Metabolomics and marker-based stability studies of methanol extract of seeds of *Syzygium cumini* L.

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Abstract: Though, herbal medicines are prone to deterioration upon storage due to their complex nature, but less attention has been paid to investigating stability of such products to assign shelf-life. Therefore, the present study aimed to assess the accelerated stability of methanolic extract of seeds of *Syzygium cumini*. The extract was kept at three different storage conditions (30°C/60% RH, 40°C/75% RH and 60°C/85% RH) for a period of 6 months. The samples withdrawn at 0 (before starting the study), 1, 2, 3, 4 and 6 months were analyzed to get UV-Visible metabolomics fingerprints and determine caffeic acid contents using RP-HPLC. The comparison of metabolomics fingerprints indicated that the extract was stable for 1 month at all the three storage conditions. However, caffeic acid contents were found to be intact for a longer period of time. Following the zero order degradation, caffeic acid was predicted to be stable for more than 3 years, if kept at 25°C. The results of the present study indicate that metabolomes of methanol extract of seeds of *Syzygium cumini* change very fast, suggesting the development of stable formulations.

Keywords: Metabolomics, caffeic acid, methanol extract, accelerated stability, *Syzygium cumini.*

INTRODUCTION

Besides other parts, the seeds of *Syzygium cumini* L. (Family: *Myrtaceae*) have been investigated for a number of pharmacological effects including anticonvulsant, antipyretic and antibacterial activities (Chaudhari *et al.*, 1990; Bhuiyan *et al.,* 1996; De Lima *et al*., 1998). We have also investigated different extracts of seeds of the plant, wherein methanol extract has shown good hepatoprotective activity (Islam *et al.,* 2015). Due to pharmacological properties, the seeds have a lot of potential of commercialization and to facilitate their utilization, stability data are needed. To the best of our literature review, such data are not available. Therefore, the present study describes the stability of methanol extract of seeds of the plant for the first time.

Unlike a well-defined modern medicine, specific protocols are required to test the stability parameters of herbal products. Since, the herbal extracts/formulations are a mixture of known as well as unknown constituents, these chemical constituents may react with each other without any perceptible change and raise many stability concerns for the regulatory authorities (Pingale *et al.*, 2008). Moreover, oxidation, hydrolysis and microbial attack may affect the chemical constituents of extracts on environmental exposure during manufacturing process (Rangari, 2008). Despite such facts, it is a common dilemma that manufacturers of herbal medicines, particularly in underdeveloped countries, do not assign shelf-life to finished products scientifically.

Usually, herbal products are presumed to be stable until there is no physical change within the formulation. On the other hand, the demand of the stability data of herbal products is increasing day-by-day as many countries of the world including Pakistan are attempting to regulate the herbal pharmaceutical industry (Hussain *et al.,* 2009). The submission of stability data was made mandatory for registration of every herbal product, with some medicinal claim in Europe (Heinrich, 2007). However, stability studies of herbal products are challenging task due to their chemical variation and complexity, and un-availability of analytical standards/markers and methods.

Nowadays, a herbal product is considered an active medicine as a whole, whether individual components are known or not. Hence, the concept of metabolomics fingerprints of such medicines may be used a tool to evaluate the stability. However, this approach cannot be used to determine chemical kinetics of the extract/product. The kinetics parameters can only be determined using a number of selected chemical constituents that can act as analytical markers. Li *et al.* (2008) described eight categories of markers, which could be used as analytical standards to develop assays. By using such assays, it is possible to generate a sound stability data of herbal products, which may help to

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improve their global acceptability (Kathrin *et al.*, 2003). Keeping these points in view, we decided to use two approaches- UV/Visible metabolomics fingerprint profiling and caffeic acid behavior - for the stability studies of methanol extract of seeds of *S. cumini* L., a plant well-known due to its fruit value and medicinal properties.

MATERIALS AND METHODS

Plant materials

Fully ripe berries of the plant were purchased in the month of May from the local market. After authentication by Prof. Dr. Zaheerud Din Khan, Department of Botany, Government College University, Lahore, Pakistan, a voucher specimen was deposited in herbarium of the university vide reference G. C. Herb. Bot. 853. The fruit pulp was removed and seeds were washed with water and dried in an oven at 50^{o}C. The dried seeds were crushed using a colloidal mill and dried under shade for 4 weeks. Finally, the material was pulverized for extraction.

Extraction

Five hundred grams of powder was extracted sequentially using solvents such as petroleum ether, chloroform and methanol by Soxhlet apparatus. The extracts were filtered and dried in *vacuo* at 40^{o}C.

Instruments

UV/Visible analysis was performed using a double beam UV/Visible spectrophotometer (Shimadzu Corporation, Kayoto, Japan), equipped with UV Probe 2.21 operating software.

HPLC analysis was performed using a liquid chromatography system (1200 series, Agilent Technologies, Waldronn, Germany) equipped with degasser (G1379 A), quaternary pump (G1310 A), auto sampler (G1329 A), column oven (G1316 A) and UV detector (G 1321 A). The data acquisition was performed by LC/MS ChemStation for Windows.

Stability study protocol

Stability of the extract was evaluated as described by ICH (2003) and EMEA (2001). Briefly, transparent screw-caped glass bottles containing the extract were kept in desiccators, containing salt solution of varying strengths to maintain relative humidity (Young, 1967; Greenspan, 1977; Marsh, 1987; ASTM, 1991). These desiccators were then stored at three different temperatures (30°C, 40°C and 60°C) for 6 months. The samples were withdrawn at 0 month (before starting the experiment), 1, 2, 3, 4 and 6 months and analyzed.

Stability using metabolomics

Preparations of samples

Stock solution of the extract having concentration 1.0 mg/mL was prepared in methanol. The working sample solution having concentration 0.1mg/mL was prepared by diluting the stock solution in methanol.

UV/Visible spectroscopy

The working sample solution of the extract was scanned in UV-Visible range (400-200nm) using methanol as a blank. UV-Visible profiles of different samples, withdrawn at different time intervals from the three storage conditions, were compared to observe changes in metabolomics.

Caffeic acid-based stability

Preparation of samples

Samples of methanol extract, taken from different storage conditions, at different time intervals, were dissolved in HPLC grade methanol to get a solution having a final concentration of 5mg/mL.

Preparation of standard solutions

A stock solution of caffeic acid having concentration 1.0 mg/mL was prepared in HPLC grade methanol. Then, working standard solutions of concentration 10.0, 20.0, 40.0, 60.0, 80.0, 100.0 and 120.0µg/mL were prepared by diluting the stock solution with methanol.

Chromatographic conditions

A volume (20µL) was eluted through a column - Eclipse X DB-C_{18} (5µm, 4.6X150cm) - using an isocratic mobile phase comprising water: acetonitrile: acetic acid (79: 20: 1, *V/V/V*) at flow rate of 1.0mL/min. The temperature of the column was maintained at 25^{o}C and detection was carried out using florescent light detector (FLD), operated at 250nm excitation and 410nm emission. The peaks were identified by comparing retention time and caffeic acid contents were determined from the calibration curve, using the linear regression equation.

Determination of kinetics parameters

Graphical method was used to determine the order of the reaction (Murphy, 1997; Pugh, 2002). Briefly, for each storage temperature, graphs were plotted for zero order (% concentration versus time), first order (LnC versus time) and second order (reciprocal of concentration versus time). The correlation coefficients of all the three graphs were compared. The plot having comparatively better linearity was considered as the order of reaction. The slope of the same plot was selected as a rate constant (K) at each storage temperature. Then, LnK values corresponding to storage temperatures were plotted against reciprocal of temperature in Kelvin (1/T). The linear regression equation obtained from this graph was used for the determination of K at 25^{o}C. Activation energy (Ea) and frequency constant (A) were determined from the slope (slope = - Ea /R, R=universal gas constant, 8.314 J. mole^{-1}. k^{-1}) and intercept, respectively. Shelf-Life (t_{90}) depends on the order of the reaction and can be calculated using different equations.

STATISTICAL ANALYSIS

The samples were analyzed in triplicates and the results were presented as mean ± SD.

RESULTS

Stability by metabolomics fingerprints

The comparison of UV-Visible metabolomics profiles of the extract, taken at different time intervals, from a sample stored at 30°C/65% RH is presented in fig. 1. These results showed that the metabolomics profiles of the extracts taken at 0 and 1 month were similar, which indicated that the extract was stable for one month. On the other hand, profiles taken at 2 and 3 months indicated a different pattern; absorbance at 370, 325, and 210nm. This changed profile indicated the changes in chemical composition that occurred in the extract. The profile taken at 4 months was further found to be changed; shift of peaks to 260 and 230nm. The profile of the sample taken after 6 months showed a significantly different profile. These results indicated that methanol extract was not stable at room temperature after 1 month.

Metabolomics profiles of the extract taken from the storage condition, 40°C/75% RH, at different intervals are given in fig. 2. The pattern observed in this case was found to be similar till 1 month; thereafter an increase in peak height and absorbance intensity took place. The profiles obtained after 2, 3, 4 and 6 months showed gradual changes in metabolomics of the extract. Higher peak height indicated that changes took place faster at higher temperature.

The comparison of metabolomics profiles of the extract taken at different intervals from a sample stored at 60°C/85% RH is given in fig. 3. These results also showed a similar pattern to that stated earlier. However, metabolomics profiles were found to be changing much faster in terms of peak height/absorbance intensity at higher temperature.

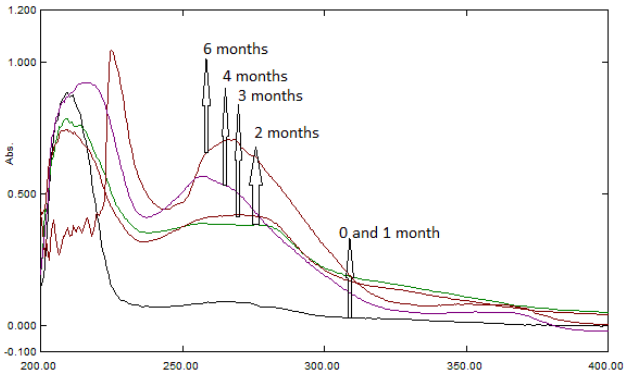

Fig. 1: The overlay UV-Visible metabolomics profiles of methanol extract of seed of *Syzygium cumini* stored at 30°C/65% RH for six months, RH (relative humidity)

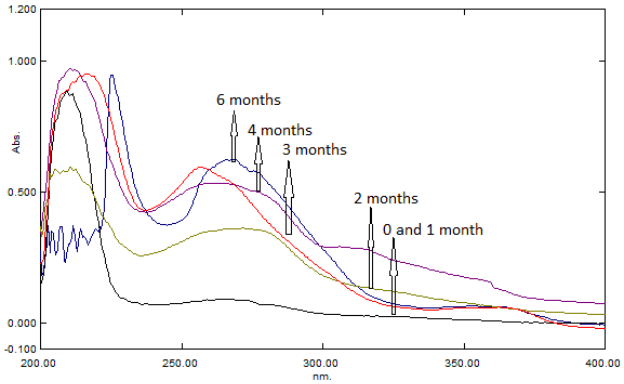

Fig. 2: The overlay UV-Visible metabolomics profiles of methanol extract of *Syzygium cumini* seeds stored at 40°C/75% RH for six months, RH (relative humidity)

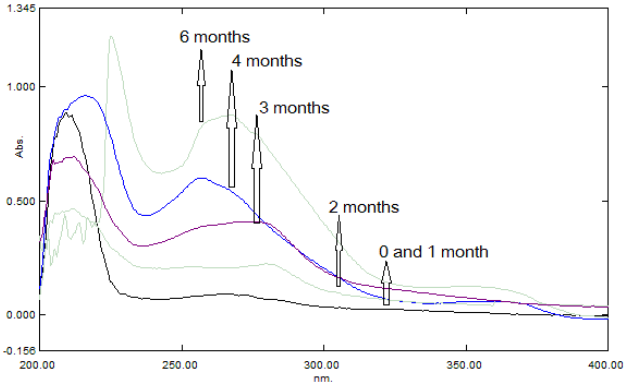

Fig. 3: The overlay UV-Visible metabolomics profiles of methanol extract of seeds *Syzygium cumini* stored at 60°C/85% RH for six months, RH (relative humidity)

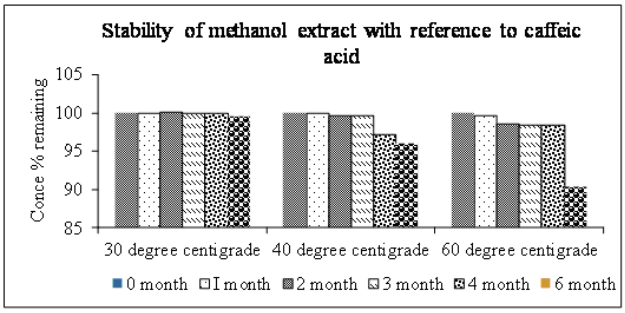

Fig. 4: Stability of methanol extract of seeds of *Syzygium cumini* with reference to caffeic acid stored at 30 °C/65% RH, 40°C/75% and 60 °C/85% RH

Caffeic acid-based stability

The samples taken at different time intervals from all the three storage conditions were analyzed by a HPLC for the determination of caffeic acid. The calibration curve was constructed at each time of the analysis and caffeic acid was quantified from the linear regression equation.

Percentage remaining of caffeic acid

The change of percentage remaining of caffeic acid in methanol extract, kept at different storage conditions, with the passage of time, is shown given in fig. 4. These

results showed that the decomposition of caffeic acid was slower at 30°C and 40°C as compared to that of the 60°C. The samples stored at 30°C (65% RH) and 40°C (75% RH) were stable for 6 months because the decrease in concentration was lower than 10%. On the other hand, the extract was not stable at 60°C/85% RH, wherein a decrease in concentration was greater than 10% in 6 months. The chromatograms of the samples stored for 6 months at different storage conditions are given in fig. 5. These results suggest a gradual decrease in the peak height of the caffeic acid.

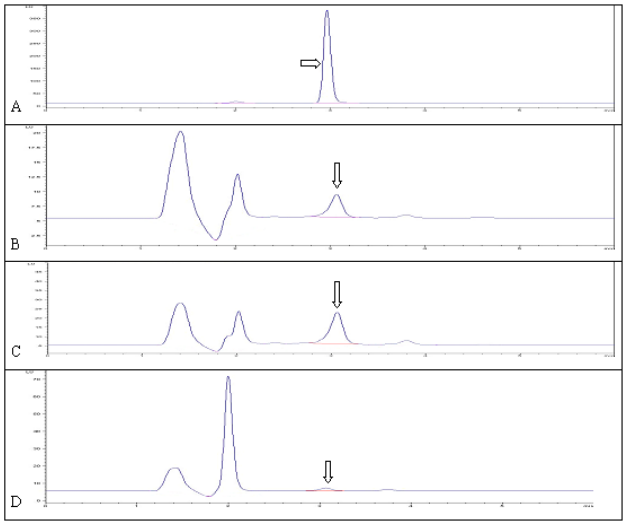

Fig. 5: Chromatograms of caffeic acid and methanol extract of seeds of *Syzygium cumini* after 6 months storage, A (caffeic acid); B (30°C); C (40°C); D (60°C); Caffeic acid (⇨)

Kinetic parameters

The plots for the determination of the order of reaction of caffeic acid in methanol extract are shown in fig. 6. These results showed that caffeic acid in the extract followed the zero order degradation. The slopes of the graph corresponding to the zero order reaction were taken as rate constants (K) for different temperatures.

Shelf-life (t_{90})

Based on the rate constants of caffeic acid in methanol extract, the shelf-life was calculated dividing 0.105 with K values. The estimated shelf-life at various storage conditions is presented in table 1. These results showed that stability was lesser at higher temperature. Furthermore, to predict the shelf-life at 25°C, K at this temperature was calculated from the linear regression equation, obtained from the graph of LnK versus 1/T (fig. 6). The calculated kinetic parameters of caffeic acid in the extract are given in table 3. These results showed that rate constants, activation energy and pre-exponential factors increased with the rise in temperature.

DISCUSSION

Stability is influenced by a number of physical and chemical factors. The temperature, moisture and light may cause hydrolysis, oxidation, polymerization and isomerisation. Moreover, temperature increases kinetic energy which in turn increases collision of molecules. Moisture is particularly important to drugs which are susceptible to hydrolysis (Waterman *et al.,* 2002). Moisture can also facilitate microbial growth which deteriorates constituents along with production of toxic chemicals. Drugs instability due to oxidation is nearly equivalent to that of hydrolysis. The temperature and light enhance the rate of oxidation. In the present study, it was noted that caffeic acid contents decreased when the products were stored at elevated temperature and higher relative humidity.

Stability testing indicates a pattern of drug degradation due to environmental factors with the passage of time (WHO, 1996). Stability testing of a product needs to be conducted for a period that corresponds to the actual storage time and temperature (Hussain *et al.,* 2011). Usually drugs are stored at room temperature, if not specified in individual monographs, and degradation is slow at this storage condition, hence, shelf-life may go up to several years. Stability testing for such a long period is time consuming and expensive. Therefore, stability studies are conducted at elevated temperatures to predict long term stability within a short time. Owing to this reason, the stability of methanol extract was investigated at accelerated conditions.

In the present study UV-Visible profiles of the sample were changed, which indicated that chemical changes too place in the extract. Therefore, extracts of the plant need to be stabilized before marketing.

As far as caffeic acid is concerned, its slow degradation at 30°C and 40°C, noted in the present study was found to be consistent with the previously reported work that indicated that decomposition increased with rise in temperature (Pourrat *et al.,* 1995; Hussain *et al.,* 2011). Pugh (2002) described that by 10°C rise in temperature resulted in 2-3 folds increase in the rate of reaction.

Table 1: Rate constant (K), activation energy (Ea) and pre exponential factor (A) of methanol extract of *Syzygium cumini* L. with reference to caffeic acid at different temperatures

Caffeic acid	K25°C	K30°C	K40°C	K60°C	Ea (J mol^{-1})25°C	A (S^{-1})
Methanol extract	0.0025	0.0032	0.0272	0.0478	69446	3.11

CONCLUSION

UV-Visible metabolomics studies indicated that extract of seed of Syzygium *cumini* L. changes chemically very fast and remains stable for one month at all the three selected storage conditions. These fast chemical changes suggest to work for the preparation of stable formulations. However, based on stability of the caffeic acid the extract is predicted to be stable for 42 months, if kept at 25°C.

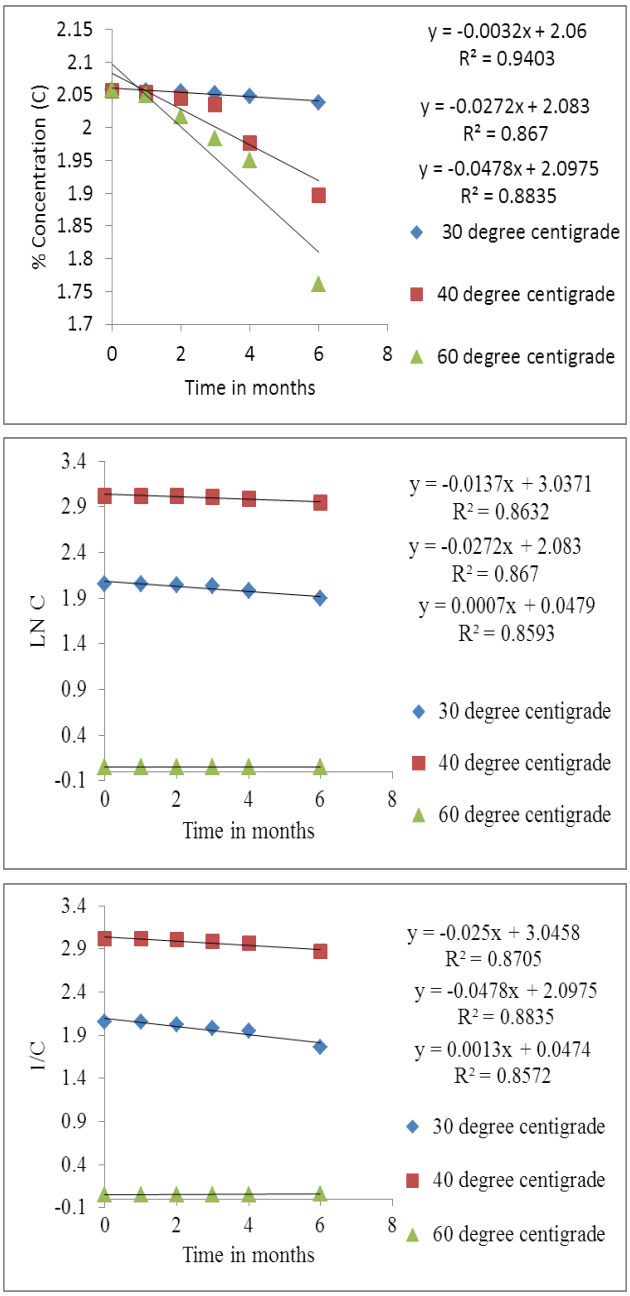

Fig. 6: Plots for the determination of order of the reaction and rate constant (K) of methanol extract with reference to caffeic acid, C (Concentration), Ln (Natural logarithm).

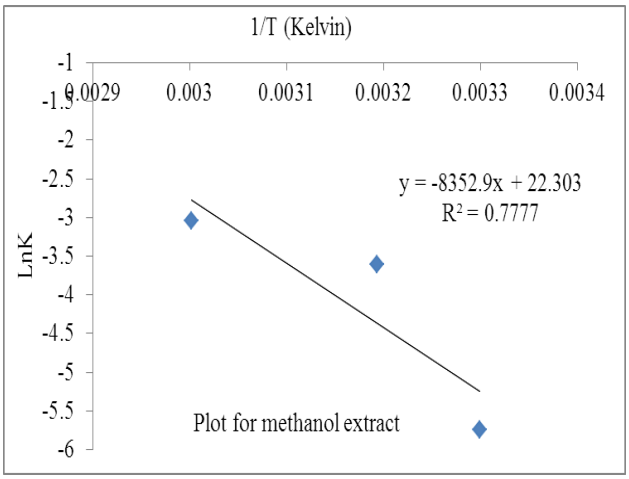

Fig. 7: Plots of LnK and 1/T of methanol extract of seeds of *Syzygium cumini* L. for the prediction of rate constant, activation energy and pre-exponential factor, K (rate constant); Ln (Natural logarithm).

REFERENCES

ASTM (1991). Annual Book of ASTM Standards: Designation. American Society for Testing and Materials. Standard practice for maintaining constant relative humidity by means of aqueous solution, Philadelphia, USA, pp.E104-185.

Bhuiyan MSA, Mia MY and Rashid MA (1996). Antibacterial principles of the seeds of *Eugenia jambolana*. *Bangladesh J. Bot.*, **25**: 239-241.

Chaudhari AKN, Pal S, Gomes A and Bhatacharya S (1990). Anti-inflammatory and related actions of *Syzygium cumini* seed extract. *Phytother. Res.*, **4**: 5-10.

De Lima TCM, Klueger PA, Pereira PA, Macedo-Neto WP and Morato GS (1998). Behavioural effects of crude and semi purified extracts of *Syzygium cumini* Linn Skeels. *Phytother. Res.*, **12**: 488-493.

EAEMP (2001). Final Proposals for Revision of the Notes for Guidance on Quality of Herbal Medicinal Products. London, pp.1-8.

Greenspan L (1977). Humidity fixed points of binary saturated aqueous solutions. *J. Res. Natl. Bur. Stand.*, **81**: 89-96.

Heinrich M (2007). Ethnopharmacy and natural product research - Multidisciplinary opportunities for research in the metabolomic age. *Phytochem. Lett.*, **1**: 1-5.

Hussain K, Ismail Z, Sadikun A and Ibrahim P (2011). Accelerated stability and chemical kinetics of ethanol extracts of fruit of *Piper sarmentosum* using high performance liquid chromatography. *Iranian J. Pharm. Res.*, **10**: 403-413.

Hussain K, Ismail Z, Sadikun A and Ibrahim P (2009). Evaluation of extracts of *Piper sarmentosum* for accelerated stability by metabolomic fingerprint profiling. *Phcog. Res.*, **1**: 185-191.

ICH (2003). Stability Testing Guidelines: Stability Testing of New Drug Substances and Products. Geneva, Switzerland, pp.1-16.

Kathrin K, Eike R and Anne B (2003). Validation of standardized high performance thin layer chromatographic methods for quality and stability testing of medicines. *J. AOAC Int.*, **86**: 909-915.

Li S, Han Q, Qiao C, Song J, Cheng CL and Xu H (2008). Chemical markers for the quality control of herbal medicines. *Chinese Med.*, **3**: 1-18.

Murphy B, Murphy C and Hathaway BJA (1997). Working Method Approach for Introductory Physical Chemistry Calculations. Royal Society of Chemistry, Cambridge, pp.113-127.

Marsh KN (1987). Recommended reference materials for the realization of physicochemical properties. (Eds.), Blackwell Scientific Publications, Oxford, pp.59-60.

Pingale SS, Pokharkar RD and Pingale MS (2008). Stability study of a herbal drug. *Pharmacology online.*, **1**: 20-23.

Pourrat H, Barthomeuf C, Pourrat A, Cottier PE and Ibrahim H (1995). Stabilization of octastatin, a somatostatin analogue. Preparation of freeze dried products for parenteral injection. *Biol. Pharm. Bull.*, **18**: 766-771.

Pugh J (2002). Kinetics and Product Stability. *In*: Aulton ME (editor). Textbook of Pharmaceutics, the Science of Dosage Form Design. 2nd ed. Churchill Livingstone, London, pp.101-112.

Rangari VD (2008). Pharmacognosy and Phytochemistry. Career Publications, 2nd ed, pp.78-100.

Thakur AK, Prasad NA and Laddha KS (2008). Stability testing of herbal products. *Pharma Review.*, **4**: 109-112.

WHO (1996). Expert Committee on Specifications for Pharmaceutical Preparation Annex 5- Guidelines for Stability Testing of Pharmaceutical Products Containing Well-Established Drug Substances in Conventional Dosage Forms. WHO Technical Report, Series No. 863, Geneva, Switzerland, pp.65-79.

Waterman KC, Adami RC, Asanate KM, Antipas AS, Arenson DR, Carrier R, Hong J, Lndis MS, Lombardo F, Shah JC, Shalev E, Smith SW and Wang H (2002). Hydrolysis in pharmaceutical formulation. *Pharm. Dev. Technol.*, **7**: 1113-1146.

Young JF (1967). Humidity control in the laboratory using salt solutions: A review. *J. Appl. Chem.*, **17**: 241-245.