Densitometric validation and analysis of biomarker β-amyrin in different *Acacia* species (leaves) grown in Kingdom of Saudi Arabia by high performance thin-layer chromatography

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Abstract: Biomarker β-amyrin was analyzed in the leaves of four different *Acacia* species (*A. salicina, A. loreta, A. hamulosa* and *A. tortilis*) grown in Kingdom of Saudi Arabia by a validated HPTLC method. The chromatography was performed on glass-backed silica gel 60 F_{254} HPTLC plates using solvents toluene: methanol (9:1, v/v) as mobile phase. The developed TLC plate was derivatized with anisaldehyde and scanned at 520nm. A sharp peak of β-amyrin was found at R_f=0.58±0.01. The r^2 and the linear regression equation for β-amyrin was found to be 0.991 and 19.913X+107.803, respectively in the concentration range of 100-800ng. The percentage of β-amyrin was found to be maximum 2.70% w/w in *A. tortilis*, 1.85% w/w in *A. loreta* and 1.80% w/w in *A. hamulosa* while it was totally absent in *A. salicina*. This study conceives maiden reporting of quantification of β-amyrin in four different species of *Acacia* by validated HPTLC method. The developed method for the analysis of β-amyrin was proved to be reproducible by statistical analysis hence it can be employed for further analysis of β-amyrin in plasma, other biological fluids and in finished products available in the market.

Keywords: *Acacia* species, β-amyrin, HPTLC, validation.

INTRODUCTION

Genus *Acacia* (Fabaceae) comprising 1350 species, distributed widely in warmer, drier parts of the World, mainly in Arabian peninsula, Australia and Africa (CSIR, 1948). Most *Acacia* species has yellow or cream-colored flowers but some has white, purple (*A. purpureopetala*) or red colour flowers (*A. leprosa*). Several *Acacia* species has medicinal and pharmaceutical applications like *A. nilotica,* which possesses antituberculer (Ali *et al*., 2012) and antimicrobial properties (Saini *et al*., 2008). A. *nilotica* was found to contain large amounts of saponins and *A. seyal* contain large amounts of phenolics, flavonoids and anthocyanins which might help these plants to be effective in the treatment of several diseases (Abdel-Farid *et al*., 2007). Isolation of large number of phenolics and flavonoidal compounds by RP-HPLC from *A. confusa*, indigenous to Taiwan makes this plant highly antioxidant in nature (Wu JH, 2005). Avicins (triterpenoid saponins) isolated from *A. victoriae* inhibit tumor cell growth and also been found to prevent chemical-induced carcinogenesis in mice (Haridas, 2001). Another species *A. salicina* was reported to be effective as antimicrobial (Boubaker, 2012), Antimutagenic and antioxidant (Boubaker *et al*., 2011; Chatti *et al*., 2011), antiproliferative (Chatti *et al*., 2009). Isorhamnetin 3-O-neohesperidoside was found to be a potent xanthine oxidase and super oxide anion scavenger's inhibitor was isolated from the leaves of *A. salicina*. It also effectively inhibited the genetoxicity causing mediators nifuroxazide and aflatoxine B1 (AFB1), which proves the cell protecting ability of *A. salicina* leaves against oxidative stress (Bouhlel *et al*., 2010). *A. tortilis* yielded 1, 3-diaryl-propan-2-ol derivatives which exhibited smooth muscle relaxant property (Hagos *et al*., 1987) and polysaccharides which exhibited a α-d-glucosidase inhibitory property and makes *A. tortilis* an useful plant for the treatment of diabetes mellitus and its complications (Bisht *et al*., 2013).

β-amyrin (fig. 1) was reported to be isolated from *A. tortilis (*Bisht *et al*., 2013; Hasan *et al.,* 2002), *A. visco* (Pedernera *et al*., 2010) and also in some other *Acacia* species (Ayoub, 1982; Anderson and McDougall, 1987). It was found to be useful as anti-inflammatory agent (Melo *et al*., 2010), as antimicrobial agent ((Jabeen *et al*., 2011), as anticancer (Lin *et al*., 2011) and inhibits collagen-induced platelet aggregation (Ching *et al*., 2010). Several chromatographic methods like HPLC-DAD (Karoune *et al*., 2015), LC-ESI-MS/MS (Maldini, 2011), GC-MS (Negi and Dave, 2010) and HPTLC (Leela *et al*., 2011; Jyoti *et al*., 2014) used for the isolation and analysis of several bioactive phytoconstituents in different *Acacia* species. As per the exhaustive literature work we did not find any validated HPTLC method reporetd for the quantitative analysis of biomarker β-amyrin in different *Acacia* species.

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MATERIALS AND METHODS

Materials

The leaves of four species of genus *Acacia* i.e. *A. salicina* (Sample 1) (Voucher no. 15007), *A. loreta* (Sample 2) (Voucher no. 15081), *A. hamulosa* (Sample 3) (Voucher no. 16221) and *A. tortilis* (Sample 4) (Voucher no. 14977) were collected from eastern region of Kingdom of Saudi Arabia and authentified by Dr. Mohammed Yusuf (Taxonomist), Pharmacognosy Department, Pharmacy College, KSU, Saudi Arab. Plant Specimens were deposited in the Department of Pharmacognosy, College of Pharmacy, King Saud University, Kingdom of Saudi Arabia.

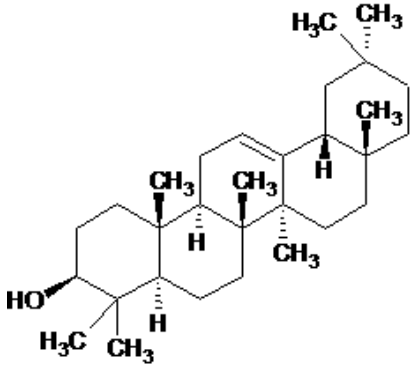

Fig. 1: Hence in this work we tried to develop and validate a HPTLC method for analysis of β-amyrin in different *Acacia* species leaves collected from eastern region of Saudi Arab.

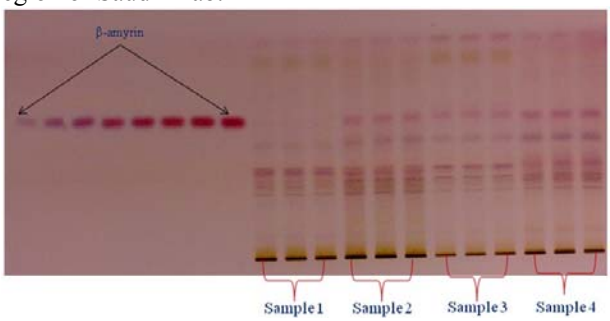

Fig. 2a: Picture of developed TLC plate derivatized with p-anisaldehyde reagent at day light; mobile phase: Toluene: Methanol (9:1)

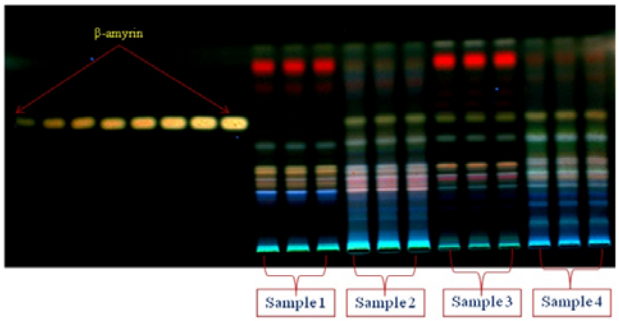

Fig. 2b: Picture of developed TLC plate derivatized with p-anisaldehyde reagent at 366 nm; mobile phase: Toluene: Methanol (9:1)

Apparatus and reagents

β-amyrin (standard) was procured from Sigma Aldrich (St. Louis, MO, USA). Toluene and Methanol (AR grade) were procured from BDH (U.K.). The HPTLC plate (Glass-backed silica gel $60F_{254}$) was procured from Merck (Germany). The standard and different extracts were applied on HPTLC plates band wise by CAMAG ATS-4 (automatic TLC sampler-4) and the HPTLC plate was then developed in ADC2 (automatic development chamber, CAMAG). The developed TLC plate was then scanned by CATS 4 (CAMAG) and documented by CAMAG TLC Reprostar 3.

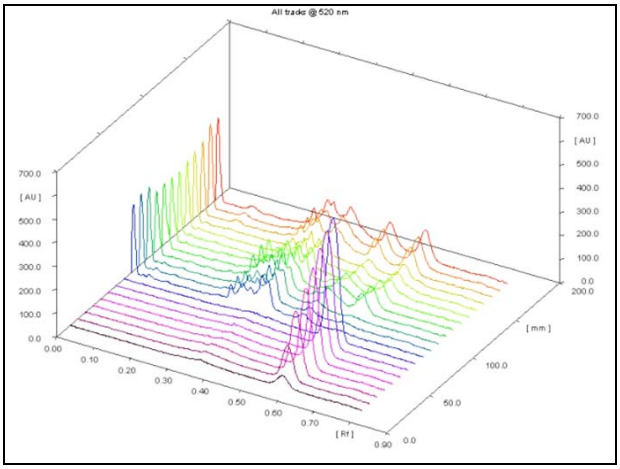

Fig. 3: 3D-display of all tracks at 520 nm; mobile phase: toluene: methanol (9:1)

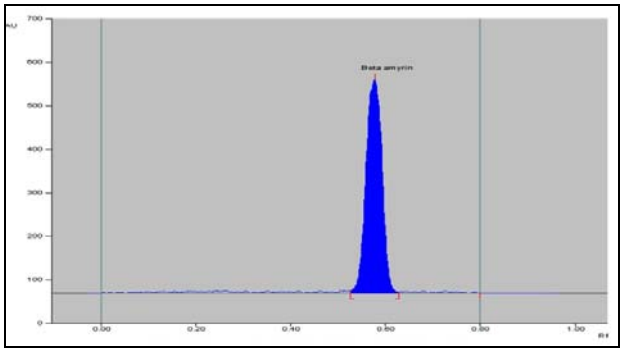

Fig. 4: Chromatogram of standard β-amyrin (700 ng spot^{-1}), Peak 1 (R_f=0.58); mobile phase: toluene: Methanol (9:1)

Preparation of standard stock solution

Stock solution of standard (β-amyrin) (1.0mg mL^{-1}) was prepared in methanol. Different dilutions of standard solution (100, 200, 300, 400, 500, 600, 700 and 800 ng/μL) were prepared in volumetric flask by using appropriate volume of stock solution and diluting it with methanol. For calibration, 1μL of each dilution of standard solution were applied to a HPTLC plate to furnish concentration in the range of 100, 200, 300, 400, 500, 600, 700 and 800ng per band, respectively.

Preparation of samples

The leaves of Samples 1-4 were dried and coarsely powdered. 250g of each powdered material was packed in the soxhlet extractor and extracted with methanol for 6 hours on a heating mantle. After the complete extraction of plant material the methanol extract was filtered by the use of what man paper no. 42. The filtered extract was then concentrated under reduced pressure by using rotavapour and finally dried. The yields were 4.5%, 5.6%, 5.8% and 6.2% w/w for samples 1-4, respectively.

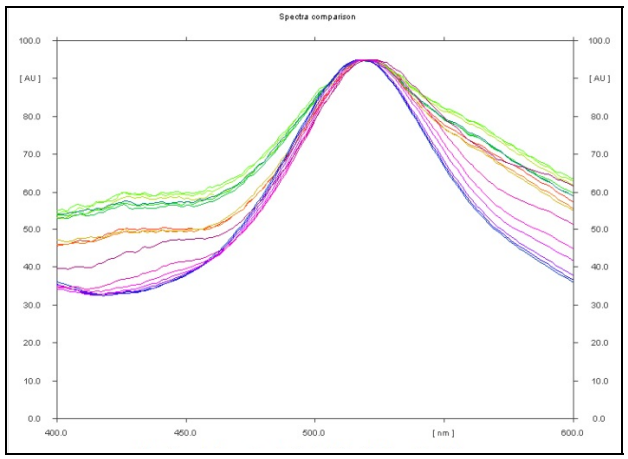

Fig. 5: spectral comparison of extracts with standard β-amyrin at 520 nm.

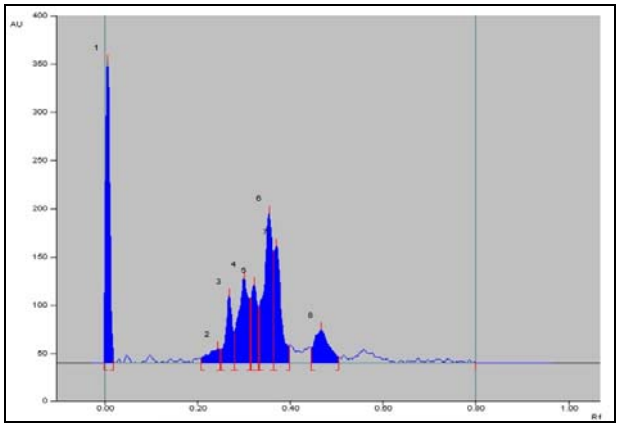

Fig. 6: Chromatogram of sample 1 scanned at 520 nm (β-amyrin is absent); mobile phase: toluene: methanol (9:1).

TLC instrumentation

The analysis of β-amyrin was done on 20×10cm normal phase HPTLC plate. The application of samples on the HPTLC plate was carried out in the form of bands of 6 mm wide and 8 mm apart by Linomat IV sample applicator. The samples were applied on plate at the rate of 160nL S^{-1}. The development of the plate was done in previously saturated twin-trough glass chamber (20×10cm) at RT (25±2°C) and RH (60±5%). After the development of HPTLC plate it was air dried and sprayed with p-anisaldehyde reagent and heated to identify compact bands.

Preparation of calibration graphs

Calibration graph for standard β-amyrin was prepared by applying a series of spots of standard with same volume from eight different dilutions of standard so as to get different amount of β-amyrin per spot. They were prepared with respect to height and area vs amount per spot.

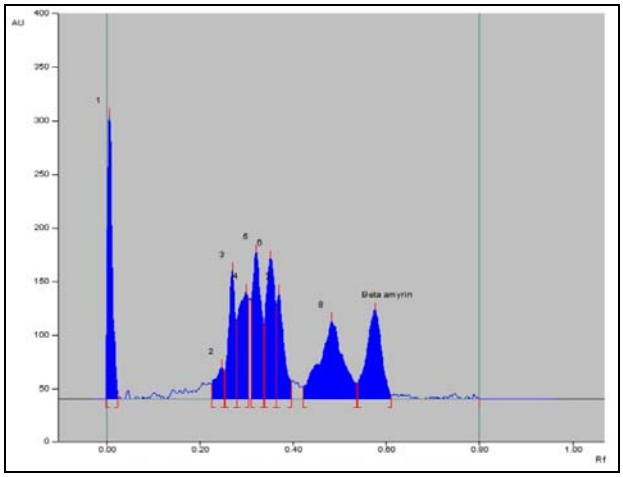

Fig. 7: Chromatogram of sample 2 scanned at 520nm (β-amyrin; R_f =0.58); mobile phase: toluene: methanol (9:1)

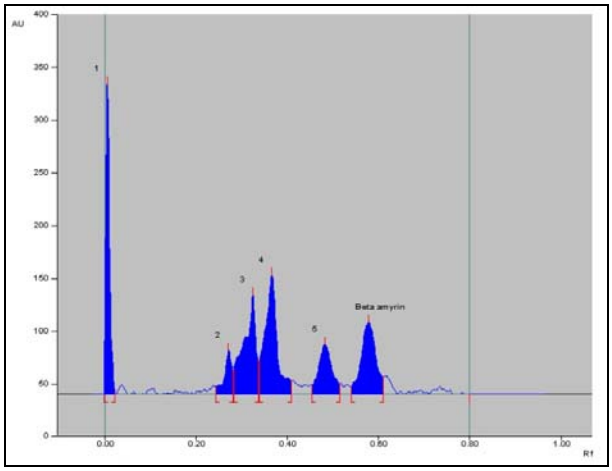

Fig. 8: Chromatogram of sample 3 scanned at 520 nm (β-amyrin; R_f = 0.58); mobile phase: toluene: methanol (9:1)

Method development

Chromatogram was developed for β-amyrin by selecting the mobile phase after trying several combinations of solvents. The best resolution was observed by using solvents tolune: Methanol in the ratio of 9:1, v/v. The same combination of solvents (tolune: methanol in the ratio of 9:1, v/v) was used for the separation of samples 1, 2, 3 and 4. The HPTLC densitometric analysis was carried out at 550nm wavelength in absorbance/reflectance mode.

Method validation

It was performed according to the International Conference on Harmonization guidelines (ICH, 2005) for

linearity range, precision, accuracy, robustness, Limit of detection, Limit of quantification and recovery studies.

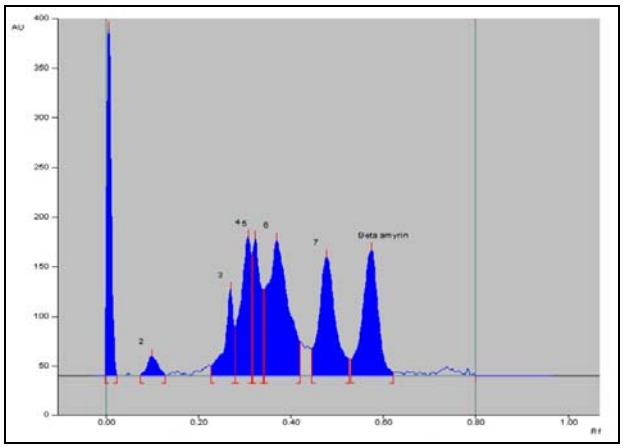

Fig. 9: Chromatogram of sample 4 scanned at 520 nm (β-amyrin; R_f =0.58); mobile phase: Toluene: Methanol (9:1)

Table 1: R_f, linear regression data for the calibration curve and sensitivity parameter for β-amyrin.

Parameter	β-amyrin
R_f	0.58±0.01
Linearity range (ng band^{-1})	100-800
Regression equation	Y=19.913X+107.803
Correlation coefficient	(r^2) 0.991
Slope ±SD	19.913±0.966
Intercept ±SD	107.803±4.803
Standard error of slope	0.394
Standard error of intercept	1.96
LOD	18.1ng band^{-1}
LOQ	54.3ng band^{-1}

β-amyrin analysis in samples 1-4

Standard β-amyrin and test samples were spotted on HPTLC plate. The percentage of β-amyrin present in test samples (sample 1 to 4) was determined by measuring area for the standard and test samples. Thereby the percentage of β-amyrin was calculated for all the four samples of *Acacia* species.

RESULTS

Method development

The developed method was found to be effective in separation of constituents present in the samples (1, 2, 3 and 4) and exhibiting sharp peaks of standard (β-amyrin) as well, with the selected mobile phase under chamber saturation conditions at a wave length of 520nm in absorbance/reflectance mode (figs. 2a, 2b and 3). Sharp and compact bands of β-amyrin were obtained at R_f =0.58±0.001 (fig. 4). The β-amyrin peaks in the different samples were compared with that of standard peak and it was found to be aligned with each other (fig. 5).

Method validation

Linearity of biomarker β-amyrin was validated by the linear regression equation and correlation coefficient. The eight-point calibration curve for β-amyrin was found to be linear in the range of 100-800ng. The r^2 and regression equation for the reference compound was found to be 0.991 and Y=19.913X+107.803, respectively which table 1). The mean value of slope and intercept (with ±SD) was found to be 19.913±0.966 and 107.803±4.803, respectively for β-amyrin. No significant difference was observed in the slopes of standard plots (P>0.05). The Intra-day and interday precisions (n=6) for β-amyrin at three quality control (QC) levels (150, 300 and 600ng band^{-1}) were found to be 1.67-1.79% and 1.72-1.87%, respectively, which demonstrated the good precision of proposed method (table 2). However, intra-day and interday accuracy of β-amyrin at three quality control (QC) levels (150, 300 and 600ng band^{-1}) were observed as 97.86-98.80% and 97.92-98.96%, respectively, which proves that the proposed method is accurate (table 2). The low value of SD and % RSD obtained after introducing small deliberate changes in the method at 300ng band^{-1} concentration level of β-amyrin indicate that the method was robust (table 3). Limit of detection and Limit of quantification were found to be 18.1 and 54.3ng 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 β-amyrin as shown in table 4. The result revealed the percentage recoveries after sample processing for β-amyrin were in the range of 97.8-98.8%.

HPTLC analysis of samples 1-4

The application of the method was assessed by using this method for the analysis of biomarker β-amyrin in the samples 1, 2, 3 and 4. Out of these four samples evaluated for the quantification of β-amyrin, three samples i.e. sample 2 (*A. loreta*), sample 3 (*A. humulosa*) and sample 4 (*A. tortilis*) were found to contain β-amyrin (fig. 7, 8 and 9). β-amyrin was found to be completely absent in sample 1 (*A. salicina*) (fig. 6). The percentage of β-amyrin found in different Acacia species are as follows: *A. loreta* (1.85%), *A. humulosa* (1.80%), *A. tortilis* (2.70%) (table 5). The authors are privileged to perform this comparative estimation of β-amyrin for the first time in four above-mentioned species of genus *Acacia* collected from Kingdom of Saudi Arabia by a validated HPTLC method.

DISCUSSION

The herbs or herbal formulations use has become 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 (Siddiqui *et al*., 2014a). These days HPTLC has been extensively used in the quality control of herbal drugs due to its several benefits over other chromatographic technique like less expensive, high sample throughput and need for minimum sample clean up (Alam *et al*., 2014). HPTLC is extensively employed for the stability studies, identification, assay, uniformity examination of raw materials and formulated products (Siddiqui *et al*., 2014b). Along with the whole world kingdom of Saudi Arabia is also facing challenges in handling the dreaded disease cancer and it is the main factor of large number of deaths every year. This is now an established fact that the herbal drugs which act as an anti-inflammatory agent may play an important role in inhibiting the cancer (Berkovich *et al*., 2012) and it is also proved from the previous literature work that some of the *Acacia* species has anticancer properties. Since, β-amyrin has good anti-inflammatory effect it might prove to be useful against cancer, which also supports the work of Linn *et al*., 2011.

The findings of this experiment may prove the existence of chemotypes present in genus *Acacia* 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 β-amyrin (*A. tortilis*) for herbal formulations and also to assure the content uniformity in other crude drug materials possessing β-amyrin.

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

Table 2: Precision and accuracy of β-amyrin

Nominal Concentration	β-amyrin Obtained (a, b)	Precision (c)	Accuracy (d) (%)
Intraday batch			
150	146.8±2.62	1.79	97.86
300	294.5±5.12	1.74	98.16
600	592.8±9.89	1.67	98.80
Interday batch			
150	146.88±2.74	1.87	97.92
300	295.62±5.35	1.81	98.54
600	593.76±10.21	1.72	98.96

[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	β-amyrin	
	SD	%RSD
Mobile phase from Toluene: MeOH (9:1,v/v)		
(8.9:1.1, v/v; 9.1:0.9, v/v; 9.2:0.8, v/v; 8.8:1.2, v/v)	4.56±0.075	1.54
Mobile phase volume (18, 20 and 22 mL)	3.81±0.062	1.28
Duration of saturation (10, 20 and 30 min)	3.13±0.054	1.05

Table 4: Recovery studies of β- amyrin

Concentration added to analyte (%)	Theoretical (ng)	Added (ng)	Detected (ng)	Recovery (%)	RSD (%)
	300				
50		450	440.1±6.01	97.8	1.42
100		600	589.8±7.56	98.3	1.34
150		750	741.0±9.21	98.8	1.31

Table 5: Estimation of β-amyrin in different *Acacia* species (samples1-4)

Sample No.	Name of *Acacia* species	β-amyrin content (%)
Sample 1	*A. salicina*	ABSENT
Sample 2	*A. loreta*	1.85%
Sample 3	*A. hamulosa*	1.80%
Sample 4	*A. tortilis*	2.70%

compensate the effect of these external and internal factors on the production of secondary plant metabolites HPTLC is used as a tool for maintaining content uniformity. The developed HPTLC method was also found to be an important analytical technique for separation, detection, identification and quantification of β-amyrin in this experiment.

CONCLUSION

Among all the *Acacia* species checked for the availability of β-amyrin in this experiment, *A. tortilis* (2.70%) was found to contain highest amount of β-amyrin among the species of Acacia and it was also found to contain highest amount of β-amyrin in comparison to other plant species reported earlier like in *Maytenus obscura* and *Maytenus parviflora* aerial parts extract (0.42 and 0.88%) (Alajmi *et al*., 2013); cabbage leaf surface extract (0.47%) (Mitja *et al*., 2007) and *Ficus nitida* leaves extract (0.54%), *Ficus palmata* leaves extract (0.31%), *Ficus carica* leaves extract (0.29%) (Basudan *et al*., 2015). The detection of β-amyrin in the extracts of *A. loreta, A. humulosa* and *A. tortilis* in this experiment justifies the use of *Acacia* species as anti-cancer agent in folk medicines. The above proposed HPTLC method is simple, precise and specific, hence it can be employed for exploration of β-amyrin in chemo taxonomically related genera of the plant kingdom. It can also be employed for further analysis of β-amyrin in plasma, other biological fluids and in finished products available in the market.

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