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COMPARATIVE PROFILING OF BIOMARKER PSORALEN IN ANTIOXIDANT ACTIVE EXTRACTS OF DIFFERENT SPECIES OF GENUS *FICUS* BY VALIDATED HPTLC METHOD

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Abstract

Background: A simple but sensitive HPTLC method was developed for the comparative evaluation of psoralen in antioxidant active extracts of leaves of five different species of genus *Ficus* (*Ficus carica*, *Ficus nitida*, *Ficus ingens*, *Ficus palmata* and *Ficus vasta*).
Materials and Methods: HPTLC studies were carried out using CAMAG HPTLC system on Glass-backed silica gel $60F_{254}$ HPTLC pre-coated plates using selected mobile phase toluene: methanol (9:1). The antioxidant activity was carried out, using DPPH free radical method.
Results: Among all the five species of genus Ficus, *F. palmata* and *F. carica* exhibited comparatively good antioxidant activity in DPPH assay. The developed HPTLC method was found to give a compact spot for psoralen (R_f = 0.55±0.001) at 305 nm. The regression equation and r^2 for psoralen was found to be Y= 4.516X+35.894 and 0.998. The quantification result revealed the presence of psoralen in only two species, *F. carica* (0.24%, w/w) and *F. palmata* (1.88%, w/w) which supported their supremacy for anti-oxidant potential over other species. The statistical analysis proved that the developed method was reproducible and selective.
Conclusion: The developed method can be used as an important tool to assure the therapeutic dose of active ingredients in herbal formulations as well as for standardization and quality control of bulk drugs and in-process formulations. This method can also be employed for the further study of degradation kinetics and determination of psoralen in plasma and other biological fluids.

Key words: *Ficus species*, *psoralen*, *antioxidant*, HPTLC, Validation.

Introduction

The genus *Ficus* includes about 800 species of woody trees, shrubs and vines and their fruits generally known as figs, distributed widely in tropical and subtropical regions (Harrison, 2005). Fresh figs, contains good poly-phenolic flavonoids whose anti-oxidant property is comparable to that of apples at 3200 umol/100 g (Ronsted et al., 2007). The various phytoconstituents reported in the genus *Ficus* are coumarins (Chunyan et al., 2009), furanocoumarin glycosides (Jain et al., 2013), Pheophytin/pheophorbide derivatives (Bafor et al., 2013), triterpene (Riaz et al., 2012), flavonoids and stilbenes (Sermboonpaisarn & Sawaadee, 2012), lactones (Shao et al., 2014), phenolics (Parveen et. al., 2014; Chen et. el., 2014), and isoflavones (Kuo and Li, 1997) and they have been used in folk medicine as antioxidant (Caliskan and Polat, 2011). Some of its species collected from different parts of the world viz. F. carica (Ali et al., 2012) has been reported to contain high antioxidant activity, F. ingens (Chauke et. al., 2012) and F. vasta (Al-Fatimi et al, 2007) showed mild antioxidant activity where as no antioxidant activity reported on F. final.

Psoralen is a naturally occurring furanocoumarin and was first discovered in *Psoralea corylifolia* and found to be active as anti-psoriatic (Zhang et. al., 2014; pavlotsky et al., 2014; Ahmed et al., 2013) and as anticancer (Wang et al., 2011; Wu et al., 2013; Jiang & Xiong, 2014) agent. It was determined in herbal drugs and poly herbal formulations by UPLC-MS/MS (Wang et al., 2014) and LC-MS/MS (Cao et al., 2008). The psoralen was reported to posses little activity against free radical reaction (Yi et al., 2013) but found in higher qunatity in the leaves of *Ficus carica* (Zaynoun et al., 1984) which was also supported by HPTLC estimation in the leaves of *Ficus carica* (Ali et al., 2011). The presence of psoralen in certain species of *Ficus* motivated the authors' establishment of a comparative profile of *Ficus* species for the psoralen content and their anti-oxidant potential. Therefore, the present study was planned to develop and validate a densitometric HPTLC method for comparative analysis of psoralen in the antioxidant active extract of leaves of five different species of genus *Ficus* grown in Kingdom of Saudi Arabia. The proposed method was validated as per ICH guideline (1996).

Materials and Methods

Materials

The leaves of *five* species of genus *Ficus* i.e. *F. Carica* (Sample 1), *F. nitida* (Sample 2), *F. ingens* (Sample 3), *F. palmata* (Sample 4) and *Ficus vasta* (Sample 5) were collected from the southern region of Kingdom of Saudi Arabia and authenticated by Dr. Mohammed Yusuf,

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Taxonomist, Medicinal Plant Collection and Survey Unit, Department of Pharmacognosy, College of Pharmacy, King Saud University, Kingdom of Saudi Arabia. Specimens of the plants were deposited in the herbarium of the Department of Pharmacognosy, College of Pharmacy, King Saud University, Kingdom of Saudi Arabia.

Apparatus and reagents

The standards psoralen and ascorbic acid were obtained from Sigma Aldrich. Analytical grade reagents and solvents (Toluene, Ethyl acetate, Chloroform and MeOH) were purchased from WINLAB and BDH (U.K.). Glass-backed silica gel $60F_{254}$ HPTLC precoated plates (20×10 cm) were purchased from Merck (Darmstadt, Germany). Chloroform solution of standard (0.1 mg/mL) and different extracts were applied to chromatographic plates bandwise, by means of a CAMAG automatic TLC sampler-4 (CAMAG, Muttenz, Switzerland) and developed in ADC2 (automatic development chamber) (CAMAG, Muttenz, Switzerland). TLC Plates were then documented by CAMAG TLC Reprostar 3 and scanned by CATS 4 (CAMAG).

Preparation of standard stock solution

Stock solution of standard (psoralen) (1 mg mL^{-1}) was prepared in chloroform. 1 ml of the stock solution was diluted in 10 mL chloroform to make the concentration 100ng/µL. For calibration, 1-8 µL standard solution was applied to a HPTLC plate to furnish concentration in the range 100-800 ng per band, respectively.

Preparation of Samples

The leaves of Samples 1-5, were air-dried and pulverised. 500 g of the powdered material were packed in muslin cloth and subjected to soxhlet extractor for continuous hot extraction with chloroform for 72 hrs. Thereafter chloroform extracts were filtered through Whatman paper no. 42 and the obtained extract was concentrated under reduced pressure and finally vacuum dried. The yield was 4.0%, 5.1%, 5.6%, 4.9% and 6.5 % w/w, respectively. Since marker compound was found to be soluble in chloroform hence the same was used for the extraction of samples.

Antioxidant activity

The antioxidant activity was carried out by scavenging of DPPH free radical as described by Brand-Williams et al. (1995). The DPPH free radical is reduced to corresponding hydrazine when it reacts with hydrogen donors. The DPPH radical is purple in color and upon reaction with hydrogen donor changes to yellow in colour. It is a discoloration assay, which is evaluated by the addition of the antioxidant to a DPPH solution in methanol or ethanol and decrease in absorbance is measured at 517nm. Various concentrations (10, 50 and 100 µg/ml) of the chloroform extract of leaves of Samples 1-5, were used. The assay mixtures contained in total volume of 100, 50 and 10 µL of the extract, 125 µL prepared DPPH and 375 µL solvent. Ascorbic acid was used as the positive control. After 30 min incubation at 25 °C, the decrease in absorbance was measured at λ=517 nm. The radical scavenging activity was calculated from the equation:

$$\% \text{ radical scavenging activity} = (A_{control} - A_{sample}) / A_{control}) \text{ X } 100$$

TLC instrumentation and chromatographic conditions:

Chromatographic analysis was done on (20 × 10 cm) HPTLC plates. Samples were applied as bands 6 mm wide and 7.7 mm apart by Linomat IV sample applicator. The application rate of sample on plate was 160 nL S^{-1}. The plates were developed in previously saturated 20 × 10 cm twin-trough glass chamber [at RT (25±2°C) and RH (60 ± 5%)]. Quantitative analysis was performed at wavelength 305 nm in absorbance/reflectance mode with CATS 4 operated by WinCATS software (Version 1.2.0).

Preparation of calibration graphs

Calibration graph for standard psoralen was prepared by applying a series of spots to standard with eight different concentrations so as to get different amount of psoralen per spot. They were prepared with respect to height and area vs amount per spot.

Method development

Chromatogram was developed for psoralen by selecting the mobile phase after trying several combinations of solvents. The best resolution was observed in the selected [(toluene: methanol (9:1, v/v/)] mobile phase. The same mobile phase has been employed for the separation of chloroform extracts of samples 1,2,3,4 and 5. The optimized saturation time was observed as 20 min. The densitometric analysis was performed at absorption maxima of wave length 305 nm in absorbance/reflectance mode.

Procedure for Method validation

Validation of the developed method has been carried out as per ICH guidelines for linearity, precision, accuracy, limits of detection (LOD) and quantification (LOQ), recovery studies and robustness.

Linearity range

For determining the linearity range of standard psoralen, a series of spots of different concentrations (100– 800 ng) of psoralen per band were applied to TLC plate. The plate was scanned and a curve was prepared with respect to height and area vs. amount per spot. The linearity data were analyzed statistically by linear regression analysis and lack of fit (LOF) test.

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Precision and accuracy

Precision (inter and intra-day) and accuracy of the assay were evaluated by performing replicate analyses (n = 6) of samples at three quality control (QC) levels i.e. low, medium and high of 150, 300 and 600 ng band^{-1}, respectively. Inter-day precision and accuracy were determined by repeating the intra-day assay on three different days. Precision was expressed as percentage coefficient of variation (% CV) of measured concentrations for each calibration level whereas accuracy was expressed as percentage recovery.

LOD and LOQ

The LOD and the LOQ were calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approaching the LOD according to the formulae: [LOD = 3.3 (SD/S) and LOQ = 10 (SD/S)]. The standard deviation of the response was determined based on the standard deviation of y-intercepts of regression lines.

Recovery studies

Recovery was studied by applying the method to drug samples to which known amounts of marker corresponding to 50%, 100%, and 150% of the psoralen had been added. Each level was analyzed in triplicates. This was to check the recovery of psoralen at different levels in the extracts.

Robustness

Robustness was studied in triplicate at 300 ng band^{-1} by making small changes to mobile phase composition, mobile phase volume, and duration of mobile phase saturation and activation of HPTLC plates. The results were examined in terms of percentage relative standard deviation (% RSD) and standard error (SE) of peak areas. Mobile phases prepared from toluene: methanol in different proportions (9:1, v/v; 9.5:0.5, v/v; 8.5:1.5, v/v; 10:0, v/v) were used for chromatography. Mobile phase volume and duration of saturation investigated were 20 ± 2 mL (18, 20, and 22 mL) and 20 ± 10 min (10, 20, and 30 min), respectively. The plates were activated at 110°c for 30 minutes before chromatography.

Assay of psoralen

Standard psoralen and test samples were spotted on HPTLC plate. The percentage of psoralen present in test samples (sample 1 to 5) was determined by measuring area for the standard and test samples. Thereby the percentage of psoralen was calculated for all the five samples of *Ficus* species.

Results

Antioxidant activity

The chloroform extracts of two plants namely, *F. palmata* (Sample 4) (93.8% and 96.7%) and *F. carica* (Sample 1) (56.6 % and 72.8%) exhibited a highly effective free radical scavenging activity in DPPH assay at somewhat low to moderate concentrations (50 and 100µg/ml), respectively. The other species i.e. *F. nitida* (Sample 2) (27% and 40.9%), *F. ingens* (Sample 3) (26.3% and 45.4%) and *F. vasta* (Sample 5) (11.5% and 28.2%) showed very mild antioxidant activity at low to moderate concentrations (50 and 100µg/ml) while the standard compound (ascorbic acid) showed 86.4% and 95.5% at 50 and 100µg/ml, respectively (Table 1). It is evident from the result that all five species of genus *Ficus* were active against free radicals but the species *F. palmata* and *F. carica* were found to be highly active. The antioxidant activities of *F. palmata* and *F. carica* were highly comparable with standard ascorbic acid as compared to other extracts tested. Hence, based on these results, we performed a comparative estimation of psoralen (an antioxidant compound) in the active extracts of different *Ficus* species by validated HPTLC method and to find out the presence and its effect in the free radical scavenging property of these species.

Method development

The developed method was found to be effective in separation of constituents present in the samples (1,2,3,4 and 5) (Fig1a & 1b) and exhibiting sharp peaks of standard (psoralen) as well, with the selected mobile phase under chamber saturation conditions at a wave length of 305 nm in absorbance/reflectance mode (Fig 2). A compact, symmetrical and high resolution band of psoralen was obtained at R_f 0.55±0.001 (Fig 3). The developed method was found to be quite selective with good baseline resolution. The identity of the bands of compounds in the sample extracts were confirmed by overlaying their absorption spectra with those of the standards (Fig 9).

Method validation

Linearity of compound psoralen was validated by linear regression analysis and LOF test. The eight-point calibration curve for psoralen was found to be linear in the range of 100-800 ng. Regression equation and r^2 for the psoralen were observed as Y= 4.516X+35.894 and 0.998, respectively, which revealed a good linearity response for developed method. The mean value with ±SD of the slope was 4.516±0.002 and intercept was 35.894±0.003 for psoralen (Table 2). No significant difference was observed in the slopes of standard plots ($P > 0.05$). The LOF test is usually used to evaluate whether the chosen regression model adequately fits the data (Loco et al., 2002). The results of linear regression analysis and LOF test are

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Table 1: Free radical-scavenging activity (DPPH-assay)

Treatment	Radical scavenging activity (%) ± SD		
	10 µg/ml	50 µg/ml	100 µg/ml
Ficus carica (Sample 1)	20.4 ± 1.7	56.6 ± 3.8	72.8 ± 5.3
Ficus nitida (Sample 2)	7.6 ± 0.5	27 ± 1.9	40.9 ± 3.0
Ficus ingens (Sample 3)	8.3 ± 0.6	26.3 ± 1.6	45.4 ± 3.1
Ficus palmata (Sample 4)	26.1 ± 1.5	93.8 ± 6.1	96.7 ± 6.5
Ficus vasta (Sample 5)	4.2 ± 0.3	11.5 ± 0.8	28.2 ± 2.2
Ascorbic acid (STD)	41.0 ± 3.1	86.4 ± 5.6	95.5 ± 6.0

Table 2: R_f, linear regression data for the calibration curve and sensitivity parameter for psoralen

Parameter	psoralen
R_f	0.55±0.01
Linearity range (ng band^{-1})	100-800
Regression equation	Y= 4.516X+35.894
Correlation coefficient	(r^2) 0.998
Slope±sd	4.516±0.002
Intercept±sd	35.894±0.003
Standard error of slope	0.004
Standard error of intercept	0.006
LOD	27 ng band^{-1}
LOQ	81 ng band^{-1}

Table 3: Data table for lack of fit test and linear regression analysis

Source	df	SS	MS	F value	P value
Regression	1	85337558	85337558	4555	<0.05
Residual	6	112408	18734		
Lack of fit	7	85000000	12000000	6.99	
Pure error	7	85449966	12207138		
Total	21	255899932	109563430		

Table 4: Precision and accuracy of psoralen

Nominal Concentration	psoralen Obtained (a, b)	precision (c)	Accuracy (d)
Intraday batch			
150	148.6	1.64	99.0
300	297.8	1.51	99.6
600	600.4	1.47	100.0
Interday batch			
150	149.1	1.86	99.4
300	299.5	1.72	99.8
600	598.6	1.63	99.7

A = Concentration in ng band^{-1}, b = Mean from six determination (n=6),
c = Precision as coefficient of variation (CV,%) = [(standard deviation) / (concentration found)] × 100, d = Accuracy (%) = [(concentration found)/(nominal concentration)] × 100

Table 5: Robustness of the method

Optimization condition	psoralen	
	SD	%RSD
Mobile phase from Toluene: MeOH		
(9:1, v/v; 9.5:0.5, v/v; 8.5:1.5, v/v; 8:2, v/v)	2.39	0.027
Mobile phase volume (18, 20 and 22 mL)	1.81	0.013
Duration of saturation (10, 20 and 30 min)	2.12	0.019
Activation of TLC plate (2, 5 and 7 min)	2.26	0.022

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Table 6: Recovery studies of psoralen

Concentration added to analyte (%)	Theoretical (ng)	Added (ng)	Detected (ng)	Recovery (%)	RSD (%)
	300				
50		450	445.5	99.0	1.36
100		600	595.8	99.3	1.13
150		750	747.0	99.6	1.02

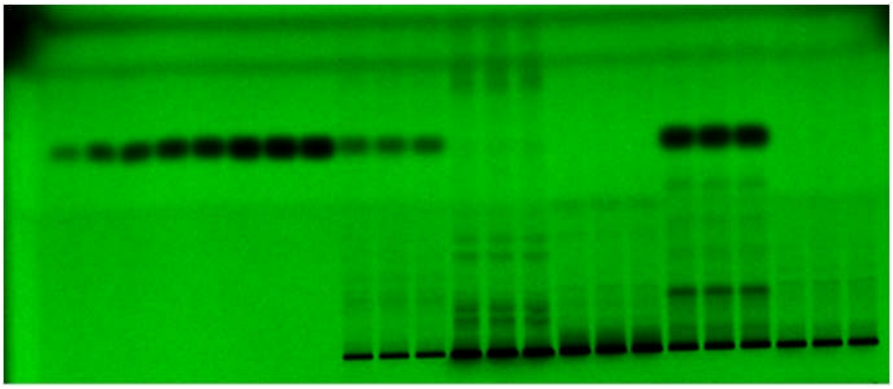

Figure 1a: Picture of developed TLC plate at 254 nm; mobile phase: Toluene: Methanol (9:1, v/v).

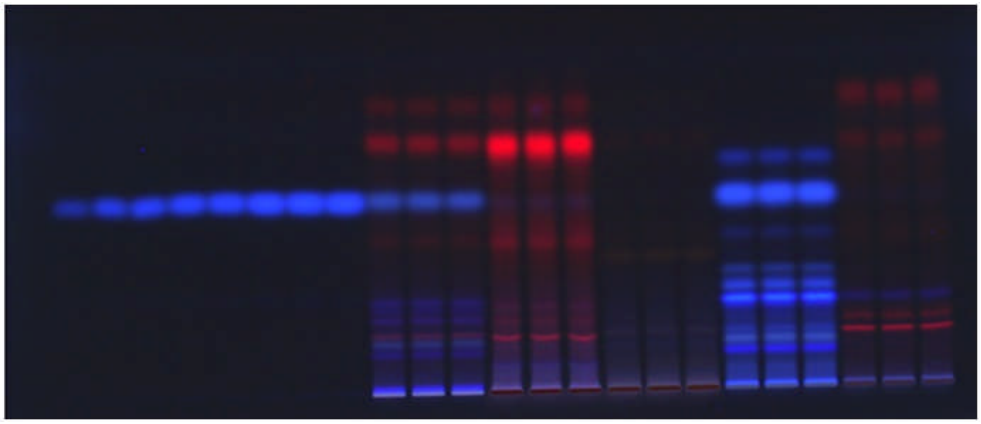

Figure 1b: Picture of developed TLC plate at 366 nm; mobile phase: Toluene: Methanol (9:1, v/v).

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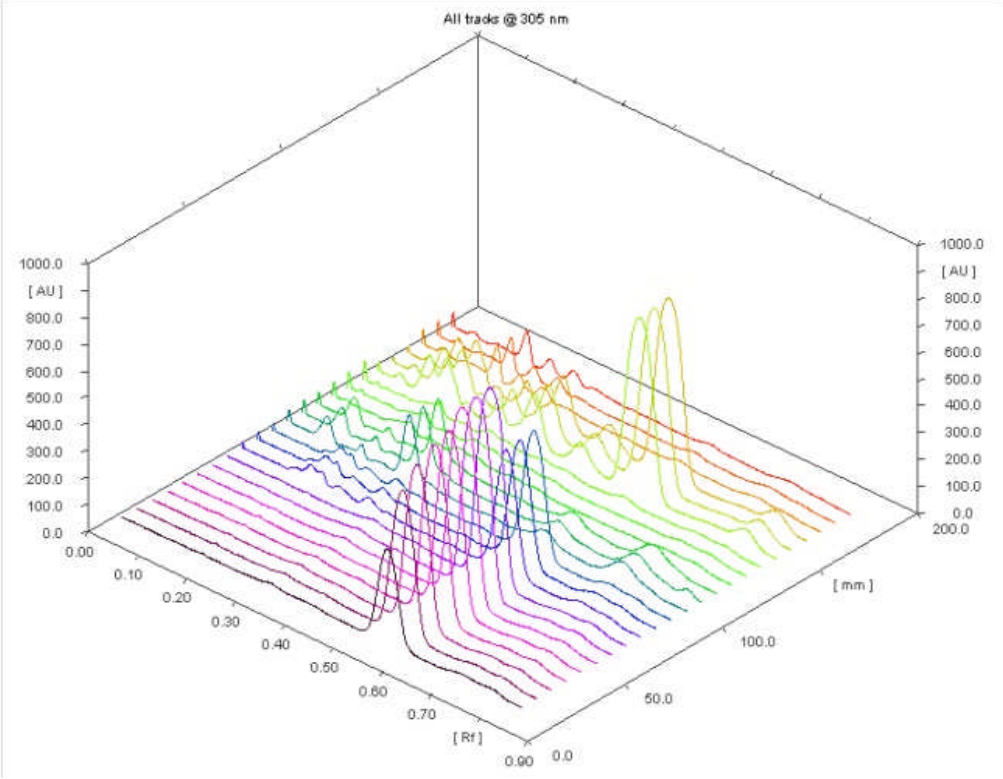

Figure 2: 3D-display of all tracks at 305 nm; mobile phase: Toluene: Methanol (9:1, v/v)

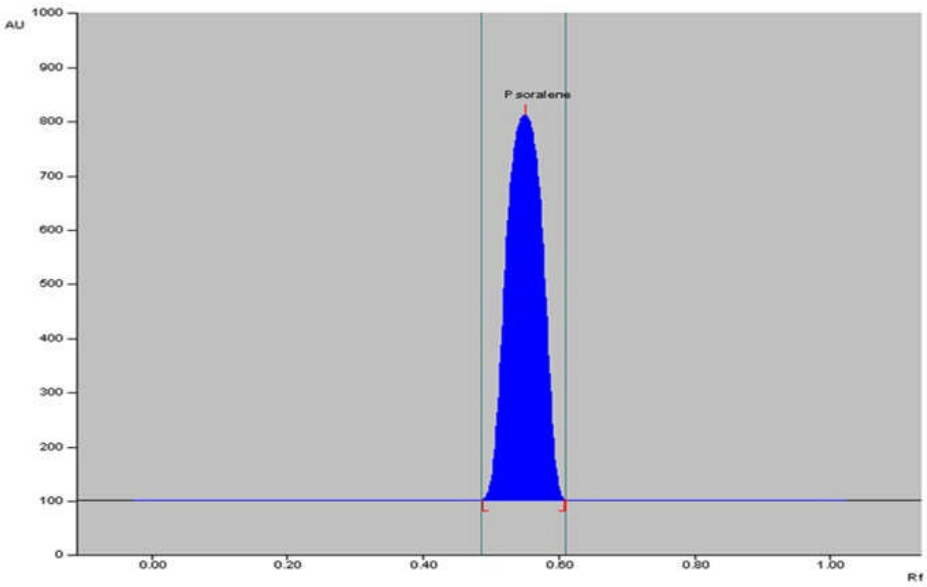

Figure 3: Chromatogram of standard Psoralen (800ng spot⁻¹), peak 1 (R_f = 0.55); mobile phase: Toluene: Methanol (9:1, v/v).

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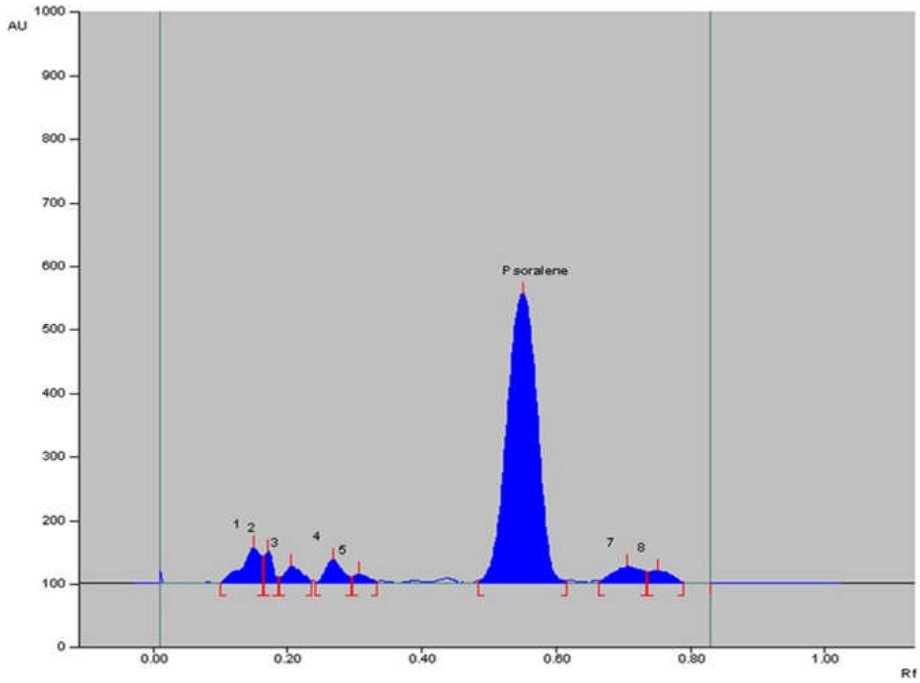

Figure 4: Chromatogram of sample 1 scanned at 305nm (psoralen; R_f = 0.55); mobile phase: Toluene: Methanol (9:1, v/v).

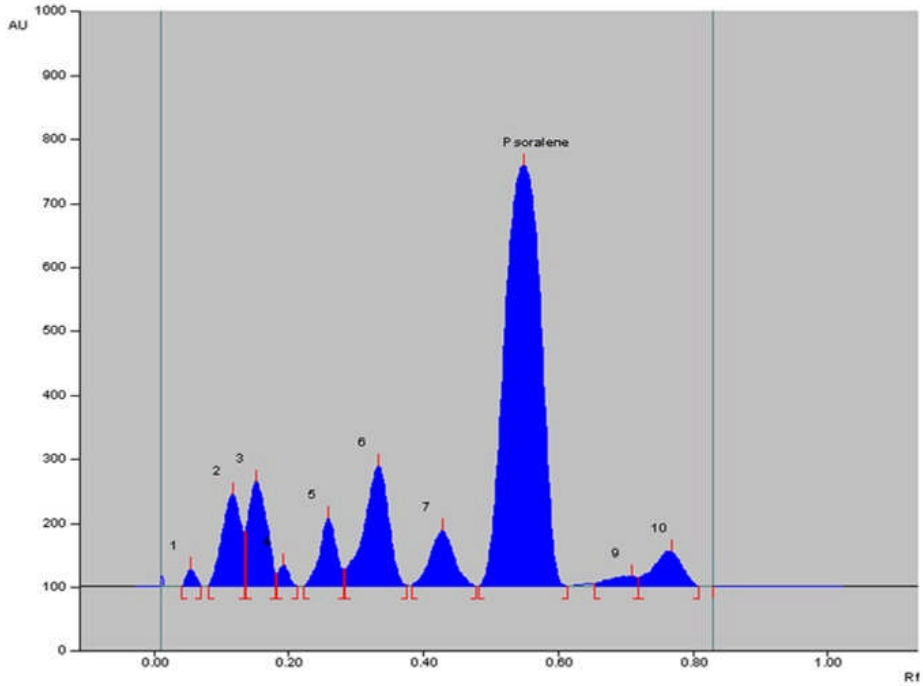

Figure 5: Chromatogram of sample 4 scanned at 305nm (psoralen; R_f = 0.55); mobile phase: Toluene: Methanol (9:1, v/v).

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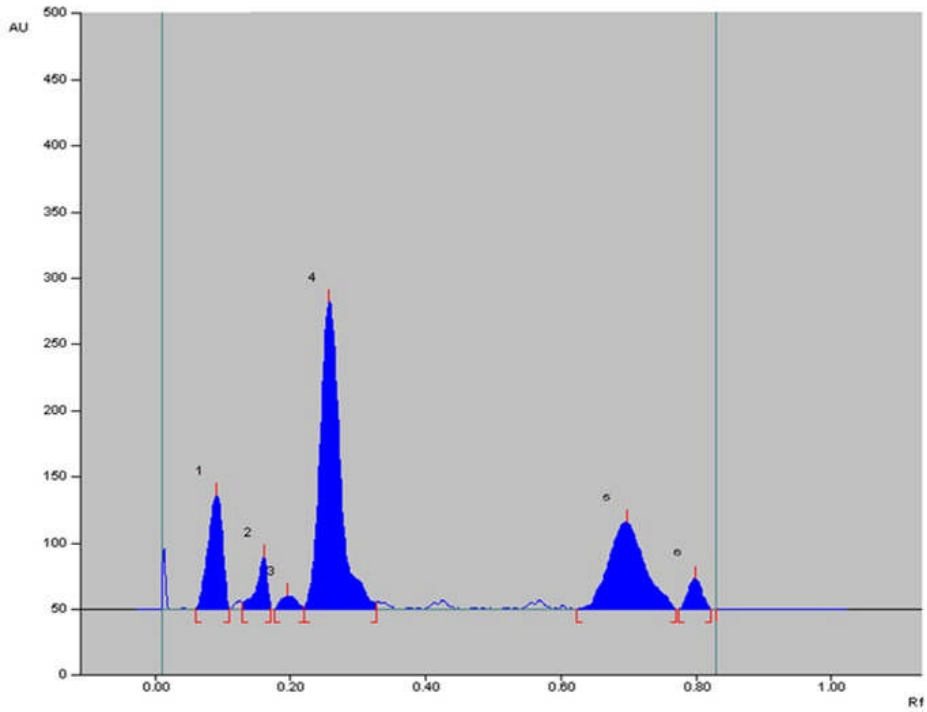

Figure 6: Chromatogram of sample 2 scanned at 305nm (psoralen is absent); mobile phase: Toluene: Methanol (9:1, v/v).

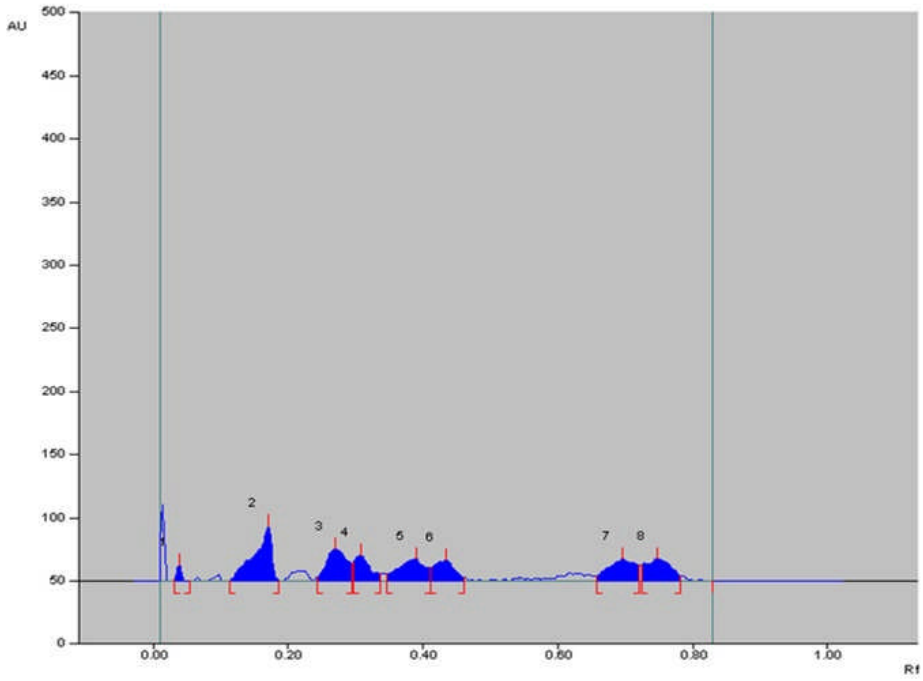

Figure 7: Chromatogram of sample 3 scanned at 305nm (psoralen is absent); mobile phase: Toluene: Methanol (9:1, v/v).

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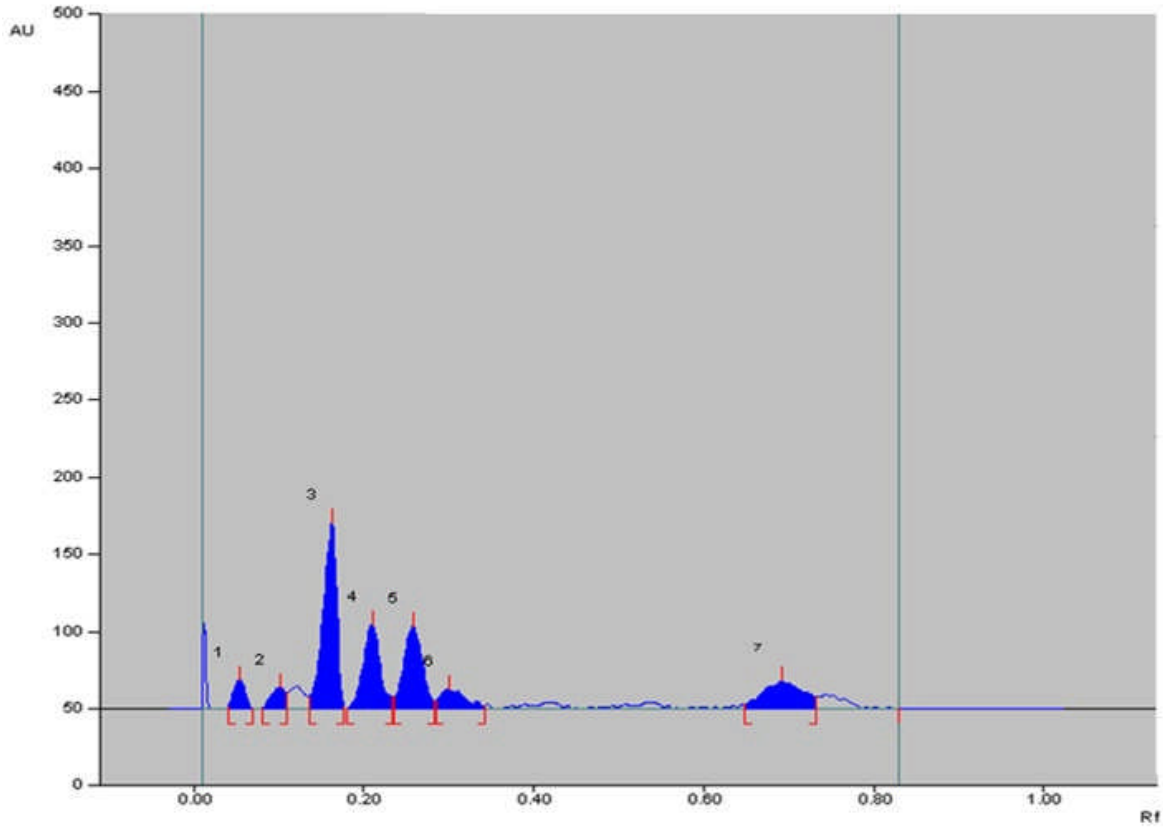

Figure 8: Chromatogram of sample 5 scanned at 305nm (psoralen is absent); mobile phase: Toluene: Methanol (9:1, v/v).

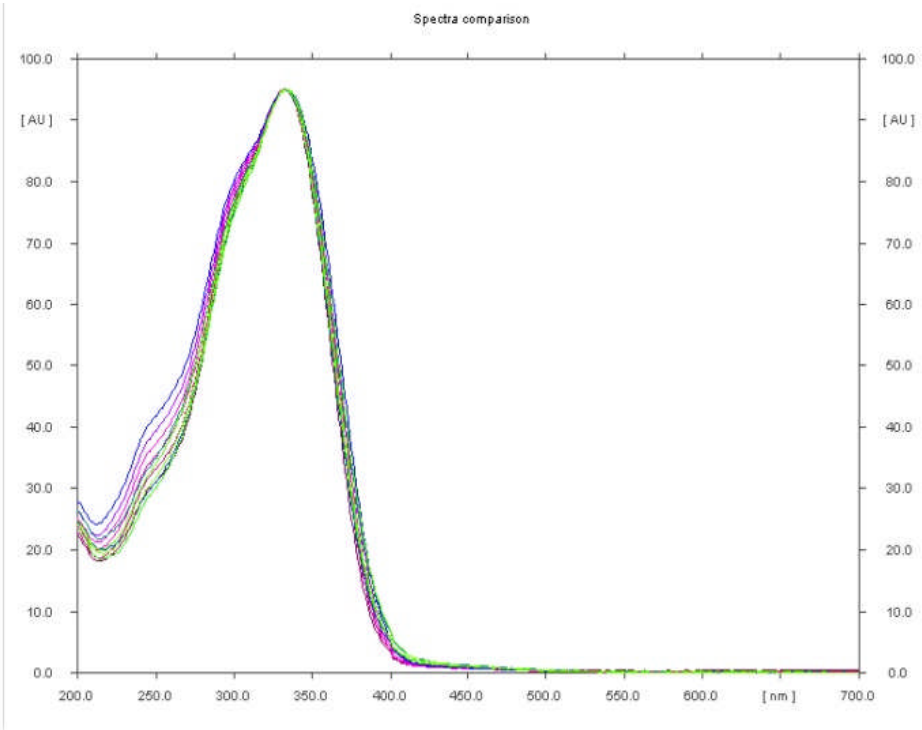

Figure 9: Spectral comparison at 305nm; mobile phase: Toluene: Methanol (9:1, v/v).

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listed in Table 3. High F value (45555) and low P value (P<0.05) for linear regression model indicated that this model adequately fits the data. However, the high values of sum of squares (SS) and F (6.99) for LOF test indicated that linear regression model was not adequate to fit the data. The SS value for pure error was also very high which again indicated there was lack of fit in the data. Hence, it is recommended to perform LOF test on each set of calibration. Table 4 presents intra-day and inter-day precision and accuracy of the assay for psoralen at three quality control (QC) levels (150, 300 and 600 ng band^{-1}). Intra-day and interday precisions (n = 6) for psoralen were found to be 1.47-1.64% and 1.63-1.86%, respectively, which demonstrated the good precision of proposed method. However, intra-day and interday accuracy of psoralen were observed as 99-100 % and 99.4-99.7 %, respectively. These results indicated the accuracy of the proposed method. The standard deviation (SD) and percentage relative standard deviation (% RSD) were also calculated at 300 ng band^{-1} concentration level of psoralen. The low value of SD and % RSD obtained after introducing small deliberate changes in the method indicate that the method was robust (Table 5). LOD and LOQ were found to be 27 and 81 ng band^{-1}, respectively (Table 2). This indicated that the proposed method exhibits a good sensitivity for the quantification of psoralen. Good recoveries were obtained by the fortification of the sample at three quality control levels of psoralen. It is evident from the results that the percent recoveries for psoralen after sample processing and applying were in the range of 99-99.6 % as shown in Table 6.

HPTLC analysis of prepared samples

The utility of the proposed method was evaluated by applying this method for the quantification of psoralen in samples 1, 2, 3, 4 and 5. Out of these five samples evaluated for the quantification of psoralen, only two samples i.e. sample 1 and sample 4 were found to contain psoralen (Fig 4 and 5). Psoralen was not found in the remaining three samples i.e. sample 2, 3 and 5 (Fig 6, 7 and 8). The content of psoralen in the sample 1 (*F. Carica*) and sample 4 (*F. palmata)* were found to be 0.24% ± 0.01% and 1.88% ± 0.03% w/w, respectively. The authors are privileged to perform this comparative estimation of psoralen for the first time in *above mentioned five* species of genus *Ficus* collected from Kingdom of Saudi Arabia by a validated HPTLC method. The outcomes of this experiment may be utilized to select the species having high content of psoralen (*F. palmata*) for herbal formulations. The formation of secondary metabolites in the plants is greatly affected by extrinsic factors (eg. climate, altitude, soil pH etc.) as well as intrinsic factors (eg. age, gender, genotype etc.), which are most often beyond our control. To compensate the effect of these external and internal factors on the production of secondary plant metabolites, the developed HPTLC method was found to be an important analytical technique for separation, detection, identification and quantification of psoralen in this experiment.

Discussion

In this experiment two species (*F. palmata* and *F. carica)* of genus *Ficus* showed good antioxidant activity in the DPPH assay in comparison to other three species i.e. *F. nitida, F. ingens* and *F. vasta*. Quantification of psoralen in all the antioxidant active species by a validated HPTLC method revealed that the psoralen was present only in two species i.e. *F. palmata* and *F. carica* and it was completely absent in *F. nitida, F. ingens* and *F. vasta*. The above finding suggested that the presence of psoralen in *F. palmata* and *F. carica* might be responsible for their high antioxidant property when compared with other three species. Biogenetically coumarins are formed from shikimate pathway and the absence of psoralen in above three species may be due to some interruption in this pathway. To confirm this biogenetic error some other shikimate derivatives need to be quantified in all the five species of *Ficus* and a comparative study can give some conclusion. The HPTLC method developed in this experiment can be used as a rational approach for quality control of natural products in more scientific and efficacious way. This HPTLC method may be applied to study the degradation kinetics of psoralen and its determination in plasma and other biological fluids. The findings of this experiment may also encourage the researchers to work on *Ficus* species for isolation of different antioxidant compounds and their possible mechanisms.

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