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HPTLC, LC-MS Aided Qualitative, Quantitative Phytochemical Screening and Immunosuppressive Activity of Medicinal Plants

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A B S T R A C T

This study analyzed major chemical constituents present in fresh leaves extracts of *Strychnus nuxvomica, Emblica officinalis, Ficus racemosa* and *Syzygium cumini* was collected, powdered and then analyzed through HPTLC (high performance thin layer chromatography), LC-MS (liquid chromatography-mass spectrometry) and bioactivity profile such as immunosuppressive activity. Preliminary phytochemical screening of aqueous leaves extract of these medicinal plants showed the presence of chemical constituents like glycosides, flavonoids, terpenoids, phenolic compounds etc. HPTLC finger print analysis of *Strychnosnuxvomica, Emblica officinalis, Ficus racemosa* and *Syzygiumcumini* showed the presence of possible number of components and determined its consistent quality of chemical constituents. The experimental conditions as well as 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 (HBsAg; 20 µg/ml) i.e. due to decrease in monocytes and granulocytes count.

Keywords: Strychnus nuxvomica, Emblica officinalis, Ficus racemosa, Syzygium cumini, immunosuppressive, LC-MS, HPTLC,

A R T I C L E I N F O

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1. Introduction

Primary metabolites of plant origin are compounds which aid in photosynthesis, respiration, growth and development. Other than primary metabolites there are compounds which are not directly involved in the survival of plants but they help them in normal growth and development. These are non-essential component for survival but rather are required for the interaction of plants with their environment e.g. survival in odd and extreme conditions, fighting against diseases etc. Terpenes, Alkaloids, Phenols etc. majority of which are ubiquitous in plant, are referred as secondary metabolites.[1,2] Therapeutic uses of these secondary metabolites are known to mankind since ages and traditional health care system in many of the developing and underdeveloped countries relies mainly on these bioactive compounds, till date. These metabolites (primary as well as secondary) obtained from various medicinal plants showed number of immune-pharmacological activities and used for various medical purposes. [3,4,5,6] Various phytochemical tests for qualitative analysis and colorimetric, spectroscopic or chromatographic methods for quantitative estimation need to be performed.[7] HPTLC and LC-MS are few of the techniques gained increasing importance in scientific fraternity due to accuracy, cost effectiveness and shorter time for analysis.[8,9] HPTLC is an advanced form of thin layer chromatography (TLC), which utilizes the chromatographic layers for utmost separation efficiency and use of state-of-the-art instrumentation for all steps in the procedure. Precise sample application, standardized reproducible chromatogram development, software controlled evaluation, rapid, accurate and cost effective analysis of the sample are some of the benefits over traditional TLC technique. HPTLC offers widely standardized methodology based on scientific facts and use of validated methods for qualitative and quantitative analysis. As a result of it HPTLC stands one of the important techniques to meet all quality requirements of today's analytical labs and due to which it is in great demand among scientific fraternity. Our study aims at the qualitative as well as quantitative phytochemical study of *Strychnus nuxvomica, Emblica officinalis, Ficus racemosa* and *Syzygium cumini.* Identification of some of the medicinally and biologically important moleculesis done on the basis of LC-MS data and determined its immunosuppressive activity.

2. Materials and Methods

Collection of plant materials: All plant leaves were collected from Nakshatra Udyan. Plants were dried at room temperature for 5 days and then in oven at 37ºC 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 the development of finger printing profile of various medicinal plants. The samples 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 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. The plate was 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 Peak densitogramswere identified.

Preparation of Extract for LC-MS

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 was macerated 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.

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

Estimation of blood counts: In order to determine its immunosuppressive activity of aqueous extract, EDTA human whole blood (n = 10; 3 ml) samples were collected from Mangal Pathology laboratory, Baramati. For these studies using 100 µl of human blood samples were taken in each falcon tube and then add without or with variable concentration of fresh leaves extracts of *Strychnus nuxvomica, Emblica officinalis, Ficus racemosa* and *Syzygium cumini*(6.25 – 25 mg, 50 µl) including HBsAg (20 µg/ml, 10 µl). Incubate these blood samples for 2 h at 4ºC and lysed with red cell lysis buffer and then washed with PBS (pH 7.2) and proceed for flow cytometric analysis for the estimation of total blood (lymphocytes, monocytes and granulocytes) counts in human whole blood.

3. Results and discussions

Qualitative as well as quantitative phytochemical investigation was carried out for *Strychnus nuxvomica, Emblica officinalis, Ficus racemosa* and *Syzygium cumini*, results of which were summarized in the **Table3**. The results revealed the presence of medically active compounds in the fourmedicinal plants studied. From the table, it could be seen thatflavonoids and terpenoidswere present in all the medicinal plants.Phenolics were present in only two plants. Saponinsis to be present in one plant, where as glycosides in two.Presence of alkaloidcould not be detected in any of the medicinal plants. Quantitative phytochemical study of the *Strychnus nuxvomica, Emblica officinalis, Ficus racemosa* and *Syzygium cumini*for total phenol, flavonoid and terpenoid content was done according to the standard procedures. Aluminum chloride colorimetric method was used for the total flavonoid determination. Gallic acid was used for the determination of total phenol content**Table 4**.

The study shows that total flavonoids content is 51.5 mg/gm, 26.75 mg/gm, 35 mg/gmand53.5 mg/gm and total terpenoid content 21 mg/gm, 17 mg/gm, 6 mg/gm, 4 mg/gm of the medicinal plants i.e. *Strychnosnuxvomica, Ficus racemosa, Emblica officinalis* and *Syzygiumcumini* respectively. Total phenolics content found to be 91.46 mg/gm, 322.35 mg/gm per sample for *Strychnosnuxvomica* and *Syzygiumcumini* respectively. It is concluded that *Syzygiumcumini* is rich with flavonoids and phenolics, supporting the claim of being medicinally important medicinal plant [17].

HPTLC analysis shows that number of phytochemicals present in the samples **Table 5, Figure 1**. The solvent systems were optimized before analysis. To identify the saponins, sample application was carried out using CAMAG LINOMAT 5 applicator, the plate was developed using Tol:MeOH:EtOAc (75:25:2) and scanned at multiple wavelength from 254-704 nm with an interval of 50 nm gives best results at 654 nm. The study furnishes, presence of 3 distinct peaks at the Rf value 0.3, 0.38 and 0.51 in Emblica officinalis corresponds to three different saponins. Analysis for terpenoids using mobile phase n-hexane: EtOAc (72:28) at 417 nm shows that single peak at Rf value 0.93, 0.97, 0.92, 0.96 for *Strychnosnuxvomica*, Emblica officinalis, Ficus racemosa, *Syzygiumcumini* respectively. Glycosides are present only in two samples having plate developed in n-BuOH:AcOH:Water (4:1:5) and scanned at 412 nm furnishing 2 peaks at Rf 0.65, 0.80 for *Ficusracemosa* and 1 peak at Rf 0.82 for *Syzygium cumini* concluding the later peaks of same compounds

The extracts of selected medicinal plants were subjected to LC-MS analysis and compounds were characterized based on their mass spectra, using the precursor ion, fragment ions,and comparison of the fragmentation patterns with molecules described in the literature. The information acquired from the LC-MS study ascertains the presence of various bioactive candidate of different class such as flavonoids, terpenoids, glycosides etc. The molecules were identified based on their *m/z* ratio. Mass spectra of representative compounds shown in **Figure 2**.

The Identification of some of the bioactive phytoconstituents was done, such as Ophiobolin Awith Rt 13.24-13.34 min and an $[M+H]^+$ion at *m/z* 400, Gibberelline A37with Rt 14.68-14.76 min and an $[M+H]^+$ion at *m/z* 387, Koparin 2'-methyl etherwith Rt 15.09-15.20 min and an $[M+H]^+$ion at *m/z* 315, Levonorgestrel acetatewith Rt 15.12-15.24 min and an $[M+H]^+$ion at *m/z* 337, Tetrahydro deoxy corticosterone with Rt 13.24-13.34 min and an $[M+H]^+$ion at *m/z* 335, Isotectorigenin with rt 18.72-18.84 min and an $[M+H]^+$ion at *m/z* 329, 3-deacetyl khivorinwith rt 23.34-23.46 min and an $[M+H]^+$ion at *m/z* 549, Neomycin Bwith Rt 24.77-24.88 min and an $[M+H]^+$ion at *m/z* 637, Arbekacinwith Rt 08.66-08.85 min and an $[M+H]^+$ion at *m/z* 553, Castasteronewith rt 25.85-25.98 min and an $[M+H]^+$ion at *m/z* 447, Triptonidewith Rt 12.80-12.88 min and an $[M+H]^+$ion at *m/z* 341, Fluoxymesteronewith Rt 12.89-13.02 min and an $[M+H]^+$ion at *m/z* 337, Gambogic acid amide with Rt 28.99-29.24 min and an $[M+H]^+$ion at *m/z* 627, 3,7-Epoxycaryophyllan-6-onewith Rt 14.09-14.17 min and an $[M+H]^+$ion at *m/z* 237, Cyclosporin Awith Rt 23.54-23.79 min and an $[M+H]^+$ion at *m/z* 1224. As per the flow cytometry results that clearly indicates its immunosuppressive effect of *Strychnus nuxvomica, Emblica officinalis, Ficus racemosa* and *Syzygium cumini*against HBsAg (i.e. decline in monocytes as well as granulocytes count, **Table 6**). Generally, monocytes represent the mediator of pro inflammatory cytokines after HBsAg-stimulation of human whole blood. Overall, this study reveals that aqueous leaves extract of these four medicinal plants showed immunosuppressive activity.

4. Conclusion

Qualitative and quantitative phytochemical credentials of *Strychnus nuxvomica, Emblica officinalis, Ficus racemosa* and *Syzygium cumini*have been investigated. Various tests as certains the presence of phytoconstituents such as terpenoids, flavonoids, saponins and glycosides and phenolics. Quantitative study reveals the presence of terpenoids, flavonoids in all plants with optimum availability whereas phenolics in *Strychnos nuxvomica* and *Syzygium cumini.* The study also shows the richness of *Syzygium cumini*with phenolic content. HPTLC analysis also confirms saponins, terpenoids and glycosides present in the samples. The identification of bioactive phytoconstituents was done based on LC-MS observations. The molecules were identified based on their *m/z* ratio and confirmed the potent molecules of biological and medicinal interest such as Ophiobolin A, Cyclosporin A, Arbekacin Isotectorigenin, Tetrahydro deoxy corticosterone etc. Flow cytometry results of aqueous leaves extract of these four medicinal plants clearly indicate its immunosuppressive effect against HBsAg*i.e.* decline in monocytes as well as granulocytes count. The given study paves a way towards the systematic investigation and analysis of various phytochemicals and facilitates tool to study their pharmacology.

Table 1: 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	90mm	80mm
Band length	8mm	7 mm
Scanned wavelength range	254-704 nm	300-500nm
Best wavelength	654nm	417nm

Table 1.1: HPTLC parameters

	Phenolics	**Glycosides**
Sample preparation	Leaves sample extracted with 70% ethanol. Heat extract at 60^{o} C, dry it and dissolved it in methanol. Centrifuge mixture at 3000rpm 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 application.
Solvent system	Tol:Acetone:Formicacid (22:22:6)	*n*-Butanol: Acetic acid: Water (4:1:5)
Solvent fornt	90mm	85mm
Band length	7 mm	7 mm
Scanned wavelength range	300-500nm	250-440nm
Best wavelength	418nm	412nm

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	SheathGasTemp	320		
5	30.00 min	95	5	SheathGas Flow	10		
				VCap	4000		
				Nozzle Voltage (V)	0		
				Fragmentor	170		

Table 3: Qualitative phytochemical screening

Sr. No.	**Botanical Name**	**Common Name**	**Alkaloid (Mayer's test)**	**Saponins** (Foam test)	**Terpenoid** (acetic anhydride)	**Flavonoids** (alkaline reagent test)	**Phenolics ($FeCl_3$ test)**	**Glycoside (Borntrang-er test)**
1.	*Strychnus nuxvomica*	Kuchla, Kajara	-	-	+	+	+	-
2.	*Emblica officinalis*	Aamla	-	+	+	+	-	-

3.	*Ficus racemosa*	Umber	-	-	+	+	-	+
4.	*Syzygium cumini*	Jamun	-	-	+	+	+	+

Table 4: Quantitative phytochemical screening

S.No	Botanical Name	Phytoconstituents		
		Flavonoids mg/gm	Phenolics mg/gm	Terpenoids mg/gm
1	*Strychnos nuxvomica*	51.5	91.46	21
2	*Ficus racemosa*	26.75	-	17
3	*Emblica officinalis*	35	-	6
4	*Syzygium cumini*	53.5	322.35	4

Table 5: Total number of peaks found for saponins, Terpenoids and Glycosides

S.No	Botanical Name	No. of peaks		
		Saponin	Terpenoid	Glycoside
1.	*Strychnus nuxvomica*	-	1	-
2.	*Emblica officinalis*	03	1	-
3.	*Ficus racemosa*	-	1	2
4.	*Syzygium cumini*	-	1	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
Strychnos nuxvomica	Control PBS	8.68 ± 1.21	2.32 ± 0.56	42.14 ± 4.56
	HBsAg, 20 µg/ml; 10 µl	11.26 ± 1.24	9.32 ± 1.02	45.6 ± 4.52
	6.25	9.26 ± 2.12	3.45 ± 0.54	20.12 ± 2.86
	12.5	14.22 ± 2.88	2.78 ± 0..48	14.72 ± 3.12
	25	21.04 ± 3.26	1.04 ± 0.004	12.24 ± 2.84
Emblica officinalis	Control PBS	8.68 ± 1.21	2.32 ± 0.56	42.14 ± 4.56
	HBsAg, 20 µg/ml; 10 µl	11.26 ± 1.24	9.32 ± 1.02	45.6 ± 4.52
	6.25	16.68 ± 3.22	6.42 ± 1.02	32.2 ± 2.48
	12.5	21.2 ± 3.74	5.12 ± 0.98	27.64 ± 4.12
	25	27.4 ± 2.88	4.14 ± 0.78	19.5 ± 3.28
Ficus racemosa	Control PBS	8.68 ± 1.21	2.32 ± 0.56	42.14 ± 4.56
	HBsAg, 20 µg/ml; 10 µl	11.26 ± 1.24	9.32 ± 1.02	45.6 ± 4.52
	6.25	13.72 ± 1.38	7.16 ± 1.10	38.4 ± 5.12
	12.5	22.14 ± 2.36	5.12 ± 0.98	34.12 ± 4.34
	25	29.34 ± 1.72	4.34 ± 1.04	26.6 ± 2.54
Syzygium cumini	Control PBS	8.68 ± 1.21	2.32 ± 0.56	42.14 ± 4.56
	HBsAg, 20 µg/ml; 10 µl	11.26 ± 1.24	9.32 ± 1.02	45.6 ± 4.52
	6.25	19.32 ± 2.32	6.78 ± 0.36	40.4 ± 4.12
	12.5	29.16 ± 4.12	4.98 ± 0.44	38.42 ± 5.12
	25	34.12 ± 5.68	3.46 ± 0.54	28.22 ± 4.68

Flow cytometric analysis of aqueous extract (6.25–25 mg/ml; 50 µl) extracted from *Strychnus nuxvomica*, *Emblica officinalis*, *Ficus racemosa* and *Syzygium cumini*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.

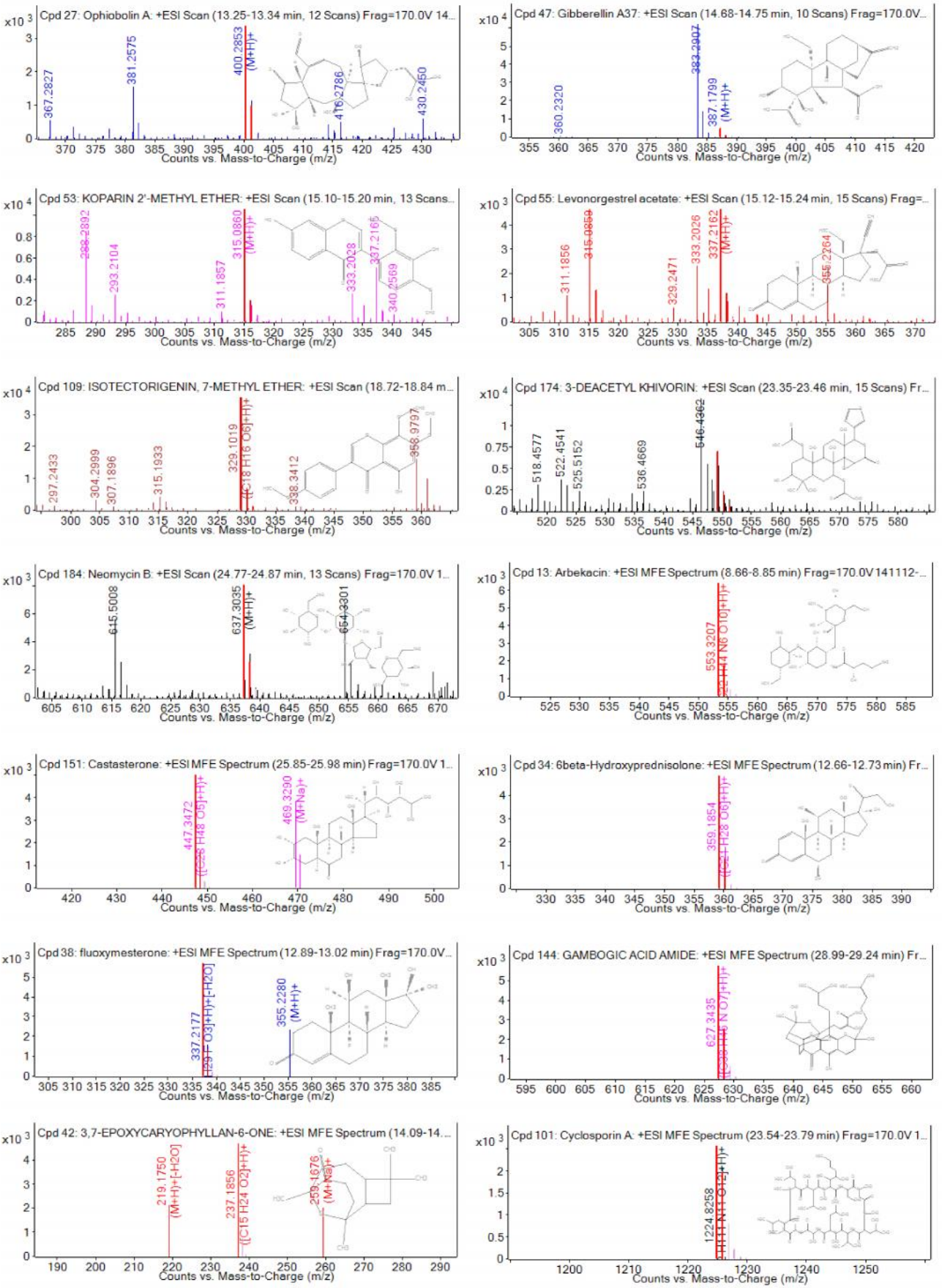

Figure 1: MS spectra for representative bioactive phytochemicals identified in *Strychnus nuxvomica*, *Emblica officinalis*, *Ficus racemosa* and *Syzygium cumini*

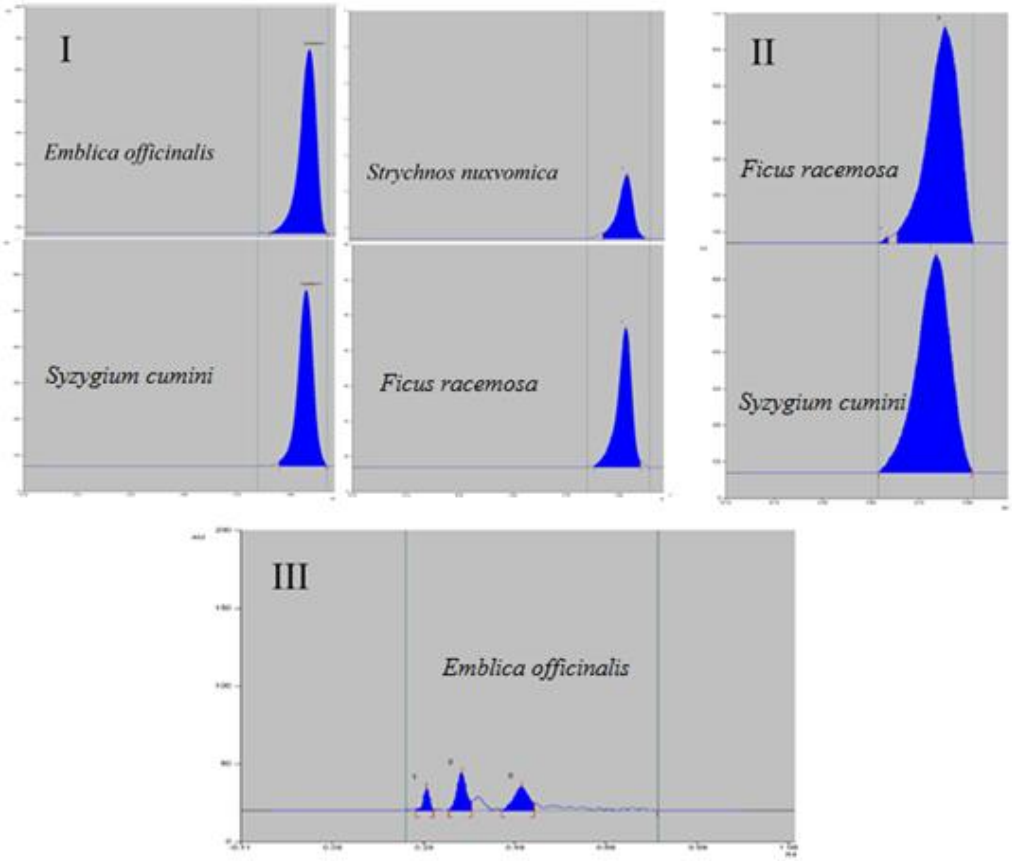

Figure 2: Typical HPTLC chromatogram for I. terpenoids, II. Glycoside, III. Saponin

5. References

[1] Krishnaiah, D., Sarbatly, R., Phytochemical antioxidants for health and medicine: A move towards nature. *Biotechnol Mol Biol Rev*., 2007, 1: 97-104.

[2] Stuffness, M., Douros, J. Current status of the NCI plant and animal product program. *Natural Products* 1982; 45(1):1–14.

[3] Brown, J.E., Rice-Evans, C.A. 1998. Luteolin rich artichoke extract protects low density lipoprotein from oxidation in vitro. *Free Radical Res*., 29: 247-255.

[4] Krings, U., Berger, R.G. 2001. Antioxidant activity of roasted foods. *Food Chem*., 72: 223-229.

[5] Salah, N., Miller, N.J., Pagange, G., Tijburg, L., Bolwell, G.P, Rice, E., Evans, C. 1995. Polyphenolic flavonoids as scavenger of aqueous phase radicals as chai breaking antioxidant. *Arc. Biochem. Broph*., 2: 339-346.

[6] Del-Rio, A., Obdululio, B.G., Casfillo, J., Main, F.G., Ortuno, A. 1997. Uses and properties of citrus flavonoids. *J. Agric. Food Chem*., 45: 4505-4515.

[7] Punit K. Jain a, V. Ravichandran a,b, Prateek K. Jain a, Ram K. Agrawal, High-performance thin layer chromatography methodfor estimation of andrographolide in herbal extract andpolyherbal formulations, *J. Saudi. Chem. Soc.* (2010) 14, 383–389.

[8] Yadav, R.N.S., Agarwala, M. Phytochemical analysis of some medicinal plants, *J. Phyt*., 2011. 3: 10-14.

[9] M.Vijayalakshmi, R.Kiruthika, K.Bharathi, K.Ruckmani, Phytochemical screening by LC-MS analysisand invitro anti- inflammatory activity of Marselia quadrifolia plant extract, *Int. J. PharmTech Res.*Vol.8, No.9, pp 148-157, 2015

[10] Sofowora, A. (1993). *Medicinal Plants and Traditional Medicine in Africa*. JohnWiley and Sons Limited, 2: 96-106.

[11] Trease, G. E., Evans, W. C.*Pharmacognosy* (15th Edn. Saunders, pp. 214-393. 2002).

[12] Trease, G. E., Evans, W. C.*Pharmacognosy* (15th Edn. Saunders, pp. 214-393. 2002).

[13] Harborne, J. B. Methods of plant analysis. In: *Phytochemical Methods* (Chapman and Hall, London. 1973)

[14] Parekh, J., Chands, S. 2008. Phytochemical screening of some plants from Western regions of India, *Plant Arch*, 8: 657 – 662

[15] Majaw,S., Moiranthem, J.Qualitative and quantitative analysis of *Clerodendron colebrookianum* Walp. Leaves and *Zinzibercassumunar* Roxb. Rhizomes Dept. of Biotech. And Bio-informatics, North Eastern Hill University, Meghalaya, India.

[16] Chia-Chi Chang, Jiing-Chaun Chem.2002. Estimation of total flavonoids content in *Propolis*

By two complementary colorimetric methods, *J. of Food & Drug Ana.* 10:178-182.

[17] Wangcharoen W., Morasuk, W. 2007. Antioxidant capacity and phenolic content of some Thai culinary plants, Mj. *Int. J. Sci. tech*, 2007, 01(02), 100-106.

[18] Baliga, M.S., Bhat, H. P., Baliga,B. R. V., Wilson, R., Palatty, P. L. Phytochemistry, traditional uses and pharmacology of Eugenia jambolana Lam. (black plum): A review *Food Res. Int.* 44 (2011) 1776–1789.