Asian Journal of Chemistry; Vol. 26, No. 3 (2014), 874-878

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.16111

Quantification of Physiologically Available Glycyrrhizin in Anti-Stress Herbal Formulations by Validated HPTLC Method

NASIR A. SIDDIQUI[1,*], PERWEZ ALAM[1], AZMAT ALI KHAN[2], ATEEQUE AHMAD[3], ADNAN J. ALREHAILY[1] and AMER M. ALANAZI[2]

[1]Department Of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh-11451, P.O.Box. 2457, Kingdom of Saudi Arabia
[2]Department Of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh-11451, P.O. Box. 2457, Kingdom of Saudi Arabia
[3]Process Chemistry and Product Development Department, CIMAP, Lucknow, India

*Corresponding author: Fax: 00966114677245; Tel: 00966544016921; E-mail: nsiddiqui@ksu.edu.sa

Received: 27 July 2013; *Accepted*: 10 October 2013; *Published online*: 30 January 2014; AJC-14657

In the present study a simple and novel high-performance thin-layer chromatography (HPTLC) method for quantitative determination of glycyrrhizin at stomach pH and its comparison with normal pH has been developed for marketed antistress liquorice root capsules and herbal tea. Chromatography was performed by using solvents including ethyl acetate:glacial acetic acid:methanol:water in proportion of 5:2:2:1, v/v/v/v as mobile phase. The developed plate was scanned and quantified densitometrically at absorption maxima 254 nm. The system was found to give compact spot for glycyrrhizin (R_f = 0.29 ± 0.001). The linearity relationship was described by the equation: Y = 2.103X + 25.289. Linearity range for glycyrrhizin was 100-1000 ng (r^2 = 0.996). The amount of glycyrrhizin was estimated by comparing the peak area of standard and the same was present in the crude extract of antistress herbal formulations. The content of glycyrrhizin in 1 g of drug material at normal pH (*i.e.* 6.8) of methanol was estimated as 7.50 % w/w and 6.05 % w/w in a dose of sample liquorice root and herbal tea, respectively. At pH 2.58 the glycyrrhizin concentration declined to 5.43 % w/w and 3.28 % w/w in sample liquorice root and herbal tea, respectively. The method was validated for precision, accuracy, recovery, robustness, specificity, detection and quantification limits in accordance with ICH guidelines.

Keywords: Antistress herbal capsule, High-performance thin-layer chromatography, Glycyrrhizin, Quality control.

INTRODUCTION

The use of herbs or herbal formulations becomes more challenging because of variability of the constituents. A well-defined and constant composition of the drug is one of the most important prerequisites for the production of a quality herbal formulation. For analytical studies of herbal products various chromatography methods are used among which HPTLC is one of the most useful method[1]. Stress is a very commonly used term in our daily life. The term stress was coined by Hans Selye in 1975[2]. The definition of stress may be given as physiological disharmony or threat to homeostasis[3]. Stress usually causes activation of HPA (hypothalamic-pituitary-adrenal) axis through unknown central afferent pathways that stimulate the hypothalamus to release multiple corticotropin (ACTH) secretagogues, corticotropin-releasing hormone (CRH), arginine, vasopressin and the resulting enhanced plasma level of cortisol causes diseases such as hypertension, osteoporosis, depression and the development of the entire spectrum of the metabolic syndrome, including visceral obesity, insulin resistance and dyslipidemia as well as the kinds of cardiovascular diseases[4]. There are many herbal drugs and their formulations like *Ocimum sanctum* leaves, *R. damacena* root and *Panax ginseng* roots have been proved their effect as anti-stress[5]. Some of the herbal drugs are very well known for their adaptogenic, immunomodulatory and antistress properties among which *Withania somnifera, Panax ginseng, Asparagus racemosus* and *Picrorhiza kurroa* roots *etc.* are prominent [6-7].

Glycyrrhizin, a triterpenoid saponin glycoside from the roots and rhizomes of genus *Glycyrrhiza* (Licorice, Family-Fabaceae). It is the major bioactive constituent of genus *Glycyrrhiza* which has been traditionally used in herbal medicine for over 4000 years for the treatment of numerous ailments including liver disorders, stress, allergy, inflammation, spasm, constipation, depression, ulcer, diabetes, dyspepsia, bronchitis, rheumatoid arthritis *etc*. Licorice is widespread throughout the mediterranean region and certain areas of Asia. Historically, the dried rhizome and root of the plant were used by the Chinese, Egyptian, Greek, Indian and Roman civilizations as expectorant and carminative[8-10].

It is well known that herbal tea (*Camellia sinensis*) have many medicinal uses due to their major phytoconstituents *i.e.*

polyphenols. Herbal teas are used as antioxidants, antimacrobials[11] and as chemopreventive agents particularly in case of prostate cancer[12], *etc*. Now days herbal teas are mixed with some other medicinally useful herbs to modulate their effect according to purpose of use. There are many examples of such drinks like throat clearing herbal teas having herbs like glycyrrhiza, sweet fennel, thyme, *etc*.[13] relaxation drink to relieve the stress which generally contain herbs like valerian, kava, *etc.* that possess tranquilizing, neurocognitive functions and have some role in sleep[14]. Several studies indicate that natural products are susceptible to enzymatic, acid, base hydrolysis and degradation by oxidation and photo oxidation it is therefore necessary to evaluate the extent of degradation particularly in case of formulations meant for oral administration. The hepatoprotective silymarin undergoes acidic and alkaline degradation and it is estimated by HPTLC method[15]. HPTLC method is also used to determine curcumin degradation under stress conditions like acidic, basic, light and oxidation[16]. Glycyrrhizin being a glycoside is also susceptible for hydrolysis at acidic pH inside stomach.

Nowadays HPTLC is becoming a routine analytical technique due to many advantages like low operating cost; high sample throughput and need for minimum sample clean up, several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis[17]. TLC and HPTLC are methods commonly applied for the identification, the assay and the testing for purity, stability, dissolution or content uniformity of raw materials (herbal and animal extracts, fermentation mixtures, drugs and excipients) and formulated products (pharmaceuticals, cosmetics, nutraceuticals)[18].

EXPERIMENTAL

The commercial formulations "Licorice root capsules" and "Herbal tea" were purchased from Riyadh, Kingdom of Saudi Arabia. These formulations are frequently used for stress relief and possess glycyrrhizin as major bioactive constituent. liquorice root capsules contains only powdered root of *Glycyrrhiza* and herbal tea contains flavoring agent, coloring agent and sweeteners along with root powder of *Glycyrrhiza* plant.

The standard glycyrrhizin was obtained from Sigma Aldrich, Bayouni Trading Co. Ltd. Al-Khobar, Saudi Arabia. The following solvents and regents were used for performing the experiments: ethanol, hexane, ethyl acetate and sulfuric acid. All reagents, chemicals and solvents were of analytical grade, purchased from WINLAB and BDH (U.K.). HPTLC was performed on 10 × 20 cm glass-backed silica gel 60F254 HPTLC plates from E. Merck (Darmstadt, Germany).

HPTLC instrumentation and conditions: Chromatography was performed, as described by Faiyazuddin *et al*.[17], 2010 on 10 cm × 20 cm glass HPTLC plates precoated with 200 µm layers of silica gel 60F254. Samples were applied as bands 6 mm wide and 8 mm apart by means of Camag Linomat IV sample applicator equipped with a 25-µL syringe. The constant application rate was 160 nL S^{-1}. Linear ascending development with solvents EA:GAA:MeOH:H_2O (5:2:2:1, v/v/v/v) as mobile phase was performed in a 20 cm × 10 cm twin-trough glass chamber (Camag) previously saturated with mobile phase for 20 min at room temperature (25 ± 2 °C) and relative humidity 60 ± 5 %. The development distance was 7.2 cm (development time 10 min) and 10 mL mobile phase was used. The plates were dried at room temperature and then heated to identify compact bands. Densitometric analysis was performed at 254 nm in absorbance/reflectance mode with a Camag TLC scanner III operated by WinCATS 4 software (Version 1.2.0). The slit dimensions were 5 mm × 0.45 mm and the scanning speed of 20 mm S^{-1}.

Extraction conditions: The protocol for preparing sample solutions was optimized for high quality fingerprinting and also to extract the marker compound efficiently. Since the marker compound was soluble in methanol, therefore methanol was used for extraction. The fingerprinting of methanol extracts of both the samples was executed by spotting with suitably diluted sample solution on a HPTLC plate. Each amount was applied in triplicate. Peak area and amounts applied were treated by linear least squares regression. The plates were developed and scanned at 254 nm in absorbance/reflectance mode with a Camag TLC scanner III operated by WinCATS 4 software (Version 1.2). The peak areas were recorded and the amount of glycyrrhizin was calculated using the calibration curve.

Preparation of sample from Licorice root capsules: C1: Two capsules of sample weighing 1000 mg powdered drug material have been extracted with 10 mL methanol by ultrasonic method for 1 h and then filtered by vacuum filtration. The same procedure was repeated again with marc to exhaust the drug material. Two filtrates were combined and concentrated to 10 mL. From this stock solution 1 mL was taken and diluted to 10 mL with the help of methanol.

C2: The procedure similar to C1 was followed and pH 2.58 was adjusted with the help of concentrated hydrochloric acid.

Preparation of sample from herbal tea: P1: Herbal tea weighing 1000 mg has been extracted with 10 mL methanol by ultrasonic method for 1 h and then filtered by vacuum filtration. The same procedure was repeated again with marc to exhaust the drug material. Two filtrates were combined and concentrated to 10 mL. From this stock solution 1 mL was taken and diluted to 10 mL with the help of methanol.

P2: The procedure similar to P1 was followed and pH 2.58 was adjusted with the help of concentrated hydrochloric acid (Conc. HCl).

Preparation of sample from standard: Stock solution of glycyrrhizin standard (1 mg mL^{-1}) was prepared in methanol and by appropriate dilution it was made to the concentration of 0.1 µg/µL. For calibration, glycyrrhizin standard solution (1-10 µL) was applied to a HPTLC plate to furnish amounts in the range 100-1000 ng $band^{-1}$.

Methanol solution (0.1 mg/mL) of standard and formulations extract (5 µL) were applied to chromatographic plates bandwise, by means of a camag automatic TLC sampler- IV and developed in ADC2 (automatic development chamber). Plates, after derivatization, were documented with the use of Camag TLC Reprostar 3 with computer program Videostore and scanned with densitometer Camag TLC scanner with computer program CATS 4 (Camag).

RESULTS AND DISCUSSION

The amount of glycyrrhizin was determined from the calibration curve (Fig. 1) obtained by plotting the concentration of glycyrrhizin standard against the peak area. The content of glycyrrhizin was estimated in the methanol extract samples of liquorice root capsules and herbal tea at normal pH by the proposed method was 7.50 % w/w and 6.05 % w/w of powdered drug material respectively. At pH 2.58 the concentration of glycyrrhizin goes down to 5.43 % w/w and 3.28 % w/w of powdered drug material of liquorice root capsules and herbal tea respectively. The percentage degradation in herbal tea was found to be more (39.48 %) than liquorice root capsules (19.38 %). The difference in the percent degradation of liquorice root capsules and herbal tea may be due to the presence of certain other constituents like coloring agents, flavoring agents, sweeteners *etc*. which were present in herbal tea while liquorice root capsules contains only root powder of glycyrrhiza. Hydrolytic effect of conc. HCl is clearly demonstrated in Figs. 3 and 4 where the difference in peak area of both the samples at two different pH can be observed. Again the selected peak of glycyrrhizin in both the samples at normal pH and at pH 2.58 shows marked difference in the peak area. Therefore this experiment proves that after oral ingestion the estimated quantity of glycyrrhizin will be the physiologically available quantity to the human body. After getting the degradation results of marker compound the quantity of powdered drug to be filled in capsules or the quantity of herbal tea to be taken as antistress can be easily be decided. It is for the first time, a simple, accurate and rapid HPTLC method has been developed for the quantification of physiologically available bioactive marker glycyrrhizin in different herbal formulations of *Glycyrrhiza* mainly prescribed as antistress agent.

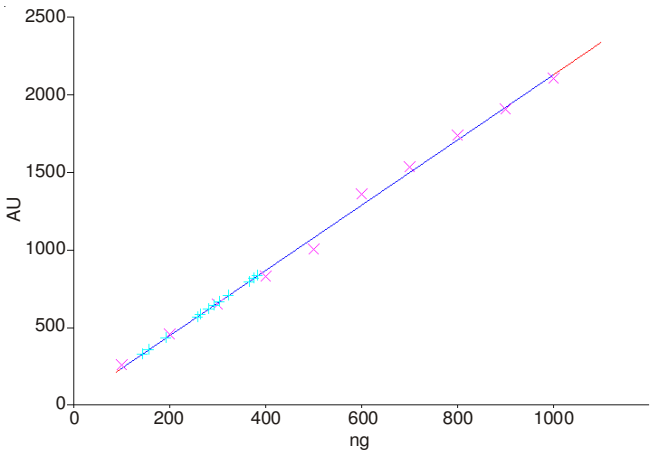


Fig. 1. Calibration curve of standard glycyrrhizin @254 nm

Chromatography: Chromatogram was developed for glycyrrhizin standard under chamber saturation conditions using solvents Ethyl acetate: GAA: MeOH: H_2O (5:2:2:1, v/v/v/v) as mobile phase (Fig. 2). The same mobile phase has been also employed for the separation of components in methanolic extracts of sample liquorice root capsules and herbal tea (Figs. 3 and 4, respectively). The optimized saturation time was found to be 10 min. UV spectra measured for the spots showed maximum absorbance at 254 nm in the absorbance/reflectance mode. Compact bands as sharp, symmetrical and with high resolution were obtained at R_f 0.29 ± 0.001.

TABLE-1
R_f, LINEAR REGRESSION DATA FOR THE CALIBRATION CURVE AND SENSITIVITY PARAMETER FOR GLYCYRRHIZIN

Parameter	Glycyrrhizin
R_f	0.29
Linearity range (ng band^{-1})	100-1000
Regression equation	Y= 2.103X + 25.289
Correlation coefficient	r^2 = 0.996
Slope ± sd	2.103 ± 0.003
Intercept ± sd	25.289 ± 0.005
Standard error of slope	0.001
Standard error of intercept	0.001
LOD	34 ng
LOQ	101 ng

Calibration curve: Linearity of compound glycyrrhizin was validated by the linear regression equation and correlation

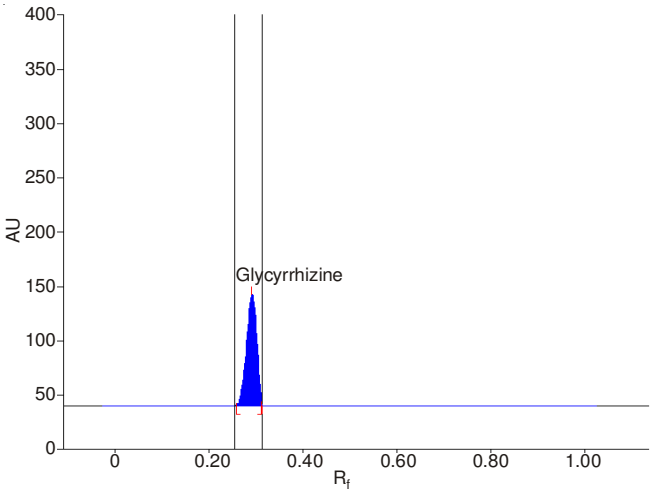
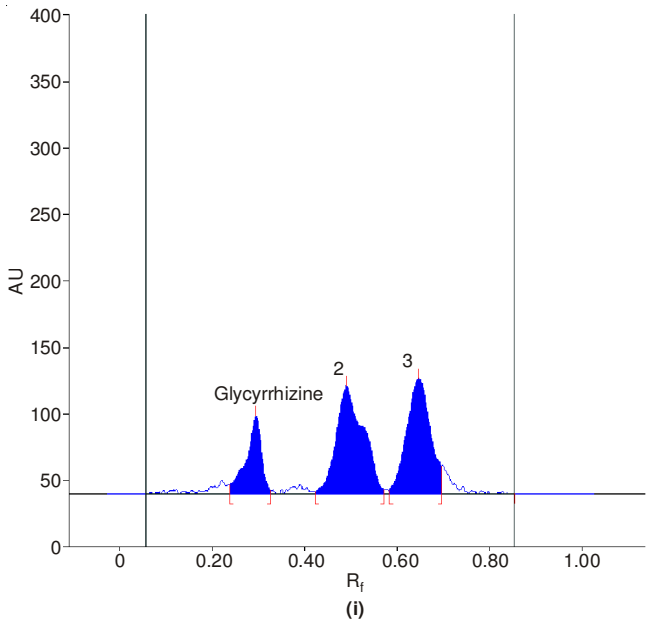


Fig. 2 HPTLC chromatogram of standard glycyrrhizin with mobile phase ethyl acetate : galacial acetic acid : methanol : water in proportion of 5:2:2:1, v/v/v/v scanned at 254 nm



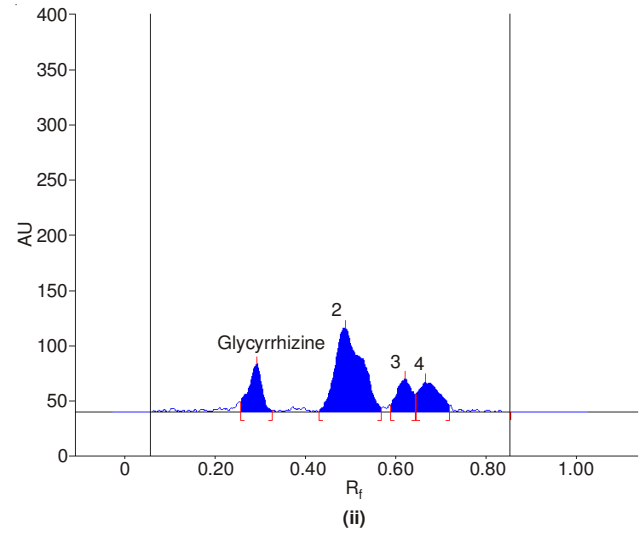


Fig. 3. (i) Components of liquorice root capsules at normal pH (ii): Components of liquorice root capsules at pH 2.58

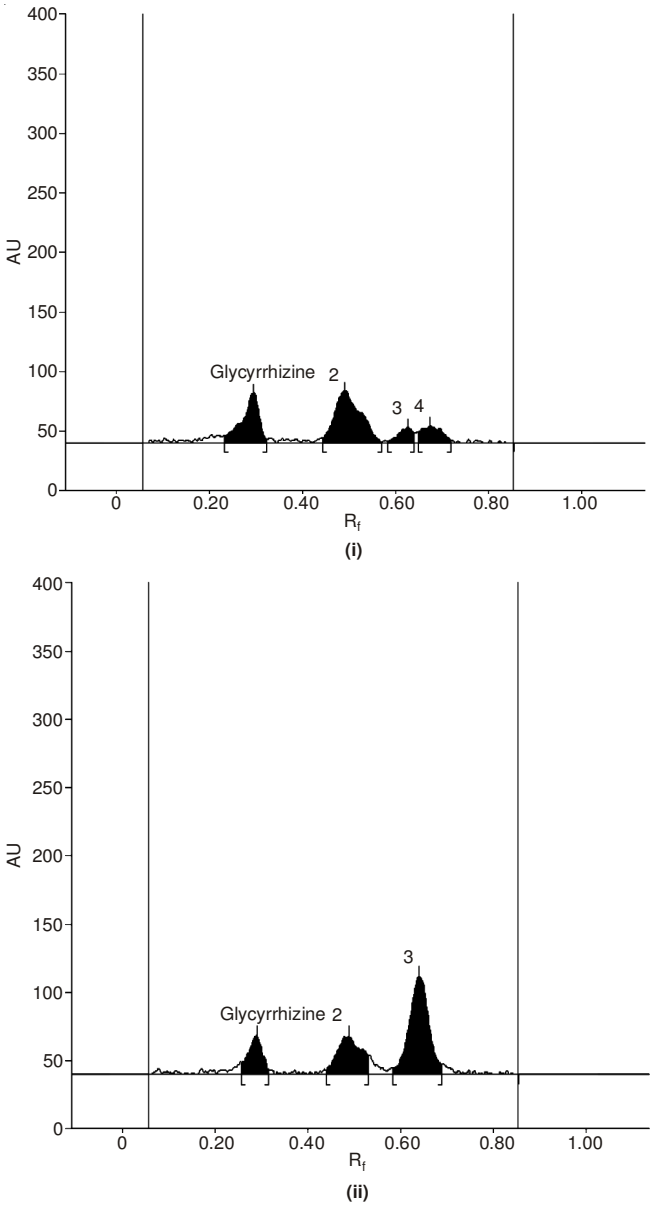


Fig. 4. (i) Components of herbal tea at normal pH (ii) Components of herbal tea at pH 2.58

coefficient. The ten-point calibration curve (Fig. 1) for glycyrrhizin was found to be linear in the range of 100-1000 ng. Regression equation and correlation coefficient for the reference compound was $Y = 2.103X + 25.289$ ($r^2 = 0.996$) which revealed a good linearity response for developed method and are presented in Table-1.

Glycyrrhizin was well resolved at R_f 0.29 from MeOH extracts of liquorice root capsules and herbal tea in the solvent system as mentioned above. The plate was visualized at wavelength 254 nm as the compound was found to absorb at this maxima. The method developed here was found to be quite selective with good baseline resolution of each compound. The identity of the bands of compounds in the sample extracts were confirmed by overlaying their absorption spectra with those of the standards at 254 nm (Fig. 5).

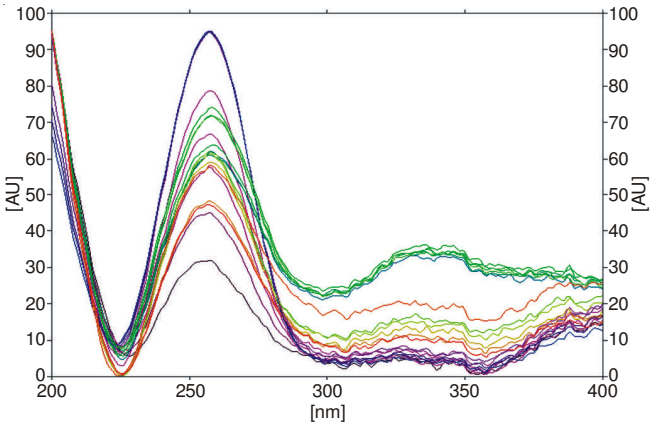


Fig. 5. Spectral comparison of glycyrrhizin with MeOH extracts of sample liquorice root capsules and herbal tea with mobile phase ethylacetate: GAA: MeOH: H_2O (5:2:2:1, v/v/v/v)

Method validation: The method adopted here was validated for precision, accuracy, robustness, sensitivity and recovery studies. Intra-day precisions (n = 6) for glycyrrhizin was found to be =1.84 %, however the inter-day precisions was =1.62 %, which demonstrated the good precision of proposed method. Intra-day accuracy of glycyrrhizin was 99.2 - 99.7 %, however interday accuracy for glycyrrhizin was 98.9-99.6 %. These values are within the acceptable range, so the method was accurate, reliable and reproducible. The SD and % RSD was calculated for glycyrrhizin. The low value of SD and % RSD obtained after introducing small deliberate changes in the method indicated that the method was robust. LOD and LOQ values for glycyrrhizin were found to be 34 and 101 ng band^{-1}, respectively, indicating adequate assay sensitivity. The LOD and LOQ were determined from the slope of the lowest part of the calibration plot. This indicated that the proposed method exhibits a good sensitivity for the quantification of above compounds. Good recoveries were obtained by the fortification of the sample at three quality control levels for glycyrrhizin. It is evident from the results that the percent recoveries for glycyrrhizin after sample processing and applying were in the range of 99.4-99.9 %.

Conclusion

A validated HPTLC method has been developed for the determination of Glycyrrhizin at normal as well as acidic pH

in different herbal formulations of *Glycyrrhiza* (liquorice root capsules and herbal tea) marketed in Kingdom of Saudi Arabia as stress reliever. The present study has clearly given an evidence of pH related degradation of the active constituents. As far as quality control of herbals are concern the formulations must be analyzed for their possible ways of degradations which may include exposure to high temperature, moisture, acids, alkali, enzymes, oxidation and photo-oxidation conditions etc.

This approach of evaluation can give an approximation about the quantity of active ingredients available physiologically to the body. Statistical analysis proved that the method is authentic and reproducible for the analysis of glycyrrhizin. To make sure the presence of therapeutic dose of active constituents in herbal formulations, HPTLC method is very useful for quantification of marker compounds in the raw material as well as in finished products. This is one of the reliable and economic methods to produce quality herbal formulations.

ACKNOWLEDGEMENTS

The authors extended their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project No. RGP-VPP-212.

REFERENCES

1. O.F. Kunle, H.O. Egharevba and P.O. Ahmadu, *Int. J. Biodivers. Conserv.*, **4**, 101 (2012).
2. H. Selye, *J. Human Stress*, **1**, 37 (1975).
3. M. Sumanth and S.S. Mustafa, *Int. J. Pharmacol*, **3**, 416 (2007).
4. R. Rosmond, *Psychoneuroendocrinology*, **30**, 1 (2005).
5. V. Singh, A. Singh, R. Nath, N. Mishra, K.S. Dixit and N. Singh, *J. Biol. Chem. Res*, **10**, 601 (1991).
6. D.C. Anderson, *Integr. Med.*, **7**, 18 (2008).
7. N.A. Siddiqi, S. Singh, M.M. Siddiqui and T.H. Khan, *Int. J. Pharmacol.*, **8**, 108 (2012).
8. Q. Afnan, M.D. Adil, A. Nissar-Ul, A.R. Rafiq, H.F. Amir, P. Kaiser, V.K. Gupta, R. Vishwakarma and S.A. Tasduq, *Phytomedicine*, **19**, 658 (2012).
9. C.V. Chandrasekaran, K. Sundarajan, A. Gupta, H.S. Srikanth, J. Edwin and A. Agarwal, *Regul. Toxicol. Pharmacol.*, **61**, 373 (2011).
10. E.W.C. Chan, P.P. Tie, E.Y. Soh and Y.P. Law, *Pharmacog. Res.*, **3**, 266 (2011).
11. J.J. Johnson, H.H. Bailey and H. Mukhtar, *Phytomedicine*, **17**, 3 (2010).
12. G. Puodziuniene, V. Janulis, A. Milasius and M. Budnikas, *Medicina (Kaunas)*, **40**, 762 (2004).
13. S. Stacy, *J. Child Adolesc. Psychopharmacol.*, **21**, 605 (2011).
14. Y.J. Li, J. Chen, Y. Li, Q. Li, Y.F. Zheng, Y. Fu and P. Li, *J. Chromatogr. A*, **1218**, 8181 (2011).
15. R. Parveen, S. Ahmad, S. Baboota, J. Ali and A. Alka, *Biomed. Chromatogr.*, **24**, 639 (2010).
16. M.J. Ansari, S. Ahmad, K. Kohli, J. Ali and R.K. Khar, *J. Pharm. Biomed. Anal.*, **39**, 132 (2005).
17. M. Faiyazuddin, J. Ali, S. Ahmad, N. Ahmad, J. Akhtar and S. Baboota, *J. Planar Chromatogr.*, **23**, 233 (2010).
18. G. Biringanine, M.T. Chiarelli, M. Faes and P. Duez, *Talanta*, **69**, 418 (2006).