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Development of HPTLC methods for isolation and characterization of botanical reference material of *Avicennia marina* stem

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ABSTRACT

In this study an attempt has been made to develop the strategy of HPTLC method forseparation – isolation of a selected botanical reference material (BRM)band from the methanol extract of *Avicennia marina* stem, followed by HPLC-MS method for further elucidation of physical identification. The HPTLC isolation method was optimized in the form of band selection, mobile phase selection and followed by preparative HPTLC parameters optimization (sample volume, TLC plate development chamber saturation time, relative humidity and temperature). The band was selected under UV – 336 nm. The band gave a compact RF max value of 0.65 with optimized chloroform: ethyl acetate (4:6) mobile phase. Optimized volume for preparative HPTLC study was found 180 µl on 160 mm the band length. The chamber saturation time optimized to be 25 minutes. All experiments were performed under temperature – 23.9 0C (constant) and relative Humidity – 69 % (constant). As a result a quick, simple, reliable, reproducible and cheap preparative HPTLC method was developed for isolation of that selected band. Further HPLC-MS analysis of that isolated band shown the presence of two major peaks (botanical reference compounds) in that separated-isolated band and revealed their specific retention time (Rt = 11.271 and 12.418 respectively) in the column and molecular mass/charge ratio (293.1521 and 548.2242 respectively) and molecular formula by the mass spectroscopic study.These selected studies will serve as the bases of robust methods for isolation and stability checking studies of botanical reference materials from this mangrove as well as other plants and will open the scope of new BRMs, further bioactivity study and their chemical structure elucidation from their extracts or other formulations.

KEY WORDS: AVICENNIA MARINA STEM, ISOLATION, SEPARATION, PREPARATIVE HPTLC, HPLC-MS, METHOD DEVELOPMENT

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INTRODUCTION

The chemicals of natural origin have always been eye catching center for drug discoveries and remediesbecause they are unique, complex, diverse, effective and strong candidate to create leading edge drugs and front line treatments (Clardy&t Walsh, 2004; Newman&tCregg, 2007;Butler & Newman, 2008 and Newman &tCragg, 2016). Howevertransform these natural products or botanicals into remedies or drugs is not easy and still a challenging job (WHO, 2001;Xie *et al.*, 2006;Bucar *et al.*, 2013).Achieve the best methodologies for separation, isolation, purification and further characterization, has always been a very critical, analytical task (Sasidharan *et al.*, 2011;Nikam *et al.*, 2012;Brusotti *et al.*, 2014).

Efficient and novel analytical strategieshave beencontinuously needed for almost every new phytocompounds for their rapid enrichment, separation and isolation (Chen *et al.*, 2014).

In this current scenario, various harmful effects like allergy, safety and microbial resistance etc. made a switch and a strong return from synthetic medicines to herbal drugs, phytotherapy and enthnomedicines (Calixto, 2000). The adverse effects of plant remedies found to be far less as compare to synthetic drugs (Ernst, 2003) but safety has always been in concern(Ernst, 2002; Chan, 2003). These reasons directed, herbal drugs towards the solution of sophisticated analytical standard methods for their proper separation, isolation – identification,safetyand expected therapeutic effects (Waksmundzka-Hajnos *et al.*, 2008).

In this outlook, HPTLC and/or HPLCare tremendously useable (Wilson,1999;Xie *et al.*, 2006;Tistaert, *et al.*, 2011; Loescher *et al.*, 2014) and their various combinations with recent analytical techniques has been extremely indispensable and very much regular now a day for herbal analysis(Morlock&tSchwack, 2006;Bimal&tSekhon, 2013; Riffault *et al.*, 2014).

FIGURE 1. *Avicennia marina* mangroves present in Godrej Marine Ecology Centre, Mumbai, Maharashtra.

Apart from traditional and folk medicinal uses,*Avicennia marina* found to be a gorgeous source of significant natural structures, among them most of observed high pharmacological values (Rui *et al.,* 2004 and Zhu *et al.*, 2009).

The *Avicennia marina* stem/bark/twig or Heartwood, has been an interesting candidate itself fora wide range of medicinal properties (Thatoi *et al.*, 2016) and reported bioactive compounds (Han *et al.*, 2007; Zhu *et al.*, 2009; Mohammed *et al.,* 2014; Jain *et al.,* 2014).

The main objective of this study was to develop the strategy ofpreparative HPTLC method forseparation – isolation of selectedbotanical reference material (BRM) from the methanol extract of *Avicennia marina* stem, followed by HPLC-MS methodfor further elucidation of physical identification. The present study may serve as the bases forthe use of HPTLC as fast, cheap, accurate and robust method for separation - isolation and stability checking studies of botanical reference materials from *Avicennia marina* stem as well as for other plants and will open the scope of discovering new BRMs and their chemical structure elucidation.

MATERIALS AND METHODS

COLLECTION AND IDENTIFICATION

The *Avicennia marina*was collected in January 2014 from the S.P.Godrej Marine Ecology Centre, Vikhroli, Mumbai city of Maharashtra, India (Geographical coordinates 19˚05'50.82˚N – 72˚56'24.06 ˚E). The plant materials were identified and authenticated by the same institute.

EXTRACTION OF PLANT MATERIAL

The stem was shed dried for 15 days and grounded by mechanical grinder into coarse particles. 500 mg of stem power was mixed with 10 ml methanol. Theextract was sonicated for 6hours until it became clear or colorless. Direct sun light and high temperature were avoided to protect heat sensitive phytochemicals. Extracts were filtered through Whitman No.1 filter.

CHEMICAL AND EQUIPMENT

CAMAG HPTLC system equipped with automatic TLC sampler LIMONATE V, TLC scanner 3, REPROSTAR 3 with 12 bit CCD camera for photo documentation analysis, winCATSsoftware. All the solvents used for HPTLC analysis were HPLC analytical grade obtained from Merck, India.

Scissor, beaker (50 ml), HB – pencil, water bath, chloroform, Ethyl acetate, methanol, sonicator, hand glows, Whitman filter paper (no. 1 and 42). HPTLC Aluminum plate silica gel $60F_{254}$precoated 20 x10 cm (Batch no. - 1.05554.0007 – HX360380).The HPLC – column was "Zorbax SB_ C 18 (2.1 X 100mm, 1.5 micron pore size)" and Mass spectroscopy (software – Agilent Mass Hunter qualitative analysis B.06.00).

HPTLC PROCEDURE

HPTLC study with different mobile phasesfor stem methanol extract were performed on commercial Aluminum – shits precoated with silica gel 60 F_{254} HPTLC plates (Merck). First of all 20 x 20 cm plates were cut down in to the 10 x 10 cm plate with the use of a scissor. Before using it for sample applicator, plates were checked in the 254nm, whether they were giving fluorescence or not and marked the limit of run and direction by a pencil. After it, a working program was generated with the use of winCATS software. Specific volume of sample was taken by the use of 100 µl Hamilton Syringe and applied (basically 10 µl) on plate as the predefined 8mm band length by the means of CAMAG LINOMATV sample applicator.

After the completion of the sample applicator program the plate was subjected for drying with the use of a drier and then placed on to CAMAG plate heater for 10 minutes for remove any water or moisture content from the plate. The specific mobile phases (Tab.3) were prepared according to maximum separation and to find better candidate for isolation of selected band. Mobile phases were separately subjected to the CAMAG Twin-Through chamber for 20 minutes. A filter papered rinsed with mobile phase was also subjected in the chamber for a uniform vapor saturation of the chamber prior adding of the sample applied plate.After the 20 min saturation of the CAMAG Twin- Through chamber, the plate was placed in it, till the solvent front reached up to the distance of 80mm (previously marked). This process took 5 – 8 minutes to develop the plate depends on the interactions of mobile phase, stationary phase and sample molecules.After development, the plate was subjected for drying by hot air device (drier) and 10 minutes on CAMAG plate heater (110 ^{0}C) at room temperature andkept it for documentation in the CAMAG TLC visualizer. This visualizer captured the images under 366nm (Fluorescence) and densitometry analysis was done under 366 nm (Hg - lamp). In the last step the plate was derivatized with AnisaldehydeSulphuric Acid reagent (ASR) in the derivatizating chamber for 3-4 seconds and air dried. After drying, the plate was heated on CAMAG plate heater for 3-4 minutes at 110 ^{0}C. Final image was quickly captured by the CAMAG TLC Visualizer under florescence (366 nm).

1. Isolation of a Botanical Reference Material (a single band) by HPTLC

A magical concept is that, different compounds can travel a different distance in the stationary phase on TLC plate, so chromatography can be used as an effective isolation procedure(Reich and Schibli, 2007). Among these separated compounds, each occupy a definite area on the TLC plate and they can be easily scraped away manually rom stationary phase and finally re dissolved it into solvent to become as sample for further analysis.

Band selection and Mobile Phase Optimization – The band was selected from the fingerprinting pattern of *Avicennia marina* stem (under – 366 nm). The band was seen only under low energy zone (UV – 366 nm) (Fig. 2).The four mobile phases (M.P.) were selected for finding desirable compact R_F max value (Tab. 3) and better separation.

Saturation Time Optimization – The optimizations were made in chamber saturation time. In the five different experiments on HPTLC plat 10 X 10 cm, the saturation time was increased in the ascending order from 10 minutes, 15 minutes, 18 minutes, 25 minutes and 30 minutes. The procedure was same.

Preparative HPTLC volume optimization – The sample volume was optimized for preparative HPTLC isolation of desired band. In this experiment, the volume of Avicennia marina stem aliquots of 5µl, 10 µl, 15 µl, 20 µl and 25 µl were loaded in the increasing order and a TLC plate 10 X 10 cm and was run with optimized parameters of mobile phase (M.P. 3) and saturation time (25 minutes). TheTemperature and relative humidity were 23.9 0C and 69 % respectively maintained constant.

Developing, Marking, Cutting and Re dissolving the compound for analysis and stability test – The plate was developed in Twin – through chamber and mobile phase was Chloroform: Ethyl acetate (4:6). Marking of desired band was done by hands, with HB - pencil under the UV-366 nm on Hg lamp (Fluorescence mode).Cutting was done by scissor.Re dissolving of isolated powered compound was done in the 50 ml beaker and cut plates were dissolved in the methanol (HPLC grade) and sonicated for 2 hours. The overnight extraction was performed. For purification Whitman no.1 filtration used for filter trace of graphite of pencil and Si gel. Whitman no. 42 filtration used for filter the Si gel and got colorless solution.

2- Dimensional Chromatography for stability detection –The possibility of sample degradation was also investigate by 2 D run. The Concept was, a stable compound will give same R_F in both two development and form a straight line connecting the application position. Stability test also give the information of the waiting time and the robustness of the method of isolation.

2. Identification with already known compounds library by GC –HR- MS and HPLC-HR-MS detection –The both studieswere carried out in SAIF, IIT Bombay. For GC, 2 µl sample was taken by the syringe (Agilent PN – 5190 – 1483, made in Australia) and applied in to

Table 1: The protocol of GC – MS working program. The temperature, time duration and the temperature increasing speed is present.

Temperature	Duration of time	Temperature increment speed
70 0C (initial temperature)	1 minutes	6 0C per minute
200 0C	3 minutes	10 0C per minute
260 0C	3 minutes	10 0C per minute
280 0C	HP 5	Neat.

the GC – applicator (Agilent Technologies 7890 A). A working program was generated (See table.3.2).

For HPLC, 5µl sample was taken by the syringeand applied in to the LC – applicator (Agilent Technologies). The column Zorbax SB_ C 18 (2.1 X 100mm, 1.5 micron pore size) was used. A working program was generated for mobile phase composition (See table.1).

Table 2: This table is showing the solvent composition and its time duration as mobile phase. Where, A – 100 % Millipore water + 0.1 % formic acid and B – 100 % acetonitrile + 10 % water + 0.1 % formic acid.

Time	Solvent composition
2 minutes	A = 95 % & B = 5 %.
25 minutes	A = 0 % & B = 100 %.
30 minutes	A = 0 % & B = 100 %.
32 minutes	A = 95 % & B = 5 %.

RESULTS AND DISSCUTION

Avicennia marina stem is a well known source of various phytochemicals and HPTLC has been found useful technique to assess the phytocompounds (Dawane *et al.*, 2016).

Previously we studied the HPTLC pattern assessment of *Avicennia marina* stem and provided the method for authentication and separation of its botanical reference materials (BRM) (Dawane *et al.*, 2016).In this present research, we further elucidated our study in the form of isolation and physical detection of selected band among separated compounds on TLC plate. The Toluene: Chloroform (6:4) solvent system has been applied initially for development of a basic chromatogram and the florescentband (under 366 nm) was selected for isolation study (Fig. 1). This band found to be prominent peak at the R_F max of 0.67 (Fig.2).

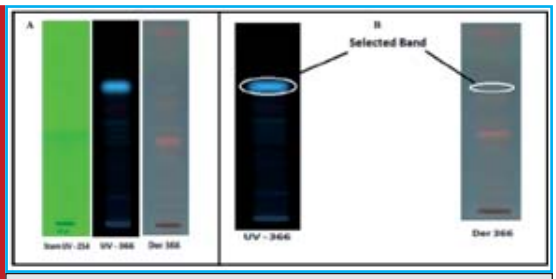

FIGURE 2. Developed plate under various energy zones. Here, A - HPTLC pattern of methanol sample in Toluene: Chloroform (6:4) and B - Selected band on TLC plate under UV- 366 nm before and after derivatization.

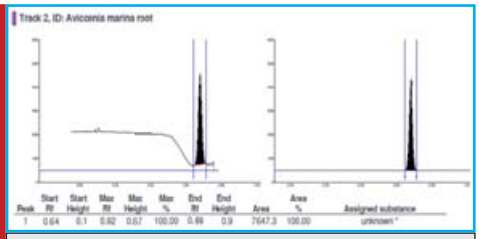

FIGURE 3. Densitogram ofselected band under UV- 366 nm and its RF max was 0.67 (in Toluene: Chloroform 6:4).

This compound may be the main component (significantly more abundant than other components) or major active principle or active ingredient or the marker compound of this extract because the detected peak has found to be prominent (Li *et al.*, 2008) under low energy zone (UV – 366 nm) and densitogram showed 7647.30 area unit (AU) concentration in 10 µl sample of 8 mm band length, other peaks were extremely minor or under below the detection limit (<500AU) (Fig.2B and 3).

The understanding about marker compound has been also very essential because from this,one can validate the effectivenessof extract as well as some times marker compound can also be acts as active principle of raw material for its correctbotanical identity check (Tistaert *et al.*, 2011; Rasheed *et al.*, 2012) but as well know single active constituent isn't always responsible for overall efficacy (Xie *et al.*, 2006) so the statement is not permanentlyright (Ruiz *et al.*, 2016).

Multiple active phyto components or poor separation also made it difficult to find and isolate markers. The certainty and probability of chromatographic outcomes has somewhat restricted, and for this reason method development remained an extensively empirical procedure (Ong, 2004;Xie *et al.*, 2006; Reich and Schibli, 2007), so four different mobile phases (Tab. 3) has been used to develop and compare the HPTLC pattern of selected band to get a good separation along with compact R_F Max value of selected band (fig. 4).

After testing these 4 mobile phases, 3rd and 4th mobile phases were chosen for further studies because these two mobile phases gave comparably better results

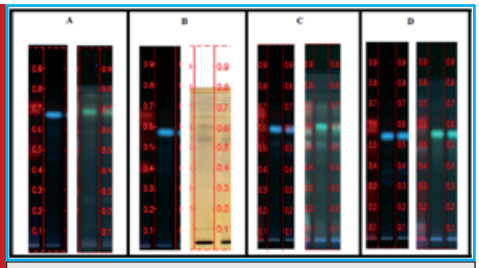

FIGURE 4. Mobile phase optimization plate. Here A, B, C, D were mobile phase 1, 2, 3 and 4 respectively. Pictures under UV – 366 nm before and after derivatization with ASR. The image B derivatized with FeCl3 under white light.

and it was also previously known that mobile phases with acidic or basic modifiersor other polar solvents (mobile phase 1 and 2), were avoided in the HPTLC isolation studies because it reflects the stability of isolated compound and may degrade the compounds sometimes as well as affects the "clarity" of generated pattern (Reich and Schibli, 2007).

Table 3: The 4 different mobile phase compositions used for optimization.

Mobile Phase	Composition
1	Cyclohexane : Ethyl acetate : Formic acid (4:6:1)
2	Toluene: Ethyl acetate : Formic acid (4:6:0.3)
3	Chloroform : Ethyl acetate (4:6)
4	N- Hexane : Ethyl acetate (1:1)

As we could see in the picture (Fig.4, 5B) one red pigment (may be chlorophyll / xanthophyll) found to bevery near from our desired band, which could be interfere in our results. So 3rd mobile phase (belonging to middle polar range) was preferred rather than 4th one (belonging to lower polar range) and optimized for isolation of selected botanical reference compound band on the TLC plate.

After mobile phase optimization the volume for preparative HPTLC was optimized to develop a sample for further analysis. The various volumes 5 µl, 10 µl, 15 µl, 20 µl, 25 µl used in the increasing order (the band width was 8 mm) and area peaks were calculated under UV-366 nm (Fig.5 and Tab.4).

With the help of densitogram the sample volume 180**µl**, was finalized with the band length of 160 mm on TLC plate (Fig. 6), and the three consecutive turns of TLC plate were used togenerate the sample for further studies.

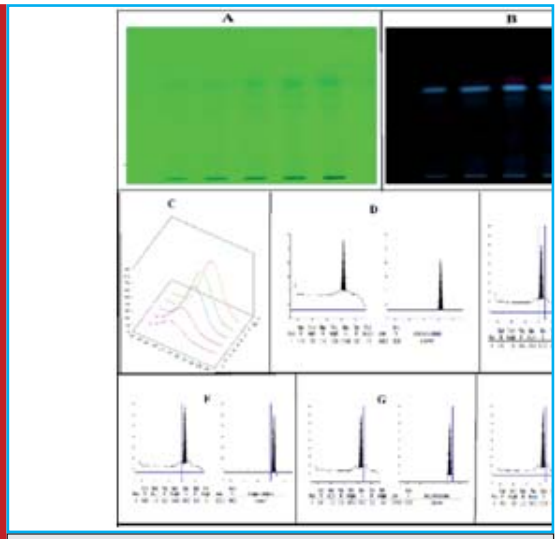

FIGURE 5. Volume optimization (in M.P. - 3). Here A and B – volume optimization plate under UV-254 nm and UV-366 nm respectively. C – Densitogram of various volumes 5 µl, 10 µl, 15 µl, 20 µl, 25 µl in the increasing order (the band width was 8 mm) and area peak is calculated at 366 nm. D,E, F, G and H were densitometrical peaks of 5 µl, 10 µl, 15 µl, 20 µl, 25 µl volume respectively. (Band length was 8 mm).

Table 4: Various volume of sample, corresponding RFvalues of selected band, max Height and Area calculation results.

Figure	Volume	RF Max	Max Height	Area
D	5 µl	6.3	179.6	4502.0
E	10 µl	6.4	296.8	7455.7
F	15 µl	6.5	534.0	12775.6
G	20 µl	6.5	637.6	15576.9
H	25 µl	6.5	691.6	17531.5

After volume optimization the preparative HPTLC on 160 mm band size clearly showed the presence of desirable band (R_F Max value 0.65) along with unwanted pigment on just top (Fig. 6). Most of times fine tuning were performed in the method development for this kind of adjustments in the generated patterns. For this, additions of modifiers in solvent systems or chambers saturation time variations often applied (Reich and Schibli, 2007). To increase the distance between the desired band and the unwanted pigment, chamber time saturation experiment was performed and as anoutcome, 25 minutes saturation gave the best results (Fig. 7).

After these, the plates were marked, Cut and re dissolved and extracted in methanol (Fig. 8). These gave a sample, how's stability and degradation were further

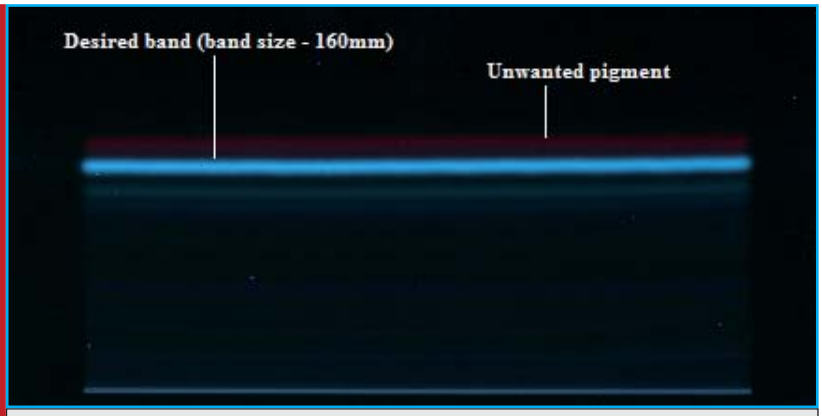

FIGURE 6. Volume optimization.Optimized volume was 180 µl and the band length is 160 mm on TLC.[Temperature – 23.9 0C (constant) and Relative Humidity – 69 % (constant)].

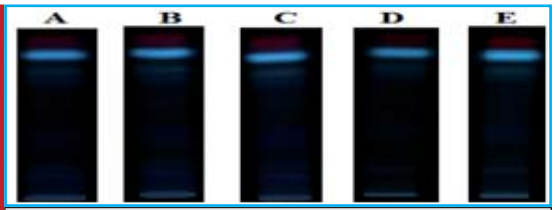

FIGURE 7. Chamber saturation time optimization plates. Here A, B, C, D and E were chamber saturation time in the ascending order from 10, 15, 18, 25 and 30 minutes respectively. [Temperature – 23.9 0C (constant) and Relative Humidity – 69 % (constant)].

FIGURE 8. Setup of extraction, isolation and purification after plate cutting.

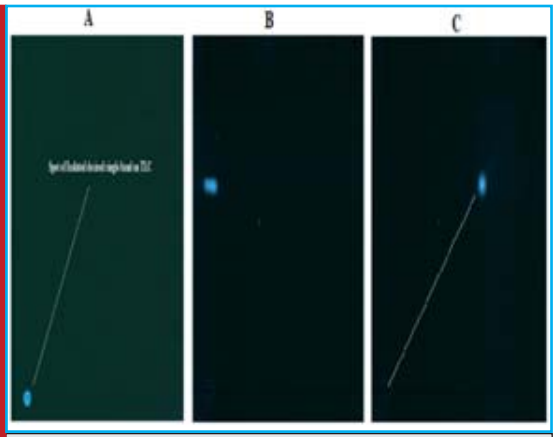

FIGURE 9. Two dimensional chromatography results.

checked with two dimensional chromatographically studies. The single band spot with two dimensional development confirmed the perfect isolation of desired band with any seen unwanted pigments (Fig.9). This kind of experiments has been extremely important because not only provide robustness of the method but also determine the proper waiting times for open handed or semi open handed analytical method like HPTLC (Reich and Schibli, 2007).

As we know HPLC and GC were extremely sensitive analytical technique and very small amount of sample can be detectable in both methods (Waksmundzka-Hajnos *et al.*, 2008; Tistaert *et al.*, 2011), sothe three runs of 180 µl sample with the 160 mm band size plates were cut and re-dissolved in the methanol to make the sample for GC-HR-MS and HPLC – HR-MS for further characterization.

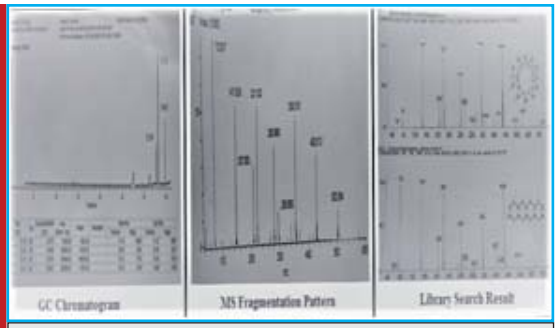

FIGURE 10. GC results. The Gas chromatogram, MS fragmentation pattern and already existing compounds library research results.

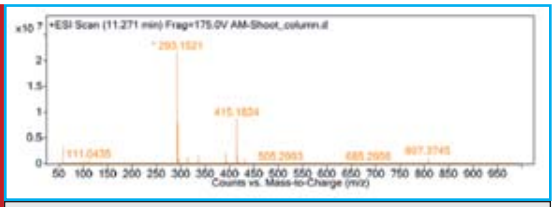

FIGURE 11. Mass spectra of 1st MS peak.

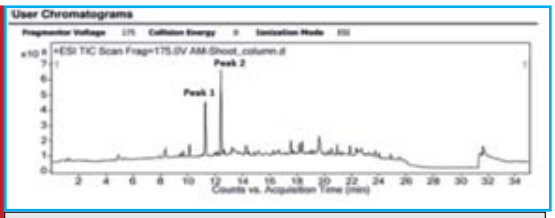

FIGURE 12. LC chromatogram.

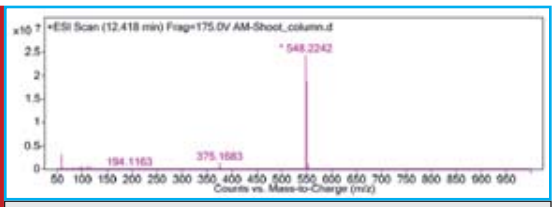

FIGURE 13. Mass spectra of 2nd MS peak.

The GC-MS is preferable choice for analysis of complex chemical identification and isolation and assess the metabolite profile of plant(Rohloff, 2015). Here, GC results revolved a more than 20 minutes noise in the chromatogram and no organic compound was detected. Only two siloxane compounds after 20 minutes were detected. This two compounds were among the base plate material of TLC plate (Tetracosamethyl –cyclododecasiloxane and Cyclononasiloxane, octadecamethyl) were detected (Fig. 10). It elucidated the possibilities on nonvolatile nature of the isolated band or also a possibility of compound's concentration under below the detection limit.

After that HPLC – MS studies were performed and method was developed. The HPLC-MS IS also very identical for isolation – purification method development of marker compounds (Cheng et al., 2007). The HPTLC chromatogram revealed two major peaks (Fig.11) and HR – MS of two detected peaks are shown (Fig. 12 and 13 respectively). This study elucidated Rt, mass / charge ratio and molecular formula results of two major peaks (Tab. 5).

Table 5: This table is showing the Rt, mass / charge ratio and molecular formula results of two major peaks detected by HPLC –HR- MS.

Attributes	Peak 1	Peak 2
Retention time (Rt)	11.271	12.418
Molecular mass to charge ratio	293.1521	548.2242
Molecular formula	$C_{18}H_{24}O_3$	$C_{24}H_{37}N_3O_9S$

The results from HPLC – MS revealed two strong marker or active compounds in one single isolated HPTLC band. It may be possible that these two compounds are coexisting in nature and providing stability to each other after isolation. It is also observed in the nature that plant's coexisting chemicals or compounds often mitigate their negative effects or unwanted side effects (Hanjos *et al.*, 2008) so the results opened the gates of further studies related to their combined and separated effects and exact chemical structures elucidation for better assessment of the nature's experiment on this coexisting compounds. Further studies are recommended to assess the identity and accurate composition of these two major peaks present in the single isolated HPTLC band, in order to obtain the their bio-chemical effects.

CONCLUSION

Variability and multiple complexities are the nature of Herbal medicines and their drugs. Hence it is very important to apply reliable chromatographic methods to study the accurate separation and isolation. For these reasons HPTLC, HPLCand their various combinations became very useful techniques for herbals now a day for patterns generation, separation and further isolation.In conclusion, very simple, quick, precise and cheap HPTLC method was developed and optimization of various HPTLC parameters has been discussed for isolation of selected botanical reference material from *Avicennia marina* stem in this study as well as optimized HPLC – HR – MS method also developed to analyze the separated band. Further studies are suggested to structure prediction and elucidation - characterization and to understand the biological and pharmacological nature of isolated compounds.

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