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Research Article

PHYTOCHEMICAL AND HPTLC STUDIES ON THREE MEDICINALLY IMPORTANT PLANTS *RAUVOLFIA TETRAPHYLLA*, *PHYLLANTHUS NIRURI* AND *CARISSA CARANDAS*

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ABSTRACT

The phytochemical investigations of methanolic extracts of the three plants *Rauvolfia tetraphylla*, *Phyllanthus niruri* and *Carissa carandas* showed the presence of major groups of secondary metabolites such as terpenoids, tannins, flavonoids, phenols and alkaloids. HPTLC chromatogram of methanolic extracts of the three plants *R. tetraphylla*, *P. niruri* and *Carissa carandas* showed eight, eight and five peaks respectively. The Densitometric scanning indicates the highest peak area which is proportional to the maximum concentration of that particular compound.

Keywords: *Rauvolfia tetraphylla*, *Phyllanthus niruri*, *Carissa carandas*, HPTLC, secondary metabolites.

1. INTRODUCTION

Medicinal plants besides therapeutic agents are also a big source of information for a wide variety of chemical constituents which could be developed as drugs with precise selectivity. These are the reservoirs of potentially useful chemical compounds which could serve as newer leads and clues for modern drug design[1]. The most important of these bioactive constituents of plants are terpenoids, alkaloids, tannins, flavonoids and phenolic compounds[2]. Correlation between the phytoconstituents and the bioactivity of plant is desirable to know for the synthesis of compounds with specific activities to treat various health ailments and chronic diseases as well [3].

Phyllanthus niruri is a plant of the family Euphorbiaceae and has about approximately 800 species which are found in tropical and subtropical countries of the world[4]. In India, *Phyllanthus niruri* is widely distributed as a weed in cultivated and waste lands[5]. It is used in several health problems such as diarrhoea, dysentery, dropsy, jaundice, intermittent fevers, urinogenital disorders, scabies and wounds[6,7,8].

Rauwolfia tetraphylla L. (Family: Apocynaceae) is a small, tree cultivated on commercial scale in India. It is an economically important medicinal plant because of the presence of various indole alkaloids in its different parts[9]. It possess various significant activities and roots of it are used to stimulate uterine contraction, decotion of shoot is used for stomachache, juice extracted from the root is used to treat muscular and rheumatism pain and Leaf decotion is used against cough and cold, leaf paste is used for skin disease among various tribes of Kerala[10].

Carissa carandas belongs to Apocynaceae. Traditionally, whole plant and its parts are used in the treatment of various ailments. The notable biological activities reported are analgesic, anti-inflammatory, anti-pyretic, anti-malarial, cardiotonic and histamine releasing. Additionally it has shown wide range of evidences for its, hepato-protective, free radical scavenging, anti-rheumatic, anti-viral and anti-convulsant activities[11,12].

HPTLC is becoming a routine analytical technique because of advantages that include the small amount of mobile phase required, the speed of the method, and the possibility of analysis of several samples simultaneously unlike HPLC, thus it reduces analysis time and cost. Automatic sample application is possible and repeated scanning can be performed on the same plat[13].

2. MATERIALS AND METHODS

2.1. Collection of plant material

Fresh leaf material of *Rauvolfia tetraphylla*, total plant material of *Phyllanthus niruri* and roots of *Carissa carandas* were collected in and around Visakhapatnam, Andhra Pradesh, India. Taxonomic identification of the collected plants was carried out with the herbarium present in the Department of Botany, Andhra University, Visakhapatnam.

2.2. Extraction of plant material

The respective plant materials of the above three plants were washed thoroughly with running tap water and air dried under shade. After complete shade drying the plant materials were grinded and the powders were kept in small plastic bags with paper labeling. The extraction was done by using soxhlation extraction method with analytical grade methanol as refluxing solvent. At the completion of extraction process, the plant extracts were recovered from the mixture by distillation and stored at 4°C until further use. Methanolic extracts were used for the preliminary screening of secondary metabolites.

2.3. Phytochemical screening

Preliminary qualitative phytochemical screening was carried out with the following methods.

Terpenoids

Two ml of each extract was added to 2 ml of acetic anhydride and concentrated H_2SO_4 separately and the formation of blue/ green rings indicates the presence of terpenoids[14].

Tannins

Two ml of each extract was added to few drops of 1% lead acetate separately and formation of yellow precipitate indicates the presence of tannins[15].

Saponins

Five ml of each extract was mixed with 20 ml of distilled water separately and then agitated in a graduated cylinder for 15minutes. Formation of foam indicates the presence of saponins[16].

Flavonoids

Aqueous extracts of the three plants were mixed with few fragments of magnesium ribbon separately and concentrated HCl was added drop wise. Appearance of pink scarlet colour after few minutes, indicate the presence of flavonoids.

Phenols

Aqueous extracts of the three plants were mixed with 2ml of 2% solution of $FeCl_3$ separately and formation of blue-green or black coloration indicates the presence of phenols.

Alkaloids

Aqueous extracts of the three plants were mixed with 2ml of 1% HCl separately and heated gently. Mayer's and Wagner's reagents were then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

2.4. Quantitative phytochemical analysis

2.4.1. Total phenolic content

The total phenolic content was determined spectrophotometrically by the method described by Sadasivam and Manickam[17] after precipitation of proteins. To 2ml of plant extract 1.0 ml of Folin Ceo-calteau reagent was added. After 3 minutes, 13 ml of distilled water was added. Later 2 ml of sodium carbonate (7.5%) solution was added and the volume was adjusted to 20 ml. The above mixture was kept for 1 hour for colour development and absorbance was recorded at 630 nm. The concentration of total phenolic content in plant extracts was calculated from the calibration curve of Gallic acid and it was expressed as Gallic acid equivalents/gram fresh weight. Each experiment has three replicates and the experiment was repeated thrice.

2.4.2. Total flavonoid content

Total flavonoid content was measured by aluminum chloride colorimetric assay described by Marinova *et al*[18]. One ml of plant extract was added to 10 ml volumetric flask containing 4 ml of distilled water. To the above mixture, 0.3ml of 5% $NaNO_2$ was added. After 5 minutes, 0.3ml of 10% $AlCl_3$ was added. At 6^{th} min, 2 ml of 1 M NaOH was added and the volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. The total flavonoid content in plant extracts was calculated from the calibration curve of catechin and it was expressed as Gallic acid equivalents/gram fresh weight. Each experiment has three replicates and the experiment was repeated thrice.

2.4.3. Tannin content

The tannin content was determined by the method given Sadasivam and Manickam[17]. To 1.0 ml of plant extract 3.5 ml of distilled water and 0.5 ml of Folin-Denis reagent was added.

Contents were allowed to mix then 1ml of saturated sodium carbonate solution was added. The final volume was made up to 10ml with distilled water. The solution was mixed well at room temperature and the absorbance was measured against prepared reagent blank at 760 nm. Tannin content in plant extracts was calculated from the calibration curve of tannic acid (10-100 µg) and it was expressed as tannic acid equivalents/gram weight. Each experiment has three replicates and the experiment was repeated thrice.

2.5. HPTLC analysis

In the present study CAMAG HPTLC system equipped with Linomat V applicator, TLC scanner 3, Reprostar 3 with 12 bit CCD camera for photo documentation, controlled by WinCATS-4 software was used. All the solvents used were HPLC grade obtained from Merck. Each extract of 100 mg was dissolved in 5 ml of methanol and the solutions were centrifuged at 3000 rpm for 5 min and used for HPTLC analysis as test solution. The samples (2µl) were spotted in the form of bands of width 5 mm with a Camag microlitre syringe on precoated silica gel glass plate 60F-254 (10 cm×10 cm) with 250 µm thickness (E-Merck, Darmstadt, Germany) using a Camag Linomat IV (Switzerland). The plates were prewashed by methanol and activated at 60°C for 5 min prior to chromatography. The sample loaded plate was kept in thin-layer chromatography (TLC) twin through developing chamber after saturated with solvent vapor with respective mobile phase and the plate was developed in the respective mobile phase up to 90 mm. The chloroform: acetone: methanol: acetic acid: water (50:20:10:10:5) was employed as mobile phase for secondary metabolites. Linear ascending development was carried out in (10 cm × 10 cm) twin trough glass chamber (Camag, Mutenz, Switzerland) saturated with the mobile phase and the chromatoplate was developed twice with the same mobile phase to get good resolution of phytochemical contents. The optimized chamber saturation time for mobile phase was 30 min at room temperature [(25 ± 2) °C]. The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images under UV light at 254 and 366 nm. The plate was photo-documented at UV 366 nm and daylight using photodocumentation (CAMAG REPROSTAR 3) chamber. Finally, the plate was fixed in scanner stage and scanning was done at 366 nm. Densitometric scanning was performed on Camag TLC scanner III and operated by CATS software (V 3.15, Camag).

3. RESULTS

The phytochemical screening and analysis of three medicinally important plants showed that leaf, whole plant and root extracts were rich in major secondary metabolites such as terpenoids, tannins, flavonoids, phenols and alkaloids (Table 1).

Table 1: Preliminary screening of secondary metabolites in *Rauvolfia tetraphylla, Phyllanthus niruri* and *Carissa carandas*

S. No	Name of the phytochemicals	*Rauvolfia tetraphylla*	*Phyllanthus niruri*	*Carissa carandas*
1	Terpenoids	+ve	+ve	+ve
2	Tannins	+ve	+ve	+ve
3	Flavonoids	+ve	+ve	+ve
4	Phenols	+ve	+ve	+ve
5	Alkaloids	+ve	+ve	+ve

Table 2: Quantitative analysis of phytochemicals in *Rauvolfia tetraphylla, Phyllanthus niruri* and *Carissa carandas*

S. No	Phytochemical	*Rauvolfia tetraphylla**	*Phyllanthus niruri* *	*Carissa carandas**
1	Phenols (mg/gm)	167.96±2.86	165.12±4.10	207.49±4.99
2	Tannins (mg/gm)	32.67±3.06	34.33±3.21	136.33±6.03
3	Flavonoids (mg/gm)	217.60±8.81	227.15±3.84	67.65±5.79

***** Each value represents mean±SD of three independent experiments and the values were significant at $p<0.05$.

Quantitative analysis of *Rauvolfia tetraphylla* reveals the phenolic content of (167.96±2.86), tannins (32.67±3.06) and flavonoids (217.60±8.81). The plant extract of *Phyllanthus niruri* showed the phenolic content of (165.12±4.10), tannins (34.33±3.21) and flavonoids (227.15±3.84). Similarly the root extract of *Carissa carandas* also showed phenolic content of (207.49±4.99), tannins (136.33±6.03) and flavonoids (67.65±5.79) respectively (Table 2).

HPTLC analysis

The mobile phase for phenolic analysis in the plant extract was chloroform: acetone: methanol: acetic acid: water (50:20:10:10:5) and the resolution was at 254 nm. The results were presented in the form of figures and tables. The methanolic leaf extract of *Rauvolfia tetraphylla* showed 8 peaks, with their corresponding Rf values 0.07, 0.24, 0.37, 0.46, 0.51, 0.66, 0.78 and 0.89. The highest peak area 5615.7 AU and lowest peak areas 276.0 AU were observed at Rf 0.24 and 0.07 respectively (Table 3 & Fig 1). The methanolic whole plant extract of *Phyllanthus niruri* showed 8 peaks, with their corresponding Rf values 0.21, 0.40, 0.51, 0.57, 0.69, 0.85, 0.90 and 0.94. The highest peak area 13564.1 AU and lowest peak areas 2022.1 AU were observed at Rf 0.69 and 0.40 respectively (Table 4 & Fig 2).). The methanolic root extract of *Carissa carandas* showed 5 peaks, with their corresponding Rf values 0.21, 0.40, 0.51, 0.57, 0.69, 0.85, 0.90 and 0.94. The highest peak area in HPTLC chromatogram of *Carissa carandas* 4463.8 AU and lowest peak areas 180.7AU were observed at Rf 0.99 and 0.01 respectively (Table 5 & Fig 3).

Table 3: HPTLC secondary metabolite profiles of leaf extract of *Rauvolfia tetraphylla*

Rauvolfia tetraphylla	Peak	Rf	Height (mm)	Area (AU)	Assigned substance
Leaf	1	0.07	26.6	276.0	unknown *
	2	0.24	147.3	5615.7	unknown *
	3	0.37	124.1	4520.9	unknown *
	4	0.46	69.1	1455.9	Quercetin
	5	0.51	140.6	2592.8	unknown *
	6	0.66	15.6	468.7	unknown *
	7	0.78	39.4	1049.7	unknown *
	8	0.89	137.7	7364.6	unknown *

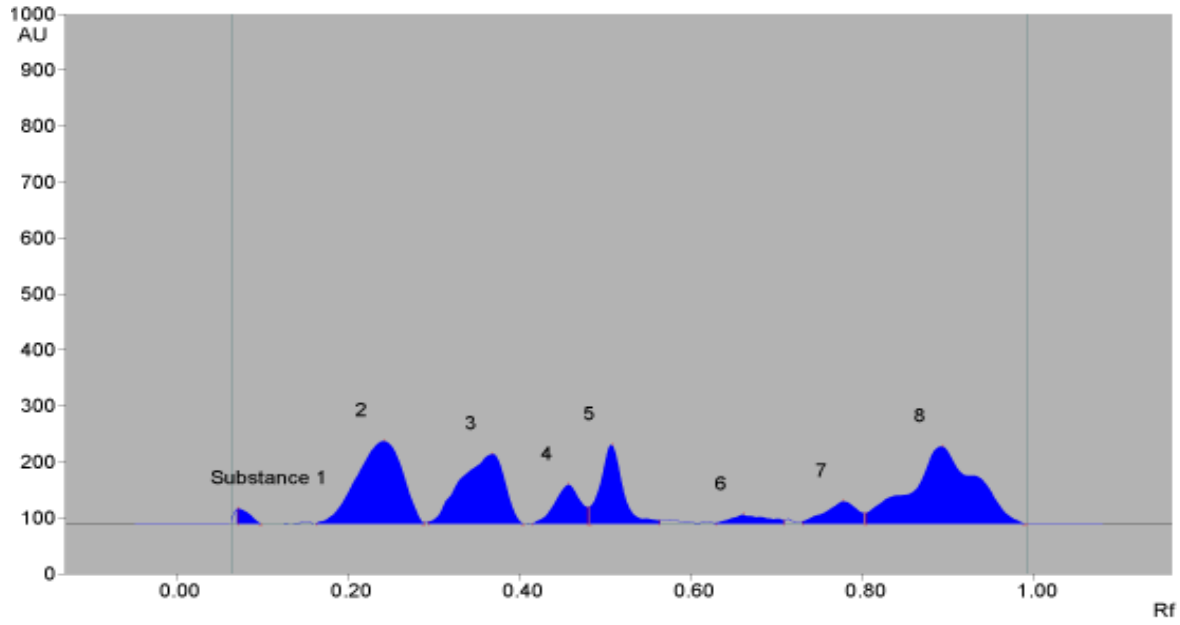

Fig. 1: Densitometric scanning of TLC plate on Camag TLC scanner III

Table 4: HPTLC secondary metabolite profiles of whole plant extract of *Phyllanthus niruri*

Parts of the *Phyllanthus niruri*	Peak	Rf	Height (mm)	Area (AU)	Assigned substance
Whole plant	1	0.21	414.3	11741.6	unknown *
	2	0.40	51.8	2022.1	unknown *
	3	0.51	372.3	10585.6	unknown *
	4	0.57	263.4	8031.9	unknown *
	5	0.69	344.0	13564.1	Caffeic acid
	6	0.85	195.8	8596.9	unknown *
	7	0.90	236.5	6752.3	unknown *
	8	0.94	209.3	5689.8	unknown *

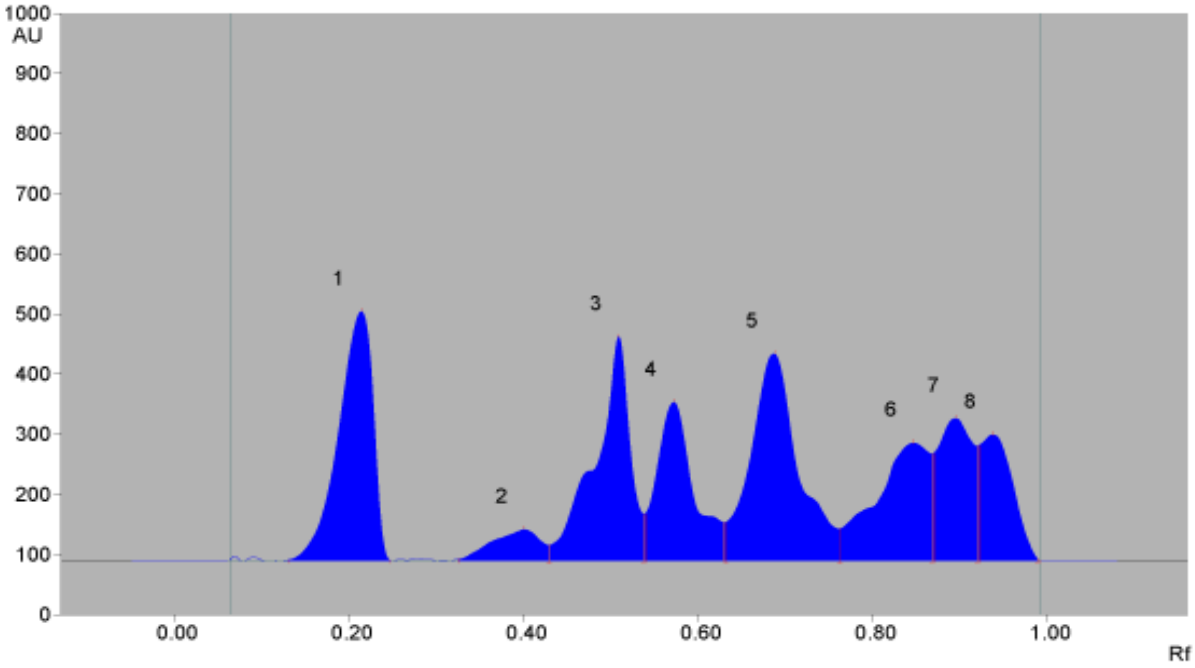

Fig. 2: Densitometric scanning of TLC plate on Camag TLC scanner III

Table 5: HPTLC secondary metabolite profile of root extracts of *Carissa carandas*

Carissa carandas	Peak	Rf	Height (mm)	Area (AU)	Assigned substance
Root	1	0.01	14.6	180.7	unknown *
	2	0.04	70.6	1286.0	unknown *
	3	0.85	107.6	2701.1	unknown *
	4	0.99	125.3	4463.8	unknown *
	5	1.03	129.4	2407.2	unknown *

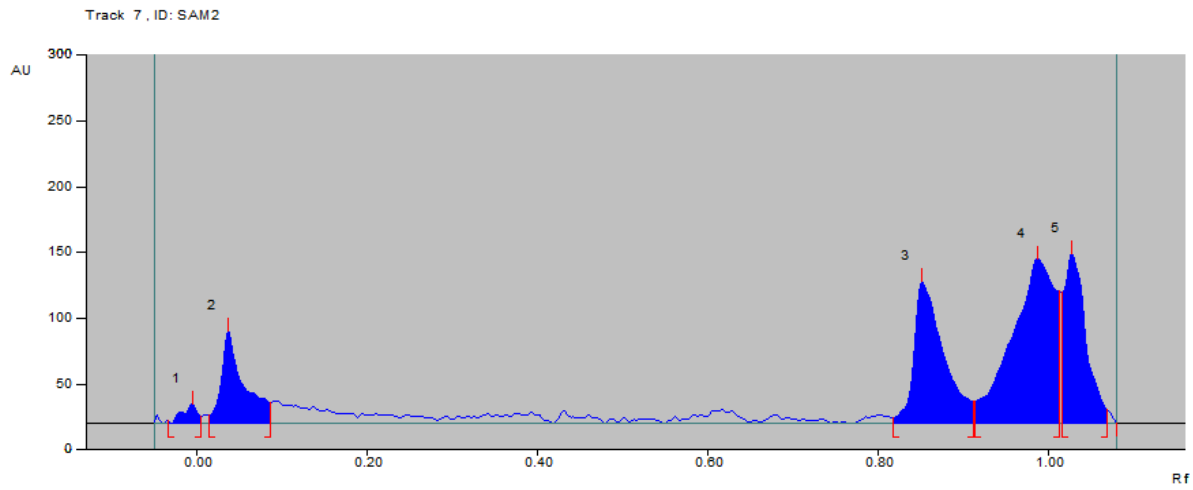

Fig. 3: Densitometric scanning of TLC plate on Camag TLC scanner III

4. DISCUSSION

Phytochemical constituents present in the plant extracts are the biologically active compounds and they are responsible for different activities such as antioxidant, antimicrobial, antifungal, and anticancer[19,20]. Most of the secondary metabolite components were isolated and identified in the polar plant crude extracts[21]. Different plant parts like leaf material of *Rauvolfia tetraphylla*, total plant material of *Phyllanthus niruri* and roots of *Carissa carandas* were used in the study as these parts were known to be rich in secondary metabolites.

The methanolic extracts from three plants revealed the presence of terpenoids, tannins, flavonoids, phenols and alkaloids (Table 1). Similar results with methanolic plant extracts were reported by Paulsamy and Jeeshna[22] in *Exacum bicolor,* Ofokansi *et al*[23] in *Bryophyllum pinnatum* and *Ocimum gratissimum,* Subhashini Devi *et al*[24] in *Boswellia serrata* and *Wrightia tinctoria,* where methanol can extract high variety of plant secondary metabolites than the other solvents did.

From the results of quantitative analysis it was observed that roots of *Carissa carandas* are rich in both phenols and tannins where as *Rauvolfia tetraphylla* and *Phyllanthus niruri* are rich in flavonoids (Table 2). *Phyllanthus niruri* (Bhui Amla) has been used worldwide as an additive in medicinal formulas. The entire plant is used for this purpose *Rauvolfia tetraphylla* is a frequently available species of *Rauvolfia* which is prevalently used in Ayurvedic and unani system of medicines and also a part of folk remedies of most Asian countries[25]. From medicinal point of view, *R. tetraphylla* is significant in the treatment of cardiovascular diseases, hypertension and a variety of psychiatric diseases[26]. *Carissa carandas* has shown wide range of evidences for its, hepato-protective, free radical scavenging, antirheumatic, antibacterial, antiviral and anticonvulsant activity[27]. From the results obtained in the present study and also through literature search confirms that several secondary metabolites synthesized in plants have significant defensive role against herbivores, pests and pathogens. The defensive role played by such secondary metabolites include deterrence activity, toxicity or acting as precursors to physical defense systems.

The importance of tannins in possessing analgesic, anti-oxidant, anti-microbial, anti-viral and anti-inflammatory activities was reported by Nand *et al*.[28] and Chung *et al*.[29]. The function of tannins as binding molecules to proline rich proteins and blocks protein synthesis was reported by Yadav and Agarwal[30]. Plant polyphenols as protective molecules against the development of cancer, cardiovascular diseases, diabetes and neurodegenerative diseases was reported by Daniel and Krishnakumari[31]. Similarly, flavonoids as inhibitors of lipid-peroxidation, platelet aggregation, capillary permeability and fragility were reported by Cook and Samman[32]; Chebil *et al*.[33] and Middleton *et al*.[34]. As the selected plants are rich in all the major classes of secondary metabolites further studies are needed to establish the role of these plants in controlling various ailments.

The peak 4 of HPTLC chromatogram of methanolic leaf extract of *Rauvolfia tetraphylla* was identified as quercetin by comparing same Rf value of quercetin standard. Similarly the peak 5 of HPTLC chromatogram of methanolic whole plant extract of *Phyllanthus niruri* was identified as caffeic acid by

comparing same Rf value of caffeic acid standard. The remaining Rf values were not coinciding with the Rf values of the standards used in this study. But the Rf values of HPTLC chromatogram of methanolic root extract of *Carissa carandas* was not coinciding with the Rf values of the standards used in this study.

The utilization of HPTLC, owing to the high automatization, can be considered as an useful tool in the analysis of complex mixtures of natural products as was stated by Marcello Nicoletti[35]. Even the technique is also used for identification and quality evaluation of vegetal raw material[36]. Keeping in view of all the advantages, HPTLC was used in the present study in order to know the important components of the selected plants.

5. CONCLUSION

The results of the present study indicate that the three plants *Rauvolfia tetraphylla*, *Phyllanthus niruri* and *Carissa carandas* are rich in potent molecules having significant pharmaceutical and medicinal properties. HPTLC analysis using solvent system for phenolic compounds indicates the presence of phenolic compounds as well as the presence of secondary metabolites other than phenolics. Hence the study will be further extended to know the presence of other secondary metabolites and also their purification using different chromatographic and spectroscopic techniques.

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