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RESEARCH ARTICLE

A HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY DETERMINATION AND QUANTIFICATION OF RUTIN IN *CARALLUMA NILAGIRIANA,* AN ENDEMIC MEDICINAL PLANT

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Abstract

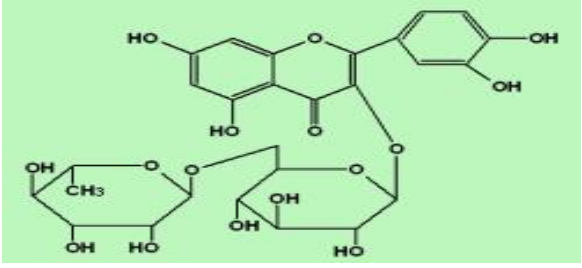
Standardization and quantification are the two essential tools in determining usefulness of a plant based drug. The present study aims to look at these factors in relation with rutin, a bioflavonoid derived from *Caralluma nilagiriana*. Many *Caralluma* species have been known to possess antipyretic, antitumor, antifungal, analgesic, diabetes and leprosy. In the current approach, rutin was extracted from shade dried aerial parts of *C.nilagiriana* using ethanol as extractant and the detection and quantification of the same were carried out subsequently using HPTLC at 366nm. The linearity of rutin obtained by the method outlined was found to be 15-200 ng/spot and the correlation coefficient was significant at 0.99365; the rutin yield at 0.018% was higher than those of many other workers too in the plant. The results thus establish, in certainty, suitability of the adopted methodology for the qualitative and quantitative analyses of rutin from any unknown plant source. The noteworthy feature exclusive to the present development, perhaps, is that the flavonoids can be identified and separated precisely from other interfering compounds at 366nm while the existing methods do not facilitate such a separation.

Keywords: HPTLC, *Caralluma nilagiriana*, rutin, quantification.

Introduction

Many plant species are being increasingly used in therapeutic compounds to treat ailments of different kinds on account of which modern approaches that describe the identification and quantification of active constituents in the plant material have come into play a vital role in standardization of herbal formulations. Rutin is a bioflavonoid occurring in various plants that are medicinally used as an antioxidant having antiinflammatory, antiulcer, and antiallergic and immunomodulation activities(Borrelli and Izzo, 2000; Miyaka and Suzuki, 1992). It is useful in the management of venous edema and capillary fragility on account of its ability in strengthening the capillaries (Couch et al., 1944)] and is proving invaluable as it also reduces the cytotoxicity of oxidized LDL cholesterol (Basarkar, 1981). Most of Rutin's benefits can be attributed to its antioxidant activity and hypotensive effect (Mazza and Oomah, 1996; SenthilNath, et al, 2013). Rutin is also used to prevent mucositis, aside produce an effect on cancer treatment(Agullo.G, et al, 1994; Deschner, 1992; Kandaswami.and Middleton, 1993; Le Marchand L, et al, 2000)] and also is an ingredient in antiageing cosmetics because it is strong ability to absorb of UV and x-rays (Arata, 1992).

Chemical Structure of Rutin



Rutin (quercetin-3-rutinoside) is a flavonol glycoside, comprised of the flavonol quercetin and the disaccharide rutinose. Though its name originates from the plant species known as *Ruta graveolens* that contains rutin, it is found in many other medicinal plants as well. (Ikram and Shafi, 1982)

Rutin is found in buckwheat, R*utagraveolens* (Rue), flower buds of *Sophara japonica*, leaves of Eucalyptus, *Dimorphandramollis* (Fava d'Anta) from the North-East of Brazil and in several others (Mabberley, 1993). Many of the *Caralluma* species are home to rutin and in India, Caralluma *altenuata* species is considered edible and is used in treating rheumatism, diabetes, leprosy, tumor, fungal diseases, and snake and scorpion bites (Jayakar, et al, 2004). Another variety, *Caralluma nilagiriana* is a succulent plant that now stands depleted because of over exploitation, lack of organized cultivation and as has completely been eaten away by sheep and goats, and the same at present has come to be classified under Indian endemic plants with its availability restricted to Nilgiris, Tamilnadu alone (Anonymous, the Indian Pharmacopoeia 1996**, ;** Balandrin, et al, 1985; Botanical Survey of India, 2002; Kumari, et al, 1976). On account of the multi-various uses of rutin as stated above, it was considered prudent to undertake study on the qualitative and quantitative analysis of rutin from unexplored plant sources – *C.nilagiriana* to be precise, aided by technologically advanced facilities on hand – like, HPTLC, for instance.

Materials and Methods

Plant material and Collection

The individuals of *Caralluma nilagiriana* were collected from foot hills of The Nilgiris, Tamilnadu and their binomial authenticity was confirmed with the voucher specimen deposit available at the Department of Botany, Government Arts College (Autonomous) Coimbatore (Prabakaran.and Kalimuthu, 2013).

Preparation of extraction

Fresh plant was dried at room temperature for two weeks, powdered with a hand mill and then stored at room temperature. About 20g of the powdered material was subsequently subjected to extraction using Soxhlet apparatus in HPLC grade ethanol (99.9%) for seven days. The extract was filtered and concentrated in rotary evaporator under reduced pressure (Vacuum 175 mbar for bp at 40^{o}C) to get 3.5g of thick, green, crude ethanolic extract (Jadge and Naik, 2009; Harbourne, 1984; Prashant Tiwari, 2011; Renuka, 2013).

Methodology

Instrumentation

TLC technique was adopted to separate the flavonoids, to identify and quantify rutin. It was done using CAMAG HPTLC system equipped with a sample applicator Linomat 5, twin trough development chamber of size (10x10) and (20x10), TLC Scanner and Visualizer together with winCATS integration software.

Reagents and Chemicals

Analytical grade ethanol, ethyl acetate, water, glacial acetic acid and formic acid were obtained from Merck and SD fine Chem. Ltd, Mumbai. The rutin standard was obtained from Sigma Aldrich, Bangalore. Precoated TLC aluminium sheets silica gel 60 F254 (10 x 10 cm, 20 x10 cm, 0.2 mm thick) were obtained from E. Merck Ltd, Mumbai.

Preparation of Test Sample

170 mg of ethanolic crude extract of the sample was diluted with ethanol up to 50 ml, sonicated for 15 min; centrifuged and 5 µL of the test sample was applied as band on plate for quantification.

Preparation of Standard Rutin Solution

10 mg of rutin was prepared with ethanol to make a stock solution of 1000 ppm strength. From this stock solution 2.5ml was diluted up to 50ml to obtain the final concentration of 50 ppm *i.e*. 50 µg/ml.

Chromatographic Conditions

Samples of ethanolic extract of *C.nilagiriana* and standard rutin were spotted on a precoated TLC aluminium sheet silica gel 60 F254 (10 x10 cm, 20 x 10 cm, 0.2 mm thick) as 8 mm wideband using automatic TLC applicator Linomat 5 at 8 mm from the bottom. The mobile phase used was ethyl acetate: water: acetic acid: formic acid (8:1:0.5:0.5 v/v) and the plates were kept for saturation in twin trough chamber for 30 min. After development, the plates were dried in air and scanned at 366 nm by using CAMAG TLC-Scanner with uv-vis spectrum,

Linomat 5 samples applicator equipped with a 100 µL syringe. A constant application rate of 5 µL s-1 was used. Automatic scanning was done with CAMAG TLC Scanner in remission absorbance mode controlled by winCATS software resident in the system. The slit dimensions were 6.00 x0.45 mm, micro and the scanning speed was 20 mm s-1. The radiation source was deuterium lamp and W emitting continuous UV radiation between 190-500 nm. The plates were photographed at 254 nm and 366 nm by using CAMAG Visualizer.

Results

Optimization of sample preparation

C.nilagiriana ethanolic shoot extract was subjected to preliminary phytochemical investigation that revealed the presence of flavonoids .Hence the extract was selected for further HPTLC analysis (Renuka, 2013; Badgujar and Jain, 2009; Chandrasekar and Rao, 2013; Subramanian sampathkumar and Ramakrishna, 2011).

Optimization of HPTLC condition

In an effort to optimize mobile phase composition *i.e.* ethyl acetate-acetic acid-formic acid-water in different proportions were investigated and the ratio [8.0:1.0:0.5: 0.5 (v/v)] yielded sharp and well defined rutin peaks of Rf 0.27 ± 0.02 (Fig 1). To get such a result the chamber had to be saturated with the mobile phase for 30 min at room temperature before developing the plate. Only at 366 nm it was possible to obtain 100% area separation of extracted sample in terms of precision and accuracy (Fig-2). The plates were photographed at 254 nm and 366 nm by using CAMAG Visualizer instrument and the typical spots were observed (Fig-3, 4).

Calibration curve for Standard Rutin

The method was found to be very specific; since the overlaying spectra of standard rutin and of sample matched perfectly (Fig-5), the above constructed standard curve can safely be treated to be very dependable for the quantification of rutin from unknown samples subject to the condition that range used in the standard curve is not deviated. The standard solutions (15-200 ng/spot) were applied on HPTLC plate and further it was developed and scanned as per the chromatographic conditions mentioned above. The calibration standards preparation and peak areas were recorded (Table-1 & 2). Calibration curve of rutin was prepared by plotting peak area against concentration of rutin applied shown in (Fig-6). Standard rutin (*Rf*: 0.26) showed same and single peak in HPTLC chromatograms. In the present analysis calibration curve of standard rutin was found to be linear (Y=-89.688+10.288 *r=0.99979 sdv=2.45). The correlation coefficient of rutin was found to be 0.99365. Based on the above curve the quantity of rutin in the analyzed sample of C.n*ilagiriana was* found to be 0.18% through HPTLC. The interpretation of results suggests that the sample contained considerable amount of rutin (Tables: 3 and 4). The derivatized HPTLC photograph of standard rutin and sample extract represented appropriate dots in yellow color (Fig-7). Photo documentation of developed photo and 3D Calibration graph is shown in Fig-8 &9

Detection of spots

The developed plate was dried by hot air to evaporate solvents followed by the spraying with1% $AlCl_3$ reagent (1g of Aluminum chloride in ethanol) and dried in hot air oven for 3 min. The plate was kept in photo-documentation chamber (CAMAG-Visualizer) and the images were captured under UV light at 366 nm showing yellow spots. The Rf values and finger print data were recorded by winCATS software.

Discussion

In many of the herbal medicines, the active components are not known; moreover, genetic and environmental factors often influence the concentration of the secondary metabolites which are usually the bioactive principles (Mukherjee, 2002; Matsumoto and Hamamoto, 1990). In practice one of those active principles is selected as a marker which is used to determine the quality of the herbal medicine. The phytochemical analysis conducted on *C.nilagiriana* extract revealed the presence of different phytoconstituents including steroidal, triterpenoidal, phenolic and flavonoidal compounds (Akerale, 1991). The curative properties of medicinal plants are due to the presence of various classes of secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, sterols *etc* (Liu, 1991; Yokoi, 1985). Owing to their membrane permeability, the flavonoids possess potential antioxidant activity that give them protective action against allergies, inflammation, free radicals, platelet aggregation, ulcers, certain microbes, hepatoxins, viruses and tumors (Abou-Karam and Shier, 1992; Skibola and Smith, 2000).

Table 1: The summary report of the calibration standards preparation

Substances-stock solution RUTIN	50.0000 mg/L	50.0000 mg/L	50.0000 mg/L	50.0000 mg/L	50.0000 mg/L	50.0000 mg/L	50.0000 mg/L	50.0000 mg/L
Preparation of concentrations	3 mg/L	5 mg/L	10 mg/L	15 mg/L	20 mg/L	25 mg/L	30 mg/L	40 mg/L
Dilution from volume	0.300mL	0.500mL	1.000mL	1.500mL	2.000mL	2.500mL	3.000mL	4.000mL
Dilution to volume mL	5.000	5.000	5.000	5.000	5.000	5.000	5.000	5.000
Application Volume	5.000 µL	5.000 µL	5.000 µL	5.000 µL	5.000 µL	5.000 µL	5.000 µL	5.000 µL
Vial	1	2	3	4	5	6	7	8

Regression via area: Linear Y=-89.688+10.288 *r=0.99979 sdv=2.45

Table 2: The summary report of the calibration standards and sample

Track	vial	Rf	Amount	Area	X(calc)	Sample
1	**1**	**0.26**	**15.00ng**	**84.05**		
2	**2**	**0.25**	**25.00ng**	**169.70**		
3	**3**	**0.26**	**50.00ng**	**396.56**		
4	**4**	**0.26**	**75.00ng**	**602.97**		
5	**5**	**0.25**	**100.00ng**	**798.38**		
6	**6**	**0.26**	**125.00ng**	**1159.65**		
7	**7**	**0.26**	**150.00ng**	**1458.53**		
8	**8**	**0.26**	**200.00ng**	**1969.49**		
9	**9**	**0.25**		**1760.75**	**179.22ng**	**plant-extract**

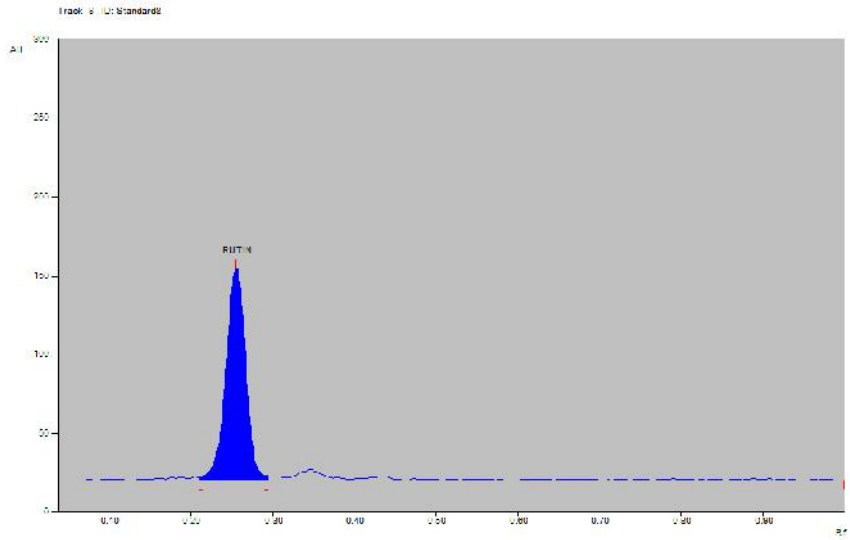
Table 3: HPTLC profile of the global peak table of standard rutin

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.20	2.5	0.26	184.0	100	0.32	3.3	1969.49	100

Table 4: HPTLC profile of the global peak table of sample caralluma nilagiriana at 366 nm

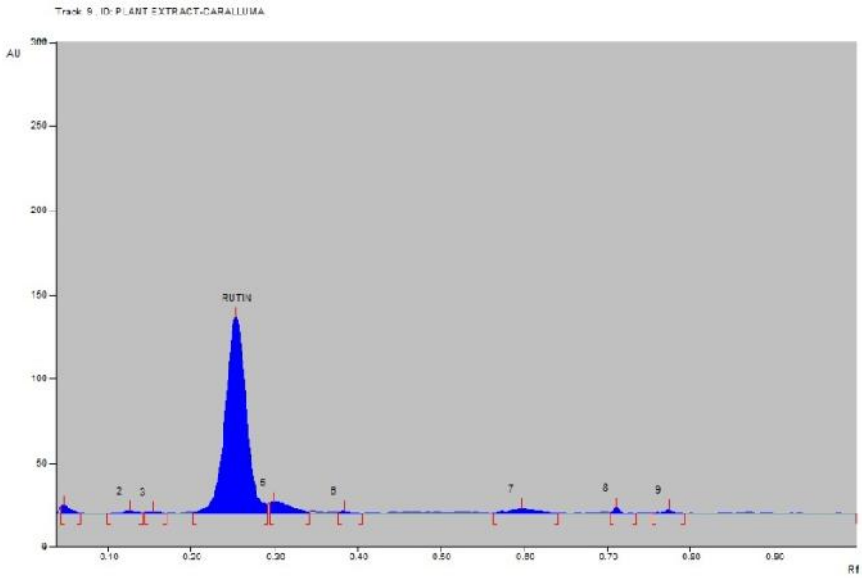
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.20	1.2	0.25	166.6	100	0.29	5.8	1760.75	100

Figure 1: The images represent the Chromatogram of standard Rutin



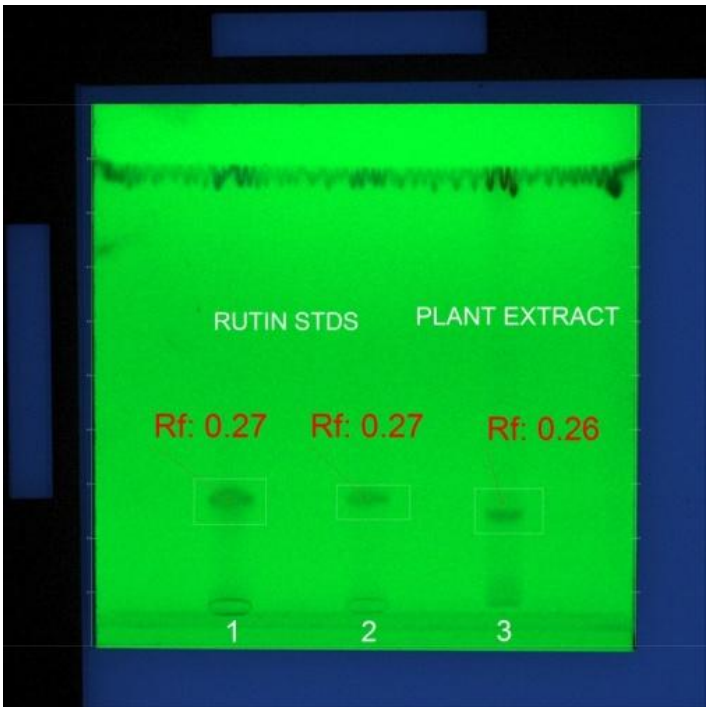
The integration area under curve is the amount of rutin using winCATS software.
Sorbent layer: HPTLC silica gel 60 F254 Aluminium sheets
Mobile phase: Ethyl acetate/water/acetic acid/formic acid (8+1+0.5+0.5 v/v)
Detection: 1% w/v aluminium chloride in ethanol
Concentration: 200 ng
Scanning: UV 366 nm absorption mode

Figure 2: The images represent the Chromatogram of sample at 366nm



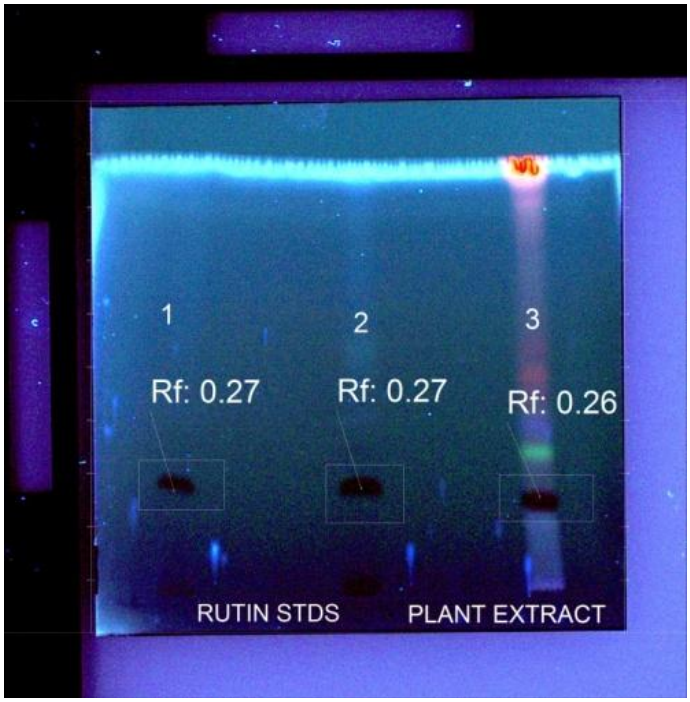
The integration of sample (*Caralluma nilagiriana*) at 366 nm area under curve is the amount of rutin using winCATS software.
Separation of Rutin in plant extract-*Caralluma nilagiriana*
Sorbent layer: HPTLC silica gel 60 F254 Aluminium sheets
Mobile phase: Ethyl acetate/water/acetic acid/formic acid (8+1+0.5+0.5 V/V)
Detection: 1% w/v aluminium chloride in ethanol
Scanning: UV 366 nm absorption mode

Figure 3: The images represent the Photo documentation of Rutin and sample at 254 nm



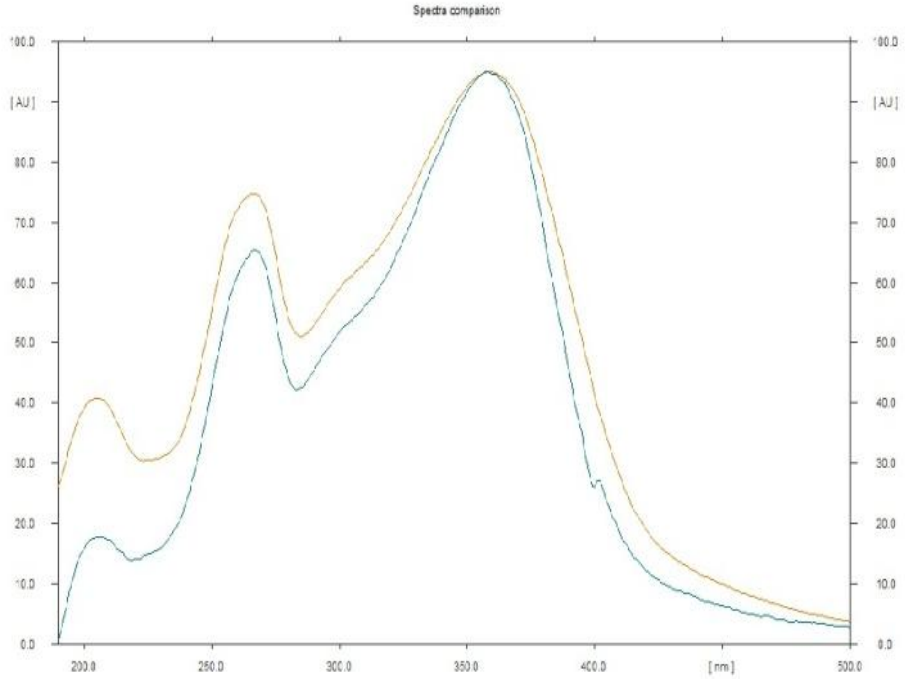
Developed image of sample and STD
The concentration (50 ppm) of rutin standards 10 µL & 5 µL are (1) & (2) respectively and their Rf values are 0.27. (3) *Caralluma* plant extract shows 0.26 Rf.
Examination: UV 254 nm

Figure 4: The images represent the Photo documentation of Rutin and sample at 366 nm



Developed image of sample and STD
The concentration (50 ppm) of rutin standards 5 µL & 10 µL are (1) & (2) respectively and their Rf values are 0.27. (3) *Caralluma* plant extract shows 0.26 Rf.
Examination: UV 366 nm (Dark spot color)

Figure 5: The Overlay uv-vis spectrum represents the Rutin and Sample



The Overlay Spectrum of standard rutin and sample at 359 nm respectively then position difference is 0.11 and correlation of the spectrum was 0.98365.

Figure 6: The calibration curve plotted against concentration and area. The Red mark shows the concentration of the sample.

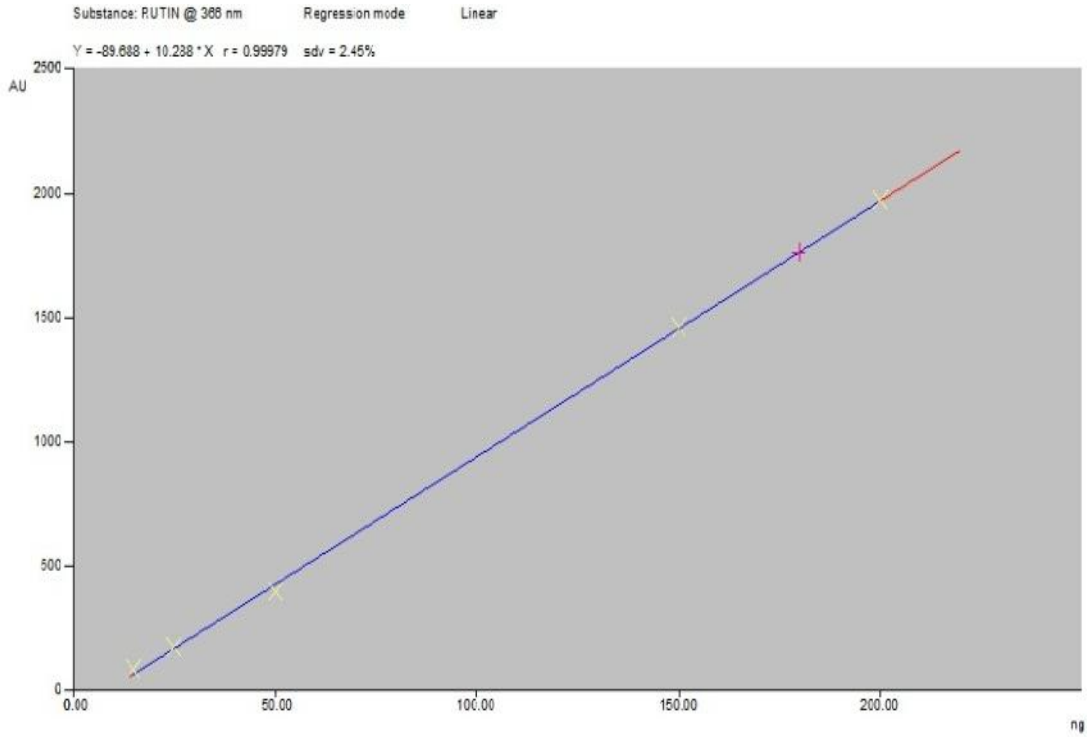


Figure 7: The images represent the Photo documentation of the derivatized image of Rutin and sample at 366 nm

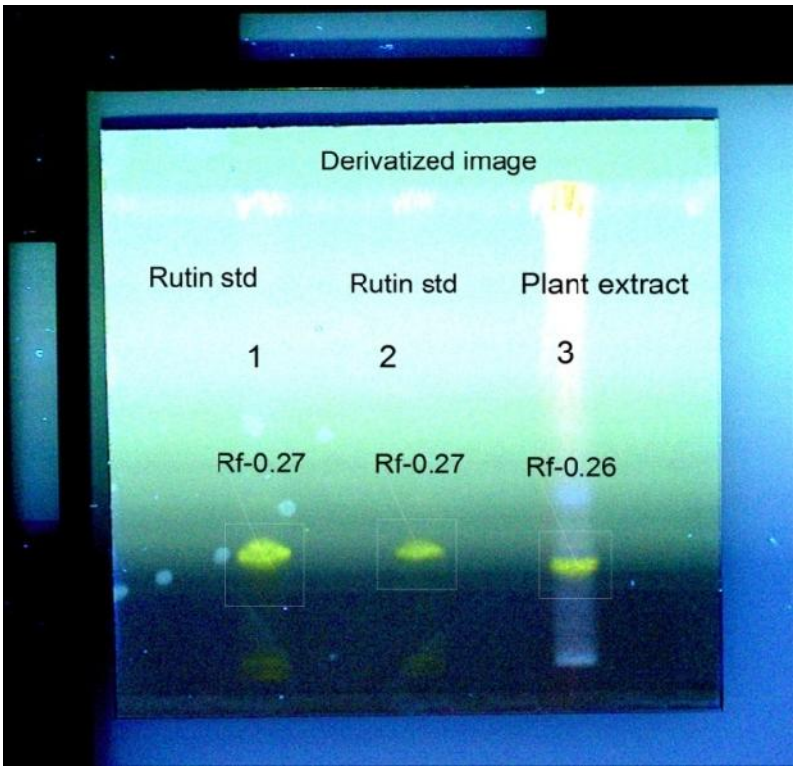
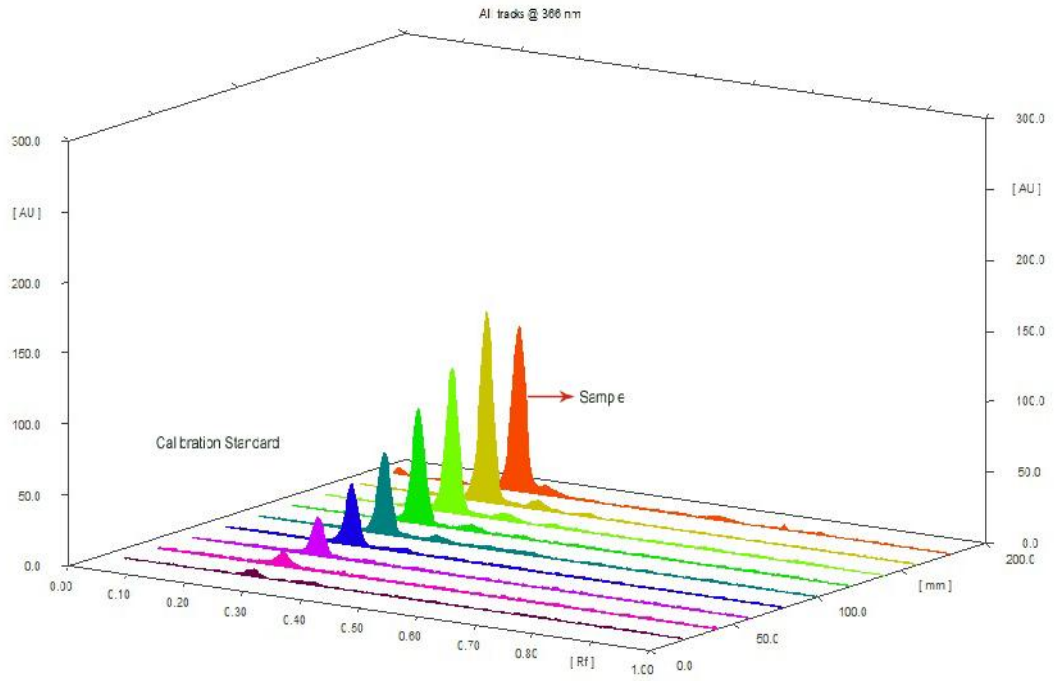


Figure 8: Developed photo of calibration of rutin standards at 254 nm

Figure 9: The overlay 3D densitogrames of calibration rutin standards at 366 nm



HPTLC technique is used to solve many qualitative and quantitative analytical problems in wide ranging fields, including medicine, pharmaceuticals, chemistry, biochemistry, food analysis, toxicology and environmental analysis (Ashok Kumar, et al., 2010; Dinesh, et al., 2012; Preetitiwari and Rakesh, 2012; Rajasekaran et al., 2011; Shubashini et al., 2011). The use of HPTLC has expanded considerably due to the development of gradient TLC methods. In the present study, different concentrations of standard solution of rutin were applied on HPTLC plates along with ethanolic extract of *C. nilagiriana*. The results obtained by adoption of methodologies outlined, clearly support that these may help in quali-quantitative analyses of rutin from any unknown plant source. Within the range of the designed standardized curve for rutin that reflects validated precision and accuracy, the presented methodology can be used with confidence. Methods that are so far in use elute several other compounds along with the flavonoids while the currently investigated technique, when carried out at 366nm, is found to be optimum for identifying and separating flavonoids. The linear relationship between the peak areas and the concentrations prove the dependability of the present method. Applying the procedural steps thus far described, it was found that the sample material contained considerable quantity (0.018%) of rutin, making *C.nilagiriana* invaluable in the fields of cosmetics and therapeutics. However, as will be required, this herbal product needs to be standardized with respect to safety before releasing the same into the market.

Conclusion

The HPTLC method developed for determination and quantification of rutin in the shoots of *C. nilagiriana* was found to be precise, simple, and specific and sensitive within the tested concentration range.

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