Quantitative Determination of Mangiferin Isolated from Leaves of Mangifera indica L. Variety Nam Doc Mai using HPTLC and Its DPPH Scavenging Activity

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Abstract

Mangiferin (2-beta-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthen-9-one) was isolated from Leaves of Mangifera indica L. variety Nam Doc Mai. It showed a potent scavenging activity on DPPH redical with an IC_{50} value of 6.38 µg/ml. A simple and cost-effective high- performance thin-layer chromatographic (HPTLC) method was developed and validated for quantitative determination of mangiferin. The method was performed on silica gel 60 F_{254} pre-coated plate with ethyl acetate-acetone-formic acid-water 8:2:1:1 (v/v) as mobile phase. Detection and quantitation was achieved by densitometric scanning at the wavelength of 320 nm. The results from validation method indicated that the proposed HPTLC method provided a good linearity at the range of 1.0 to 3.0 µg/band, accuracy (107.89 ± 0.29%), precision (intra-day RSD 0.51-1.71% and inter-day RSD 1.52%), and specificity (sharp peak at R_f value of 0.48).

Keywords: Mangiferin / HPTLC / DPPH radical

Introduction

Mango (Mangifera indica L) belonging to the family Anacardiaceae is known as Ma-mung in Thai. It is a popular and economically important fruit which is widely cultivated throughout the country. There are numerous varieties grown in Thailand and Nam Doc Mai is a mainly requirement variety because of its sweetly taste and fragrance. Phytochemical investigation of different parts of M. indica revealed many types of compounds such as phenolic acids, phenolic esters, flavonols and mangiferin, the C-glucoxanthone (Andreu et al., 2005; Hernandez et al., 2007; Ribeiro et al., 2008; Ling et al, 2009). Mangiferin, $C_{19}H_{18}O_{11}$ (2-beta-D-glucopyranosy l-1, 3, 6, 7-tetrahydroxyxanthen-9-one) has been reported for its significant pharmacological properties such as antioxidant (Garrido et al., 2004; Sanchez et al, 2000; Martinez et al, 2001), antitumor (Leiro et al., 2003; Sarkar et al., 2004; Yoshimi et al, 2001), antidiabetic (Garcia et al, 2003; Miura et al., 2001), vascular modulatory (Beltran et al, 2004), immunomodulatory, and antiviral activities (Dar et al., 2005; Nong et al., 2005; Ribeiro et al., 2008). Our previous study has shown that leaf of Nam Doc Mai is an excellent source of mangiferin and an uncomplicated method for isolation and purification of mangiferin has been also reported (Jutiviboonsuk et al., 2010).

Standardization is an important process for assuring the quality, efficacy and safety of herbal raw materials and products. However, quality control methods for single synthetic drugs cannot simply be used for quality control of herbal raw materials and products which are complex mixtures of substances. High-performance thin-layer chromatography (HPTLC) is a separation technique based on the different affinities of the components of a mixture to two immiscible phrases, stationary and mobile phrases. HPTLC is a planar chromatography which the stationary phase is a flat and thin layer coated onto an inert support such as glass, aluminum, or polyester. Silica gel is most commonly used as stationary phase. The smaller particle size and narrow particle size distribution of silica gel are important properties for the separation efficiency. Silica gel for TLC has an average particle size of 15 to 20 µm whereas HPTLC material is smaller particles of 5 to 7 µm that gives better separation and reproducibility. Since silica gel is porous material therefore the pore diameter and pore size distribution can affect on chromatographic behavior of silica gel. Generally the average pore size of silica gel used for TLC is 6 nm, equal to 60 A°. Silanol groups (Si-OH), typically found about 5 to 7 µmole on 1 m^2 surface of silica gel, are the active sites which interact with analyte molecules by dipole-dipole or Lewis acid-base interaction, hydrogen bonding, or ionic interaction. Due to the nature of silica gel that has a high affinity to water so the water vapor from the surroundings is easily adsorbed. The higher humidity, the more water molecules are adsorbed and a consequence the less active the silica gel becomes which affects the retardation factor (R_f) value of the analyte. Heating a silica gel plate to 120 °C in an oven can completely remove adsorbed water from silica gel and the active sites are maximized. Mobile phase is the liquid that migrates through the stationary phase. It is responsible for the selectivity of the separation. The term solvent strength is used to describe the effect of a mobile phase on the retention of analyte. In normal phase chromatography, solvent strength is regarded as polarity where mobile phase is less polar than stationary phase. Increasing of solvent strength by increasing polarity of mobile phase can decrease retention and increase the R_f value. The R_f value is a relative measure of the substance position in the chromatogram with respect to the position of the solvent front.

$$R_f = Z_i / (Z_f - Z_0)$$

Where Z_i is migration distance of analyte (mm), Z_f is migration distance of front measured from the immersion line (mm), and Z_0 is distance between immersion line and sample application position (mm).

R_f value is typically given with two significant digits, $0 < R_f < 1$. At $R_f = 0$, the analyte does not migrate and no separation is achieved. At $R_f = 1$, the analyte moves in the solvent front and is not separated. Therefore the position of the analyte in the chromatogram must be optimized and the R_f value should be 0.2 to 0.8. Densitometry is the detection of analyte on TLC/HPTLC plate and generating analog curve of the chromatogram track for qualitative and quantitative evaluation. In the densitometer, a light beam of defined shape is directed vertically onto the plate. The light that comes back to the detector, placed

at a 45 degree angle, is measured. If there is an analyte which can absorb at the wavelength of the light, the signal generated in the detector will be lower. The chromatogram data can be integrated and evaluated to produce peak area and peak height. When a set of calibration standards has been chromatographed together with the analytes, a calibration function can be calculated. Moreover, UV spectra of separated analytes can be recorded for identification purposes using multi-wavelength scanning (Reich et al., 2006).

HPTLC is a rapid, precise, and cost-effective method for determination of bioactive compounds in medicinal plants. Many samples can easily be compared on the same time and multiple detections without repeating the chromatography are achievable (Schibli et al., 2005). And HPTLC uses the off-line principle because the process is separated into individual steps (Figure 1). The last few years applications of HPTLC for quality control of medicinal plants have been increase; for example, quantitative estimation of 14-deoxy-11,12-didehydroandrographolide in Andrographis paniculata (Kapadi et al., 2010), an identification of green tea and green tea extract (Reich E et al., 2006), quantitative analysis of artemisinin in dried Artemisia annua L. (Widmer et al., 2007), and detection of adulteration of black cohosh (Cimicifuga racemosa) with other Cimicifuga species (Anki et al., 2008).

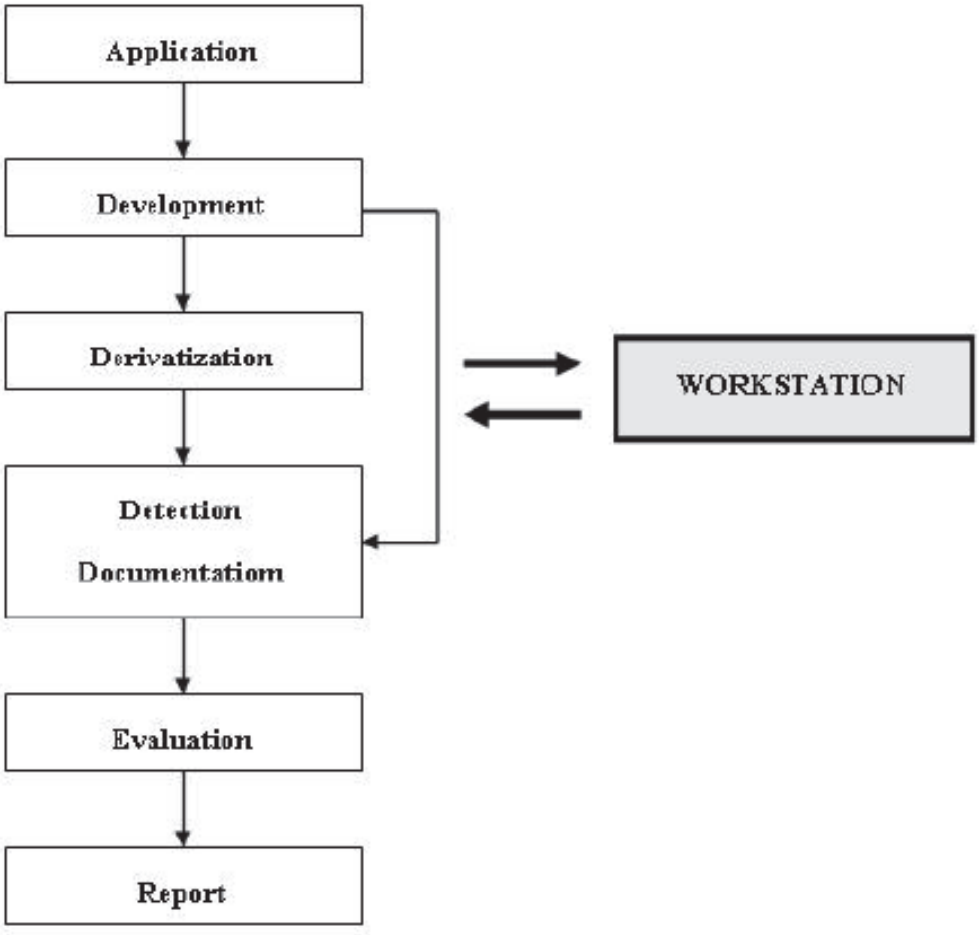

Figure 1. The off-line principle in TLC/HPTLC (Reich et al., 2006).

The present study aims to isolate mangiferin from leaves of Nam Doc Mai, to develop and validate the quantitative determination of isolated mangiferin using HPTLC method, and to evaluate DPPH (2, 2-diphenyl-1-pricrylhydrazyl) scavenging activity of the compound.

Material and Methods

Reagents

All chemicals and reagents used were of analytical reagent grade. DPPH (2, 2-dipheny-l-1-pricrylhydrazyl), L-ascorbic acid, and Trolox ((±)-6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxy lic acid) were purchased from Sigma-Aldrich. Mangiferin from Mangifera indica L. stem bark was purchased from Sigma-Aldrich (Cat. No. M3547, USA).

Plant materials

The leaves of mango variety Nam Doc Mai were collected from Kram sub-district, Klaeng district, Rayong province, Thailand. After cleaning by rinsing with tap water, the leaves were dried at 40-50°C in an oven and ground to powder with a cutting mill. The powdered leaves were stored at room temperature in dark and dry place.

Equipments

Vacuum rotary evaporator (Buchi Rotavapor® model R-210, Switzerland), magnetic stirrer with heating (Heidolph® model MR 3001, Germany), UV/VIS spectrophotometer (JASCO® model V-630, Japan), Camag HPTLC system with Linomat 5 applicator, Camag TLC scanner 3, and WinCATs software version 1.4.4 (Switzerland).

Isolation of mangiferin

Mangiferin was isolated from leaves of mango variety Nam Doc Mai according to the method described by Jutiviboonsuk et al. with some modifications. The dried powdered leaves (1.5 kg) were macerated with 85% ethanol (1.0 L) at room temperature for 7 days, the extraction solvent was changed everyday, and semisolid extract (0.5133 kg) was obtained after concentrated under reduced pressure. The extract was resuspended in 50% ethanol (1.0 L) and partitioned with dichloromethane (500 ml). Pale yellow precipitate (0.032 kg) appeared when the ethanol fraction was concentrated. The precipitate was confirmed as mangiferin by TLC analysis.

HPTLC procedure

Chromatography was performed on 20 x 10 cm aluminum plates coated with 0.2 mm layers of silica gel 60 F_{254} (E. Merck, Germany). The samples and standards were applied on the plates as 6 mm wide bands, positioned 15 mm from lower edge of the plate using Linomat 5 applicator with a nitrogen flow providing delivery from the syringe at a speed of 150 nLs^{-1}. The mobile phase was ethyl acetate-acetone-formic acid-water 8:2:1:1 (v/v). Saturation time was 30 minutes at room temperature and

ascending mode was used for development of the plates with a distance of 80 mm from lower edge of the plate. The developed plates were scanned at wavelength of 320 nm with Camag TLC scanner 3 controlled by WinCATs 1.4.4 software. The source of radiation was deuterium lamp and the absorbance mode was used. Slit dimension was 4.0 x 0.3 mm micro and scanning speed was 20 nms^{-1}. These parameters were maintained for all quantitative analysis and validation method.

Preparation of mangiferin standard solution and calibration curve

Accurately weighed 5 mg of mangiferin standard was dissolved in 10 ml of methanol in a volumetric flask and sonicated for 5 minutes. A calibration curve was established over five concentrations of mangiferin (1.0, 1.5, 2.0, 2.5, and 3.0 µg/band) in triplicate by applying 2.0, 3.0, 4.0, 5.0, and 6.0 µl of the standard solution on TLC plate. The plate was developed and scanned using the previously described procedure. The calibration curve was plotted between amounts of mangiferin versus average peak area.

Preparation of sample solution

The isolated mangiferin from leaves of mango variety Nam Doc Mai was accurately weighed and dissolved in methanol to obtain 1 mg/ml sample solution. The solution was sonicated for 5 minutes and subjected for further analysis.

Method validation

Specificity

The specificity of the method was assessed by analysis of standard and sample solutions. The band of mangiferin from the sample solution was confirmed by comparisons of Rf values and UV spectra of the sample with mangiferin standard.

Linearity and range

The standard calibration curve was used for evaluation of the linearity of the method. The equation of linear regression curve and correlation coefficient (r) were determined.

Precision and accuracy

Three different concentrations at 1.0, 2.0, and 3.0 µg/band of mangiferin standard were used in the test and expressed as %RSD and recovery. For the intra-day repeatability test, each concentration was applied for 5 times. The inter-day repeatability test, concentration of 2.0 µg/band of mangiferin standard was applied for 6 times on three separate days.

Application of HPTLC procedure

In order to test the application of the HPTLC method, the methanolic solution of isolated mangiferin from leaves of mango variety Nam Doc Mai at concentration of 0.5 mg/ml was analyzed (2.5 µl/band of the solution, n = 3).

Determination of antioxidant activity with DPPH radical scavenging method

The antioxidant activity of isolated mangiferin from leaves of mango variety Nam Doc Mai was measured in terms of radical scavenging ability using the stable radical, DPPH (2,2-diphenyl-1-pricryl-hydrazyl). The solution of DPPH radical in methanol produces a deep violet color, characterized by an absorption band at a wavelength of 517 nm. The color is disappeared when DPPH radical is converted to reduced form, diphenylpicrylhydrazine, by an antiradical compound (Brand-Williams et al., 1995). Different concentrations (2.0-8.0 µg/ml) of isolated mangiferin were reacted with 200 µl of freshly prepared DPPH radical in methanol (2.0 mM). The absorbances were recorded at 517 nm after 20 minutes of reaction. The mixture of methanol (2.8 ml) and 2.0 mM DPPH radical solution (200 µl) was used as negative control and methanol was used as blank. Ascorbic acid and trolox were used as reference antioxidants. The experiment was done in triplicate for each substance. The percentage of scavenging activity (AA%) was calculated by the equation: AA% = (1-As/Ac) x 100 where As is the absorbance value of the substance and Ac is the absorbance value of the negative control. Results were expressed as IC_{50} value, the concentration (µg/ml) of substance that causes 50% loss of DPPH radical activity. It was calculated by linear regression plotted between AA% and used concentration of substances.

Results and Discussion

Pale yellow precipitate of mangiferin was isolated from leaves of mango variety Nam Doc Mai with the percentage yield of 2.13 (w/w). HPTLC chromatogram of isolated mangiferin showed peak with R_f value of 0.48 at the wavelength of 320 nm which was corresponding to the peak of mangiferin standard. The UV spectra acquired from the mangiferin peaks obtained from sample and standard were identical indicating the specificity of the method (Figure 2). The linear relationship between the peak area and the concentration of mangiferin determined a good linearity at the range of 1.0 to 3.0 µg/band. The equation of linear regression curve was Y = 6284.6X + 7086.5 and correlation coefficient (r) was 0.9953 (sdv = 2.85%).

The intra-day repeatability test was investigated with 5 replicates of three different concentrations of mangiferin standard and the percentage relative standard deviations (%RSD) were in the range of 0.51-1.71%. The inter-day repeatability test was investigated with 6 replicates of same concentration of mangiferin standard on three separate days and the %RSD was 1.52%. The RSD values were found to be below 2%, indicating the precision of the method was good (Table 1). Accuracy of the method was studied using standard addition method. The result indicated a good accuracy of the method with the percentage recovery as 107.89 ± 0.29 (Table 2).

Table 1. Precision of the method.

Amount of mangiferin (µg/band)	Intra-day repeatability (n = 5)		Inter-day repeatability (n = 6)	
	Mean area ± SD	%RSD	Mean area ± SD	%RSD
1.0	12890.00 ± 220.83	1.71	17479.58 ± 265.35	1.52
2.0	18771.12 ± 94.96	0.51		
3.0	23553.91 ± 320.43	1.36		

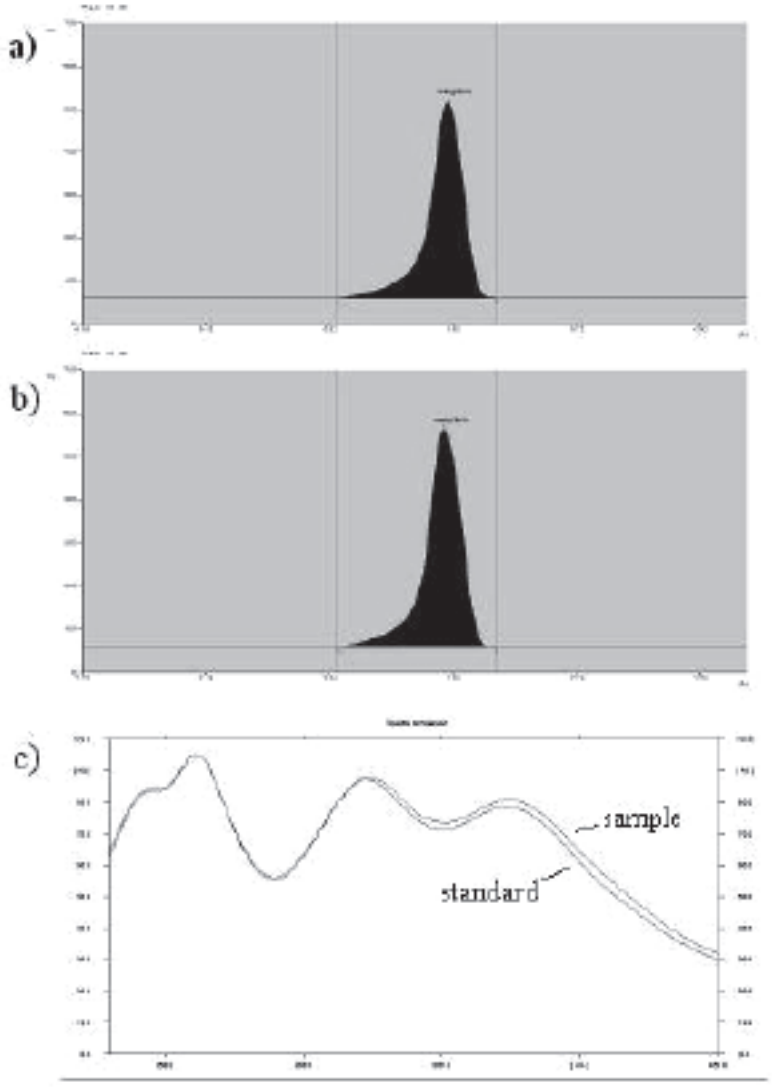

Figure 2. Comparison of HPTLC chromatograms and UV spectra of isolated mangiferin and mangiferin standard. a) HPTLC chromatogram of mangiferin standard; b) HPTLC chromatogram of isolated mangiferin and c) overlay absorption spectra of isolated mangiferin and mangiferin standard.

Table 2. Accuracy of the method.

Amount of mangiferin (µg)			Recovery (%)
Amount in sample	**Amount added**	**Amount detected in mixture**	
1.176	1.000	2.275	108.28
1.176	1.000	2.267	107.48
1.176	1.000	2.269	107.68
1.176	1.000	2.273	108.08
1.176	1.000	2.272	107.98
1.176	1.000	2.271	107.88
		Average ± SD	**107.89 ± 0.29**

Isolated mangiferin from leaves of mango variety Nam Doc Mai was assayed using the HPTLC method in this work. The amount of mangiferin was calculated as 93.15 %. Figure 3 showed the HPTLC chromatogram of standard at different concentrations and sample with UV spectra for peak purity. Moreover isolated mangiferin was determined for antioxidant activity using DPPH radical scavenging method. The result showed the potent antioxidant activity with an IC_{50} value of 6.38 µg/ml where as ascorbic acid and trolox produced IC_{50} values of 5.24 and 7.89 µg/ml, respectively (Table 3).

Table 3. DPPH scavenging activity of isolated mangiferin, ascorbic acid and trolox.

Concentration (µg/ml)	AA% * ± SD		
	Ascorbic acid	**Isolated mangiferin**	**Trolox**
2.67	24.79 ± 0.52	21.31 ± 1.01	14.29 ± 1.05
4.00	38.21 ± 0.48	31.46 ± 0.16	25.41 ± 0.15
5.33	51.41 ± 1.33	42.64 ± 1.31	34.06 ± 0.50
6.67	66.53 ± 1.00	52.48 ± 0.84	43.48 ± 0.49
7.33	73.05 ± 2.65	57.36 ± 0.12	ND
8.00	ND	63.86 ± 0.50	53.88 ± 1.29
8.67	ND	67.62 ± 0.44	ND
9.33	ND	ND	63.75 ± 1.54
10.67	ND	ND	74.83 ± 1.00
IC50 (µg/ml)	5.24	6.38	7.89

* AA% = the percentage of scavenging activity, ND = not determined

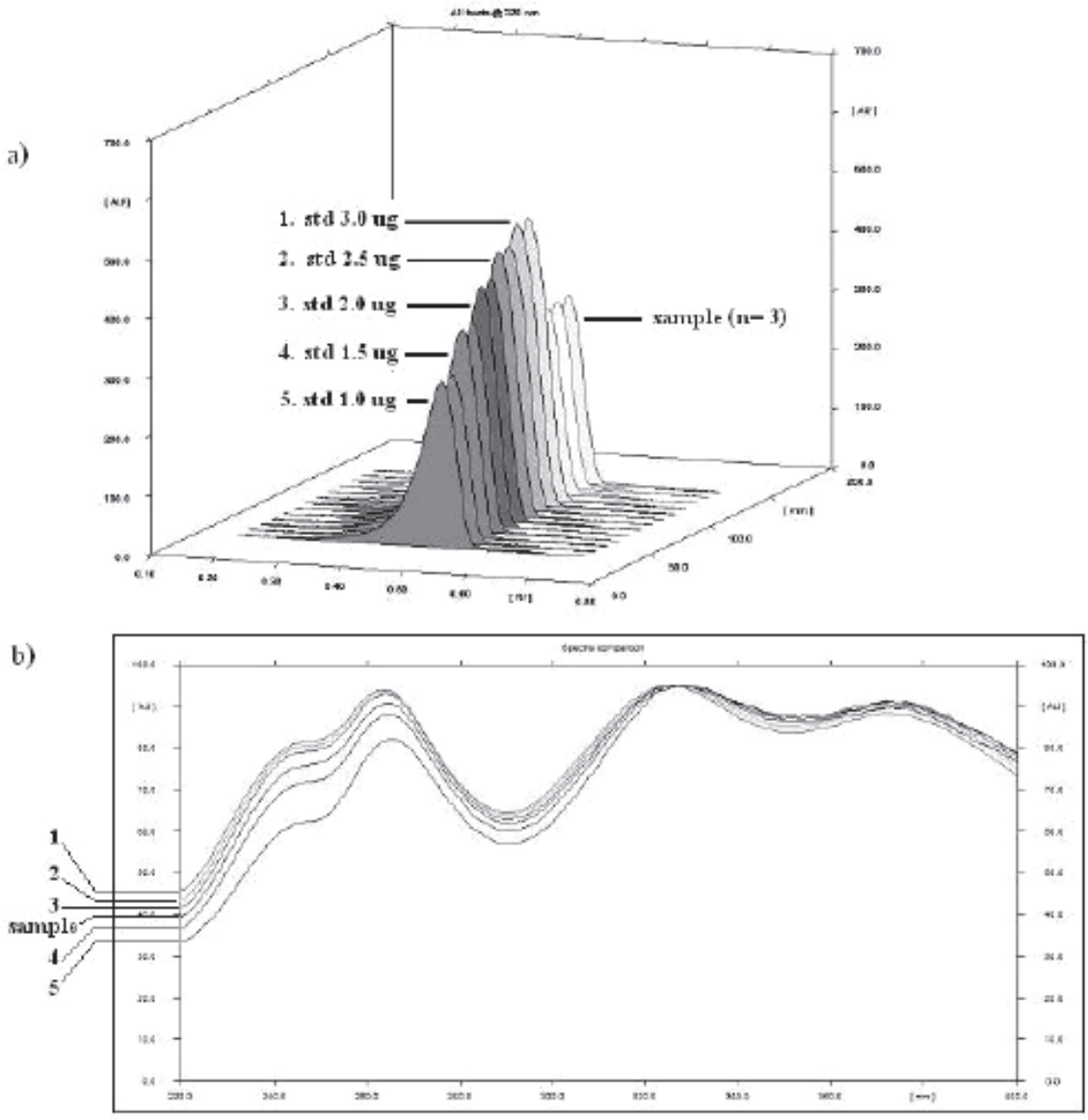

Figure 3. HPTLC chromatogram of mangiferin standard at different concentrations and isolated mangiferin with UV spectra for peak purity. a) HPTLC chromatogram of mangiferin standard and isolated mangiferin; b) overlay absorption spectra.

Conclusion

Mangiferin, the C-glucoxanthone isolated from leaves of Mangifera indica variety Nam Doc Mai, showed a potent antioxidant activity as good as ascorbic acid and trolox. HPTLC method on silica gel 60 F_{254} pre-coated plate with ethyl acetate-acetone-formic acid-water 8:2:1:1 (v/v) as mobile phase and densitometric evaluation at wavelength of 320 nm was used for quantitative determination of mangiferin. The method was validated and found to be in linearity, accuracy, precision, and specificity.

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