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SIMULTANEOUS QUANTIFICATION OF RUTIN AND ISOQUERCITRIN FROM *JASMINUM SAMBAC* AIT., BY HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

A normal phase high performance thin layer chromatography (HPTLC) method has been developed and validated for simultaneous quantitative determination of two flavonoids viz. rutin and isoquercitrin from dried leaf powder of *Jasminum sambac* Ait. The analysis was performed on TLC aluminum plates precoated with silica gel 60F254. Linear ascending development was carried out in a twin-trough glass chamber saturated with mobile phase comprised of ethyl acetate: methanol: formic acid (10.0:2.0:1.0 v/v/v) at room temperature 25±2ºC. The densitometric scanning was done at λ=366nm for both the components in reflectance/absorbance mode. The detector response was linear for concentrations ranging from 0.20µg/band to 0.65µg/band and 0.30µg/band to 1.2µg/band for rutin and isoquercitrin respectively with correlation coefficient of 0.999 for both the components. The method was precise as the value of percent relative standard deviation was found to be less than 2. The amounts of rutin and isoquercitrin in the dried leaf powder of *Jasminum sambac* Ait. were found to be 0.4962mg/g and 0.6461mg/g respectively. The accuracy of the developed HPTLC method was checked by carrying out the recovery experiment at three different levels, by using standard addition method. The values of percent recovery were found to be 97.60 and 98.84 for rutin and isoquercitrin respectively. This developed HPTLC method for simultaneous determination and quantification of rutin and isoquercitrin present in the dried leaf powder of *Jasminum sambac* Ait. is simple, rapid , precise and can be used for routine quality control.

INTRODUCTION: *Jasminum sambac* Ait. (Family-Oleaceae) is commonly known as Mogra. It is a famous fragrant plant widely cultivated in all over the world. The flowers of this plant are used in the preparation of an essential oil and for making jasmine tea [1]. In addition to this, the plant has many medicinal properties. The flowers are useful as a drug for the treatment of diarrhea, abdominal pain, conjunctivitis and dermatitis [1]. The leaves are used to heal the wounds [2]. Charak included this herb for the treatment of insanity and epilepsy[3]. Phytochemiclas such as rutin, quercitrin, isoquercitrin, quercitrin-3-dirhamnoglycoside, and kaempherol-3-rhamnoglycosides, α-amyrin and β-sitisterol are reported to be present in its leaves [3].

Flavonoids are a group of polyphenolic compounds widely distributed throughout the plant kingdom. Flavonoids have been referred to as "nature's biological response modifiers" because of strong experimental evidence of their inherent ability to

modify the body's reaction to allergens, viruses, and carcinogens [4]. They show anti-allergic [5], anti inflammatory [6], antimicrobial [7] and anticancer activity [6].

In the present research work, two flavonoids viz. rutin and isoquercitrin are simultaneously quantitated from dried leaf powder of *Jasminum sambac* Ait by HPTLC technique.

In literature, HPTLC methods have been reported for simultaneous analysis of rutin and isoquercitrin from *Annona squamosa* Linn.[8], *Calendulae officinalis* flos.[9] and also from *Stachys recta* subsp. *Recta* [10].

However, no HPTLC method is reported for the simultaneous quantification of rutin and isoquercetrin from leaves of *Jasminum sambac* Ait.

Thus, precise and accurate HPTLC method has been developed and validated using International Conference on Harmonization (ICH) guidelines for simultaneous determination and quantification of rutin and isoquercetrin from dried leaf powder of *Jasminum sambac* Ait.

EXPERIMENTAL METHODS

Materials:

1. **Standard, Reagents and Chemicals:** The reference standards rutin hydrate (purity 94.0% HPLC Grade) and isoquercitrin (purity 90.0% HPLC Grade) were purchased from Sigma-Aldrich Chemie GmbH (Aldrich Division, Steinbeim, Germany). All other chemicals, methanol (99.9%), ethyl acetate (99%), formic acid (99%) were procured from Qualigens Fine Chemicals (Mumbai, India). TLC plates precoated with silica gel $60F_{254}$ used were obtained from E. Merck (India).

2. **Plant Material:** The leaves of *Jasminum sambac* Ait., were collected from Keshav Srushti, Mumbai, India. Herbarium of the plant was prepared and authenticated from Botanical Survey of India (BSI), Pune, India. A duplicate herbarium was prepared and preserved in Ramnarain Ruia College. The leaves of *Jasminum sambac* Ait., were washed with water to remove soil particles, dried at 45±2^{o}C [11], powdered and then sieved through BSS mesh size 85 and stored in an airtight container at room temperature (25 ± 2^{0} C).

Preparation of solutions:

1. **Preparation of stock and working standard solutions of rutin:** About 10.0 mg of rutin was accurately weighed and transferred to 10.0 mL volumetric flask. 5.0 mL of methanol was added and the contents were sonicated in an ultrasonic bath (Model: TRANS-O-SONIC, Frequency: 50 Hz) for 5 minutes for complete dissolution of rutin. The contents were then diluted up to the mark with methanol to obtain a solution of rutin containing 1000 µg/mL of rutin. 0.25 mL of above stock solution of rutin was then transferred to 10.0 mL volumetric flask and the contents of volumetric flask were diluted up to 10.0 mL by methanol to obtain stock solution of rutin containing 25.0 µg/ mL of rutin.

2. **Preparation of stock and working standard solutions of Isoquercitrin:** About 10.0 mg of isoquercitrin was accurately weighed and transferred to 10.0 mL volumetric flask. 5.0 mL of methanol was added and the contents were sonicated for 5 min for complete dissolution of isoquercitrin. The contents were then diluted up to the mark with methanol to obtain a solution of isoquercitrin containing 1000 µg/mL of isoquercitrin. 0.50mL of above stock solution of isoquercitrin was then transferred to 10.0 mL volumetric flask and the contents of volumetric flask were diluted up to 10.0 mL by methanol to obtain stock solution of isoquercitrin containing 50.0 µg/ mL of isoquercitrin.

3. **Preparation of Sample Solution:** About 1.0g of finely powdered leaf powder of *Jasminum sambac* Ait., was accurately weighed in a 20mL stopper test tube. 10mL of methanol was added to it and the tube was sonicated in an ultrasonic bath for 15 minutes. The tube was then shaken at 50 rpm, on a test tube rotator overnight at room temperature (25 $\pm$ 2^{o}C). Further, sample was filtered through whatmann filter paper no.41. The filtrate was then finally filtered using 0.45 µm nylon filters (Millipore) before the analysis.

4. **Preparation of Mobile Phase:** The mobile phase used in the present research work for simultaneous quantification of rutin and isoquercitrin from the leaf powder of *Jasminum sambac* Ait., was prepared by mixing ethyl acetate: methanol: formic acid (10.0:2.0:1.0 v/v/v) in a test tube. It was then sonicated for 5 minutes.

HPTLC conditions: Chromatography was performed on 5.0 cm x 10.0 cm TLC plates which were cut from 20.0 cm x 20.0 cm TLC aluminum plates precoated with 200 µm layers of silica gel $60F_{254}$ (E. Merck, Mumbai, India). The plates were prewashed with methanol and activated at 105-110ºC for 15 minutes before analysis. Standard and sample solutions were applied to the plates as 7mm bands, 6mm from each other and 10 mm from bottom edge of the plate, under a continuous supply of nitrogen by means of a CAMAG (Muttenz, Switzerland) Linomat V Automatic TLC sample applicator fitted with a 100µL syringe (Hamilton, Bonaduz, Switzerland).

Linear ascending development was carried out in a twin-trough glass chamber (Camag, Muttenz, Switzerland) saturated with mobile phase comprised of ethyl acetate: methanol: formic acid (10.0:2.0:1.0 v/v/v). The optimized chamber saturation time for the mobile phase was 20 minutes at room temperature (25±2^{o}C). The plates were developed to a distance of 90 mm from the bottom edge of the plate. After development the plates were dried in air and densitometric scanning was performed at λ=366 nm in reflectance/absorbance mode using CAMAG TLC scanner III with Win CATS software version 1.4.2.

Method Validation:

Linearity:

1. **Preparation of calibration curve of Rutin:** Calibration curve of rutin was prepared by applying 8µL, 10µL, 12µL, 14µL, 16µL, 18µL, 20µL, 22µL, 24µL, 26µL from stock solution of 25.00µg/mL of standard rutin on TLC plate to obtain concentrations of 0.20µg/band to 0.65µg/band.

2. **Preparation of calibration curve of isoquercitrin:** Calibration curve of isoquercitrin was prepared by applying 6 µL, 8µL, 10µL, 12µL, 14µL, 16µL, 18µL, 20µL, 22µL, 24µL from stock solution of 50.00µg/mL of standard isoquercitrin on TLC plate to obtain concentration in the range of 0.30µg/band to 1.2µg/band.

Each concentration of rutin and isoquercitrin were applied, in triplicate, on three different plates and developed and scanned at λ=366 nm under the specified chromatographic conditions described above. The densitograms were then acquired and the peak areas were recorded for each applied concentration of both rutin and isoquercitrin. The calibration curves of both rutin and isoquercitrin were obtained by plotting graphs of mean peak areas vs. corresponding concentrations. The results listed in Table 1.0, show that within the concentration range indicated, there was a good correlation between mean peak area and concentration of standards.

Limit of Detection (LOD) and Limit of Quantification (LOQ): The limit of detection (LOD) and limit of quantification (LOQ) were determined at signal to noise ratios of 3:1 and 10:1, respectively. The LOD and LOQ values obtained for both the components are listed in **Table 1**.

Precision: The method was validated in terms of instrumental precision, repeatability, and intermediate precision.

- Instrumental precision was studied by applying rutin and isoquercitrin standard solutions of concentration 0.50 µg /band and 0.70 µg /band respectively in ten replicates, on TLC plates under the specified conditions. The peak areas of rutin and isoquercitrin were recorded.

- The repeatability was evaluated by triplicate analysis of three sample solutions i.e. methanolic extract of the dried leaf powder of *Jasminum sambac* Ait., was applied on TLC plate in triplicates on the same day in the same laboratory under the specified chromatographic conditions. The peak areas of rutin and isoquercitrin were recorded.

- The Intermediate precision of the method was evaluated by analyzing the sample solution in triplicate on three different days, in the chromatographic system, under the specified chromatographic conditions. The peak areas of rutin and isoquercitrin were recorded.

The precision results were expressed as percentage relative standard deviations of peak areas of rutin and iso-quercitrin and are listed in **Table 1**. The results indicate that the proposed method is precise and reproducible.

TABLE 1: METHOD VALIDATION DATA FOR SIMULTANEOUS QUANTIFICATION OF RUTIN AND ISOQUERCITRIN

Parameters	Results	
	Rutin	Isoquercitrin
Linear range (n=3) µg/band	0.2-0.65	0.3-1.2
Correlation coefficient r^2	0.999	0.999
LOD µg/ band	0.033	0.066
LOQ µg/ band	0.102	0.202
Instrumental precision % R.S.D. (n=10)	0.74	0.49
Repeatability % R.S.D. (n=3) (on the same day)	0.60	0.52
Intermediate precision % R.S.D. (n=3) (Mean percent R.S.D. for three successive days)	0.52	0.42

System Suitability: System suitability was carried out to verify that resolution and reproducibility of the system were acceptable for the analysis. System suitability test was carried out by applying standard solutions of rutin and isoquercitrin of concentrations of 0.50µg/band and 0.70µg/band respectively on TLC plate in five replicates under specified chromatographic conditions. The chromatograms were recorded. The values of percent relative standard deviations of peak area and retention factor of standards were taken as an indicator of system suitability and are less than 2, indicating that the method is suitable for analysis.

Specificity: The specificity of the proposed HPTLC method was ascertained by comparing UV absorption spectra of rutin and isoquercitrin standards with those in the sample. The spectras were compared at three different positions namely at the peak start, peak centre and peak end. Good correlation was obtained between UV spectra obtained from rutin and isoquercitrin standards and rutin and isoquercitrin from the sample at all the positions. The rutin and isoquercitrin peaks were not masked by any peaks arising due to other components or impurities present in the sample solution (**Figure 1 and 2**).

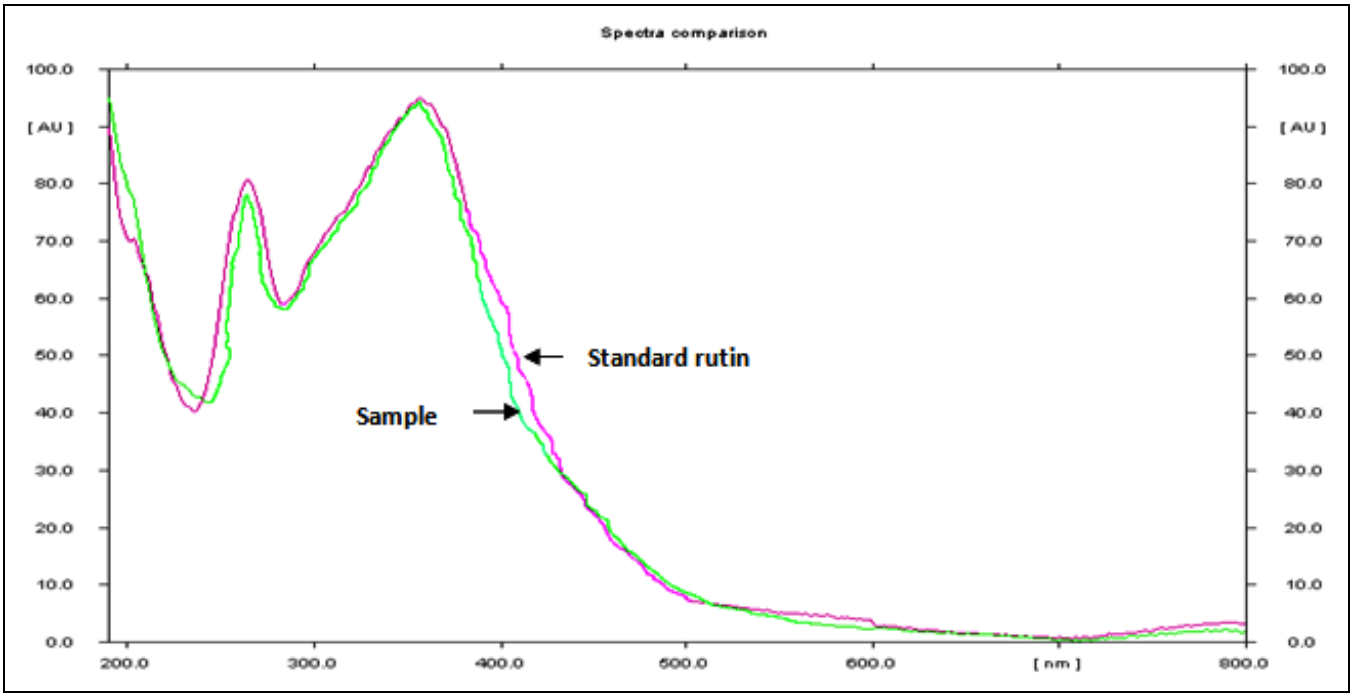

FIGURE 1: OVERLAY SPECTRA OF RUTIN STANDARD AND RUTIN PRESENT IN THE DRIED LEAF POWDER OF *JASMINUM SAMBAC* AIT.

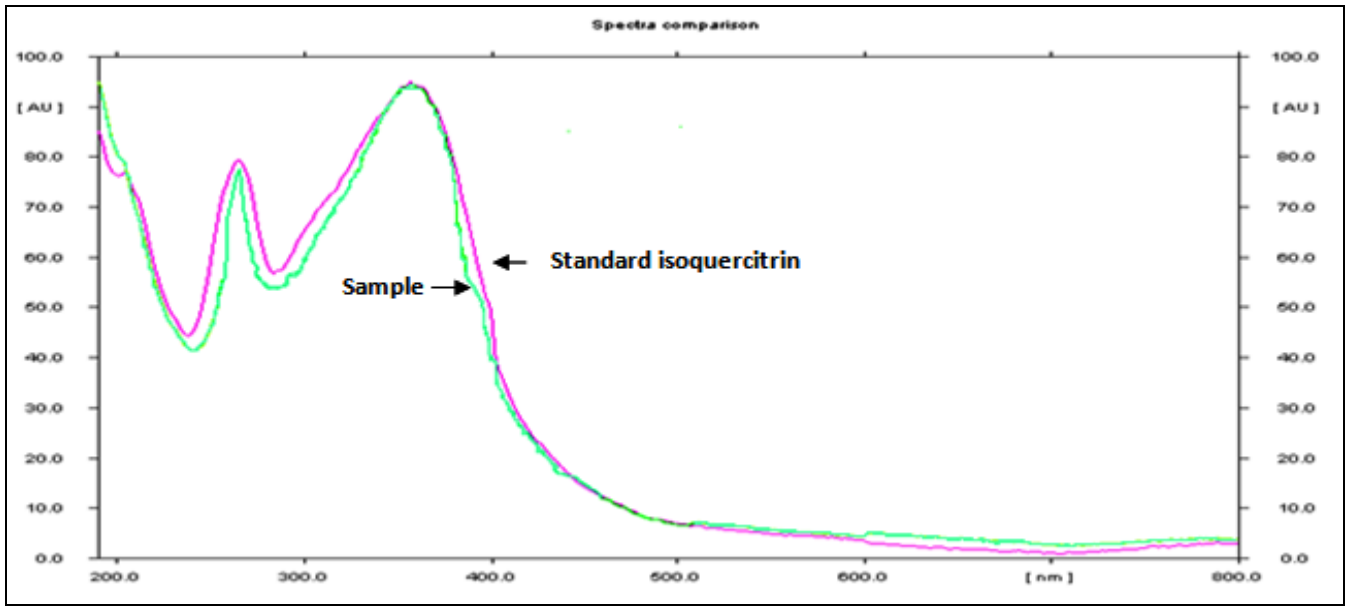

FIGURE 2: OVERLAY SPECTRA OF ISOQUERCITRIN STANDARD AND ISOQUERCITRIN PRESENT IN THE DRIED LEAF POWDER OF *JASMINUM SAMBAC* AIT.

Assay procedure: The developed and validated HPTLC method was used for quantification of rutin and isoquercitrin from the methanolic extract of dried leaf powder of *Jasminum sambac* Ait. 10µL of methanolic extract of the dried leaf powder of *Jasminum sambac* Ait., was applied as a band on the TLC plate (n=7). The plate was developed and scanned under the specified chromatographic conditions. The chromatograms were recorded.

Amounts of rutin and isoquercitrin present in the sample solution were determined from the calibration curve, by using the peak area of rutin and isoquercitrin in the sample. Mean contents of rutin and isoquercitrin in methanolic extract of the dried leaf powder of *Jasminum sambac* Ait., are found to be 0..4962mg/g and 0.6461mg/g respectively.

Accuracy: The accuracy of the method was established by performing recovery experiment by using standard addition method at three different levels. To accurately weighed about 1.0g of dried leaf powder of *Jasminum sambac* Ait., known amounts of standard rutin (0.025mg, 0.050mg and 0.075mg) and isoquercitrin (0.1mg, 0.2mg, 0.3mg), were added, and extracted using methanol. Each of the three different levels containing sample solution and standard was applied in seven replicates; the plates were developed and scanned under the specified chromatographic conditions, as described above. The rutin and isoquercitrin contents were quantified by the proposed method and the percentage recovery was calculated. The values of percent recoveries obtained were 97.60 and for 98.84 for rutin and isoquercitrin respectively. The results of accuracy are listed in **Table 2.**

TABLE 2: RESULTS OF RECOVERY STUDY FOR SIMULTANEOUS HPTLC QUANTIFICATION OF RUTIN AND ISOQUERCITRIN FROM METHANOLIC EXTRACT OF DRIED LEAF POWDER OF *JASMINUM SAMBAC* AIT.

Level	Amount of sample (g)	Amount of standard added to sample (mg)	*Mean amount of standard found (mg)	Percent recovery
		Rutin		
0	1.005	0.0000	0.4989±0.0008	97.60
1	1.001	0.025	0.5249±0.0004	
2	1.006	0.050	0.5475±0.0002	
3	1.003	0.075	0.5727±0.0002	
		Isoquercitrin		
0	1.005	0.0000	0.6518± 0.0006	98.84
1	1.001	0.1000	0.7601± 0.0010	
2	1.006	0.2000	0.8521± 0.0010	
3	1.003	0.3000	0.9506± 0.0010	

*Average ± S.D. (n=7)

Solution Stability: The stabilities of standard rutin and isoquercitrin solution were determined by comparing the peak areas of rutin and isoquercitrin solution, of concentration 0.5µg/band and 0.7µg/band, at different time intervals, for a period of minimum 48 hrs, at room temperature. The results showed that the peak areas of rutin and isoquercitrin almost remained unchanged (values of percent relative standard deviation were less than 2) over a period of 48 hrs, and no significant degradation was observed within the given period, indicating the stability of standard solutions of rutin and isoquercitrin for minimum 48 hrs.

RESULTS AND DISCUSSION: Different mobile phases were tried for simultaneous HPTLC separation of rutin and isoquercitrin from other components of the dried leaf powder of *Jasminum sambac* Ait. and good separation was achieved by using ethyl acetate: methanol: formic acid (10.0:2.0:1.0 v/v/v) as mobile phase. Detection was carried out densitometrically using a CAMAG TLC Scanner at λ = 366 nm as both rutin and isoquercitrin showed maximum response at this wavelength. The identity of the bands of rutin and isoquercitrin in the sample solutions was confirmed by comparing their Rf values in sample with that of reference standards. **Fig. 3** represents a typical TLC plate showing separation of rutin and isoquercitrin. The R_f values for rutin and isoquercitrin were 0.29 and 0.59 respectively.

Figure 4 shows typical HPTLC chromatograms of standard rutin, standard isoquercitrin and methanolic extract of dried leaf powder of *Jasminum sambac* Ait.

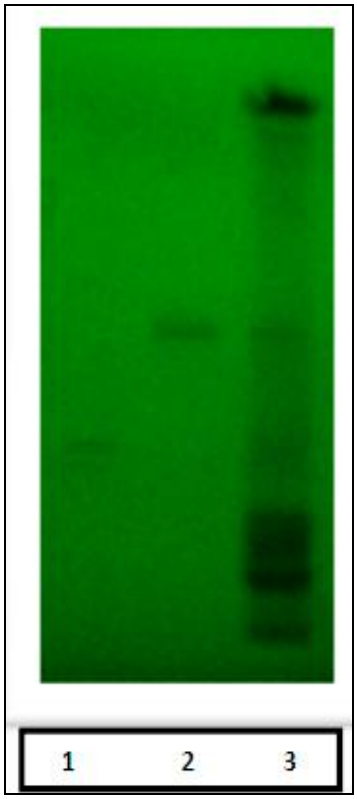

FIGURE 3: HPTLC PLATE SHOWING SEPARATION OF STANDARD RUTIN (1), STANDARD ISOQUERCITRIN (2) AND METHANOLIC EXTRACT OF DRIED LEAF POWDER OF *JASMINUM SAMBAC* AIT. (3)

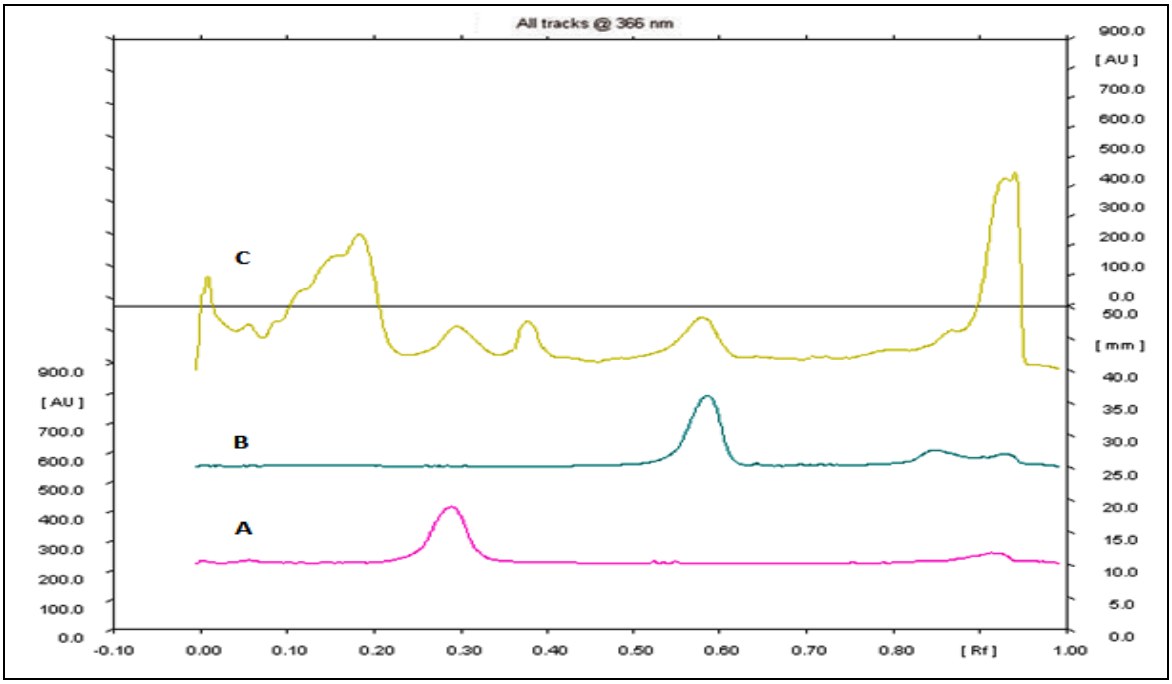

FIGURE 4: HPTLC CHROMATOGRAMS OBTAINED FOR STANDARD RUTIN (A), STANDARD ISOQUERCITRIN (B) METHANOLIC EXTRACT OF DRIED LEAF POWDER OF *JASMINUM SAMBAC* AIT. (C)

A good linear relationship was observed for rutin and isoquercitrin in the concentration in the range of 0.2µg/band to 0.65µg/band and 0.3µg/band to 1.2µg/band respectively with correlation coefficient of 0.999 for both the components (Table 1). When the method was validated for instrumental precision, repeatability and intermediate precision, the values of percentage relative standard deviations were less than 2, indicating the proposed method is precise and repeatable (Table 1). The mean amounts of rutin and isoquercitrin from the methanolic extract of dried leaf powder of *Jasminum sambac* Ait. were found to be 0.4962mg/g and 0.6461mg/g respectively. The values of percent recoveries of rutin and isoquercitrin at three levels were 97.60 and 98.84 respectively indicating accuracy of the method (Table 2).

A normal phase HPTLC method has been used for simultaneous quantification of rutin and isoquercitrin from leaves of *Annona squamosa* Linn. using mobile phase comprised of ethyl acetate: formic acid: glacial acetic acid: ethyl methyl ketone: water in the volume ratio of 5.0:0.7:0.3:3.0:0.10 at λ=366nm[8].

Rutin and isoquercitrin were also simultaneously determined from *Calendulae officinalis* flos. by using toluene: ethyl acetate: formic acid: water in the volume ratio of 1:9:2.5:2 as a mobile phase at λ=366nm [9].

Simultaneous quantification of rutin and isoquercitrin from *Stachys recta* subsp. *recta* was carried out by using ethyl acetate: acetic acid: formic acid: water in the volume ratio of 10:1.1:1.1:2.6 as a mobile phase at λ=366nm [10].

The mobile phase selected for the present research study is ethyl acetate: methanol: formic acid (10.0:2.0:1.0 v/v/v). Rutin and isoquercitrin are strongly polar compounds [12] hence polar mobile phase comprising of ethyl acetate and methanol was used. The addition of formic acid to mobile phase helped to improve the peak shapes of rutin and isoquercitrin. The selected mobile phase in the present research work is advantageous as compared to the reported mobile phases.

In all the methods reported above, water has been used as one of the solvent in the mobile phase but in the developed method the use of water in the mobile phase is avoided since, normal phase chromatographic technique is used. If the mobile phase contains water as one of the components, it takes longer time to ascend the polar stationary phase and drying of the plate also takes a longer time.

CONCLUSION: The developed HPTLC technique is precise, specific and accurate and can be used for the routine quality control analysis and simultaneous quantitative determination of rutin and isoquercitrin from the dried leaf powder of *Jasminum sambac* Ait.

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