 | 7 ± 0.02 | 7.1 ± 0.01 | 7 ± 0.01 |

Table 6 — Results of preservative effectiveness test

| Microorganism | log reduction (cfu/mL) | | | | | |
|---|---|---|---|---|---|---|
| | day 14 | | | day 28 | | |
| | test group (product) | control group (simple syrup) | control group (product without preservative) | test group (product) | control group (simple syrup) | control group (product without preservative) |
| *Escherichia coli* | 4 | 3 | 3 | 4 | 3 | 4 |
| *Pseudomonas aeruginosa* | 3 | 2 | 2 | 3 | 2 | 3 |
| *Staphylococcus aureus* | 3 | 2 | 3 | 3 | 2 | 3 |
| *Candida albicans* | 3 | growth | 3 | 3 | growth | 3 |
| *Aspergillus niger* | 3 | growth | 3 | 3 | growth | 3 |

## Conclusion

The results of this study showed that the raw material was in compliance with the quality control standards and the high amount of spinosin was detected by validated HPTLC method in Soxhlet extract. In addition, the isosbestic point occurs at a wavelength of 259 nm that was different from $\gamma_{max}$ 334 nm. The hydrolysis of syrup can be controlled by choosing suitable buffer (phosphate buffe, pH 7). This syrup due to its physicochemical stability, acceptable reduction in spinosin content and preservative free formulation can be considered as a potential medicine for industrial production.

## Acknowledgment

This work was supported by the Research Affairs of the Mashhad University of Medical Sciences as a part of PhD Thesis [grant numbers 922165].

## Conflict of Interest

The authors declare that they have no conflict of interest.

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