Pharmacognosy Research [Phcog Res.]
Vol 1, Issue 4, Jul-Aug, 2009 Page 219-225
(An Official Publication of Pharmacognosy Network Worldwide)
Received: 14 April, 2009
Modified: 02 June, 2009
Accepted: 03 June, 2009

PHCOG RES.: Research Article

Evaluation of extracts of *Piper sarmentosum* for accelerated stability by metabolomic fingerprint profiling

Hussain K*, Ismail Z, Sadikun A, Ibrahim, P.

School of Pharmaceutical Science, Universiti Sains Malaysia, 11800, Penang, Malaysia.

Corresponding author: *Department of Pharmaceutical Chemistry, Universiti Sains Malaysia, 11800, Penang, Malaysia. Phone and Fax +6046563443, email: hussain_761@yahoo.com*

ABSTRACT

Unlike pharmaceuticals, precise stability assessment of herbal products is challenging because of their complex nature. A new trend in natural products is that the extract is considered active as a whole whether active constituents are known or not. Hence, the stability of all the constituents must be taken into account, which is possible by analyzing metabolomic fingerprint profiles. Therefore, present study aimed to evaluate ethanol extracts of fruit of *Piper sarmentosum,* an important medicinal plant, for accelerated stability using metabolomic fingerprint profiling. The extract was exposed to three storage conditions of different temperature and humidity and analyzed at 0, 1, 2, 4 and 6 months by Fourier transform infrared (FTIR) spectroscopy and high performance thin layer chromatography (HPTLC) to get metabolomic fingerprints. FTIR fingerprints in combination with chemometrics indicated the changes in metabolomics, stirring with the passage of time at all storage conditions. Visual inspection of HPTLC densitograms revealed metabolomic changes in the extracts stored for 6 months at 60 °C and 85% relative humidity. The results of the study indicate that the products made from this plant ought to be stored at room temperature, below 30 °C and 45% relative humidity, and excessive heating must be avoided during manufacturing process. Moreover, the method may be used by natural product industry as a tool of identification, classification and discrimination (ICD).

Keywords: *Piper sarmentosum; Piperaceae*; Stability; Metabolomic fingerprint profiling

INTRODUCTION

Stability is a time during which a drug retains its chemical integrity and labeled potency within the specified limits. The stability of a pharmaceutical preparation is its degree of resistance to chemical and physical changes. A product must be consistent in efficacy and claimed potency or may possibly change only within the limits specified by legal provisions until expiry date (1). Stability studies provide information about storage conditions where product is intended to retain its efficacy within specified limits. Moreover, it is the most vital factor which determines whether a compound or a mixture of compounds can be developed into a pharmaceutical product. The stability is affected by physical factors such as temperature, moisture and light, and chemical factors such as hydrolysis, oxidation, isomerization and polymerization etc. The stability testing involves the examining of quality and potency of a product at suitable time intervals is conducted for a period corresponding to the normal time that the product is likely to remain in stock or in use. Degradation is usually slow at room temperature and shelf life may go up to several years. Since the period can be as long as two years, stability testing for this period will be time consuming and expensive. Therefore, accelerated stability testing is devised, which enables a rapid prediction of long term stability of a product.

Stability studies of natural products are difficult as compared to pharmaceuticals because these are a complex mixture of various types of unlimited constituents. Different types of strategies such as marker compounds and metabolomic fingerprint profiling may be applied to assess the stability of natural products (2, 3). In the former only marker compound(s) is considered ignoring other constituents, which may synergize the activity or buffer side effects, while the later includes inimitable patterns designating the presence of particular molecule(s), based on specialized analytical techniques, which can be used to identify active components, contaminants or other chemicals present in extracts (4). Therefore, in present study, we used FTIR spectroscopy and HPTLC to get fingerprints for the evaluation of stability of extract of an important medicinal plant, *Piper sarmentosum*.

Piper sarmentosum Roxb. (*Piperaceae)* is cultivated or found wild under shady trees in South East Asian region and is well recognized due to culinary and medicinal properties. As a traditional medicine, the extracts of different parts of the plant are being used to cure a number of ailments (5, 6, 7, 8). The plant has also been investigated for a number of pharmacological activities such as anti-amoebic (9), antibacterial (10), anti-neoplastic (7), neuromuscular blocking (11), hypoglycemic (12), anti-malarial (13), antioxidant (14, 15, 16), anti-TB (17) and antiangiogenic (18). Due to these pharmacological properties, nowadays different products are being manufactured from the plant and marketed without any stability information.

Therefore, present study was undertaken to investigate the extracts of the plant for accelerated stability using metabolomic fingerprint profiling, which may provide beneficial information to natural product industry about the storage of raw materials and finished products of the plant for safe delivery of product to the consumer. Moreover, to see whether the method can be applied as an effective tool of identification, classification and discrimination (ICD)?

MATERIALS AND METHODS

Plant material and extraction

The fruit of the plant was collected from the Botanical Garden of the School of Pharmaceutical Sciences, Universiti Sains Malaysia and authenticated by Prof. Dr. Zhari Ismail, Herbal Secretariat, School of Pharmaceutical Sciences of the university where a voucher specimen was deposited vide reference No. 0071/06. The fruit was cleaned, sliced into small pieces, dried at 40 °C and pulverized. The pulverized material (50 g) was extracted twice with 300 ml ethanol by reflux for 1 h. The extract was filtered and dried in vacuo at 40 °C.

Stability study protocol

Study protocol of the International Conference on Harmonization (ICH) as suggested by the Working Party of Herbal Medicinal Products (WPHMP) of the European Agency for the Evaluation of Medicinal Products (19, 20) was applied. The extracts were kept in screw caped transparent glass bottles and exposed to three different storage conditions of temperature and relative humidity such as 30 °C/60% RH, 40 °C/75% RH, 60 °C/85% RH, for 6 months. The humidity was controlled by saturated salt solution (21, 22, 23, 24).

Instruments - FTIR spectra were recorded using FTIR spectrometer (Thermo Nicolet, USA) equipped with software OMNIC version 6.0 a.

HPTLC analysis was performed on system comprising of densitometer (CAMAG Model-3 TLC scanner) equipped with winCATS 4 software and semi- automatic sampler (Linomat-5). The documentation was performed at 254 or 366 nm using CAMAG PROSTER 3 (CAMAG, Berlin, Germany).

Analysis of extracts using FTIR spectroscopy and principal component analysis

The potassium bromide (KBr) disk technique was used for sample preparation as 2 mg sample was mixed with 100 mg KBr and grounded as fine powder. The mixture was placed in a small mold and pressed for 5 min to make disk. Then the disk was removed and placed in a sample holder and FTIR spectrum was recorded in the mid-IR region 4000-400 cm^{-1} at resolution 4 cm^{-1} and 16 scans. The samples stored at different storage conditions were analyzed in triplicate and the spectra were analyzed by principal component analysis (PCA) using PerkinElmer software (Spectrum QUANT + v4.51).

Analysis of extracts using HPTLC

The samples (2 mg/ml in methanol) were applied on silica gel $60F_{254}$ TLC plates (10 x 20 cm; 0.25 mm layer thickness; Merck) by Linomat 5 in duplicate as 2 µl/ application, band length 6 mm, 10 mm from x-axis and 8 mm from y-axis. The plate was developed up to 8 cm in saturated horizontal chamber with solvent system comprising of toluene : ethyl acetate (1 : 1 v/v). The plate was dried with gentle warm air and scanned at 260 nm using densitometer equipped with winCATS 4 software.

Statistical analysis

FTIR spectra were analyzed by principal component analysis (PCA) and results were compared with respect to principal component (PC) 1 and 2.

RESULTS

The FTIR spectra obtained at 0, 1, 2, 4 and 6 months from the extracts exposed to different storage conditions were compared. It was found that the spectra of the samples stored at 30 °C/45% RH and 40 °C/75% RH for 6 months were relatively similar indicating the integrity of constituents. The FTIR profiles of the samples stored at 60 °C/85% RH and analyzed at different time intervals (Figure 1) clearly indicated the changes in metabolomics due to high temperature and humidity. The FTIR profiles of the samples taken at 4 and 6 months from the storage condition, 60 °C/85% RH, varied significantly from those taken at 0, 1 and 2 months indicating that metabolomic changes started after 2 months.

Besides visual comparison, FTIR metabolomic fingerprint profiles were analyzed by principal component analysis, a multi variant technique, to compare the profiles in fingerprint region (1800-800 cm^{-1}) using PerkinElmer software. The results of PCA (Figure 2) indicated the differentiation of samples into three main groups. This differentiation designated the changes in metabolomics, stirring at all storage conditions with the passage of time.

HPTLC analysis in stability testing based on visual comparison of densitogram fingerprints in which minor changes can not be noticed but major changes can be observed was used (25). Densitograms of the samples stored at specific condition were recorded at different time intervals at 260 nm and compared to observe the effects of different storage conditions with the passage of time. Three dimensional comparisons of densitigram profiles of different samples (Figure 3) indicated the changes in metabolomics at high temperature and relative humidity. One dimensional dentistogram fingerprint profiles of the samples stored at 60 °C/ 85% RH at different time intervals (Figure 4) indicated significant changes, which took place with the passage of time. These results indicated that significant chemical changes took place in extracts stored at 60 °C/ 85% RH in 6 months.

DISCUSSION

Stability studies provide evidences on how the quality of a drug substance varies with the passage of time under the influence of environmental factors (26). Stability studies before developing a dosage form are the first quantitative assessment of chemical constancy of a product. These studies are also useful to recommend storage conditions and predict shelf life of medicinal products.

Temperature enhances the rate of degradation of ingredients due to increase in kinetic energy resulting

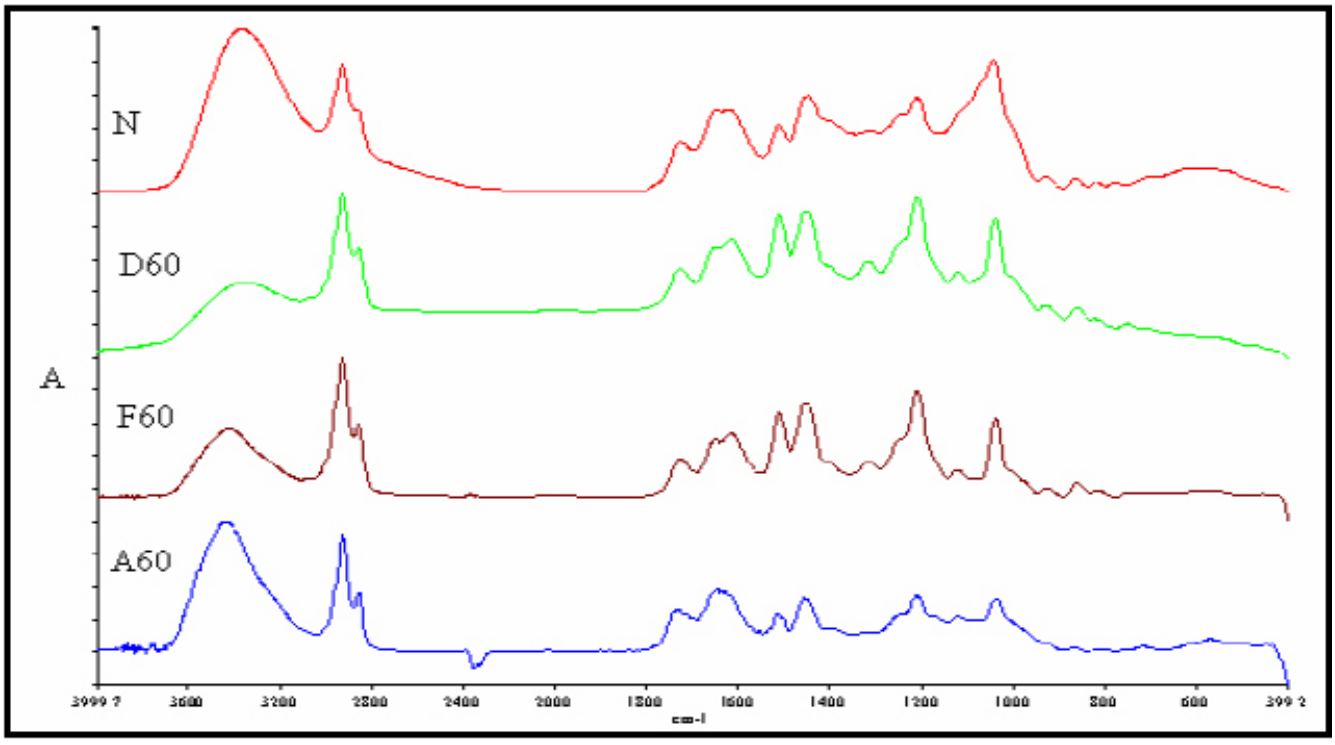

Figure 1. FTIR spectra of fruit ethanol extract of *Piper sarmentosum* stored at 60 °C/85% RH, N (0 month), D60 (1 month), F60 (2 months) and A60 (6 months)

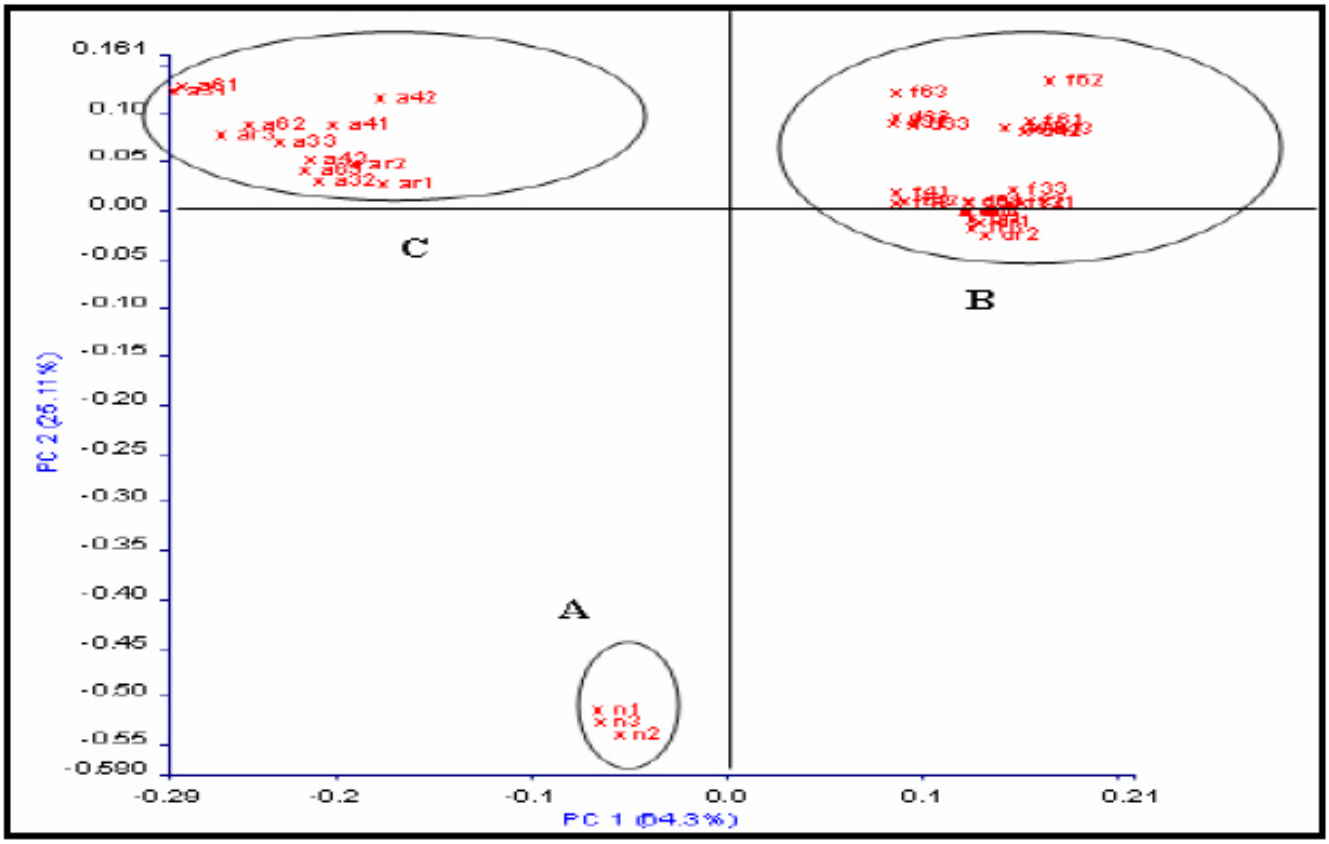

Figure 2. Analysis of FTIR fingerprints of fruit ethanol extracts of *Piper sarmentosum* stored at different conditions by principal component analysis, A (0 months), B (1 and 2 months); C (4 and 6 months)

raise in the fraction of colliding molecules with sufficient energy to cross the energy barrier, activation energy . Moisture content amplifies the rate of decomposition and makes the product susceptible to hydrolysis (27). In case of herbal crude powders or extracts, it also facilitates the growth of microbes, which not only deteriorate the constituents but may produce toxic substances. Hence, moisture content should be controlled within the formulation and in storage environment. Decomposition of pharmaceutical preparation due to oxidation is nearly as probable as that with hydrolysis and the rate of oxidation is temperature dependent. For example peroxidation of fatty acids, break down of fatty acids into aldehydes and ketones, accelerates as the temperatures exceeds 50 °C (28). Polymerizations, addition of similar molecules, and isomerisation, change in isomeric forms, are additional factors affecting the stability. There are many reports about the effect of sunlight on the stability of pharmaceuticals (29), which being a form of energy can initiate and accelerates decomposition. Exposure to sunlight is particularly important in extracts containing photolabile and volatile constituents (28, 30).

Metabolomics, a system of cell biology, comprise of all the compounds other than proteins, DNA and RNA. Metabolomics, detect, quantify and catalogue the time related metabolic processes of an integrated biological system under specified conditions (31). Meatbolomic fingerprints can be obtained using various analytical techniques. In present study, we used FTIR spectroscopy and HPTLC to get fingerprint profiles for stability assessment of extracts. FTIR spectroscopy is a useful non-destructive and convenient technique which offers to collect data in variety of states such as on solid, liquid and gas. Though, the coexistence of different chemical constituents usually causes the overlap of spectra, which make the qualitative and quantitative analysis difficult. Nevertheless, FTIR spectra in combination with chemomatrics, a multivariate statistical technique, proved to be a powerful tool of identification, classification and discrimination.

HPTLC is another important tool to obtain qualitative information about the constituents of herbal preparations by densitogram fingerprint evaluation. HPTLC allows the analysis of complex herbal products efficiently and

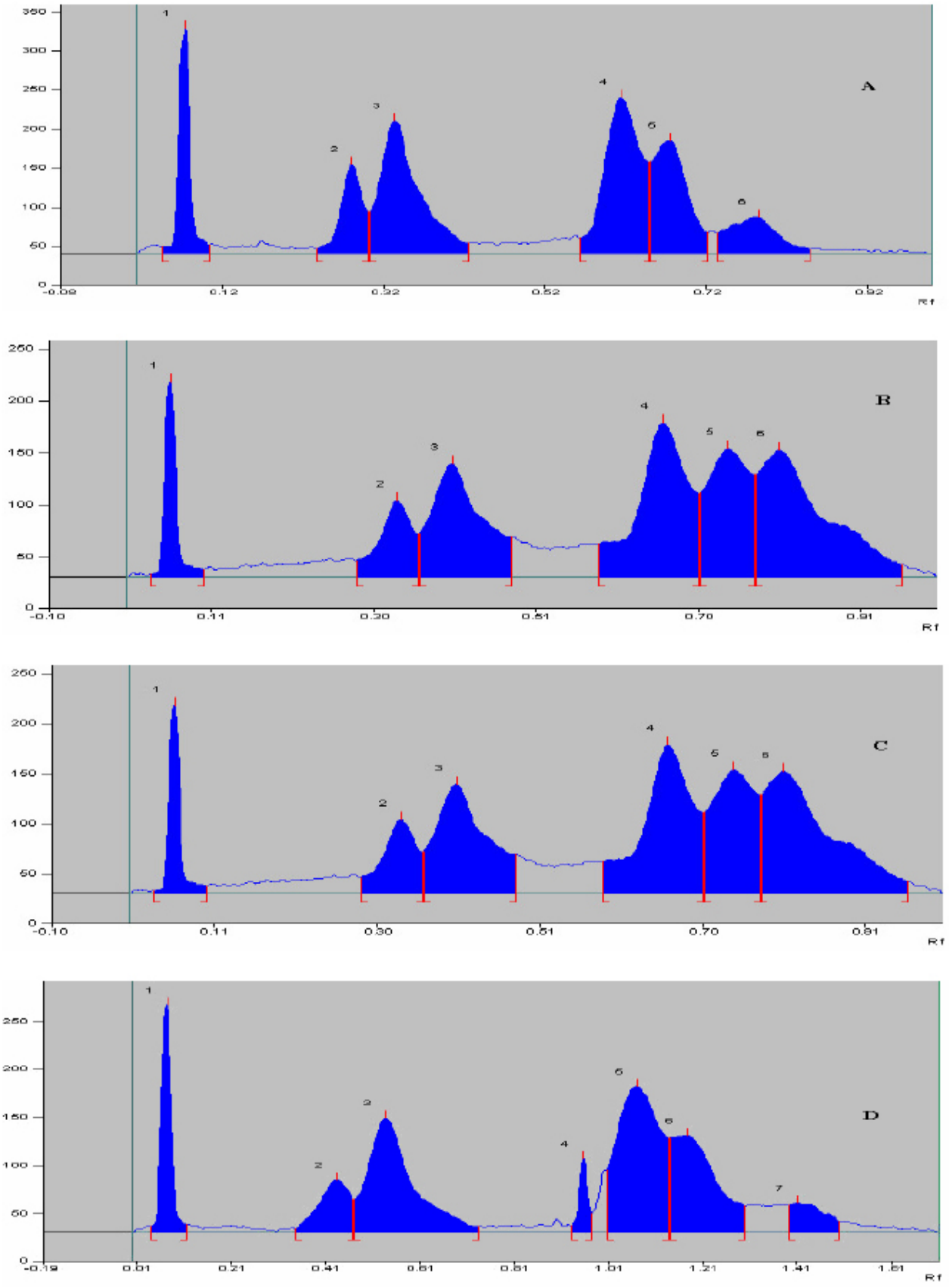

Figure 3. HPTLC 1D densitograms of fruit ethanol extracts of *Piper sarmentosum* stored at 60 °C/85% RH at different intervals, A (0 time), B (1 month), C (2 months), D (6 months)

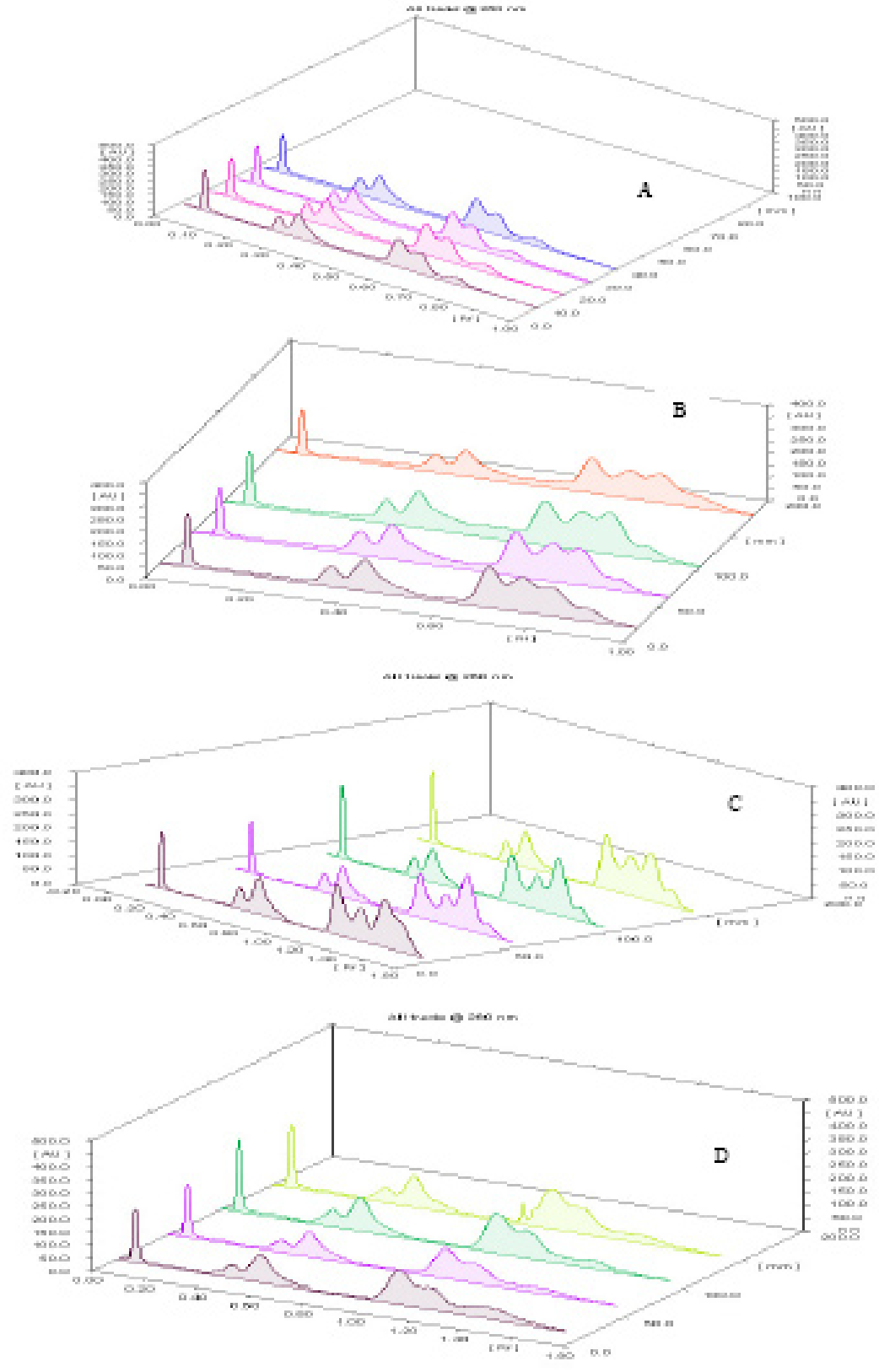

Figure 4. Three dimensional densitogram profiles of fruit ethanol extracts of *Piper sarmentosum* exposed to different storage conditions for six months, A (0 month), B (1 month), C (2 months), D (6 months), track 1 and 2 (30 °C/45% RH), track 3 (40 °C/75% RH) and track 4 (60 °C/85% RH)

many samples can be applied on a single plate to facilitate comparison.

It is evident from the results of the study that the suitable storage condition of extracts or products prepared from *Piper sarmentosum* is below 30 °C and 45% relative humidity. Exposure to high temperature and humidity deteriorates the constituents of the extracts, which is more pronounced at 60 °C. Moreover, the natural product manufacturer may use metabolomic fingerprints for the assessment of quality and stability of herbal products.

ACKNOWLEDGMENTS

Corresponding author would like to acknowledgment with thanks the Govt. of Malaysia for providing scholarship under Commonwealth Scholarship and Fellowship Plan and authorities of the University of the Punjab, Lahore, Pakistan for granting study leave.

REFERENCES

1. Racz I. *Drug Formulation*. New York: John Wiley and Sons. (1989).
2. Hussain K., Majeed M.T., Ismail Z., Sadikun A. and Ibrahim P. Complementary and alternative medicine: quality assessment strategies and safe usage. *S. M. R*, **1**(2), 19–23 (2009).
3. Li S., Han Q., Qiao C., Song J., Cheng C.L. and Xu H. Chemical markers for the quality control of herbal medicines: an overview. *Chinese Med.*, **3**, 7–24 (2008).
4. Fan X.H., Cheng Y.Y., Ye Z.L., Lin R.C., Qian Z.Z. Multiple chromatographic fingerprinting and its application to the quality control of herbal medicines. *Anal. Chim. Acta*, **555**: 217–224 (2008).
5. Perry L.M.. *Medicinal plants of East and Southeast Asia*. MiT Press, Cambridge. (1981).
6. Duke J. A. and Ayensu E. S. Medicinal Plants of China. In: *Medicinal Plants of the World*, no. **4**, Reference Publications Inc., Algonac, MI, USA. (1985).
7. Toong V.Y. and Wong B.L.. Phytochemistry of Medicinal plants, *Piper sarmentosum*. *Proceedings: Traditional Medicine, Kuala Lumpur*. pp. 280, Institute of Advance Studies, University of Malaya, Kuala Lumpur. (1989).
8. Wee Y.C. *A Guide to Medicinal Plants*. Singapore Science Centre. (1992).
9. Sawangjiaroen N., Sawangjiaroen K. and Poonpanang P. Effects of *Piper longum* fruit, *Piper sarmentosum* root and *Quercus infectoria* nut gall on caecal amoebiasis in mice. *J. Ethnopharmacol*. **91**(2–3): 357–360 (2004).
10. Masuda T., Ingumi A., Yamada Y., Padolina W.G., Kikuzaki H. and Nakatani N. Antimicrobial phenyl propanoids from *Piper sarmentosum*, *Phytochemistry*. **30**(10): 3227–3228 (1991).
11. Rididtid W., Rattanaprom W., Thaina P., Chittrakaran S. and Sunbhanich M. Neuromuscular blocking activity of methanolic extract of *Piper sarmentosum* leaves in the rat phrenic nerve hemi diaphragm preparation. *J. Ethnopharmacol*. **61**(2): 135–142 (1998).
12. Peungvicha P., Thirawarapan S.S., Temsiririrkkul R., Watanabe H., Kumar P.J. and Kadota S. Hypoglycemic effect of the water extract of *Piper sarmentosum* in rats. *J. Ethnopharmacol*. **60**(1): 27–32 (1998).
13. Najib N.A., Rehman N., Furuta T., Kojima S., Takane K. and Ali M.M. Anti-malarial activity of extracts of Malaysian medicinal plants. *J. Ethnopharmacol*. **64**(3): 249–254 (1999).
14. Vimala, S. Mohd-Iiham, A. Abdul, R.A. and Rohana, S. Natural antioxidants: *Piper sarmentosum* (Kadok) and *Morinda elliptica* (Mengkudu). *M. J. Nutr*. **9**(1): 41–51 (2003).
15. Hutadilok, N.T., Chaiyamutti, P. Panthong, K. Mahabusarakam, W. and Rukachaisirikul, V. Antioxidant and free radical scavenging activities of some plants used in Thai folk medicine. *Pharmaceut. Biol.* **44**(3): 221–228 (2006).
16. Hussain, K. Ismail, Z. Sadikun, A. and Ibrahim, P. Antioxidant, anti-TB activities, phenolic and amide contents of standardized extracts of *Piper sarmentosum* Roxb. *Nat. Prod. Res*. **23**(3): 238–249 (2009).
17. Hussain, K. Ismail, Z. Sadikun, A. and Ibrahim, P. Analysis of proteins, polysaccharides, glycosaponins contents of *Piper sarmentosum* Roxb. and anti-TB evaluation for bio-enhancing/interaction effects of leaf extracts with isoniazid (INH). *N. P.R.* **7**(5): 204–208 (2008).
18. Hussain, K. Ismail, Z. Sadikun, A. Ibrahim, P. and Malik, A. *In vitro* anti-agiogenesis activity of standardized extracts of *Piper sarmentosum* Roxb. *J. Ris. Kim*. **1**(2):146–150 (2008).
19. EMEA. Final proposals for Revision of the notes for Guidance on Quality of Herbal Medicinal Products. The European Agency for the Evaluation of Medicinal Product. London, CPMP/QWP/2819/00. (2001).
20. International Conference of Harmonization (ICH). Q1A (R2). Stability testing guidelines: Stability Testing of New Drug Substances and Products. London. CPMP/ICH/2736/99. (2003).
21. Young, J. F. Humidity control in the laboratory using salt solutions a review. *J. Appl. Chem*. **17**, 241–245(1967).
22. Greenspan, L.J. Humidity fixed points of binary saturated aqueous solutions. *J. Res. Natl. Bur. Stand*., **81A**, 89 (1977).
23. Marsh, K. N. *Recommended Reference Materials for the Realization of Physicochemical Properties*. Blackwell Scientific Publication, Oxford, UK. (1987).
24. ASTM. Standard practice for maintaining constant relative humidity by means of aqueous solution, ASTM Standard. (1991).
25. Sherma, J. and Fried, B. *Handbook of Thin-Layer Chromatography*, 3rd ed. New York Marcel Dekker Inc. (2003).
26. WHOGuidelines for stability testing of pharmaceutical products containing well-established drug substances in conventional dosage forms. WHO Technical Report Series, No. 863. (1996).
27. Waterman, K. C. and Adami, R. C. Accelerated aging: Prediction of chemical stability of pharmaceuticals. *Intl. J. Pharmaceut*, **293**, 101–125 (2005).
28. Rawlins, E.A. *Bentley's Text Book of Pharmaceutics*. London, Bailliere Tindall. (1977).
29. Tonnesen, H.H. *Photostability of Drugs and Drug Formulations*. 2nd ed. London. Taylor and Francis Ltd. (2004).
30. Pugh, J. Kinetics and product stability. In *Text Book of Pharmaceutics, the Science of Dosage Form Design*, (ed) Aulton, M.E., 2nd ed., London, Churchill Livingstone. (2002).
31. Zhou M., Liu P., Jia W., Zhao A., Qui M., Qui Y. and Su M. Evaluation of therapeutics effects of TCM based on metabolic variations. *World Science and Technology-Modern and Traditional Chinese Medicine and Materia Medica*, **8**: 113–119 (2006).