

HPTLC pattern assessment of *Avicennia marina* stem and spectrometric analysis of the separated phyto-constituents

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ABSTRACT

The scientific evaluation, validation and authentication of medicinal plants are highly essential and key steps to achieve effective phytochemicals and drugs for disease prevention. The checklist of medicinal plants especially mangroves along with their scientific validation is still biased. The traditional methods are poor, time consuming as well as less scientific, so there is a need to use emerging technological knowledge and sophisticated analytical methods to fulfill this knowledge gap. Therefore, present study is an attempt to study the phytochemistry and recognize HPTLC pattern of *Avicennia marina* stem and spectrum analysis of resolved compounds in three different extracts. As a result, presence of different class of phytochemicals were detected. HPTLC determination densitometry analysis of 10 µl sample size under UV- 258 nm of stem extract – 1 (5 % NH₃) showed the presence of 2 components with R_f values ranging from 0.76– 0.90; the stem extract – 2 (methanol alone) revealed the presence of 4 components with R_f values in the range of 0.03 – 0.90; and the stem extract – 3 (5 % HCl) revealed presence of 7 components with R_f values in the range of 0.03 – 0.90. As result, extract – 3 (Slightly acidic) gave maximum number of separated compounds and characteristic fingerprinting pattern and may be useful as a biochemical integrity check marker for this plant as well as to assess the diversity and further characterization of phyto-compounds present in *Avicennia marina* stem.

KEY WORDS: AVICENNIA MARINA, BIOCHEMICAL MARKERS, EXTRACT, FINGERPRINTING, PHYTOCHEMICALS, HPTLC, STEM

INTRODUCTION

Plants are the chemical factories of nature and enormous sources of organic chemical entities on this planet (Balandrin *et al.*, 1985; Marderosian, 1987; Pavinato *et al.*,

2008; Udayaprakash *et al.*, 2013). Coastal marine flora, specially mangroves are too an ultimate and rich source of biochemically unique chemicals and have broader degree of valuable phytochemicals (Bandarnayake, 2002; Boopathy and Kathiresan, 2010; Patra and Tha-

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toi, 2011) useful in ethno medicine and pharmacology (Bandarnayake, 1998; Ravindran *et al.*, 2005; Revathi *et al.*, 2013) and as significant role in the field of natural product research and drug discovery since ancient times (Kathiresan and Bingham, 2001; Bandarnayake, 2002; Saikia *et al.*, 2013).

Among all true mangrove species of the world, *Avicennia marina* is a valuable mangrove because of its abundant distribution (Duck *et al.*, 1998), availability and useful medicinal values (Bandarnayake, 1998) of its different parts like leaves, stems, roots, flowers and also as a whole plant formulation, (Ishrak *et al.*, 2003).

The stem of *Avicennia marina* possess various pharmacological and bioactive activities including anticancer (Prakash *et al.*, 2013), antimalarial (Sundaram, 2010 and Rafat *et al.*, 2012), anti-inflammatory (Vinod and Guruvayoorappan, 2012), antioxidant (Thatoi *et al.*, 2014), antibacterial (Sharief *et al.*, 2014), antifungal (Mariam *et al.*, 2006) and antiviral (Ishrak *et al.*, 2003). These and several other studies have made this mangrove a precious and preferable source of new and already existing phytochemicals for disease prevention.

In the field of herbal treatment standardization of plant materials is the essential requirement of the day (Folashade *et al.*, 2012) and the WHO has also emphasized the need to ensure the proper authentication and quality assessment of medicinal plants, their products, extracts along with their formulations (Calixto, 2000; Shrikumer *et al.*, 2006) by applying modern sophisticated techniques for their proper use in medication (Gautam *et al.*, 2010). For this reason HPTLC becomes a routine technique because of its uniqueness, specificity and simplicity for cheap, fast, accurate and reliable identification, authentication and fingerprinting of herbals now a days, (Reich and Schibli, 2007).

The main objective of this study is to stabilize HPTLC fingerprint pattern for *Avicennia marina* stem by compare three different formulations of same methanol extracts of the stem. The present study may serve as a basis for medicinal use of *Avicennia marina* stem and for making strategies related to compounds separation, characterization and isolation from its extracts or formulations.

MATERIAL AND METHODS

Avicennia marina was collected in January 2014 from the S.P. Godrej Marine Ecology Centre, Vikhroli, Mumbai Maharashtra, India (Geographical coordinates 19°05'50.82"N-72°56'24.06"E). The plant materials were identified and authentication certificate issued by the same institute.

The stem was shed dried for 15 days and grounded by mechanical grinder into coarse particles. For extract - 1, the 500 mg of stem power was mixed with 10 ml methanol along with 5 % NH₃. For extract - 2, 500 mg of stem power was mixed with 10 ml methanol. For extract - 3, the 500 mg of stem power was mixed with 10 ml methanol along with 5 % HCl. All three extracts were sonicated for 4 hours until the extract was clear or colorless. Direct sun light and high temperature was avoided to protect heat sensitive phytochemicals. Extracts were filtered through Whitman No.1 filter.

CAMAG HPTLC system equipped with automatic TLC sampler LIMONATE V, TLC scanner 3, REPROSTAR 3 with 12 bit CCD camera was used for photo documentation analysis along with winCATS 4 CAMAG Planer Chromatography software. All the solvents used for HPTLC analysis were of HPLC analytical grade obtained from Merck, India.

Preliminary phytochemical analysis was performed for establishing the phyto-constituents (phenols, alkaloids, terpenoids, steroids, carbohydrates, proteins, amino acids, tannins, saponins, and flavonoids, Gums and Mucilage) present/absence in the extracts according to the standard methods (Evans, 1998; Khandelwal, 2007).

Optimized HPTLC studies were carried out following the methods of Wagner *et al.*, (1996), Harborne (1998) Reich and Schibli (2007) and Mona *et al.*, (2014). The protocol for preparing sample solutions was optimized for high quality and accurate TLC pattern generation.

HPTLC of three different extracts were performed on 10x10 cm commercial Aluminum - sheets precoated with 0.2 mm layer of silica gel 60F₂₅₄ (E. Merck Ltd, Darmstadt, Germany) batch number - 1.05554.0007 stored in a desiccator. Sample extracts were applied as bands 6 mm wide by means of CAMAG Linomat V sample applicator equipped with a 100 µL syringe, programmed through winCATS software. The plate was prewashed with methanol and dried in an oven at 60°C for 5 minutes. The sample loaded plate was kept in TLC Twin Trough Chamber (10x10cm) for 20 min. saturation with the solvent vapors with respective mobile phase. The plate was developed in a linear ascending mode upto 90 mm.

The optimized mobile phase Toluene:Chloroform:n - Propanol: Ethanol (4:4:1:1) were used (Reich and Schibli, 2007). After development, the plate was subjected for drying by hot air device (drier) and 10 minutes on CAMAG plate heater (110 °C) at room temperature and kept it for documentation in the CAMAG TLC visualizer. The CAMAG TLC visualizer capture the images under 254 nm (UV range), 366nm (Fluorescence) and 540nm (White R). After it, Densitometry evaluations were carried out under 258 nm (Deuterium lamp) with the use

of CAMAG Scanner 3 and spectral analysis is performed under D₂ – lamp (range 190 nm – 400 nm). Finally, R_F values calculations, peak areas calculations were analyzed for interpretation and HPTLC Fingerprinting pattern profile generation.

In the last step the plate was derivatized with specific derivatizing agent for visual identification. In this experiment the derivatizing agent was Anisaldehyde Sulphuric Acid reagent (ASR) in the CAMAG derivatizing chamber for 2-3 seconds and air dried.

After drying, the plate was heated on CAMAG plate heater for 3-10 minutes at 110 °C until the color bands can be seen visually. Final images were quickly captured by the CAMAG TLC Visualizer under visible white light and fluorescence (366 nm). After this, UV – 258 nm scanning profile is used as final interpretation for HPTLC fingerprinting development of three different extracts.

RESULTS AND DISCUSSION

Preliminary phytochemical analysis of these extracts showed the presence of major secondary metabolites in their extracts with minor differences. All three extracts showed the absence of steroids and proteins and amino acids. Extract – 1 (NH₃) showed the absence of tannins and saponins also. Apart from that all metabolites were present in three extracts. Extract – 3 (HCl) showed high presence of tannins and phenols (Table.1).

Table 1: Preliminary phytochemical bioassay results. + = presence and - = absence.				
Phytochemical	Test	Results		
		NH ₃	M	HCl
Phenols	Folin-Ciocalteu reagent test	+	+	++
Flavonoids	Shenoda test	+	+	+
Alkaloid	Mayer's test	+	+	+
Cardial Glycosides	Keiler – Killani test	+	+	+
Anthraquenes Glycosides	Borntrager's test	+	+	+
Tannins	FeCl ₃ test	-	+	++
Terpenoids	Noller's test	+	+	+
Steroids	Liebermann's test	-	-	-
Saponins	Foam test	-	+	+
Carbohydrates	Fehling's test	+	+	+
Gums and Mucilage	Swelling test	+	+	+
Proteins and amino acids	Ninhydrin test	-	-	-

HPTLC photo documentation results

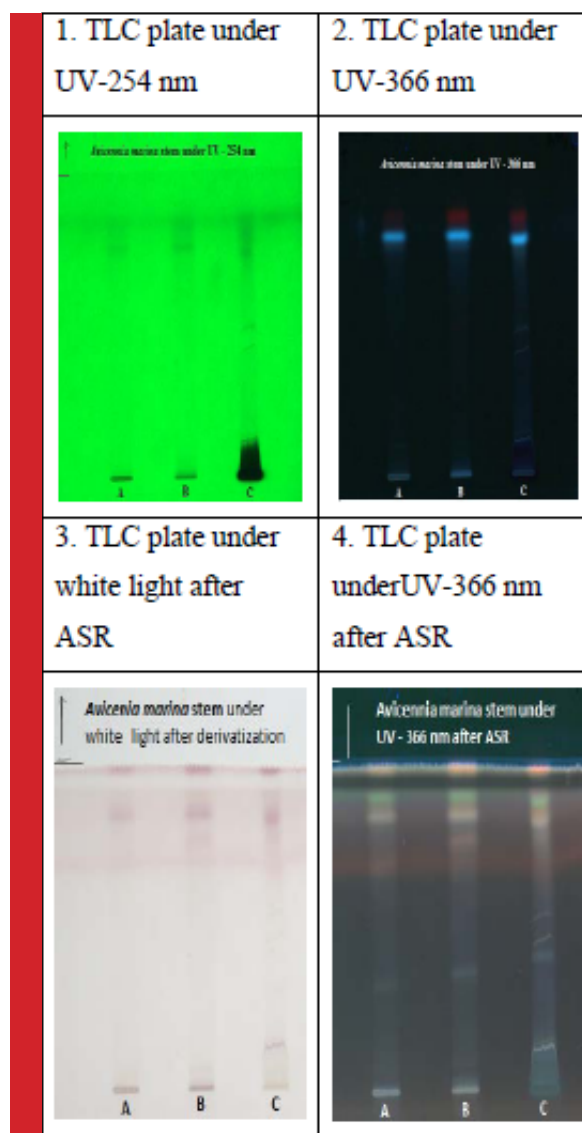


FIGURE 1: Figure showing the developed TLC plate under UV – 254 nm (1) & – 366 nm (2), under white light after derivatization with ASR (3) and under UV – 366 nm after ASR (4). Here, A = extract – 1, the 500 mg of stem powder was mixed with 10 ml methanol along with 5 % NH₃. B = extract – 2, 500 mg of stem powder was mixed with 10 ml methanol. C = extract – 3, the 500 mg of stem powder was mixed with 10 ml methanol along with 5 % HCl.

HPTLC scanning results (under 258 nm)

The HPTLC densitogram under UV – 258 nm of three extracts revealed several peaks which are presented in Fig. 2.

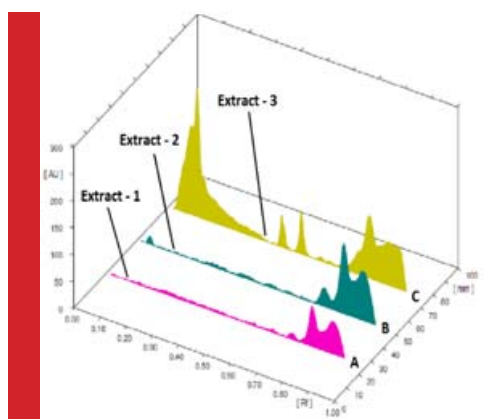


FIGURE 2: Figure is showing densitogram results of developed TLC plate under UV – 258 nm.

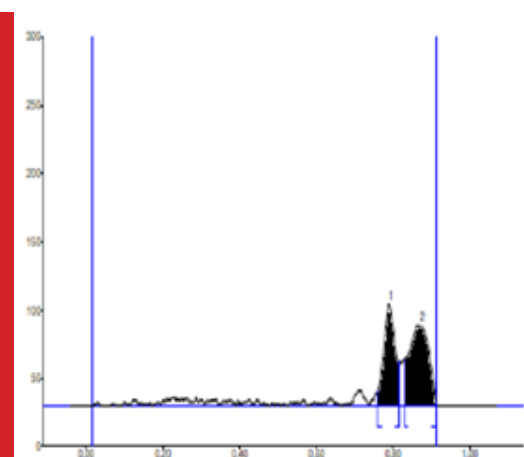


FIGURE 3: Extract.1 HPTLC chromatogram.

The densitometry results of extract – 1, under UV – 258 nm revealed 2 spots with Rf max values 0.79 and 0.87 (Fig. 3 and Table. 2). Both 2 spots are principal components of extract – 1 alongwith the percentage area of 41.93 and 58.07 respectively.

The densitometry results of extract – 2, under UV – 258 nm revealed the presence 4 spots with Rf max values 0.05, 0.72, 0.79 and 0.86 (Fig. 4 and Table. 3). Two new spots were detected at the Rf max 0.05 and 0.72, which were not present on extract – 1.

Table 2: Number of detected Peaks, their corresponding Rf values, Height – Area calculation results of Extract – 1.

Track	Peak	Rf Max	Max Height	Max %	Area (AU)	Area %
A	1	0.79	75.4	55.45	1897.6	41.93
A	2	0.86	60.6	44.55	2627.9	58.07

Among these 4 detected spots, 2 spots (spot no. 3 and 4) may be principal components of extract – 2 alongwith the very high percentage area of 45.20 and 42.04 respectively. Other than these major peaks, the spots at Rf max 0.05 and 0.72 contain very less percentage area of 2.29 and 10.47 respectively.

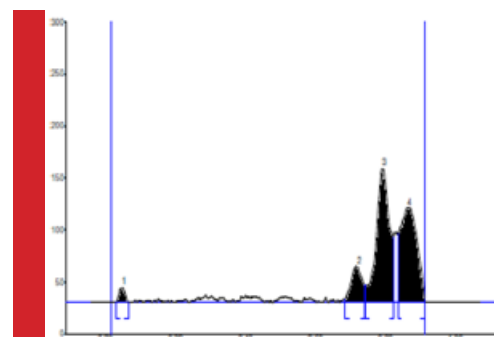


FIGURE 4: Extract. 2 HPTLC chromatogram.

Table 3: Number of detected Peaks, their corresponding Rf values, Height – Area calculation results of Extract – 2.

Track	Peak	Rf Max	Max Height	Max %	Area	Area %
B	1	0.05	15.4	5.65	203.8	02.29
B	2	0.72	35.2	12.92	933.3	10.47
B	3	0.79	130.0	47.70	4029.3	45.20
B	4	0.86	92.0	33.73	3747.5	42.04

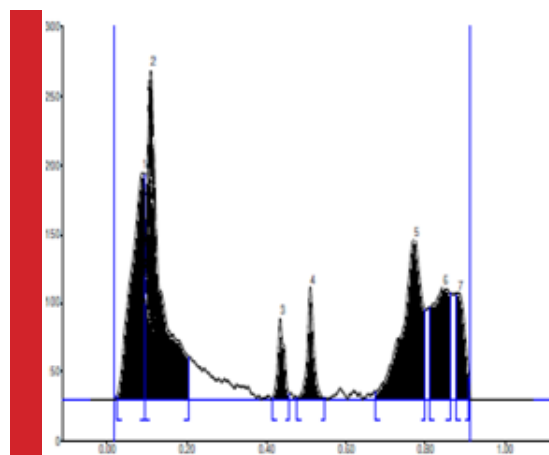


FIGURE 5: Extract – 3 HPTLC chromatogram

The densitometry results of extract – 3, under UV – 258 nm revealed the presence 7 spots with Rf max values 0.09, 0.11, 0.44, 0.51, 0.77, 0.85 and 0.89 (Fig. 5 and Table. 4). Among all 7 detected peaks, 3 spots (spot no. 1,

2 and 5) with corresponding R_f max 0.09, 0.11 and 0.77 may be principal components of extract – 3 because they contain comparatively the very high percentage area of 19.97, 31.38 and 21.49 respectively. Other peaks at the R_f max 0.44, 0.51, 0.85 and 0.89 contain very less percentage area of 3.64, 5.07, 12.23 and 6.22 respectively.

Table 4: Number of detected Peaks, their corresponding R_f values, Height – Area calculation results of Extract – 3.

Track	Peak	R _f Max	Max Height	Max %	Area (AU)	Area %
C	1	0.09	165.8	20.17	4525.0	19.97
C	2	0.11	240.3	29.24	7110.0	31.38
C	3	0.44	59.1	7.19	823.90	03.64
C	4	0.51	81.3	9.89	1147.8	05.07
C	5	0.79	116.8	14.21	4868.5	21.49
C	6	0.86	81.3	9.89	2771.8	12.23
C	7	0.89	77.3	9.41	1408.9	06.22

HPTLC spectra results –

So we can see that the developed chromatograms were specific with selected mobile phase Toluene: Chloroform: n – Propanol: Ethanol (4:4:1:1), R_f values and can serve as critical tool for analysis and standardization of the phytochemicals/drugs from *Avicennia marina* stem. Among all three extracts, extract – 1, 2 and 3 revealed the 2, 4 and 7 spots respectively. It means extract – 3 is comparatively good for phytochemicals studies for *Avicennia marina* stem because maximum number of peak separation is achieved under UV – 258 nm (high energy zone). The spectra analysis showed that the two spots R_fmax values 0.79 and 0.86 are same components and detected in all three samples. Two spots R_fmax values 0.05 and 0.72 are uniquely detected only in sample no. –

2 and among seven detected spots, five spots R_fmax values 0.09, 0.11, 0.44, 0.51 and 0.89 are uniquely detected in the sample no. – 3 only.

The spectra analysis and pattern comparisons elucidated further conformation about the uniqueness and similarities of the compounds present in these extracts (Table.5 and Figure. 6).

CONCLUSION

In conclusion, this optimized HPTLC method for *Avicennia marina* stem can provide standard fingerprints and can be used as a reference. Among three different extracts of *Avicennia marina* stem the extract – 3 is comparatively good for evaluation of phytochemical fingerprinting profile.

This characteristic and optimized HPTLC pattern assessment method for *Avicennia marina* stem is a fast, cheap, accurate, precise and reliable method for the identification, fingerprinting, authentication, and quality control. It can also provide the basic information useful for the isolation and purification of existing or new bioactive compounds because the unique spectra along with corresponding R_f values can provide a basis and better insides for further analysis.

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Table 5: Table of spots detected their R_f, position, wavelength and comparative presence or absence in samples.

Detected spot RF	Position (MD mm)	Correspo-ndding wavelenghts (Maximum @)	Presence of spot in the sample		
			stem extract – 1 (5 % NH3)	stem extract – 2 (metha-nol alone)	stem extract – 3 (5 % HCl)
0.05	11.5	208 nm	-	yes	-
0.09	14.4	291 nm	-	-	Yes
0.11	15.9	293 nm	-	-	Yes
0.44	39.4	190 nm	-	-	Yes
0.51	44.8	201 nm	-	-	Yes
0.72	59.9	195 nm	-	yes	-
0.79	64.4	198 nm	yes	yes	yes
0.86	69.8	197 nm	yes	yes	yes
0.89	71.8	198 nm	-	-	yes

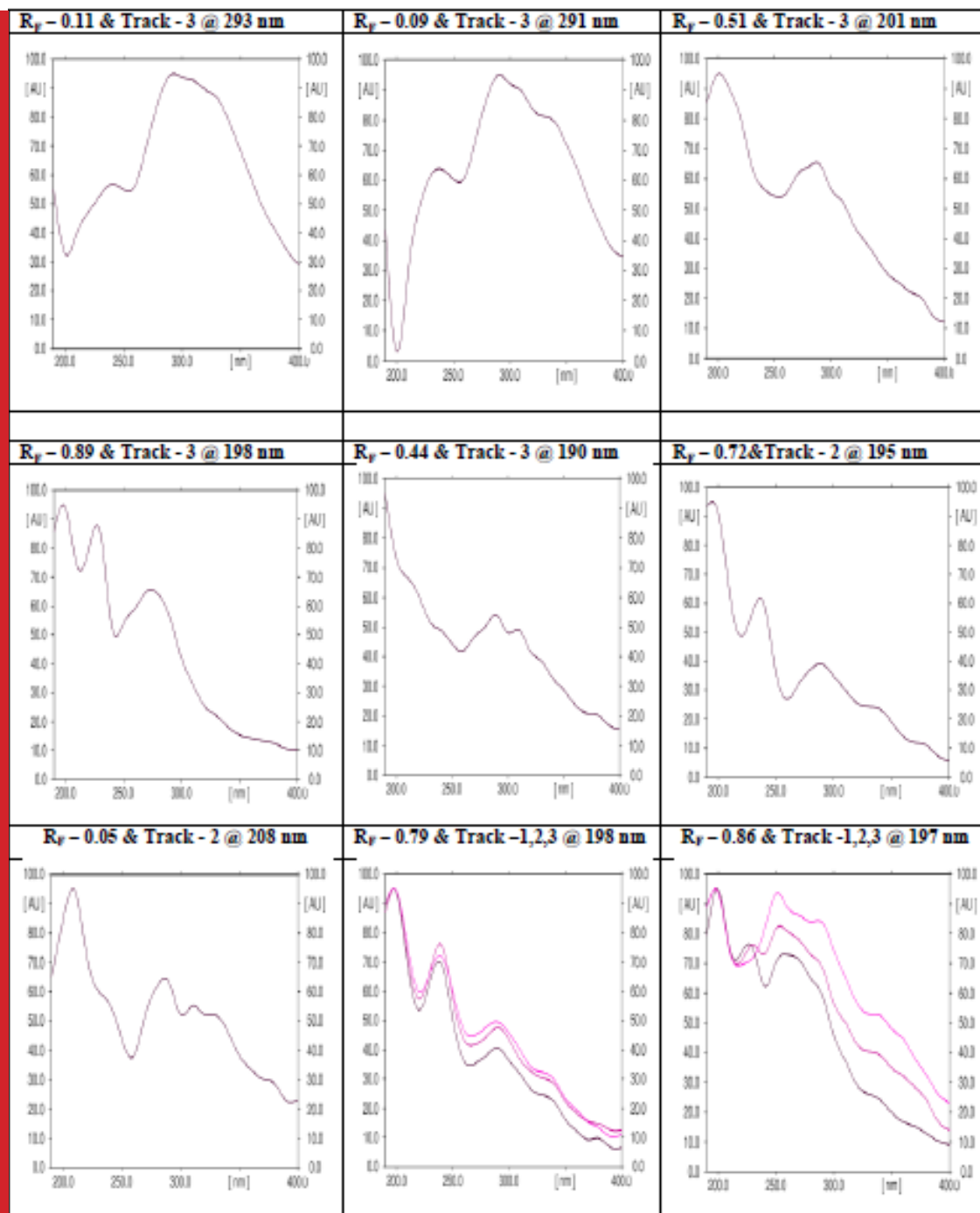


FIGURE 6: This figure is showing the spectra patterns of all assigned peaks present in the three different sample extracts of *Avicennia marina* Stem sample. Their RF and the corresponding wavelengths are also acknowledged.

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