



Original Research Article (Experimental)

WHO prescribed shelf life assessment of *Syzygium cumini* extract through chromatographic and biological activity analyses



Jasmeen Kaur, Gulshan Bansal*

Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala, 147002, India

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ABSTRACT

Background: Regulatory guidelines recommend shelf life of herbal products to be established through systematic stability studies.

Objective: The study was designed to establish shelf life of *Syzygium cumini* extract through accelerated and long-term stability testing as per WHO guidelines.

Material and methods: The extract was stored under accelerated (40°C/75 %RH) and long-term (25°C/60 %RH) stability conditions for 6 and 30 months, respectively. Samples were withdrawn at periodic intervals and analysed through two validated HPLC-UV methods (I and II) for fingerprint and quantitative analysis of markers. Antidiabetic activity of control and stability samples was evaluated by α -glucosidase inhibitory model.

Results: Method I generated a well resolved fingerprint of the control sample that was found to contain gallic acid (GA, 1.45 % w/w) and ellagic acid (EA, 3.97 % w/w). The content of GA did not change under both the stability conditions, but that of EA varied insignificantly (3.97–4.77 % w/w) under long-term conditions up to 24 months and subsequently decrease to 3.15 % w/w after 30 months. There was no visible change in LC-UV fingerprint of any stability sample with respect to control. α -Glucosidase inhibitory activity of all stability samples also remained unaltered as compared to control sample (IC₅₀ 1.48 mg/mL). GA and EA did not elicit any activity at the concentrations present in the extract.

Conclusion: Phytochemical composition and antidiabetic efficacy of *S. cumini* extract remain unchanged during its storage under both accelerated and long-term stability conditions, which suggest its shelf life to be 30 months. Also, GA and EA are not appropriate anti-diabetic markers.

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1. Introduction

Antidiabetic herbal drug products form a major part of the herbal drug market. About 800 plant species have been reported to possess antidiabetic properties and are used as antidiabetic remedies in Ayurveda [1,2]. *Gymnema sylvestre*, *Syzygium cumini*, *Trigonella foenum graceum*, *Momordica charantia*, *Panax ginseng*, *Morus alba*, *Withania somnifera*, *Aegle marmelos*, *Ocimum sanctum*, *Azadirachta indica*, *Andrographis paniculata* and *Murraya koenigii* are most widely used plants in traditional system of medicine, and also in antidiabetic herbal drug products. *S. cumini* (Family- Myrtaceae) is amongst the most commonly used herb in different antidiabetic

herbal products available in market. Though all parts of *S. cumini* plant such as leaf, bark, stem and fruit are known to possess antidiabetic activity, and are used in traditional medicines [3–7], but its fruit (both pulp and seed) exhibit maximum antidiabetic activity [8–10]. Various phytoconstituents belonging to categories of glycosides (bergenin), alkaloids, phenolic acids (gallic acid, ellagic acid), tannins, steroids, flavonoids (myricetin, kaempferol, quercetin), triterpenes (acetyl oleanolic acid, betulinic acid, lupeol) and phytosterols (stigmasterol, β -sitosterol) are empirically considered as the constituents responsible for its different biological effects [11–13]. Some reports have attributed its antidiabetic potential partially to the presence of phenolic compounds such as gallic acid, ellagic acid, quercetin, cinnamic acid and ferulic acid [14,15]. The extracts of *S. cumini* containing higher phenolic content exhibit higher antidiabetic activity [14] and its seeds contain higher content of polyphenols than the fruits [16]. Therefore, seeds are the most active part of *S. cumini* for antidiabetic activity. It exhibits its

* Corresponding author.

E-mail: gulshanbansal@rediffmail.com

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antidiabetic activity by blocking the activity of α -glucosidase enzyme [17], which is present in intestinal brush border and is responsible for hydrolyses of di- and oligosaccharides to glucose. Inhibition of the enzyme slows down the process of digestion and absorption of carbohydrates, and thus decreases the post-prandial blood glucose levels in diabetic patients.

A large number of herbal products containing *S. cumini* are available in market. Many of these products are consumed by diabetic patients without any prescription and monitoring especially in developing countries. These products bear a shelf life of 2–3 years, as suggested in Drugs and Cosmetics (Amendment) Rules 2016 in India for various types of herbal formulations, irrespective of their chemical composition [18]. The international drugs regulatory agencies such as World Health Organization (WHO), International Conference on Harmonisation (ICH) and European Medicines Agency (EMA) have laid down various guidelines for establishing quality, safety and efficacy of herbal products through systematic stability studies [19–24]. The WHO guidelines also mention the monitoring of specific biological activity during shelf life assessment. Bansal et al. have highlighted various issues in stability testing of herbal medicinal products, and have proved that the assessment of biological activity during stability testing of a herbal product is as important as that of physico-chemical parameters [25]. However, there is only one report wherein Das has studied the physicochemical changes in *S. cumini* fruit beverages stored at room temperature for 6 months [26]. Till date, there are no reports available on stability testing of *S. cumini* extract that complies with recommended stability testing protocol. Hence, the present study is designed to conduct stability testing on *S. cumini* extract under the accelerated and long-term conditions as recommended in WHO guidelines, in order to establish their shelf life through scientifically generated data, and to evaluate therapeutic efficacy during the proposed shelf life.

2. Experimental

2.1. Material

A hydroalcoholic extract of seeds of *S. cumini* (Batch no. SC/11LOT001, Mfg. Aug 2013) was obtained from Natural Remedies (Bangaluru, India) as a gift sample in July 2014. Acetonitrile (HPLC grade) and potassium dihydrogen phosphate were purchased from Merck Specialist Pvt. Ltd. (Mumbai, India). Methanol (HPLC grade), acetic acid (HPLC grade) and maltose monohydrate were purchased from S.D. Fine-chem Ltd. (Mumbai, India). Gallic acid and formic acid were purchased from Loba Chemie Pvt. Ltd. (Mumbai, India). Ellagic acid was purchased from TCI Chemicals Pvt. Ltd. (Chennai, India).

Rat intestinal acetone powder, acarbose, ferulic acid, sinapic acid and kaempferol were purchased from Sigma–Aldrich (Bangaluru, India). Cinnamic acid was purchased from Fisher Scientific (Mumbai, India). Quercetin was purchased from Otto Chemie Pvt Ltd (Mumbai, India). Glucose level was estimated by using commercially available kit (Erba diagnostics Mannheim GmbH, Germany). Triple distilled water was produced in laboratory for preparation of all the solutions.

2.2. Instruments

The HPLC system consisted of binary pumps (515), photodiode array detector (2998) and Rheodyne manual injector (Waters, Milford, MA, USA). The data was acquired and processed in Empower 3 software (Waters, Milford, MA, USA). The chromatographic analysis of the samples was carried out on Reliant C18 (250 mm \times 4.6 mm, 5 μ) column. The mobile phase was filtered

through nylon membrane (0.45 μ m) using Millipore filter assembly and was degassed using transonic sonicator bath (570/H ELMA, Germany). Stability chamber I (Rolex Scientific Engineers, Ambala Cantt, India) and stability chamber II (AD/SC-20, Allyone, Nasik, India) both capable of controlling temperature and humidity within range of ± 2 °C and ± 5 %RH, respectively were used for long-term and accelerated stability studies, respectively. The spectrophotometric analysis was achieved on UV spectrophotometer (U-2900, Hitachi High Technologies Co-operation Tokyo, Japan). Digital pH meter (Easy five kit, Mettler Toledo AG, Analytical, Schwerzenbach, Switzerland) was used for adjusting the pH of buffer solution.

2.3. Methods

2.3.1. Stability studies

The extract (2 g) sealed in tightly screwed glass vials was placed in stability chamber I maintained at long-term condition (25 ± 2 °C ; 60 ± 5 %RH) as well as in stability chamber II maintained at accelerated condition (40 ± 2 °C; 75 ± 5 %RH) in Aug 2014. The accelerated stability samples were withdrawn after 1, 3 and 6 months, and long-term stability samples after 3, 6, 9, 12, 18, 24 and 30 months of storage as recommended in WHO guideline [27]. The tolerance period for withdrawal of stability sample at each time interval was 48 h. The control (0 month) and all stability samples were stored at -20 °C under nitrogen till analysis and biological evaluations. Each of the stability and control samples was analysed for changes in chromatographic fingerprint using a validated HPLC method. The HPLC methods were also used for determination of content of Gallic acid (GA), ellagic acid (EA), ferulic acid (FA), sinapic acid (SA), cinnamic acid (CA), quercetin (Qtn) and kaempferol (Kmp) taken as markers for the evaluation of chemical stability of the extract.

2.3.2. HPLC method

Solutions of control as well as each stability sample of the extract (2 mg/mL) were prepared in 80% methanol by sonication for 5 min, followed by filtration through 0.45 μ m nylon membrane. Each solution was analysed for the content of GA, EA, SA, CA and FA on a Reliant C18 (250 mm \times 4.6 mm, 5 μ) column. The samples and reference standards were chromatographed by gradient elution with mobile phase composed of acetonitrile (A) - phosphate buffer (pH 2.5, 20 mM) (B) flowing at a rate of 1 mL/min. The solvent gradient used was: 0–5 min, 5% A; 5–20 min, 5–20% A; 20–35 min, 20–40% A; 35–40 min, 40–5% A (method I). The eluent was detected at 270 nm for GA and 257 nm for EA, FA, SA and CA. For quantification of Qtn and Kmp, the samples and references standards were analysed on the same column by chromatographic conditions as reported by Priya et al. [28], wherein the samples were chromatographed with mobile phase composed of water:acetic acid (98:2; % v/v) as solvent A and methanol:acetic acid (98:2; % v/v) as solvent B flowing at a rate of 1 mL/min. The solvent gradient used was 0–15 min, 15% B; 16–20 min, 50% B; 21–35 min, 70% B; 36–50 min, 100% B (method II). The eluent was detected at 365 nm. The injection volume was fixed at 20 μ L in both the methods.

2.3.3. HPLC method validation

The method I was validated by evaluating various parameters such as linearity, LOD, LOQ, accuracy, precision and robustness in accordance with ICH guideline Q2 (R1) [29]. For evaluation of linearity, standard solutions of GA (1–100 μ g/mL) and EA (1–200 μ g/mL) were analysed by the optimized chromatographic conditions in increasing order of their concentrations. Calibration curves were plotted in triplicate to calculate slope, intercept and correlation coefficient. LOD and LOQ of each marker were determined by calibration curve method using equations: $LOD = 3.3 \sigma/S$

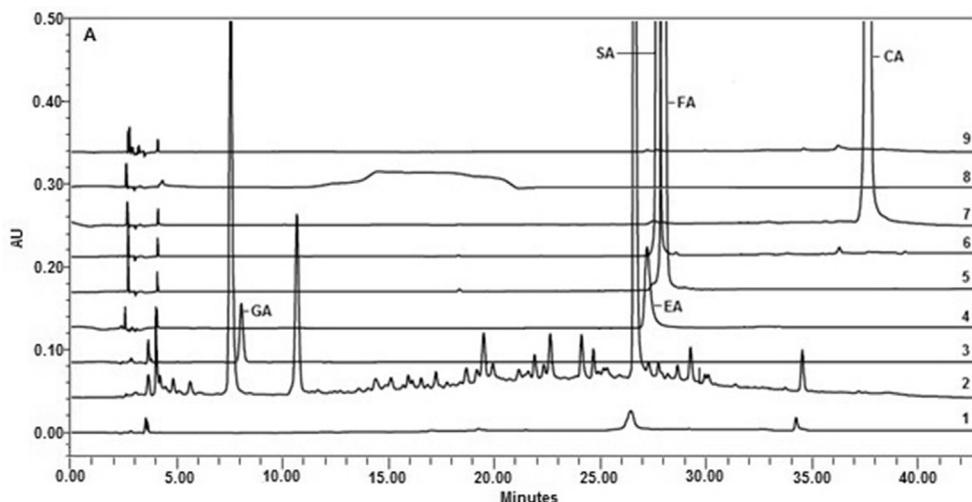


Fig. 1. Overlaid LC-UV chromatograms (A) of blank (1), *S. cumini* extract control (2), GA (3), EA (4), FA (5), SA (6), CA (7), Qtn (8) and Kmp (9) using method I.

and $LOQ = 10 \sigma/S$; where σ and S are standard deviation of slope and mean intercept, respectively determined from respective calibration equation. For the evaluation of accuracy, initially the control sample solution was diluted appropriately so that concentration of GA and EA was $5 \mu\text{g/mL}$. Each of these control sample solutions was mixed with an equal volume of 80% methanol to produce dilute control samples so that the concentration of GA and EA was $2.5 \mu\text{g/mL}$. These dilute control samples were marked as unfortified sample solutions for evaluating accuracy of the method in terms of recovery of GA and EA from fortified control samples. For GA, the same control sample solution was mixed with equal volumes of standard GA solutions ($5, 10,$ and $100 \mu\text{g/mL}$) solutions so that GA concentrations in the control sample solution were fortified by $2.5, 5$ and $50 \mu\text{g/mL}$, respectively. For EA, the fortified samples were prepared similarly as for GA using the same concentrations. Each fortified and unfortified solution for GA and EA was analysed three times and accuracy was expressed as % recovery of GA and EA from fortified solutions vis-à-vis the unfortified solution. The intra-day precision was determined by analysing three different concentrations of GA ($10, 50,$ and $100 \mu\text{g/mL}$) and of EA ($5, 50,$ and $100 \mu\text{g/mL}$) spread over their linearity range on the same day. The inter-day precision was evaluated by analysing the same concentration on three different days. Each concentration was analysed in triplicate,

and precision was expressed as %RSD of the calculated concentration. The robustness of the method was evaluated by making small and deliberate changes in various parameters of optimized chromatographic conditions such as pH of mobile phase (± 0.1), flow rate ($\pm 0.1 \text{ mL/min}$) and column brand. The content of markers and changes in retention time (Rt) were determined vis-a-vis the optimized chromatographic conditions.

2.3.4. α -Glucosidase inhibitory activity

2.3.4.1. Isolation of α -glucosidase enzyme. Rat intestinal acetone powder (200 mg) was suspended in 10 mL phosphate buffer (pH $6.9, 0.1 \text{ M}$), homogenized and centrifuged at 8000 rpm for 25 min at 4°C . The supernatant obtained was used as enzyme solution.

2.3.4.2. Assay method. An assay for α -glucosidase inhibitory activity was initially developed for acarbose (standard) using glucose method as reported by Vogel [30] with some modifications. A mixture of $50 \mu\text{L}$ acarbose solution at varying concentrations ($1\text{--}200 \mu\text{g/mL}$ in water) and $50 \mu\text{L}$ maltose solution (20 mM in phosphate buffer) was incubated at 37°C for 5 min . The enzyme solution ($50 \mu\text{L}$) was added to initiate the reaction and the mixture was incubated at 37°C for 30 min . The reaction was quenched by boiling the solution for 5 min . The solution was brought to room

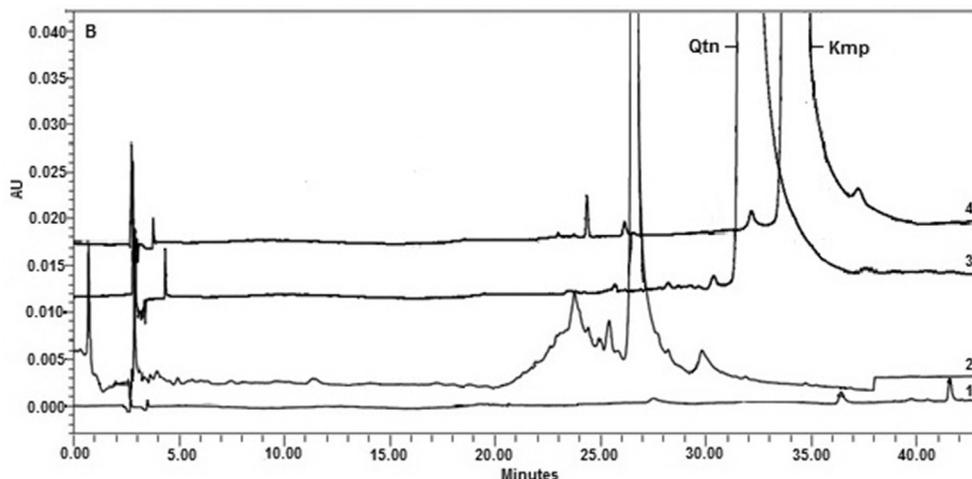


Fig. 2. Overlaid LC-UV chromatograms (B) of blank (1), *S. cumini* extract control (2), Qtn (3) and Kmp (4) using method II.

Table 1
Validation data of HPLC method for gallic acid and ellagic acid.

Validation parameter	Gallic acid			Ellagic acid		
Calibration equation ($y = mx + c$); r^2	$y = 149,012 (\pm 2168.48)x + 264,887 (\pm 3599.26)$; 0.9991			$y = 179,290 (\pm 4726.30)x + 143,116 (\pm 4245.47)$; 0.9977		
Accuracy studies						
Conc. added ($\mu\text{g/mL}$)	5	10	100	5	10	100
Conc. found ($\mu\text{g/mL}$) (Mean; %RSD)	5.10; 0.03	10.87; 0.03	105.81; 0.01	5.02; 0.07	9.66; 0.09	101.34; 0.01
%Recovery	102.09	108.70	105.80	100.30	96.60	101.34
Precision studies						
Actual conc. ($\mu\text{g/mL}$)	10	50	100	5	50	100
Calculated conc. ($\mu\text{g/mL}$)						
Intra-day (Mean; %RSD)	10.31; 0.03	49.07; 0.01	100.17; 0.02	4.91; 0.10	53.01; 0.02	103.86; 0.01
Inter-day (Mean; %RSD)	10.49; 0.04	48.09; 0.03	101.49; 0.01	4.62; 0.12	52.86; 0.01	102.37; 0.01
Robustness	Rt (min)	Content (% w/w)		Rt (min)	Content (% w/w)	
Optimized condition	7.45	1.38		26.92	3.96	
Mobile phase (pH = 2.4)	7.68	1.44		26.75	4.04	
Mobile phase (pH = 2.6)	7.56	1.18		26.69	3.81	
Flow rate (0.9 mL/min)	8.15	1.44		27.59	4.20	
Flow rate (1.1 mL/min)	7.07	1.43		26.03	3.87	
Column Nucleodur	7.32	1.32		26.72	3.91	

temperature, and a 20 μL portion of the solution was mixed with 2 mL of glucose reagent and incubated at 37 °C for 15 min. Absorbance of the solution was read at 490 nm. For blank, acarbose and enzyme solution were replaced with equal volume of water and phosphate buffer, respectively. For control, acarbose was replaced with equal volume of phosphate buffer. The percent inhibition (I %) of α -glucosidase was calculated as follows:

$$I\% = (A_c - A_s)/A_c \times 100$$

where, A_c is the absorbance of control and A_s is the absorbance of standard acarbose.

The same method was found suitable for evaluation of α -glucosidase inhibitory activity of *S. cumini* extract. The activity of control sample of the extract was evaluated over a concentration range of 0.5–5 mg/mL in 80% methanol. IC_{50} values of both acarbose and control samples were calculated using BLeSq software. Solutions of each long-term and accelerated stability sample of the extract were prepared having concentration almost equal to IC_{50} of control sample and evaluated for α -glucosidase inhibitory activity by the method as described above. The activity was also evaluated for GA and EA in the concentration range of 0.05–10 mg/mL and 1–500 $\mu\text{g/mL}$ in 80% methanol, respectively.

3. Results

3.1. HPLC methods

The markers GA, EA, FA, SA and CA were eluted at 7.8, 26.6, 27.9, 27.7 and 37.6 min, respectively by method I (Fig. 1) whereas Qtn and Kmp were eluted at 32 and 34.5 min, respectively (Fig. 2) by method II. The method I was validated for GA and EA. It was linear for GA in the concentration range of 1–100 $\mu\text{g/mL}$ and for EA in the concentration range of 1–200 $\mu\text{g/mL}$, and highly sensitive (LOD 0.03 and 0.11 $\mu\text{g/mL}$, and LOQ 0.08 and 0.33 $\mu\text{g/mL}$, respectively for GA and EA). Good recoveries of GA (102–109 %) and EA (96–102 %) at each fortification level were achieved with RSD less than 2 %. It suggested that the method is sufficiently accurate for the quantification of both GA and EA. No significant variation in the calculated GA and EA concentration was observed on the same day as well as on different days, which proved that the method was sufficiently precise for determining the concentration of GA and EA with RSD less than 2 %. There was no significant change in retention time (Rt) as well as % change in content of GA and EA with deliberate but small changes in pH and flow rate of mobile phase (Table 1) which indicated that the method was sufficiently robust for quantitative analysis of markers in the extract.

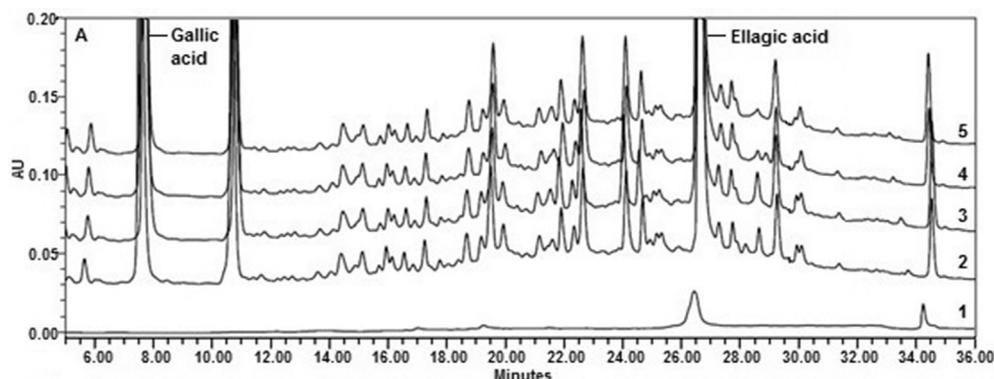


Fig. 3. Overlaid LC-UV chromatograms (A) of control and accelerated stability samples of *S. cumini* extract: blank (1), *S. cumini* extract control (2), stability sample - 1 month (3), 3 months (4) and 6 months (5).

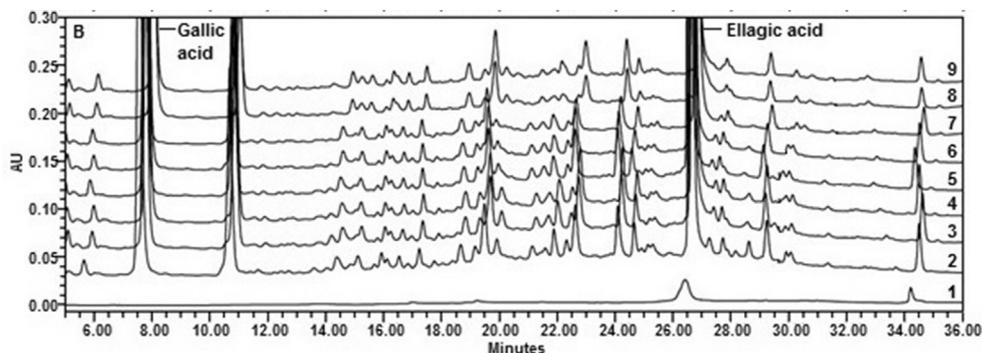


Fig. 4. Overlaid LC-UV chromatograms (B) of control and long-term stability samples of *S. cumini* extract: blank (1), *S. cumini* extract control (2), stability sample - 3 months (3), 6 months (4), 9 months (5), 12 months (6), 18 months (7), 24 months (8) and 30 months (9).

3.2. Stability studies

An optimally better resolved fingerprint of *S. cumini* extract was generated by method I than by method II (Fig. 1 versus Fig. 2). Moreover, Qtn and Kmp were not found in the extract. Hence, method I was used to analyse control and all stability samples of the extract. Comparison of LC-UV chromatograms of control and stability samples through overlay revealed that there was no visible change in LC-UV fingerprint of any of the stability samples with respect to that of the control sample (Figs. 3 and 4). The contents of GA and EA in control sample were found to be 1.45 ± 0.11 and 3.97 ± 0.07 % w/w, respectively. The content of GA in each stability sample was found to remain significantly unchanged (Table 2). However, the content of EA was found to vary insignificantly up to 24 months of storage under long-term condition, and significantly decreased in 30 months sample.

3.3. α -Glucosidase inhibitory activity

The IC_{50} values of acarbose, control sample of extract, and of GA for α -glucosidase inhibition were found to be 3.79 ± 0.56 μ g/mL, 1.48 ± 0.01 mg/mL and 0.75 ± 0.02 mg/mL, respectively (Fig. 5). EA was found inactive against α -glucosidase up to a concentration of 500 μ g/mL. On the basis of IC_{50} value, α -glucosidase inhibitory activity of control and all stability samples of the extract were evaluated at 1.5 mg/mL. It was found that the activity of all stability samples remained statistically similar to that of the control sample (Table 2). The activity of GA was also evaluated at 0.02 mg/mL, the

concentration present in 1.5 mg/mL of the extract. But GA was found to be inactive at this concentration.

4. Discussion

4.1. Chromatographic methods

Initially, attempts were made to develop HPTLC method for efficient monitoring of stability samples in terms of markers contents and fingerprints. However, no concrete and reliable method was developed even up to three months of initiation of stability studies. Thereafter, attempts were made to develop an isocratic HPLC method with inputs from the reported methods [31,32]. However, none the reported method as well as several trials could provide isocratic chromatographic conditions for simultaneous fingerprint and marker analyses. Subsequently, an HPLC method reported by Balyan and Sarkar [33] was employed as lead to obtain fingerprint of the extract. The trials started by running control sample on a C18 column by mobile phase acetonitrile (A) and 0.1% formic acid (B) in gradient mode (0–20 min 5–30 % A, 20–22 min 30–15 % A, 22–24 min 15–10 % A, 24–26 min 10 % A, 26–28 min 10–5 % A, 28–30 min 5 % A). The LC-UV chromatogram showed that all components in the extract eluted as a cluster of peaks within 20 min. In order to resolve the peaks, gradient program was altered to 0–20 min 5–20 % A, 20–30 min 20–40 % A, 30–35 min 40–5 % A. The chromatogram revealed that the peaks in first 7 min get collapsed and others were resolved. To achieve resolution in early phase of elution, acetonitrile proportion was decreased which resulted in resolution of peaks up to 7 min but latter peaks get collapsed. In order to obtain a resolved fingerprint with a balanced resolution of both early and late eluting components, numerous modifications were made in the chromatographic conditions such as replacement of formic acid with phosphate buffer, changing the columns (Nucleodur C18 250 mm \times 4.6 mm, 5 μ ; Chromolith C18 100 mm \times 4.6 mm, 5 μ ; Waters Reliant C18 250 mm \times 4.6 mm, 5 μ), pH of phosphate buffer (pH 2.5, 3 and 4), using different gradients, and different flow rates (1, 1.2 and 1.5 mL/min). An optimally resolved fingerprint of the extract was obtained on Reliant C18 (250 mm \times 4.6 mm, 5 μ) column with mobile phase composed of acetonitrile (A) and phosphate buffer pH 2.5 (B) flowing at a rate of 1 mL/min with gradient programmed as: 0–5 min, 5 % A; 5–20 min, 5–20 % A; 20–35 min, 20–40 % A; 35–40 min, 40–5 % A. This method I, though resolved the markers GA, EA, FA, SA and CA, but did not elute Qtn and Kmp. Therefore a method II, as reported by Priya et al. [27], was employed for elution of Qtn and Kmp. However, a comparison of HPLC-UV chromatograms of Qtn and Kmp with that of the extract (Fig. 2) revealed that these markers were not present in the extract and the components in the extract were

Table 2
Contents of GA and EA in stability samples and α -glucosidase inhibitory activity of the stability samples of *S. cumini* extract.

Sample	Marker concentration (% w/w) (Mean \pm SD)		% Inhibition of α -glucosidase (Mean \pm SD)
	GA	EA	
Control	1.45 \pm 0.11	3.97 \pm 0.07	48.96 \pm 1.65
Accelerated study			
1 month	1.38 \pm 0.04	3.92 \pm 0.09	52.20 \pm 3.15
3 month	1.41 \pm 0.04	4.38 \pm 0.12	57.46 \pm 2.02
6 month	1.48 \pm 0.02	4.41 \pm 0.29	55.63 \pm 3.21
Long-term study			
3 month	1.54 \pm 0.03	4.48 \pm 0.01	46.82 \pm 1.47
6 month	1.53 \pm 0.04	4.77 \pm 0.04	53.08 \pm 3.43
9 month	1.42 \pm 0.11	4.68 \pm 0.05	54.20 \pm 3.27
12 month	1.45 \pm 0.03	4.39 \pm 0.15	51.68 \pm 3.64
18 month	1.53 \pm 0.02	4.54 \pm 0.14	51.63 \pm 3.81
24 month	1.42 \pm 0.02	4.71 \pm 0.38	53.54 \pm 3.56
30 month	1.39 \pm 0.05	3.15 \pm 0.20	53.43 \pm 3.18

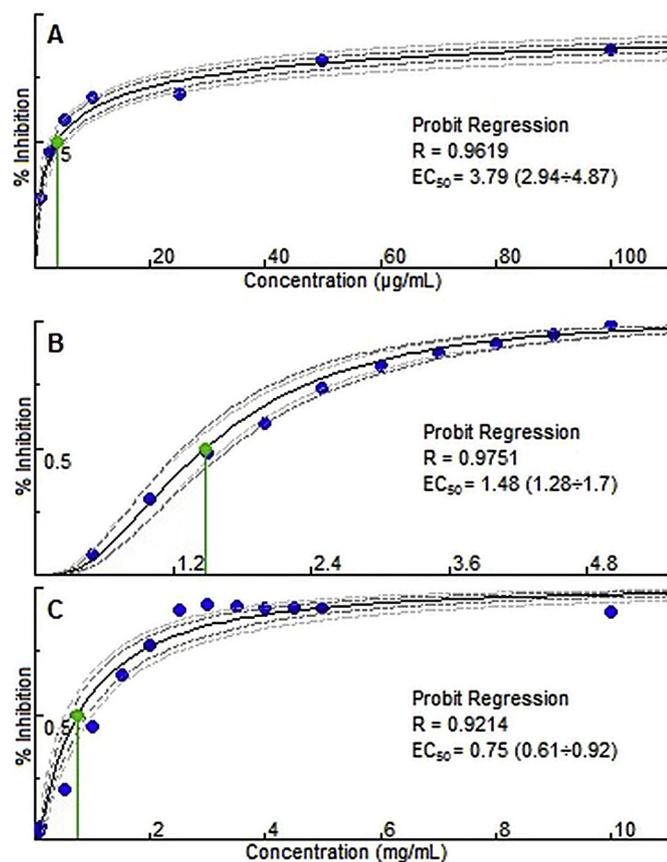


Fig. 5. IC_{50} value determination of acarbose (A), control sample of extract (B) and GA (C) using BLeSq software.

also not resolved. Out of the other markers eluted in method I, only EA was eluted with method II but its peak was not resolved from other constituents of the extract. Because only GA and EA were found present in control sample of the extract, the method I was validated to ensure its reproducibility, accuracy and robustness for quantitative determination of GA and EA.

4.2. Stability studies

The regulatory guidelines recommend the stability studies on drug products in the intended container closure system but selection of containers for testing on active pharmaceutical ingredients (APIs) remains unspecified. In the present study, stability study has been conducted on the extract, which is an API here. Hence, borosilicate glass vials were employed for the study to provide an inert container. The control and stability samples withdrawn at pre-decided intervals were stored at -20°C under nitrogen to prevent any chemical change in the samples till these were used for the analysis and evaluations. The driving forces for storing the samples were the non-availability of (i) reliable chromatographic method to analyse the control and stability samples, and (ii) standardized method for monitoring biological activity of the samples. The stability samples were analysed in one go after completion of the study using method I. Comparison of fingerprints of stability samples with the control sample suggested that the phytochemical composition of the extract remained unaltered during storage under accelerated as well as long-term stability conditions. The contents of GA and EA in the extract has been found to be much lower in comparison to that reported by Balyan and Sarkar [33] i.e., 9.08 and 3.06 % w/w,

respectively. This significant variation in the contents of the markers may be due to biochemical variability which is the inherent attribute of herbal raw material. While GA has been found to remain stable but EA is suggested to undergo some reversible changes due to lactone rings in its structure during the storage.

4.3. α -Glucosidase inhibitory activity

Insignificant difference in the activity of stability samples with respect to control sample has suggested that the antidiabetic activity of the extract remains unchanged during its storage under accelerated condition for 6 months as well as long-term stability condition for 30 months. IC_{50} value of GA is almost two times lower than that of the extract, which indicated that GA may be a reliable marker for stability testing on *S. cumini* seed extract. Therefore, the activity of GA was also evaluated at 0.02 mg/mL, the concentration equivalent to IC_{50} of the extract, to ascertain its suitability as therapeutic marker. However, despite having IC_{50} lower than the extract, GA was found to be inactive at the tested concentration. The findings indicated that both GA and EA are not responsible for the activity of *S. cumini* extract, and hence cannot be taken as therapeutic markers for stability studies of the extract.

5. Conclusion

Accelerated as well as long-term stability studies were conducted on extract of *S. cumini* for 6 months and 30 months, respectively. An HPLC-UV method was developed to quantify various markers in the extract. The same method was also used to develop a fingerprint of the extract. Off the various markers reported in *S. cumini*, only GA and EA were found present in the control sample. The method was validated as per ICH guideline Q2 (R1) and applied to analyse all the stability samples. There was no visible change in the fingerprint of any of the stability sample with respect to control. There was no significant change in content of GA and EA in stability samples. These data suggest that the phytochemical composition of the extract remains unaltered during storage under accelerated and long-term stability conditions. The α -glucosidase inhibitory activity of all stability samples was also found to remain significantly unchanged, with respect to control sample, which suggest that antidiabetic activity of *S. cumini* extract does not change with storage. Both, GA and EA were found inactive at the concentration equivalent to that in the extract, and thus these are not suggested to be responsible for the antidiabetic activity of *S. cumini* extract. These findings preclude their use as therapeutic markers for conducting stability testing of the extract, and indicate towards some unidentified compounds as therapeutic markers of the extract. Further studies involving isolation and characterization of the active compounds in *S. cumini* extract may help to identify therapeutic markers to monitor the stability of the extract as per drugs regulatory guidelines.

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Conflicts of interest

None.

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