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Identification of structurally unique molecules, phytochemical and immunological activity of medicinal plants

Ajam C. Shaikh, Amit Gupta and Sushama R Chaphalkar*

Vidya Pratishthan's School of Biotechnology (VSBT, Research Centre affiliated to Savitribai Phule Pune University), Baramati, Maharashtra, India

A B S T R A C T

In the present study, our group analyzed major chemical constituents that are present in fresh aqueous leaves extracts and analyzed through HPTLC (high performance thin layer chromatography), LC-MS (liquid chromatography-mass spectrometry) and determined its immuno pharmacological (i.e. immunosuppressive) activity. During preliminary studies, phytochemical screening was carried out and showed the presence of chemical constituents like glycosides, flavonoids, terpenoids, phenolic compounds etc. HPTLC finger print analysis of *Acaica catechu, Santalum album, Bambusa arundinacea, Mesua ferrea, Ficus religiosa and Ficus benghalensis* showed the presence of possible number of components and determined its consistent quality of chemical constituents. As per the experimental conditions including general comments on the application of chromatographic fingerprint analysis are discussed. In addition, aqueous extract of these medicinal plants also responsible for immunosuppressive activity against specific protein antigen (hepatitis B vaccine antigen, HBsAg; 20 µg/ml) i.e. due to decrease in monocytes and granulocytes count.

Keywords: *Acaica catechu, Santalum album, Bambusa arundinacea, Mesua ferrea, Ficus religiosa, Ficus benghalensis*

A R T I C L E I N F O

CONTENTS

1. Introduction 223
2. Materials and Methods 224
3. Results and discussion 224
4. Conclusion 229
5. References 229

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***Corresponding Author**
Dr. Sushama R Chaphalkar
Vidya Pratishthan's School of Biotechnology
Baramati, Maharashtra, India
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1. Introduction

Phytoconstituents are isolated or purified from various medicinal plants are of utmost importance for developing and under developed countries due to traditional health care system totally relies on such crude medication. Efficacy of

these crude medicinal plants attributed to the bioactive phytoconstituents in it. These phytoconstituents are nothing but the secondary metabolites which are not directly involved in plant survival rather helps to communicate with external environment in the event of extreme conditions such as diseases/defense mechanisms, growth, agents of symbiosis between microbes and plants, nematodes, insects etc. [1] The most important of these bioactive constituents of plants i.e. alkaloids, tannins, flavonoids and phenolic compounds. Because of varying effectiveness of same medicinal plants i.e. due to quality of the crude sample, it is warranted to establish the scientific basis to these traditional drugs and their crude formulations by identification and standardization using bio-analytical tools such as HPTLC, LC-MS, HPLC [2,3,4] Systematic evaluation of the quality control parameters of the plant material and its formulation (aqueous extract) using modern analytical techniques i.e. chromatography and spectroscopy can be productive in validation of herbal drugs. Thus, standardization is an essential criterion for herbal medicines with respect to the betterment of animal and human health.

HPTLC is being one of the major analytical tools for analysis having advantages such as ability to analyze or estimated various samples simultaneously using minor quantity of mobile phase, reduced analysis time, reduction in cost, less exposure risks and significantly reduced disposal problems of toxic organic effluents and result reproducibility with same or different parameters. [5,6,7]. Likewise LC-MS is an analytical chemistry laboratory technique for identification, quantitation and mass analysis of materials. This technique allows for the structural elucidation of unknown molecules through fragmentation. [8,9]This study aims at the qualitative, quantitative, analytical and immunological study of *Acacia catechu, Santalum album, Bambusa arundinacea, Ficus religiosa, Mesua ferrea, Ficus benghalensis,* using various phytochemical tests, spectroscopic techniques (i.e. HPTLC and LC-MS) and flow cytometric analysis.

2. Materials and Methods

Assemblage of plant materials

All plant leaves were assembled from Nakshatra Udyan. Plants were dried at room temperature for 5 days and then in oven at 37 ºC for 15 min. Leaves became powdered and sieved.

Qualitative estimation of phytoconstituents

Chemical tests for the screening and identification of various phytochemicals in the medicinal plants under consideration were carried out in powder specimens using standard procedures [10, 11, 12, 13].

Quantitative estimation of phytoconstituents

For quantitative studies, one gm plant sample extracted with 25 mL 95% ethanol at 200 rpm for 24 h., filtered and used for further analysis of terpenoids [14], flavonoids and phenolics [15,16] by using standard procedures.

HPTLC Analysis for various phytoconstituents

Densitometric HPTLC analysis was done for initiation of finger printing profile of various medicinal plants. The samples (leaves aqueous extract) were loaded in the form of band length on precoated silica gel $60F_{254}$ TLC plate [Merck] using Hamilton syringe and CAMAG LINOMAT 5 instrument. The samples (aqueous extract) loaded plate was kept in TLC twin trough developing chamber (after saturation with solvent vapor) with respective mobile phase **(Table 1).** The developed plate was dried using hair drier to evaporate solvents from the plate. Afterwards, the plate kept in Photo-documentation chamber (CAMAG Reprostar 3) for documentation. Finally, the plate was fixed in scanner stage and scanned at multiple wavelengths (CAMAG Scanner 3). The win Cats 1.2.3. Version software used for HPTLC. The Peak table, Peak display and Peakdensito grams were identified.

Preparation of Extract for LC-MS and analysis

Freshly harvested plant leaves were washed with tap water. Thereafter, leaves were air dried and cut into small pieces and maceration was done with liquid nitrogen (-196 ºC) for one minutes to prepare fine powder. Weigh 8 g of leaves powder (*Acacia catechu, Santalum album, Bambusa arundinacea, Ficus religiosa, Mesua ferrea, Ficus benghalensis*) was macerated or liquefied in 80 mL PBS (phosphate buffered saline) using mortar and pestle at room temperature for two minutes with occasional stirring. Thereafter, the aqueous extract of leaves was filtered and filtrate collected was kept in refrigerator at 4ºC. All the extracts were subjected to LC-MS analysis. All MS acquisitions were performed in the positive electro spray ionization mode. The capillary voltage, cone voltage, fragmentor voltage were 4 kV, 45V and 170V, respectively. The gas temperature was set at 325 ºC. Data was acquired at scan rate of 3Hz in mass range 100-100 *m/z*. Further data was analyzed with Mass hunter qualitative software and METLIN database.

LC-MS specification

LC: Agilent 1260 binary LC System (**Table 2**)

Column: Agilent Zorbax SB 18 RRHT column (100×2.1 mm, 1.8μm); Flow rate: 0.3mL/min; Run time: 30 min; Injection Vol: 1μL and MS: 6540 ultra-high definition accurate mass QTOF LC/MS system

Mobile phase A: Water (0.1% Formic acid)

Mobile phase B: Acetonitrile

Immunological activity

Human whole blood (samples collected from pathology lab) were treated with variable concentration (6.25 – 25 mg/ml; 50 µl) of aqueous leaves extract of *Acacia catechu, Santalum album, Bambusa arundinacea, Ficus religiosa, Mesua ferrea, Ficus benghalensis* for 4 h incubation. After incubation, blood was lysed with red cell lysis buffer (containing ammonium chloride, potassium bicarbonate and EDTA) and washed with PBS (pH, 7.2). The resulting stained cell pellet was resuspended in 500 µl of PBS and run on a FACS Calibur flow cytometer [17].

3. Results and Discussion

*Acacia catechu*is known for its antioxidant, anti-inflammatory, and chemo protective properties [18, 19]; *Santalum album* for antipyretic effect, astringent activity[20], desiccant [21]; *Bambusa arundinacea* for antiulcer, anti-diabetic activity [22]; *Ficus religiosa* for

gynecological problems, dysentery, diarrhea, nervous disorders [23]; *Mesua ferrea* for antifungal, anthelmintic, hypotensive, antispasmodic, antianaphylactic [24]; *Ficus benghalensis*foranti-septic and anticancer agent [25]. Our aim is to systematically evaluate the presence of bioactive phytoconstituents present in aqueous leaves extract of these medicinal plants based on HPTLC and LC-MS data as well as spectroscopic methods to quantify them and determined its immunological (immunosuppressive) cavity. Qualitative phytochemical screening showed the presence of terpenoids and flavonoids in all medicinal plants whereas saponins, phenolics and glycosides are found in some of the plants **Table 3**. Alkaloids did not find in any of the plants. Quantitative investigation furnished that *Mesua ferrea* and *Ficus benghalensis* are rich with phenolics (503.10 mg/gm and 244.92 mg/gm respectively), *Ficus religiosa* with flavonoid (104 mg/g), whereas in *Acacia catechu*found major content of terpenoids (131 mg/g) as shown in Table 4. The densitometric HPTLC fingerprint profile of these medicinal plants showed the existence of phytochemicals in the medicinal plant extracts. The solvent systems were optimized before analysis. The systematic analysis shows the presence of terpenoids in all plants. The chromatogram developed for saponins, phenolics and glycosides contains number of peaks confirming presence of number of corresponding components (Table.5& Figure.1).The extract of medicinal plants under study were subjected to LC-MS analysis and compounds were distinguished based on their mass spectra, using the precursor ion, fragment ions,and comparison of the fragmentation patterns with molecules described in the literature. LC-MS data identified many compounds which are structurally unique and architecturally challenging to synthesize, such as Nystatin A3 an antibiotic [26], Sapindoside A; an anti-inflammatory agent [26], Triptonide; with antileukemic and anti tumor activities [28], Gibberelline A36, Elephantopin; a tumor inhibitor [29], Punctaporin B, Harderoporphyrin, Cyclosporin A; an immunosuppressant [30], Oleandolide an antibiotic [31], Khayasin C (Figure 2). These medicinal plants under study, found to have unique feature to produce such bioactive and architecturally complex secondary metabolites. In the initial screening of these medicinal plants, our group used aqueous leaves extract and determined its immunosuppressive activity against HBsAg. In this study, treatment of aqueous leaves extract on human whole blood which is exposed with HBsAg with or without variable concentration of aqueous leaves extract and showed immunosuppressive properties because of rapid decline in monocytes and granulocytes count at higher doses. Overall, these medicinal plants in the form of aqueous leaves extract showed immunosuppressive activity against HBsAg.

Table 1A: HPTLC parameters

	Saponin	**Terpenoid**
Sample preparation	2.0g sample was taken in 15ml 70% ethanol and refluxed for 20 min at 100-105 ºC. Solution is filtered and filtrate is used as a sample for HPTLC to detect saponins.	0.5g sample was taken in 10ml methanol and centrifuged at 3000rpm for 5min. Supernatant is taken for HPTLC analysis.
Solvent system	Tol:MeOH:EtOAc (75:25:2)	*n*-Hexane:EtOAc (72:28)
Solvent fornt	90 mm	80 mm
Band length	8 mm	7 mm
Scanned wavelength range	254-704 nm	300-500 nm
Best wavelength	654 nm	417 nm

Table 1B: HPTLC parameters

	Phenolics	**Glycosides**
Sample preparation	Leaves sample extracted with 70% ethanol. Heat extracts at 60^{o} C, dry it and dissolved it in methanol. Centrifuge mixture at 3000 rpm for 5 min. Use supernatant for sample application.	Leaves sample extracted with 25ml 70% ethanol. Extract kept in rotary shaker (120 rpm) for 8 hours. Lead acetate is added to the filtrate based on the volume and centrifuged at 5000 rpm/10 min. The supernatant was further centrifuged by adding 3ml 6.3% Na_2CO_3 at 10000 rpm for 10min (Approximately for 20 ml filtrate adds 3ml of Na_2CO_3). The retained supernatant is dried, re-dissolved in chloroform and used for sample applications.
Solvent system	Tol:Acetone:Formic acid (22:22:6)	*n*-Butanol: Acetic acid: Water (4:1:5)
Solvent fornt	90 mm	85 mm
Band length	7 mm	7 mm
Scanned wavelength range	300-500 nm	250-440 nm
Best wavelength	418 nm	412 nm

Table 2: Gradient, parameter and acquisition mode

	Time	% A	%B	Parameter	Value	Acquisition Mode MS1	
1	5.00 min	95	5	Gas Temp (°C)	325	Min Range (*m/z*)	50
2	18.00 min	5	95	Gas Flow (l/min)	10	Max Range (*m/z*)	1700
3	27.00 min	5	95	Nebulizer (psig)	20	Scan Rate (spectra/sec)	2.00
4	27.10 min	95	5	Sheath Gas Temp	320		
5	30.00 min	95	5	Sheath Gas Flow	10		
				VCap	4000		
				Nozzle Voltage (V)	0		
				Fragmentor	170		

Table 3A: Qualitative phytochemical identification

S.No.	Botanical Name	Common Name	Alkaloid (Mayer's test)	Saponins (Foam test)
1.	*Acacia catechu*	Khair	-	-
2.	*Santalum album*	Chandan	-	+
3.	*Bambusa erandinasia*	Velu	-	-
4.	*Ficus religiosa*	Pimpal	-	+
5.	*Messua ferrea*	Naagkeshar	-	-
6.	*Ficus bengalensis*	Vad	-	+

Table 3B: Qualitative phytochemical identification

S.No	Botanical Name	Common Name	Terpenoid (acetic anhydride)	Flavonoids (alkaline reagent test)	Phenolics ($FeCl_3$ test)	Glycoside (Born-tranger test)
1.	*Acacia catechu*	Khair	+	+	+	-
2.	*Santalum album*	Chandan	+	+	-	-
3.	*Bambusa erandinasia*	Velu	+	+	+	-
4.	*Ficus religiosa*	Pimpal	+	+	+	-
5.	*Messua ferrea*	Naagkeshar	+	+	+	+
6.	*Ficus bengalensis*	Vad	+	+	+	+

Table 4: Quantitative phytochemical identification

Botanical Name	Phytoconstituents		
	Flavonoids mg/gm	Phenolics mg/gm	Terpenoids mg/gm
Acacia catechu	60	107.82	131
Santalum album	ND	-	7
Bambusa arundinacea	ND	44.80	11
Ficus religiosa	104	180.07	52
Mesua ferrea	39	503.10	23.3
Ficus benghalensis	35	244.92	10

Table 5: Total number of peaks found for saponins, terpnoids, glycosides and phenolics

Botanical Name	No. of peaks			
	Saponin	Terpenoid	Glycoside	Phenolics
Acacia catechu	-	1	-	ND
Santalum album	3	1	-	-
Bambusa arundinacea	-	2	-	ND
Ficus religiosa	5	1	-	1
Mesua ferrea	-	1	1	ND
Ficus benghalensis	2	1	ND	1

Table 6: Effect of variable doses of aqueous extract on human blood counts using flow cytometry

Plant material	Doses (mg/ml; 50 µl)	Lymphocytes	Monocytes	Granulocytes
Acacia catechu	Control PBS	5.28 ± 0.36	1.78 ± 0.04	39.23 ± 3.12
	HBsAg, 20 µg/ml; 10 µl	9.26 ± 1.04	7.98 ± 0.88	43.62 ± 5.56
	6.25	8.38 ± 1.06	3.02 ± 0.76	32.4 ± 4.02
	12.5	10.64 ± 2.38	2.52 ± 0.08	21.88 ± 2.64
	25	14.88 ± 2.76	0.82 ± 0.002	14.08 ± 3.06
Santalum album	Control PBS	5.28 ± 0.36	1.78 ± 0.04	39.23 ± 3.12
	HBsAg, 20 µg/ml; 10 µl	9.26 ± 1.04	7.98 ± 0.88	43.62 ± 5.56
	6.25	7.98 ±1.68	8.02 ± 1.26	30.82 ± 4.14
	12.5	11.36 ±2.56	4.04 ± 0.06	24.48 ± 3.66
	25	19.24 ± 4.02	1.02 ± 0.001	13.45 ± 2.44
Bambusa arundinacea	Control PBS	5.28 ± 0.36	1.78 ± 0.04	39.23 ± 3.12
	HBsAg, 20 µg/ml; 10 µl	9.26 ± 1.04	7.98 ± 0.88	43.62 ± 5.56
	6.25	14.34± 2.18	8.18 ± 1.38	40.46 ± 5.78
	12.5	26.86 ± 5.68	4.98± 0.76	22.16 ± 3.14
	25	31.84 ± 5.14	1.34 ± 0.04	15.42 ± 2.04
Ficus religiosa	Control PBS	5.28 ± 0.36	1.78 ± 0.04	39.23 ± 3.12
	HBsAg, 20 µg/ml; 10 µl	9.26 ± 1.04	7.98 ± 0.88	43.62 ± 5.56
	6.25	8.22± 0.46	7.04 ± 1.06	34.28 ± 5.06
	12.5	12.24 ± 1.78	4.16± 0.14	25.88 ± 3.44
	25	17.86 ± 3.28	1.34 ± 0.002	13.28 ± 2.18
Mesua ferrea	Control PBS	5.28 ± 0.36	1.78 ± 0.04	39.23 ± 3.12
	HBsAg, 20 µg/ml; 10 µl	9.26 ± 1.04	7.98 ± 0.88	43.62 ± 5.56
	6.25	10.28 ± 1.84	9.12 ± 1.34	38.18 ± 4.88
	12.5	17.24 ± 2.64	5.82 ± 0.78	31.23 ± 6.42
	25	27.22 ± 3.82	2.12 ± 0.04	26.98 ± 2.58
Ficus benghalensis	Control PBS	5.28 ± 0.36	1.78 ± 0.04	39.23 ± 3.12
	HBsAg, 20 µg/ml; 10 µl	9.26 ± 1.04	7.98 ± 0.88	43.62 ± 5.56
	6.25	18.16 ± 2.98	7.04 ± 0.56	34.28 ± 7.34
	12.5	26.74 ± 3.42	4.26 ± 0.34	23.58 ± 4.62
	25	33.12 ± 4.82	2.98 ± 0.02	11.78 ± 3.18

Flow cytometric analysis of aqueous extract (6.25 – 25 mg/ml; 50 µl) extracted from *Acacia catechu, Santalum album, Bambusa arundinacea, Ficus religiosa, Mesua ferrea and Ficus benghalensis* for determine its effect in human whole blood against HBsAg containing lymphocytes, monocytes and granulocytes count. Data acquisition of 10000 events and fraction or separation of cell populations representing different phenotypes analyzed using cell quest software

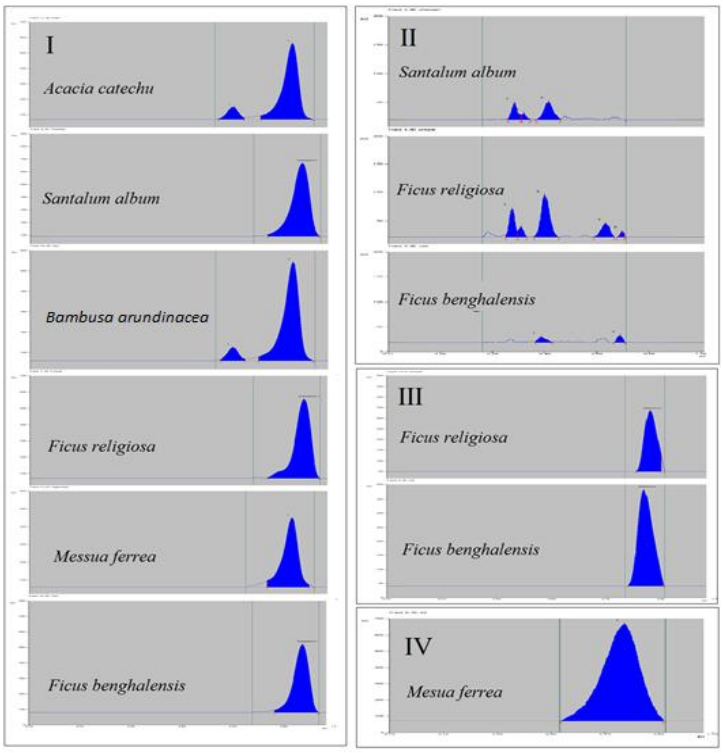

Figure 1: Typical HPTLC chromatogram for I. terpenoids, II. Saponin, III. Phenolics, IV. Glycoside

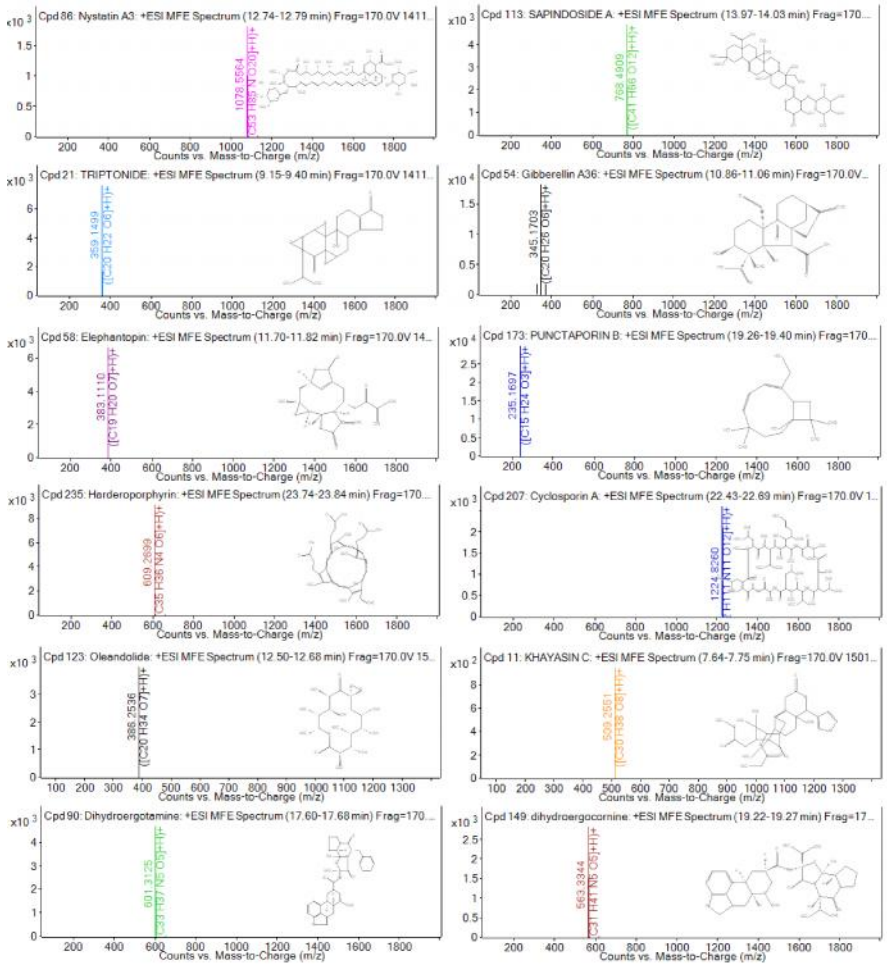

Figure 2: MS spectra for representative structurally unique and architecturally challenging bioactive phytochemicals identified in *Acacia catechu*, *Santalum album*, *Bambusa arundinacea*, *Ficus religiosa*, *Messua ferrea*, *Ficus benghalensis*

4. Conclusion

Present article describes qualitative and quantitative phytochemical study of *Acacia catechu, Santalum album, Bambusa arundinacea, Ficus religiosa, Mesua ferrea, Ficus benghalensis* using standard test, spectroscopic method, HPTLC and LC-MS. Qualitative test identifies the various phytoconstituents present in the extract such as saponin, terpenoids, glycosides, flavonoids etc. Quantitative tests shows terpenoids present in all the plants having major quantity in *Acacia catechu.* Phenolic compounds are being most ubiquitous among others, also found in major quantity in these medicinal plants with highest in *Mesua ferrea* whereas, flavonoidsare present in selected plants. HPTLC fingerprinting analysis is also reaffirms the presence of different phytoconstituents evident from the obtained chromatograms. LC-MS study identifies structurally unique and architecturally challenging molecules from the extract, such as Sapindoside A, Triptonide, Cyclosporin A etc. These molecules are considered to be therapeutically important bioactive secondary metabolites and showed immunosuppressive activity against specific protein antigen. The given study not only accounts presence of various phytochemicals but also identifies them, which would certainly help in standardizing the drug formulation and also provide scientific basis to the traditional health care system.

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