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DEVELOPMENT OF VALIDATED HPTLC METHOD FOR SIMULTANEOUS QUANTIFICATION OF RUTIN AND QUERCETIN FROM BARK OF *ANOGEISSUS LATIFOLIA*

Pradeep Hulikare Ananth[1], Saleemulla Khan[2], Raghu Chandrasekhar[2] and Mohammed Ibrahim[1*]

[1]Department of Pharmacognosy and Biotechnology, Nizam Institute of Pharmacy, Deshmukhi, Pochampally (Mandal), Near Ramoji Film City, Nalgonda 508284, Andhra Pradesh & Centre for liver Research and Diagnosics, Deccan College of Medical Sciences & Allied Hospitals, Kanchanbagh, Hyderabad 500058, Andhra Pradesh, India.
[2]Manipal College of Pharmaceutical Sciences, Dept of Pharmacognosy and Biotechnology, Manipal, Karnataka & JSS College of Pharmacy, Dept of Phytopharmacy & Phytomedicine, TIFAC CORE, Ooty, Tamilnadu

***Corresponding author e-mail:** ibrahim_cce@rediffmail.com

ABSTRACT

A simple and fast method was developed for simultaneous quantitative determination of two biologically active flavonoid compounds i.e. quercetin and rutin in bark of *Anogeissus latifolia* using High-Performance Thin-layer Chromatography. The separation was performed on TLC aluminium plates precoated with silica gel 60 F_{254}. Good separation was achieved in the mobile phase of Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:26, *v*/*v*) and densitometric determination of these compounds was carried out at 366 nm in reflection/absorbance mode. The rutin and quercetin content of hydroalcohol bark extract of *Anogeissus latifolia* were found to be 0.1617% w/w and 1.875%w/w respectively. The linear regression analysis data for the calibration plots showed a good linear relationship with *r*=0.9997 and r= 0.9942 for rutin and quercetin, respectively. The average recovery of rutin and quercetin was 99.98 % and 100.11%, respectively indicating the excellent reproducibility. Statistical analysis of the data showed that the method is reproducible. The present method is being reported first time and may be used for routine quality control of the bark of *Anogeissus latifolia.* This HPTLC method was found to be simple and convenient for rapid screening of active compounds and quantification of the investigated flavonoids in *Anogeissus latifolia.*

Keywords: HPTLC, *Anogeissus latifolia* bark, Quercetin and Rutin

INTRODUCTION

Flavonoids comprise a vast array of biologically active compounds ubiquitous in plants, many of which have been used in traditional medicine for thousands of years[1]. Over 4000 structurally unique flavonoids have been identified in plant sources[2]. Flavonoids belong to a group of polyphenolic compounds, which are classified as flavonols, flavonones, flavones, flavanols, flavan-3-ols and isoflavones according to the positions of the substitutes present on the parent molecule. Flavonoids of different classes have several pharmacological activities[3]. Flavonoids, Rutin (5,7,3',4',tetrahydroxy flavonol -3- rhamnoglucoside) and Quercetin (5,7,3',4', tetrahydroxy flavonol) have been known to posses biochemical effects, which inhibit a number of enzymes such as aldose reductase[4], xanthine oxidase[5], phosphodiesterase[6], Ca^{+2}-ATPase[7], lipoxygenase[8], cycloxygenase[9], *etc* . They also have a regulatory role on different hormones like estrogens, androgens[10] and thyroid hormone[11]. In view of their wide pharmacological and biological actions[12] they seem to be having a

great therapeutic potential. High performance thin layer chromatography HPTLC method is the most suitable method for estimation of chemical constituents present in plant materials. So, the quality control and quantitative analysis of flavonoid compounds in traditional herbal medicines have become a necessary undertaking.

The genus *Anogeissus* (*Combretaceae*) is widely distributed in most tropical and subtropical countries of the world. *Anogeissus* is a genus of trees, shrubs or small trees. The genus has eight species, five native to South Asia, two indigenous to the southern Arabian Peninsula, and one native to Africa. Plants of this genus have been used in folk medicine for centuries to treat a broad spectrum of disorders. Chemical constituents isolated from genus *Anogeissus* so far include amino acids, terpenoids (and their glycoside derivatives), steroids, flavonoids and their glycosides, tannins and other phenolic compounds and lignan[13].

Anogeissus latifolia, *(A.latifolia)* (*Combretaceae*), locally known as Dhava, is a moderate sized tree characteristic of dry deciduous forests and available throughout India. In phytotherapy, the bark, leaves, heartwood and roots of the plant is traditionally used for the treatment of dysentery, snakebite, leprosy, wounds and ulcers, skin diseases including diabetes and jaundice[14]. The bark is reported to have potent antioxidant activity[15] and possess several biological activities like antiulcer, antimicrobial[16], wound healing[17], chemoprotective[18] and hepatoprotective activity[19]. A variety of chemical constituents which might contribute to various therapeutic activities have been identified in the plant. The bark of *A. latifolia* is reported to contain phenolic compounds like gallic acid, ellagic acid, chebulic acid and flavonoids like rutin and quercetin[20,21] which are potential antioxidants[22-25]. The different biological activities of the bark are mainly attributed to antioxidant constituents like gallic acid, ellagic acid, chebulic acid, quercetin and rutin of the bark. In this contest, *A.latifolia* an important medicinal and economic plant was selected to develop a better method for analysis of its flavonoid compounds by HPTLC. Densitometric HPTLC has been widely used for phytochemical evaluation of drugs and formulations. Hence, a densitometric HPTLC method has been developed in the present work for the quantification of rutin and quercetin in *A.latifolia* bark extract. The bark of the plant was standardized for the presence of Gallic acid and Ellagic acid (0.95% w/w and 0.25%w/w respectively) using HPTLC by Govindrajan et al, 2006. However, no repot is available on the quantitative studies of rutin and quercetin. Keeping this in view, the present study was carried out for simultaneous determination and quantification of rutin and quercetin (Fig 1) in hydroalcohol extract of bark of *A.latifolia* using HPTLC.

MATERIALS AND METHODS

Reagents, Standard Solutions, and Materials: All chemicals and solvents used were of analytical grade and obtained from E.Merck (Darmstadt, Germany). Flavonoid standards (rutin and quercetin) were purchased from Lobo Chemie, Mumbai, India (purity >97%). Stock solutions (1 mg/ml) of the standards were prepared daily in methanol immediately before use. TLC aluminum plates pre-coated with silica gel 60 F_{254} (10x 10 cm, 0.2 mm thick) used were obtained from E. Merck Ltd (Mumbai, India).

Plant material: Bark of *A.latifolia* was collected from Chikmagalure District, Karnataka, India, during the month of May. It was authenticated from Botanical Survey of India, Coimbatore, Tamilnadu, India (No.BSI/SC/5/23/06-07/Tech.880).

Extraction of plant material for HPTLC analysis:
The bark was shade dried and powdered coarsely. The coarse powder (250g) obtained was extracted with n-Hexane to remove the fatty substances; the marc was further extracted exhaustively with 70% ethanol in Soxhlet apparatus and filtered. The extract was concentrated under reduced temperature and pressure to get dry residue (26.8g) and stored in the dessicator was used for subsequent experiments. Preliminary phytochemical screening revealed the presence of Polyphenols, flavonoids and carbohydrates.

Preparation of Standard and Sample solutions: Standard stock solutions of rutin and quercetin were prepared by dissolving 10mg of rutin and quercetin in 10ml of methanol. From this 3µl each of these solutions was applied using Linomat applicator. 100mg of hydroalcoholic extract of *A.latifolia* was dissolved in 10ml of methanol and filtered. The filtrate (10mg/ml) was used for the HPTLC chemoprofiling.

Chromatographic conditions: Chromatography was performed on pre-activated (at 110^{o}C) silica gel 60 F_{254} HPTLC plates. Sample(8µl) and standard (3µl each) compounds were applied to the layer as 8 mm wide bands, positioned 10 mm from the bottom of the plate, using an automated TLC applicator Linomat IV (Camag, Muttenx, Switzerland) with nitrogen flow providing delivery from the syringe. These critical

parameters were maintained for all analyses performed.

Detection and quantification of compounds: TLC was performed with Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:26, *v*/*v*) as mobile phase. Chromatograms were developed at room temperature (24 ± 1°C) in glass twin-trough chambers (10 cm × 10 cm, with metal lids; Camag, Switzerland) previously saturated with mobile phase vapor for 30 min. The development distance was 80 mm. Ascending mode was used for development of thin layer chromatography.

Following the development, the TLC plate was dried in a current of air with the help of an air dryer at 110°C for 10min, and immediately scanned at λ = 366 nm and the chromatograms were obtained with CAMAG TLC scanner equipped with CATS 4 software (Camag) in absorption reflection scan mode.

The presence (or absence) of the investigated compounds was determined according to their Rf values and fluorescence colors. Estimation of retention factor (Rf) and Area Under Curve (AUC) were done by Integration Software 4.05. Calculations for percentage were done considering standard and sample Rf, AUC and dilution factor. Integrated legends are presented as fig 1-4. For validation of the method, calibration curve was obtained by plotting peak area Vs concentration of rutin and quercetin and checked for reproducibility, linearity and validating the proposed method. Spectra of samples and standard rutin and quercetin were matched[26].

Validation of HPTLC method

Linearity: A calibration curve of standard rutin and quercetin were obtained by plotting peak area of rutin and quercetin against the different concentration. Stock solutions of rutin and quercetin were prepared in methanol and different amounts 1, 2, 4, 6, 8, and 10 µg/spot of these were loaded onto a TLC plate for preparing calibration curves. There was a good linear relationship between peak area and concentration in the range 1-10µg per zone (Table1). The experiment was performed in triplicate.

Accuracy (% Recovery): This was determined by addition of standard marker. To a fixed amount of preanalysed sample, certain amount of standards were added in the below and above level from normal level expected in sample. For this two concentrations in triplicate were used. The average recovery of rutin and quercetin was 99.98% and 100.11%, respectively indicating the excellent reproducibility (Table 2).

RESULTS AND DISCUSSION

HPTLC is primarily used as an inexpensive method for separation, qualitative identification, or semi-quantitative visual analysis of samples[27]. Recent reviews show that the HPTLC techniques can be used to solve many qualitative and quantitative analytical problems in a wide range of fields, including medicine, pharmaceuticals, chemistry, biochemistry, food analysis, toxicology and environmental analysis[28].

The use of HPTLC has expanded considerably due to the development of forced flow (FF) and gradient TLC methods, improved stationary and mobile phase selection, as well as new methods of quantitation methods[29]. To estimate rutin and quercetin in hydroalchoholic extract of *A. latifolia* bark the composition of the mobile phase was optimized by testing different solvent compositions of varying polarities. The best results were obtained using Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:26, *v*/*v*) as mobile phase.

The selected mobile phase showed good resolution. Standard rutin and quercetin showed single peak in HPTLC chromatogram (Fig.1 and 2). After development the plate was scanned at 366 nm. The compound with R_f value 0.38 and 0.97 were identified as rutin and quercetin respectively. The rutin content of hydroalcoholic extract of *A.latifolia* bark was 0.1617% w/w and quercetin content was found to be 1.875%w/w. The peak areas for different concentration of rutin and quercetin were found to be linearly dependent on the concentration in the range of 1-10 µg per spot. According to the area of peak, the regression equation and correlation coefficient were from calibration curves, Y= 6.1496 x + 85, r = 0.9997 for rutin and Y = 6.9601 x + 5.18, r = 0.9942 for quercetin. As the quercetin content is quit high it can serve as commercial source. Average recovery of rutin and quercetin was 99.98 % and 100.11 %, respectively indicating the excellent reproducibility Drug shows good amount of flavonoids and phenolic compound, therefore it will prove better in antioxidant profile also.

CONCLUSION

HPTLC method mentioned here represented an excellent technique for simultaneous determination of rutin and quercetin in the bark extract of *A.latifolia* with good sensitivity, precision and reproducibility. Running time and cost per analysis are relatively low in comparison with other methods. Furthermore, the method can be used as quality control for flavonoids

in *A.latifolia* and will play a reference role on the determination of flavonoids in other medicinal plants or pharmaceutical herbal preparations.

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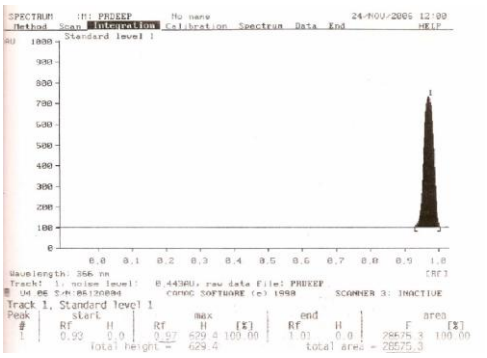

Fig 1: HPTLC chromatogram of quercetin

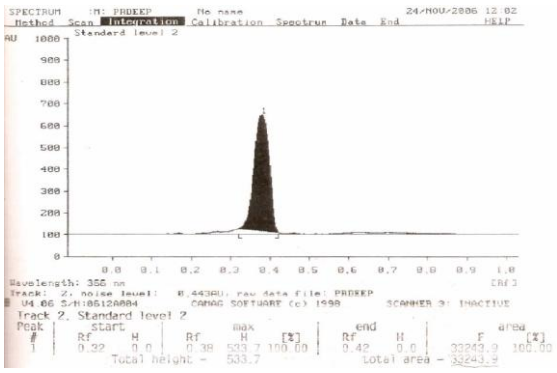

Fig 2: HPTLC chromatogram of Rutin

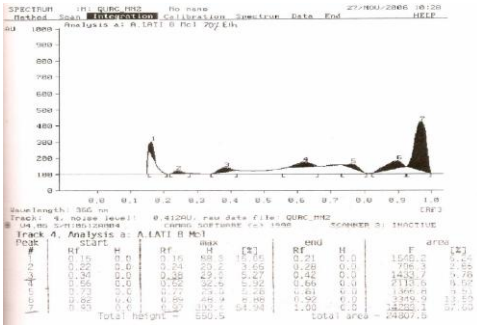

Fig 3: HPTLC Chromatogram of A. latifolia bark extract

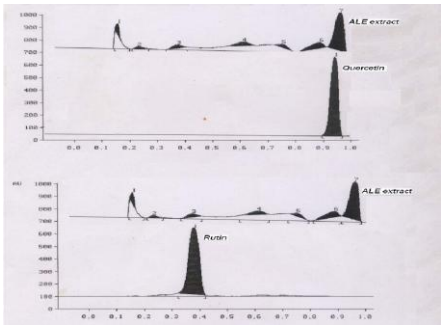

Fig 4. Chromatogram of Std. and sample track

Table 1: *R*f and linear regression equation of quercetin and rutin

Compound	R_f	Regression equation	r*
Rutin	0.38	$y = 6.1496\ x + 85$	0.9997
Quercetin	0.97	$y = 6.9601\ x + 5.18$	0.9942

* Correlation coefficient

Table 2: Accuracy (%Recovery) for Rutin and Quercetin by HPTLC

Compound	Amount of compound in bark (mean,mg/100mg	Amount of Std added(mg)	Amount of Std found in mxture(mg)	Recovery (%)
Rutin	161	161	325.8	100.19±0.86
		322	471.0	99.77±0.91
Quercetin	1875	1875	3752.19	100.11±1.12
		3750	5626.60	100.12±0.44

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