Estimation of Germanicol by Validated HPTLC Densitometric Method in Leaves of Different Species of Genus *Ficus*

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A simple and sensitive HPTLC method was developed for estimation of germanicol in the leaves of five different species of genus *Ficus* (*Ficus carica*, *Ficus nitida*, *Ficus ingens*, *Ficus palmata* and *Ficus vasta*) grown in Kingdom of Saudi Arabia. The chromatography was performed on glass-backed silica gel 60 F_{254} HPTLC plates with solvents hexane: chloroform (55:45, v/v) as mobile phase. After the development of HPTLC plate it was derivatized with *p*-anisaldehyde reagent to give well resolved and compact spot of germanicol. The developed method was found to give a compact spot for germanicol ($R_f = 0.39 \pm 0.01$) at 525 nm. Germanicol was found to be present in four species *i.e.*, *F. carica* (0.21%, w/w), *F. nitida* (0.22%, w/w), *F. palmata* (1.06%, w/w) and *F. vasta* (0.27%, w/w) while it was absent in the *F. final*. The proposed method can be used in study of degradation kinetics of germenicol and its determination in plasma.

Keywords: *Ficus* species, Leaves, Germanicol, HPTLC, Validation.

INTRODUCTION

The genus *Ficus* includes about 800 species[1] of woody trees, shrubs and vines and their fruits generally known as figs, distributed widely in tropical and subtropical regions[2]. The common fig (*Ficus carica*) originated in the Old World Tropics-Asia Minor and the Mediterranean region. Figs are oblong or pear-shaped syconium fruit of a cultivated tree of the mulberry (Moraceae) family. Figs have been used as a sweetener for thousands of years and are good from nutrition point of view and health benefits[3]. Though figs are seasonal fruits but they are available throughout the year in dried form. Fresh figs, contain poly-phenolic flavonoids whose antioxidant property is comparable to that of apples at 3200 µmol/100 g. From ancient time figs were used as food and traditional medicine, contains laxative substances, flavonoids, sugars, vitamins A and C, acids and enzymes[4]. Some other important phytoconstituents reported in the genus *Ficus* are coumarins[5], furocoumarin glycosides[6], isoflavones[7], lignans[8], sterols and/or terpenes[9] and chromone[10]. *Ficus* species have been used in folk medicine as anticancer and antiinflammatory[11], antiepileptic[12], antioxidant[13], antihepatotoxic[14], gastroprotective[15], antidiabetic[16] and as antimalarial[17]. Dried figs are concentrated sources of energy (100 g dried figs provide 249 calories)[18].

Germanicol is a pentacyclic triterpene occurs in numerous plants[19-21]. It was reported to posses antibacterial[22], antifungal[23] and anti-inflammatory[24] activities. Extensive literature survey revealed that not much analytical methods have been tried for the identification as well as quantification of germanicol in plant extracts and in formulations. Only one method reported for its identification by GC/MS[25]. Also a validated HPTLC method is not yet reported for the quantification of germanicol in different species of genus *Ficus* grown in Kingdom of Saudi Arabia. Therefore, the aim of present study was to develop and validate a sensitive and cost effective HPTLC densitometric method for the quantitation of germanicol in methanol extracts of leaves of five above mentioned of species *Ficus* grown in Kingdom of Saudi Arabia. The proposed method was validated as per ICH guideline, 1996[26].

EXPERIMENTAL

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 Southern region of Kingdom of Saudi Arabia and authentified by Dr. Mohammed Yusuf, Field 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 Department of Pharmacognosy, College of Pharmacy, King Saud University, Kingdom of Saudi Arabia.

Germanicol (standard) was obtained from Sigma Aldrich. Analytical grade reagents and solvents (toluene, methanol) were purchased from Winlab and BDH (U.K.). Glass-backed Silica gel 60 F_{254} HPTLC plates (20 × 10 cm) were purchased from Merck (Darmstadt, Germany). Methanol solution of standard 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 (germanicol) (1 mg mL^{-1}) was prepared in choloroform. 1 mL of the stock solution was diluted in 10 mL choloroform to make the concentration 100 ng/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 sample 1, sample 2, sample 3, sample 4 and sample 5 were air-dried and pulverized. 500 g of the powdered material were packed in muslin cloth and subjected to soxhlet extractor for continuous hot extraction with methanol for 72 h. Thereafter methanol 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 , 5.1, 5.6, 4.9 and 6.5 % w/w, respectively.

TLC instrumentation: Chromatographic analysis was done on (20 cm × 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 room temperature (25 ± 2 °C) and relative humidity (60 ± 5 %)]. The plates were dried at room temperature and then derivatized with *p*-anisaldehyde reagent and heated to identify compact bands.

Preparation of calibration graphs: Calibration graph for standard (germanicol) was prepared by applying a series of spots of germanicol with eight different volumes so as to get different amount of germanicol per spot. They were prepared with respect to area *vs.* amount per spot.

Method development: Chromatogram was developed for germanicol by selecting the mobile phase after trying several combinations of solvents. The best resolution was observed in the selected [hexane: chloroform (55:45, v/v)] mobile phase. The same mobile phase has been employed for the separation of methanol 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 525 nm in absorbance/reflectance mode with CATS 4 operated by WinCATS software (Version 1.2.0).

Method validation: Method validation was carried out as per international conference on harmonization (ICH) guidelines for linearity range, precision, accuracy, robustness, LOD, LOQ and recovery.

Precision (inter and intraday) 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. Interday precision and accuracy were determined by repeating the intra-day assay on three different days. Precision was expressed as per cent coefficient of variation (% CV) of measured concentrations for each calibration level whereas accuracy was expressed as percentage recovery.

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 TLC plates. The results were examined in terms of per cent relative standard deviation (% RSD) and standard error (SE) of peak areas. Mobile phases prepared from hexane: chloroform in different proportions (55:45, v/v; 50:40, v/v; 53:47, v/v; 58:42, 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 min before chromatography.

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

RESULTS AND DISCUSSION

The developed method was found to be effective in separation of constituents present in the samples (1, 2, 3, 4 and 5) and exhibiting sharp peaks of standard (germanicol) as well, with the selected mobile phase under chamber saturation conditions at a wavelength of 525 nm in absorbance/reflectance mode (Fig. 1). Compact, symmetrical and high resolution bands of germanicol were obtained at R_f 0.39 ± 0.001 (Fig. 2).
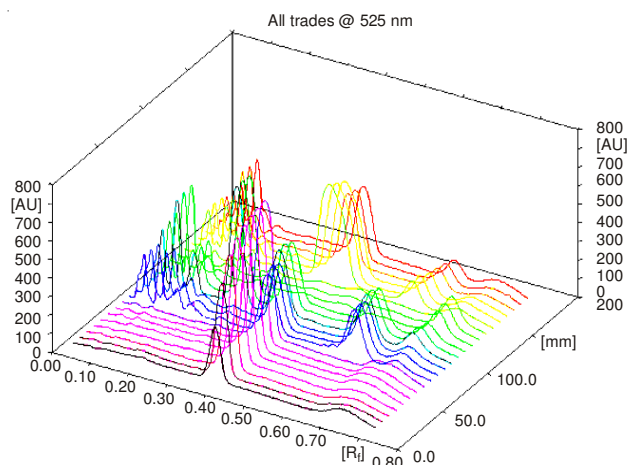

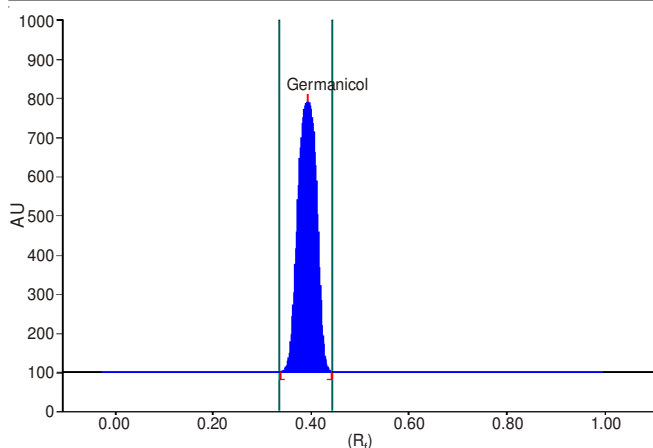Fig. 1.　3D-display of all tracks at 525 nm; mobile phase: hexane: chloroform (55:45, v/v)

Fig. 2. Chromatogram of standard germanicol (800 ng spot^{-1}, R$_f$ = 0.39), peak 1; mobile phase: hexane: chloroform (55:45, v/v)

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. 3).
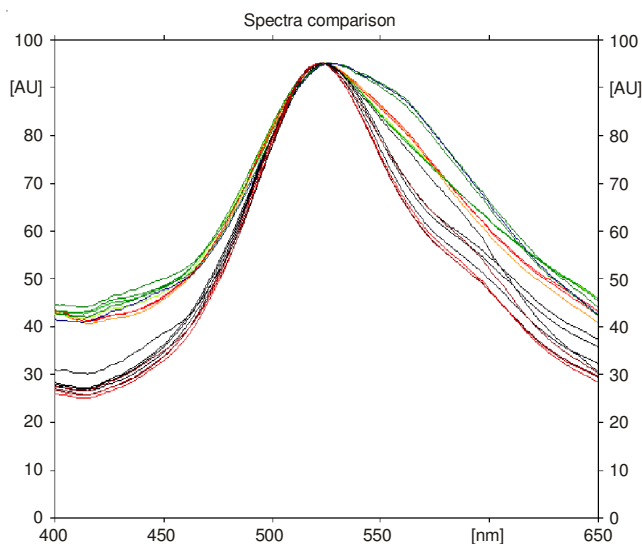

Fig. 3. Spectral comparison at 525 nm; mobile phase: hexane: chloroform (55:45, v/v)

Method validation: Linearity of germanicol was validated by the linear regression equation and correlation coefficient. The eight-point calibration curve for germanicol was found to be linear in the range of 100-800 ng. Regression equation and r^2 for the reference compound were observed as Y = 2.895X + 29.694 and 0.998, respectively which revealed a good linearity response for developed method (Table-1). The mean value with ±SD of the slope was 2.895 ± 0.004 and intercept was 29.694 ± 0.003 for germanicol. No significant difference was observed in the slopes of standard plots (P > 0.05). Table-2 presents intra-day and inter-day precision and accuracy for the assay of germanicol at three quality control (QC) levels (150, 300 and 600 ng band^{-1}). Intra-day and inter-day precisions (n = 6) for germanicol were found to be 1.46-1.63 and 1.61-1.84 %, respectively, which demonstrated the good precision of proposed method. However, intra-day and

interday accuracy of germanicol were observed as 99.3-99.9 and 99.2-99.6 %, respectively. These results indicated the accuracy of the proposed method. The standard deviation (SD) and per cent relative standard deviation (% RSD) were also calculated at 300 ng band^{-1} concentration level of germanicol. The low value of SD and % RSD obtained after introducing small deliberate changes in the method indicate that the method was robust (Table-3). LOD and LOQ were found to be 33 and 99 ng band^{-1}, respectively (Table-1). This indicated that the proposed method exhibits a good sensitivity for the quantification of above compound. Good recoveries were obtained by the fortification of the sample at three quality control levels of germanicol. It is evident from the results that the per cent recoveries for germanicol after sample processing and applying were in the range of 99.3-99.8 % as shown in Table-4.

HPTLC analysis of prepared samples: The utility of the proposed method was evaluated by applying this method for the quantification of germanicol in the samples 1, 2, 3, 4 and 5. Out of these five samples evaluated for the quantification of germanicol, four samples *i.e.*, sample 1 (*F. carica*), sample

TABLE-1
R$_f$, LINEAR REGRESSION DATA FOR THE CALIBRATION CURVE AND SENSITIVITY PARAMETER FOR GERMANICOL

Parameter	Germanicol
R$_f$	0.39 ± 0.01
Linearity range (ng band^{-1})	100-800
Regression equation	Y = 2.895X + 29.694
Correlation coefficient	(r^2) 0.998
Slope±sd	2.895 ± 0.004
Intercept±sd	29.694 ± 0.003
Standard error of slope	0.004
Standard error of intercept	0.003
LOD	33 ng band^{-1}
LOQ	99 ng band^{-1}

TABLE-2
PRECISION AND ACCURACY OF GERMANICOL

Nominal concentration	Germanicol obtained (a, b)	Precision (c)	Accuracy (d)
Intra-day batch			
150	148.9	1.63	99.3
300	297.8	1.49	99.6
600	599.4	1.46	99.9
Inter-day batch			
150	148.8	1.84	99.2
300	298.2	1.70	99.4
600	597.6	1.61	99.6

^{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-3
ROBUSTNESS OF THE METHOD

Optimization condition	Germanicol	
	SD	% RSD
Mobile phase from hexane: chloroform (55:45, v/v; 50:40, v/v; 53:47, v/v; 58:42, v/v)	2.47	0.022
Mobile phase volume (18, 20 and 22 mL)	1.79	0.011
Duration of saturation (10, 20 and 30 min)	2.15	0.016
Activation of TLC plate (2, 5 and 7 min)	2.34	0.019

TABLE-4
RECOVERY STUDIES OF GERMANICOL

Concentration added to analyte (%)	Theoretical (ng)	Added (ng)	Detected (ng)	Recovery (%)	RSD (%)
–	300	–	–	–	–
50	–	450	446.8	99.3	1.32
100	–	600	597.0	99.5	1.14
150	–	750	748.5	99.8	1.07

2 (*F. nitida*), sample 4 (*F. palmata*) and sample 5 (*Ficus vasta*) were found to contain germanicol (Figs. 4-7) while it was completely absent in sample 3 (*F. ingens*) (Fig. 8). The content of germanicol in the *F. carica*, *F. nitida*, *F. palmata* and *F. vasta* were found to be 0.21 ± 0.01 %, 0.22 ± 0.03 %, 1.06 ± 0.01 %, 0.27 ± 0.01 %, w/w, respectively. The authors are privileged to perform this comparative estimation of germanicol for the first time in five above mentioned species of genus *Ficus* collected from Kingdom of Saudi Arabia by a validated HPTLC method. The findings of this experiment may prove
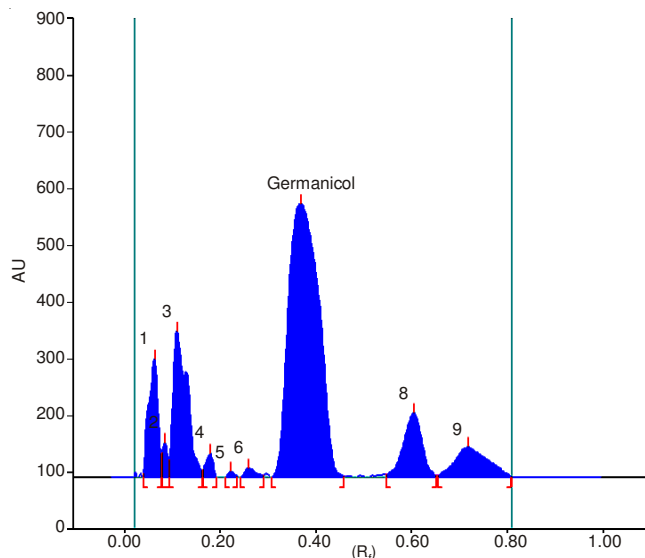

Fig. 6.   Chromatogram of sample 4 scanned at 525 nm (Germanicol; R_f = 0.39); mobile phase: hexane: chloroform (55:45, v/v)
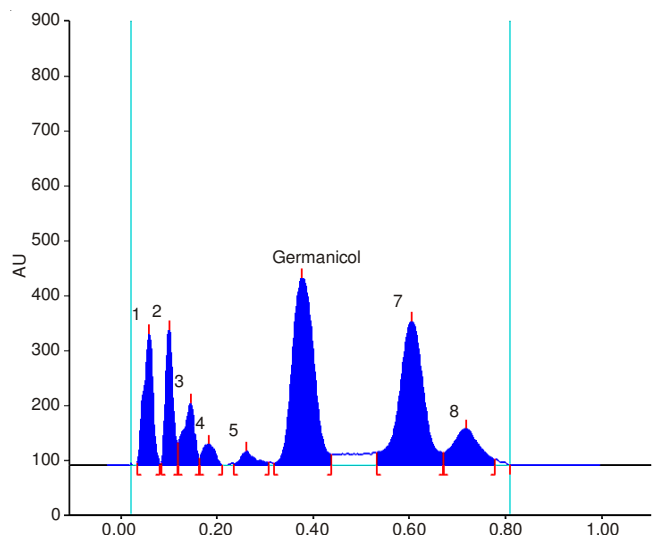

Fig. 4.   Chromatogram of sample 1 scanned at 525 nm (germanicol; R_f = 0.39); mobile phase: hexane: chloroform (55:45, v/v)
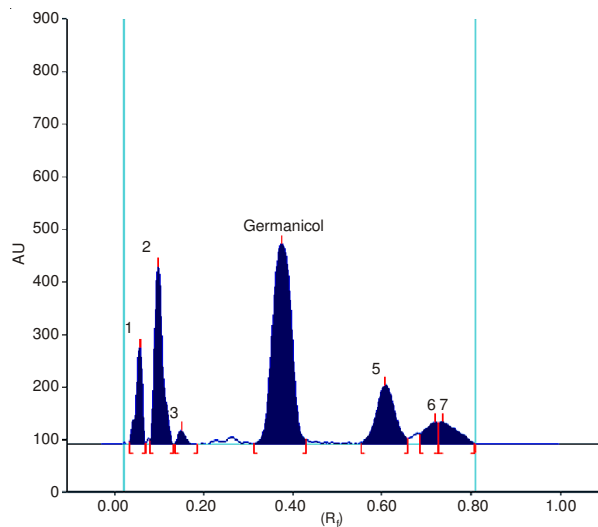

Fig. 7.   Chromatogram of sample 5 scanned at 525 nm (germanicol; R_f = 0.39); mobile phase: hexane: chloroform (55:45, v/v)
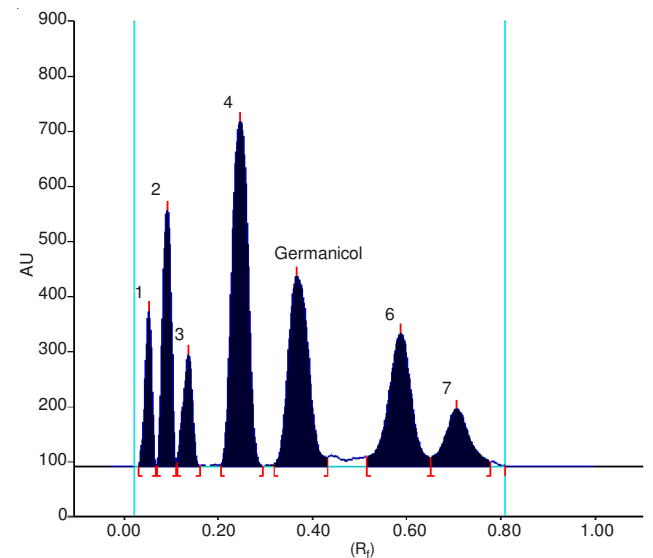

Fig. 5.   Chromatogram of sample 2 scanned at 525 nm (germanicol; R_f = 0.39); mobile phase: hexane: chloroform (55:45, v/v)
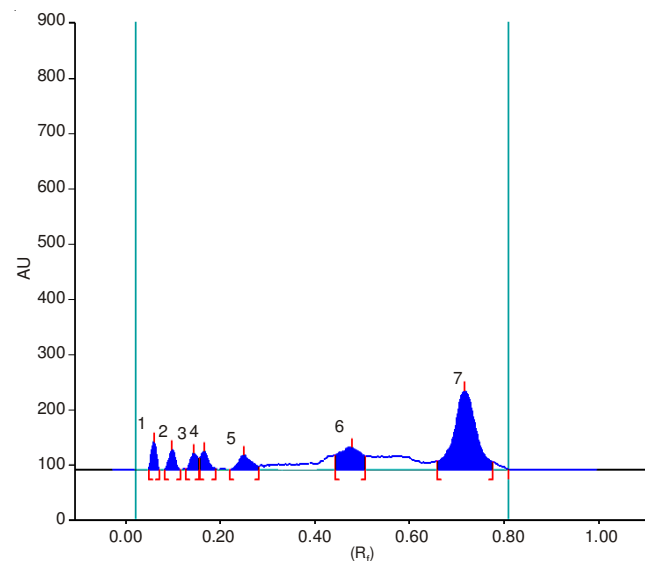

Fig. 8.   Chromatogram of sample 3 scanned at 525 nm (germanicol is absent); mobile phase: hexane: chloroform (55:45, v/v)

the existence of chemotypes present in genus *Ficus* that may be due to some external or internal factors. The outcomes of this experiment may be utilized to select the species having high content of germanicol (*F. palmata* and *F. vasta*) for herbal formulations and also to assure the content uniformity in other crude drug materials possessing germanicol.

Triterpenoids are secondary metabolites and plays a vital role in health care system. There are two known major pathways for the biosynthesis of the isoprene unit, one based on mevalonic acid and 1-deoxyxylulose[27]. The formation of secondary metabolites in the plants is greatly affected by extrinsic (*e.g.*, climate, altitude, soil pH *etc.*) as well as intrinsic factors (*e.g.*, 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 germanicol in this experiment.

Conclusion

In recent years, because of its several benefits like low operation cost, high sample throughput and need for minimum sample clean up, the high performance thin layer chromatography (HPTLC) has become a conventional analytical approach for the quality control of herbal drugs[28]. It is widely used for the identification, 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 and nutrients)[29]. Cancer is the most dreaded disease in the world as well as in Kingdom of Saudi Arabia and causes large number of deaths every year. It is now a well established fact that the herbal drugs having antiinflammatory potential may play a role to inhibit cancer[30] and it is also reported that the *Ficus* species were used traditionally as an anticancer drug[31]. Since, germanicol has good anti-inflammatory effect[24], it can also be active against cancer. The detection of germanicol in the extracts of four species of *Ficus* in this experiment justifies the use of *Ficus* as anti-cancer drug in folk medicines. Further studies can be designed to explore the mechanism of action, its bioavailability (concentration in blood plasma) and degradation kinetics of germanicol. The above proposed HPTLC method can also be employed for exploration of germanicol in chemotaxonomically related genera of the plant kingdom.

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